Distinct activities of Scrib module proteins organize epithelial polarity

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Edited by Margaret T. Fuller, Stanford University School of Medicine, Stanford, CA, and approved April 15, 2020 (received for review October 25, 2019)

A polarized architecture is central to both epithelial structure and function. In many cells, polarity involves mutual antagonism between the Par complex and the Scribble (Scrib) module. While molecular mechanisms underlying Par-mediated apical determination are well-understood, how Scrib module proteins specify the basolateral domain remains unknown. Here, we demonstrate dependent and independent activities of Scrib, Discs-large (Dlg), and Lethal giant larvae (Lgl) using the *Drosophila* follicle epithelium. Our data support a linear hierarchy for localization, but rule out previously proposed protein–protein interactions as essential for polarization. Cortical recruitment of Scrib does not require palmitoylation or polar phospholipid binding but instead an independent cortically stabilizing activity of Dlg. Scrib and Dlg do not directly antagonize atypical protein kinase C (aPKC), but may instead restrict aPKC localization by enabling the aPKC-inhibiting activity of Lgl. Importantly, while Scrib, Dlg, and Lgl are each required, all three together are not sufficient to antagonize the Par complex. Our data demonstrate previously unappreciated diversity of function within the Scrib module and begin to define the elusive molecular functions of Scrib and Dlg.

epithelia | polarity | *Drosophila* | Par complex | Scrib module

Cell polarity is defined by the coexistence of two distinct spatial identities within the confines of a single plasma membrane. This process is critical for many cell types, including stem cells, epithelial cells, migratory cells, and immune cells, to carry out their physiological functions (1, 2). Despite the distinct manifestations of polarity in these specialized cells, polarity in each is generated by a common pathway involving a set of conserved protein modules (3–5). Foremost among these are the Par and Scrib modules, consisting of Par-3, Par-6, and atypical protein kinase C (aPKC) for the former and Scribble (Scrib), Discs-large (Dlg), and Lethal giant larvae (Lgl) for the latter (3, 4). These proteins play crucial roles in diverse biological processes and have also been implicated in numerous pathologies, from congenital birth defects to cancer (3, 4, 6). Thus, uncovering their molecular activities is essential to a mechanistic understanding of cell, developmental, and disease biology.

A number of studies have provided important insight into the molecular function of the Par module and each of its individual components (7–11). Much of this work derives from *Drosophila* epithelial cells and neural stem cells, where the Par module regulates the apical domain and the Scrib module is required to specify the basolateral domain. The core distinction of cortical domains arises from mutual antagonism between the two modules, centering around interactions between aPKC and Lgl (Fig. 1*A*). In the apical domain, aPKC phosphorylates Lgl on three residues within a polybasic domain, causing it to dissociate from the plasma membrane (12–14). Conversely, Lgl inhibits aPKC kinase activity and localization along the basolateral cortex (15–17). Many details of Par protein activities and their outcomes are now understood, including specific protein–protein interactions in dynamic complexes, their structural basis, post-translational modifications, and the kinetic order of events during apical polarization (18, 19).

In contrast to the wealth of mechanistic information about the Par complex, and despite the discovery of the relevant genes decades ago, the molecular mechanisms of basolateral domain specification by the Scrib module are still unknown. All three genes encode large scaffolding proteins containing multiple protein–protein interaction domains and lack obvious catalytic activity (13, 20–22). Recent studies have identified novel interacting partners of Scrib module proteins, but few of these interactors have been implicated as regulators of cell polarity themselves (23, 24). Moreover, few studies have focused on the regulatory relationships within the Scrib module itself, and beyond the well-characterized aPKC-inhibiting function of Lgl, the fundamental molecular activities of Scrib and Dlg remain unknown. In this work, we identify distinct activities of Scrib, Dlg, and Lgl that are required but not sufficient for basolateral polarization, shedding light on the mechanisms that restrict the Par complex to partition the epithelial cell membrane.

Results

A Linear Hierarchy for Localization but Not Function of Basolateral Polarity Regulators. We used the conserved epithelial features of *Drosophila* ovarian follicle cells to study regulation of the basolateral cortical domain (25) (*SI Appendix*, Fig. S1 *A–C*). Cells mutant for null alleles of *scrib*, *dlg*, or *lgl* encoding severely truncated or nonfunctional proteins lose polarity, characterized by mixing of apical and basolateral domains and cells form multilayered masses at the poles of the egg chamber (Fig. 1 *B–E* and *SI Appendix*, Fig. S1 *I–K*) (20, 21, 26). Importantly, we focused our analysis on the central follicle epithelium, where polarity-deficient cells retain relatively normal morphology that

Significance

To enable their physiological functions, cells must polarize their plasma membrane. In many epithelia, polarity is regulated by balanced activity of the apical Par complex and basolateral Scribble module. While the former is understood in molecular detail, little is known about how the latter works. We identify distinct functions of the three Scribble module proteins, separating independent roles in a localization hierarchy from cooperative roles in apical polarity antagonism, and showing that they are not together sufficient to specify basolateral identity. This work establishes an essential basis for a mechanistic understanding of this core polarity machinery that controls processes ranging from stem cell divisions to organ morphogenesis across animal species.

Author contributions: M.J.K. and D.B. designed research; M.J.K. performed research; M.J.K. analyzed data; and M.J.K. and D.B. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1918462117/-/DCSupplemental.

First published May 15, 2020.

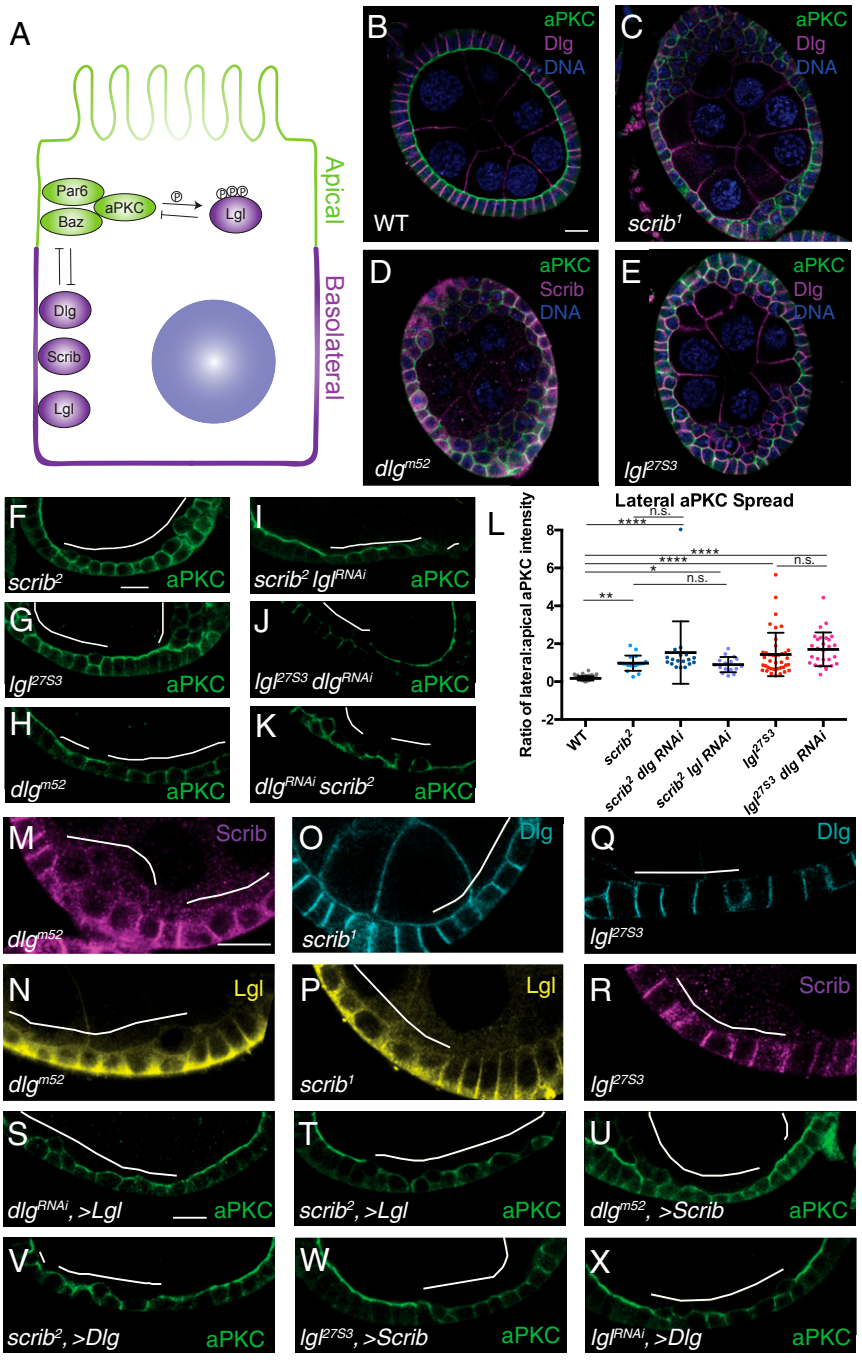

Fig. 1. Functional relationships within the Scrib module. (*A*) Simplified schematic representation of epithelial polarity interactions. Polarity phenotypes of WT (*B*), *scrib* (*C*), *dlg* (*D*), and *lgl* (*E*) follicle cells: Mutants exhibit polarity loss, characterized by mixing of apical and basolateral domains and multilayering of the epithelium. Compared to single mutants (*F–H*), double-depleted combinations (*I–K*) do not show an enhanced apical expansion phenotype. (*L*) Quantification of aPKC mislocalization phenotype in single and double mutants. aPKC spread represented as ratio of lateral:apical intensity in single cells. Localization of Scrib module proteins: Both Scrib and Lgl show hazy, cytoplasmic mislocalization in *dlg* mutant cells (*M* and *N*). In *scrib* mutant cells, Dlg localization is normal (*O*), while Lgl is mislocalized (*P*). In *lgl* mutants, both Scrib and Dlg localizations are unchanged (*Q* and *R*). Overexpression of Lgl does not rescue apical polarity defects in *dlg* or *scrib* mutants (*S* and *T*). Scrib overexpression cannot rescue *dlg* mutants (*U*) nor can Dlg overexpression rescue *scrib* mutants (*V*). *lgl* mutants are not rescued by Scrib or Dlg overexpression (*W* and *X*). (Scale bars, 10 μm.) One-way ANOVA with Tukey's multiple comparisons test. Error bars represent SD, data points are measurements from single cells. White line indicates mutant cells and/or overexpression clones in this and all subsequent figures. Follicles in *D*, *F*, and *R* are stage 5; *H*, *J*, *P*, and *S* are stage 7; *K* and *O* are stage 8; all others are stage 6. n.s. (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

allows accurate monitoring of protein localization. We first asked whether Scrib, Dlg, and Lgl have independent as well as shared functions in epithelial polarity. We generated follicle cells simultaneously mutant for one of the genes and expressing a validated RNAi transgene targeting a second gene. However, we saw no differences between single mutant cells and cells double-depleted for *scrib dlg*, *scrib lgl*, and *lgl dlg*, and lateral aPKC spread was not enhanced in double mutants (Fig. 1 *F–K*,

quantified in Fig. 1*L*). These phenotypes are consistent with Scrib, Dlg, and Lgl regulating polarity through a single, common pathway.

We next defined regulatory relationships between Scrib module components. Previous work in several organs has documented mutual dependence for localization, but also significant differences in their interrelationships (27–29). In *dlg* mutant follicle cells, both Scrib and Lgl are mislocalized and exhibit hazy, cytoplasmic distributions (Fig. 1 *M* and *N*). In *scrib* mutant follicle cells, although Lgl is mislocalized as in *dlg* mutants, Dlg maintains normal basolateral localization (Fig. 1 *O* and *P*). Moreover, in *lgl* mutant follicle cells, both Scrib and Dlg maintain normally polarized cortical domains (Fig. 1 *Q* and *R*). We note that for both Scrib and Lgl, mislocalization reflects a partial and somewhat variable rather than complete cortical loss, consistent with other observations (13, 30, 31). We observe a similar hierarchical relationship in the embryonic epithelium, as suggested previously (*SI Appendix*, Fig. S1 *D–H*) (27). These results suggest a linear pathway whereby Dlg localizes independently to the cell cortex, Scrib localization requires Dlg, and Lgl localization is dependent on both other Scrib module proteins.

We then asked whether elevated levels of one protein in this pathway could compensate for loss of another, using previously validated transgenes (15, 32–34). We first tested overexpression of Lgl in *scrib* or *dlg* mutant cells and found that this did not modify the phenotype of either mutant (Fig. 1 *S* and *T*). Similarly, Scrib overexpression did not modify the *dlg* mutant phenotype, and Dlg overexpression did not modify the *scrib* mutant phenotype (Fig. 1 *U* and *V*). Moreover, neither Scrib nor Dlg overexpression was able to modify the *lgl* mutant phenotype (Fig. 1 *W* and *X*). These data suggest that, despite the linear localization hierarchy, regulation of basolateral polarity in the follicle epithelium involves relationships that cannot be bypassed by simple overexpression of one Scrib module component.

Dlg Stabilizes Scrib at the Cortex. Since Dlg is required for Scrib cortical localization, we investigated the underlying mechanism. We used fluorescence recovery after photobleaching (FRAP) assays to compare the stabilities of each protein at the cell cortex, using functional GFP-tagged versions expressed from endogenous loci. In WT cells, Scrib::GFP was highly stable, whereas Dlg::GFP was intermediately dynamic and Lgl::GFP was comparatively mobile (Fig. 2*A*). Strikingly, in *dlg*-depleted cells, Scrib::GFP exhibited an approximately fourfold increase in recovery kinetics, consistent with the loss of cortical localization also seen in fixed tissue (Fig. 2*B*). In contrast, although Dlg::GFP in *scrib* and *lgl* mutant cells remained localized at the cortex and mobile fractions are not changed, it also exhibited increased recovery kinetics (*SI Appendix*, Fig. S2 *A* and *B*), perhaps reflecting increased in-plane mobility due to defective septate junction formation (35, 36). Importantly, however, Scrib::GFP was unchanged in *lgl* mutant cells (*SI Appendix*, Fig. S2*C*). Thus, FRAP assays support an important role for Dlg in stabilizing Scrib on the cell cortex.

One mechanism that could localize Scrib to the cortex is a phospholipid-binding polybasic motif (PBM), as seen in other polarized proteins, including Lgl and aPKC (13, 14, 30). However, an obvious PBM is not seen in the Scrib protein sequence. PBMs directly bind polar phospholipids, but mutating PI4KIIIα or expressing dominant-negative PI3K (Δp60), which deplete PIP2 and PIP3, respectively, did not alter Scrib cortical localization (*SI Appendix*, Fig. S3 *A* and *B*) (37, 38). Additionally, ATP depletion by antimycin A treatment, which reduces PIP levels and is sufficient to delocalize Lgl::GFP, did not alter Scrib::GFP localization (*SI Appendix*, Fig. S3 *C–F*) (13).

An alternative mechanism by which Dlg could regulate Scrib cortical localization is via physical binding. The conserved colocalization and shared functions of Scrib module proteins has led to propositions that they function as a macromolecular complex. The Dlg GUK domain, which binds to the Dlg SH3 domain in an autoinhibitory manner, is the central mediator (39, 40). The GUK domain is reported to interact directly with Lgl and also indirectly with Scrib PDZ domains through the protein Gukholder (Gukh) at synapses and in vitro; a recent study further suggests a role for Gukh in epithelial development (40–43). We tested the requirement for these GUK-mediated interactions in vivo by analyzing a hypomorphic *dlg* deletion allele (dlg^{v59}) that removes the C-terminal two-thirds of the GUK domain (Fig. 2*C*) (21). Apicobasal polarity and aPKC localization remained unchanged in central follicle cells mutant for this GUK-deficient allele, as did cortical localization of Lgl (Fig. 2 *D*, *F*, *G*, and *I*). A significant loss of cortical Scrib localization was seen, although this may be due to reduced levels of mutant Dlg (Fig. 2*H* and *SI Appendix*, Fig. S4 *C* and *F*) (21), as the additional GUK-truncating allele dlg^{1P20} (Fig. 2*C*) shows unaffected Scrib as well as aPKC and Lgl localization in follicle cells (*SI Appendix*, Fig. S4 *D–F*). As with the GUK-deficient *dlg* alleles, no polarity defects were seen in follicle cells homozygous for a *scrib* allele that truncates the protein before the PDZ domains ($scrib^{4}$), or in maternal and zygotic (*m*/*z*) mutant embryos (Fig. 2 *C*, *J*, and *K* and *SI Appendix*, Fig. S4 *M–P*) (33). In contrast, a missense mutation in the Dlg SH3 domain (dlg^{m30}, L632P), which does not alter Dlg protein levels or localization, was sufficient to cause mislocalization of Scrib, as well as both Lgl and aPKC, in a manner indistinguishable from null alleles in follicle cells and *m*/*z* mutant embryos (Fig. 2 *C*, *L*, and *M* and *SI Appendix*, Fig. S4 *A* and *F–L*) (21). These results reveal a role for the SH3 domain in regulating Scrib localization as well as apical domain antagonism, but show that the GUK domain is not required for epithelial polarity.

A third mechanism that can localize cytosolic proteins to the cortex is via posttranslational attachment of lipophilic groups. To measure protein acylation, we performed acyl-biotin exchange (ABE), which converts thioester-linked acyl groups to biotin that can be detected by Western blot (44). To avoid the significant germline contribution of Scrib and Dlg in the ovary, we analyzed larval lysates by ABE and found that Scrib::GFP is acylated in *Drosophila* (Fig. 3*A*), consistent with a previous report (45). Recent work has shown that mammalian Scrib is S-palmitoylated on two conserved N-terminal cysteine residues, and this modification is required for Scrib cortical localization and function (46). We generated a Scrib::GFP transgene in which these conserved palmitoylated cysteines are changed to alanine ($Scrib^{C4AC11A}$::GFP). Surprisingly, this protein localizes appropriately to the plasma membrane and rescues *scrib* mutant polarity phenotypes (Fig. 3 *B–D*). ABE showed that these mutations are not sufficient to abolish all acylation, suggesting that Scrib can be palmitoylated on additional nonconserved residues (Fig. 3*A*). We then inhibited palmitoyltransferases either pharmacologically, using 2-bromopalmitate (2-BrP) or by knocking down the *Drosophila* homolog of the Scrib-regulating palmitoyltransferase, ZDHHC7 (Flybase, CG8314), and found that both of these approaches failed to impact Scrib localization (*SI Appendix*, Fig. S5). Finally, we asked whether Dlg might regulate Scrib through influencing its palmitoylation. However, in *dlg* tissue no change in the acylation of Scrib::GFP could be detected by ABE (Fig. 3*A*). Thus, palmitoylation itself is not sufficient to localize Scrib to the cortex; instead Dlg must regulate Scrib localization by an independent mechanism.

To test whether cortical Scrib stabilization is the sole function of Dlg in epithelial polarity, we made use of a nanobody-based system for relocalizing GFP-tagged proteins within the cell (47). We tethered Scrib::GFP to the cortex via interactions with a uniformly distributed transmembrane anchor and examined apicobasal polarity in the absence of *dlg*. However, aPKC mislocalized to the lateral membrane and Lgl was displaced to the cytoplasm in these cells, as in cells depleted of *dlg* alone (Fig. 3 *E–J*). As a complementary approach, we generated a constitutively membrane-tethered version of Scrib via

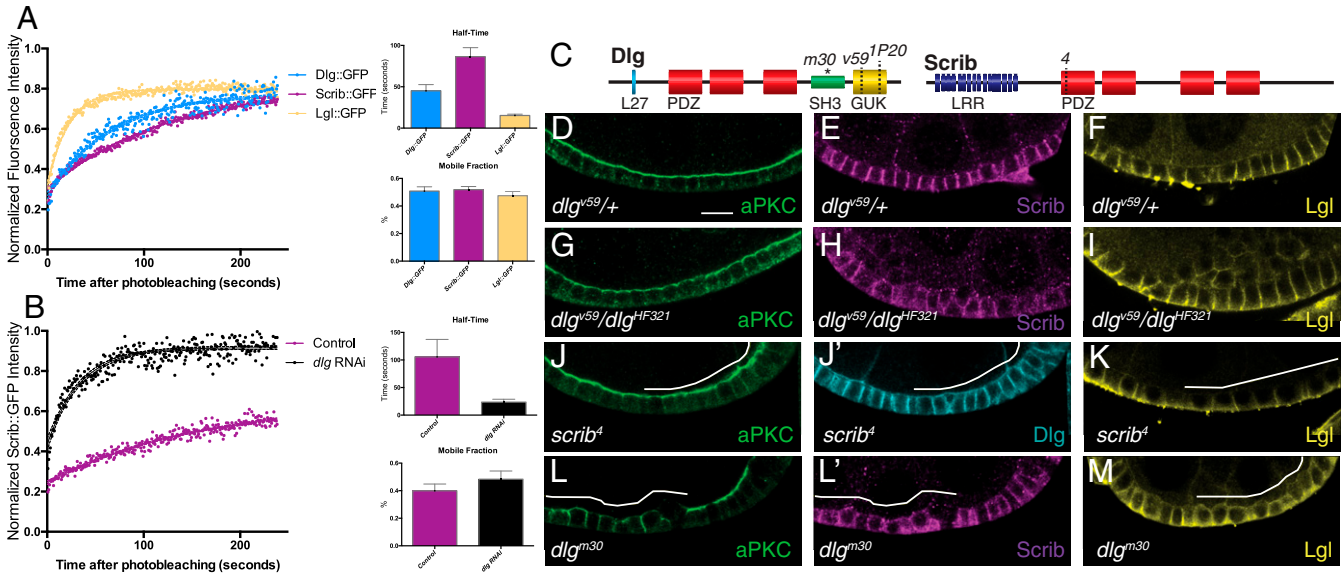

Fig. 2. Dlg regulates cortical Scrib stability. FRAP assay (*A*) shows distinct mobility of Scrib, Dlg, and Lgl in WT cells; Scrib is most stable and Lgl is most dynamic. (*B*) Scrib shows an approximately fourfold increase in recovery kinetics in *dlg* RNAi cells. (*C*) Schematic of the mutant *dlg* alleles and *scrib* alleles used in *D*–*M*. dlg^{m30} harbors a point mutation in the SH3 domain (L632P, asterisk). dlg^{v59} contains a deletion resulting in frameshift and truncation of the GUK domain. dlg^{1P20} is a nonsense mutation truncating the GUK domain. $scrib^{4}$ truncates all four PDZ domains. Dotted lines indicate truncations. Compared to WT (*D*–*F*), aPKC (*G*), and Lgl (*I*) localizations are unaffected in cells mutant for a *dlg* GUK-deficient allele. Scrib localization (*H*) is partially affected, although this may be due to reduced stability of mutant Dlg. aPKC (*J*), Dlg (*J'*), and Lgl (*K*) localizations are unaffected in cells mutant for a *scrib* allele lacking PDZ domains. (*L*) aPKC mislocalizes laterally in cells mutant for a *dlg* SH3 point mutant allele. Scrib (*L'*) and Lgl (*M*) also exhibit cytoplasmic mislocalization in these cells. (Scale bars, 10 μm.) Error bars represent 95% confidence intervals. All follicles are stage 7, except *K* and *M*, which are stages 8 and 6, respectively.

attachment of an N-terminal myristoylation sequence. This myr-Scrib transgene was capable of rescuing polarity defects in *scrib* mutant follicle cells (*SI Appendix*, Fig. S6 *A*–*E*). However, in *dlg*-depleted cells expressing myr-Scrib, in which myr-Scrib remains cortical, neither aPKC nor Lgl mislocalization was rescued (Fig. 3 *K* and *L*). The lack of rescue in these experiments suggests that Dlg has polarity functions in addition to Scrib localization and that Scrib and Dlg act in parallel to regulate apicobasal polarity.

Scrib and Dlg Are Not Regulated by, and Do Not Directly Regulate, aPKC. We then examined the relationship between the Scrib module and aPKC. A central feature of this relationship is the exclusion of Lgl from aPKC-containing cortical domains, due to direct phosphorylation; when follicle cells are depleted of *apkc*, Lgl can reach the apical domain (Fig. 4*A*) (34, 48, 49). We asked if Scrib and Dlg also exhibit aPKC-dependent apical exclusion, but the juxtaposition of the nurse cell membrane, which also expresses Dlg, to the apical domain of the follicle cells obscures accurate measurement. Dlg and Scrib remain localized to the basolateral cortex in *apkc*-depleted follicle cells (Fig. 4 *B* and *C*), as well as in cells mutant for null alleles of the Par complex genes *par-6* and *cdc42* (*SI Appendix*, Fig. S7 *A* and *B*). Cells also retain basolateral Scrib and Dlg when aPKC is depleted within *lgl* mutant cells (Fig. 4 *D*, *E*, *G*, and *H*). Furthermore, overexpression of a constitutively active form of aPKC ($aPKC^{\Delta N}$) does not displace Scrib or Dlg from the cell cortex (Fig. 4*M*). Thus, localization of Scrib and Dlg depends on cues independent of aPKC activity.

The inhibitory relationship between aPKC and Lgl is well established, but it is not known whether Scrib and Dlg might also be direct inhibitors of aPKC (12, 15, 16). Notably, when aPKC mislocalizes laterally in *lgl* mutant cells, it colocalizes with Scrib and Dlg, which are not displaced (Fig. 4*J*). This lateral aPKC is active because it can recruit Patj, whose localization is dependent on aPKC activity, to ectopic sites (Fig. 4 *F* and *I* and *SI Appendix*, Fig. S7 *C* and *D*) (9, 50). Additionally, a constitutively active, membrane-targeted aPKC isoform ($aPKC^{CAAX}$) that is sufficient to respecify the entire cortex as apical (shown by Patj recruitment) (*SI Appendix*, Fig. S7 *C* and *D*), can colocalize with Scrib and Dlg at the basolateral membrane (*SI Appendix*, Fig. S7*E*). These results rule out Scrib and Dlg as intrinsic inhibitors of aPKC, suggesting they work through Lgl to block the spread and apicalizing activity of aPKC, and that the aPKC mislocalization seen in *scrib* and *dlg* mutant cells (Fig. 4 *K* and *L*) reflects a weakening of Lgl inhibitory activity in the absence of either Scrib or Dlg.

Scrib and Dlg Are Both Required to Stabilize and Enable Lgl Activity. If Scrib and Dlg do not directly inhibit aPKC, how do they participate in apicobasal antagonism? FRAP measurements of Lgl::GFP show that in *dlg*- and *scrib*-depleted follicle cells, a clear increase in recovery kinetics and decrease of the mobile fraction compared to WT is seen (Fig. 5 *A* and *B*). Whereas Lgl::GFP becomes cytoplasmic in *dlg* RNAi cells, an endogenously expressed, nonphosphorylatable Lgl fusion protein (Lgl^{S5A}::GFP) remains cortically associated in *dlg* RNAi cells (Fig. 5 *C* and *D*) (13). Moreover, codepleting aPKC in *dlg* RNAi cells restores Lgl cortical localization (Fig. 5 *E* and *F*). These results are consistent with dynamic exchange of Lgl between an hypophosphorylated membrane-associated pool and an aPKC-hyperphosphorylated cytoplasmic pool, and suggest that Scrib and Dlg stabilize the former.

Cortical association of Lgl depends on interactions between polar phospholipids and charged residues within the Lgl PBM (13, 14). PIP2 and PIP3 show apicobasally polarized distributions in epithelial cells of *Drosophila* as well as vertebrates, raising the possibility that Dlg and Scrib could regulate Lgl function by altering the distribution of PIP species at the basolateral membrane (51, 52). However, using genetically encoded reporters, we

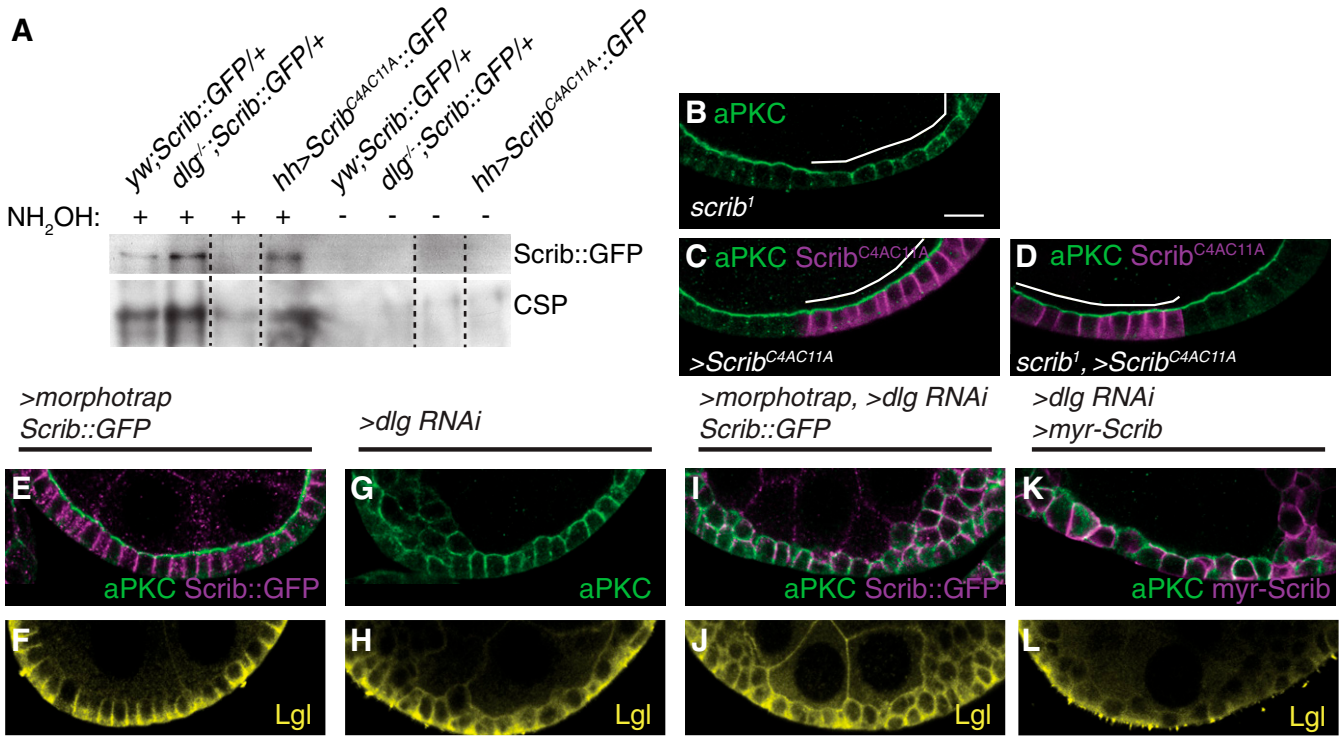

Fig. 3. Cortical Scrib still requires Dlg for function. (*A*) ABE demonstrates that Scrib is palmitoylated in vivo in larval tissues, but this is not detectably altered in *dlg* mutant animals. Mutating two conserved cysteines (Scrib^{C4AC11A}) does not abolish Scrib palmitoylation. Cysteine string protein (CSP) serves as a control palmitoylated protein. Non-NH_2OH–treated lanes control for biotinylation specificity to palmitoylated residues. Dotted lines in lanes 3 and 7 indicate data excluded due to an experimental error in protein loading. (*B–D*) Scrib^{C4AC11A} can fully rescue polarity loss in *scrib* mutant follicle cells, and localizes appropriately to the basolateral membrane. Compared to WT and *dlg* RNAi alone (*E–H*), membrane tethering Scrib by Morphotrap (*I* and *J*) or N-terminal myristoylation signal (*K* and *L*) in *dlg* RNAi cells does not rescue aPKC spread or Lgl mislocalization. (Scale bars, 10 μm.) All follicles are stage 6, except *B–D* and *G*, which are stage 7.

did not detect differences in PIP2 distribution or levels in *dlg* or *scrib* mutants, consistent with a recent report (*SI Appendix*, Fig. S8 *A–D*) (31, 53, 54). While there was a slight increase in cortical PIP3 levels in *scrib* and *dlg* mutants (*SI Appendix*, Fig. S8 *E–H*, quantified in *SI Appendix*, Fig. S8 *I* and *J*), this is unlikely to have an impact on Lgl, which does not exhibit physiologically relevant differences in binding preference to different PIP species (14).

An alternative mechanism by which Scrib and Dlg could ensure antagonism of apical identity is by simply promoting Lgl cortical localization. We therefore tested whether cortical localization of Lgl was sufficient to bypass loss of *scrib* or *dlg* function in follicle epithelia. However, in our hands overexpression of a constitutively membrane-tethered Lgl (myr-Lgl) did not alter polarity defects in *scrib*- or *dlg*-depleted follicle cells, nor did it cause polarity defects in WT follicle cells (*SI Appendix*, Fig. S6 *F–J*) (48). In contrast, a mutant Lgl protein with only the most C-terminal aPKC phosphorylation site present (Lgl^{S656A,S660A}, hereafter Lgl^{AAS}) was suggested to be a dominant inhibitor of aPKC (34). We confirmed that Lgl^{AAS} expression in otherwise WT follicle cells causes dominant phenotypes, including multilayering and loss of apical aPKC staining (Fig. 5*G*). We note that although Lgl^{AAS} localizes uniformly to the cortex, including the apical domain (*SI Appendix*, Fig. S6*L*), and can displace aPKC (Fig. 5*G*), it cannot establish an ectopic basolateral domain at the former apical site, as it does not recruit Scrib (Fig. 5*J*).

To determine whether Lgl^{AAS} is a bona fide aPKC inhibitor, we compared the phenotype of Lgl^{AAS}-expressing cells with *apkc* RNAi-expressing cells, using Bazooka (Baz, *Drosophila* Par-3) localization as a phenotypic readout (7, 11). Baz is an aPKC substrate, and preventing phosphorylation via *apkc* depletion or expression of nonphosphorylatable Baz results in formation of several large aggregates in the cell, visible in maximum-intensity projections of the apical surface of single follicle cells (Fig. 5*L*) (8, 9, 11). Interestingly, Lgl^{AAS} was also capable of inducing Baz aggregates in follicle and embryonic epithelia (Fig. 5*M* and *SI Appendix*, Fig. S9 *B–E*), and codepletion of *apkc* did not modify the follicle phenotype (Fig. 5 *N* and *W* and *SI Appendix*, Fig. S9*A*). Furthermore, while expression of an activated form of aPKC caused Baz to localize in a larger number of smaller, fragmented puncta, similar to the adherens junction (AJ) fragments described previously in basolateral mutants (Fig. 5 *O* and *P* vs. Fig. 5 *S* and *T*) (20, 55), coexpression of Lgl^{AAS} resulted in aggregates indistinguishable from those caused by expression of Lgl^{AAS} alone (Fig. 5 *Q*, *R*, and *W* and *SI Appendix*, Fig. S9*A*). We also directly examined the effect of Lgl^{AAS} on aPKC kinase activity by staining follicle clones with an antibody specific for S980 phosphorylated Baz (p-S980 Baz) (9). We observed loss of p-S980 Baz staining in 48.2% of Lgl^{AAS}-expressing clones, compared to 91.7% of *apkc* RNAi-expressing clones (*SI Appendix*, Fig. S9 *F* and *G*). These data are consistent with a model in which Lgl^{AAS} has enhanced aPKC-inhibiting properties compared to WT Lgl, but is not entirely equivalent to aPKC loss of function.

We then asked whether the dominant effects of Lgl^{AAS} depend on Dlg or Scrib activity. In *dlg* RNAi or *scrib* mutant cells, Lgl^{AAS} retained the ability to create several Baz aggregates, although an increased number and intermediate size suggested incomplete epistasis (Fig. 5 *S–W* and *SI Appendix*, Fig. S9*A*). Coexpression of Lgl^{AAS} also reduced the lateral expansion of aPKC seen in cells depleted of either *dlg* or *scrib* (Fig. 4 *K* and *L*

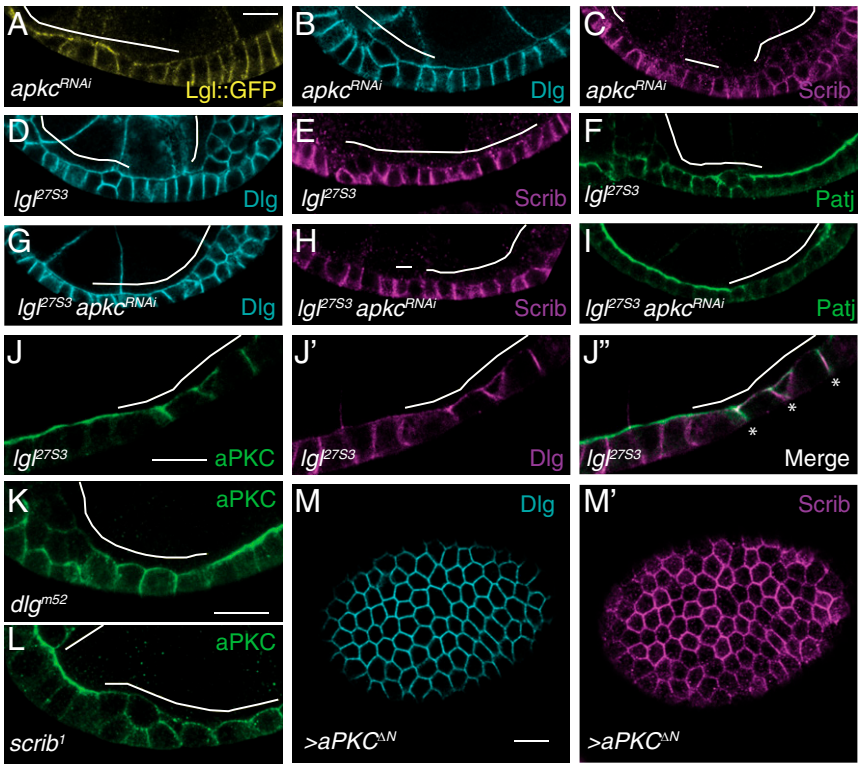

Fig. 4. Scrib and Dlg do not directly antagonize aPKC. (*A*) In *apkc*-depleted follicle cells, Lgl reaches the apical membrane, but Dlg (*B*) and Scrib (*C*) remain basolaterally localized. (*D*, *E*, *G*, and *H*) Scrib and Dlg localization is unaffected when apicobasal antagonism is eliminated by codepletion of *apkc* and *lgl*. Laterally mislocalized aPKC in *lgl* mutant cells (*F*) is active, as it recruits Patj to these sites (*I*). (*J*) aPKC spreads along the basolateral membrane in *lgl* mutant cells, where it colocalizes with Dlg (*J′* and *J′′*, asterisks). (*K* and *L*) aPKC mislocalization is also seen in *dlg* (*K*) and *scrib* (*L*) mutant cells. (*M* and *M′*) Expression of a constitutively active aPKC (aPKC$^{\Delta N}$) does not displace Scrib or Dlg. (Scale bars, 10 μm.) *B–D*, *F*, *G*, and *K* are stage 6; *A*, *E*, *H*, *I*, *L*, and *M* are stage 7; *J* is stage 8.

vs. Fig. 5 *G–I*). These results suggest that many apical-inhibiting effects of Lgl^{AAS} do not strictly depend on Scrib or Dlg.

The Entire Scrib Module Is Necessary but Not Sufficient for Basolateral Polarity. The fact that the activity of WT Lgl but not Lgl^{AAS} requires Scrib and Dlg suggests that Scrib and Dlg could enhance Lgl's ability to antagonize aPKC at the basolateral cortex, perhaps by protecting Lgl from aPKC phosphorylation. A model where both Scrib and Dlg are required would be consistent with the inability of either single protein to bypass loss of the other (Fig. 1 *R–W*). To test if ectopic apical localization of Scrib and Dlg together would therefore allow Lgl to inhibit aPKC, we used a combination of apical domain-specific nanbody tethering and overexpression to simultaneously mislocalize one, two, or all three Scrib module proteins (47). However, despite robust colocalization at the apical cell surface, no effects were seen in any case on aPKC, apicobasal polarity, or epithelial architecture (Fig. 6). We conclude that, despite the necessity for each component in basolateral domain identity, even the entire Scrib module taken together is not sufficient to inhibit apical polarity determinants.

Discussion

Despite being central regulators of cell polarity in numerous tissues from nematodes to mammals, the mechanisms of Scrib module activity have remained obscure. Our work highlights previously unappreciated specificity in these activities, and begins to define the molecular functions of Scrib, Dlg, and Lgl. Our data focus on the *Drosophila* follicle epithelium, as well as in some cases *Drosophila* embryos, but it is important to note that tissue contexts can differ in polarity programs (18, 56): For example, in the adult *Drosophila* midgut epithelium, where Scrib module proteins are dispensable for epithelial organization (29). We failed to detect phenotypic enhancement in double-mutant follicle cells, compared to single mutants, which together with the complete penetrance of single-mutant phenotypes suggest full codependence of function rather than functional overlap. Moreover, we were unable to bypass Scrib module mutants in any combination by overexpression of other genes in the module, consistent with unique roles for each protein. Thus, while Scrib, Dlg, and Lgl act in a common "basolateral polarity" pathway, they each contribute distinct functions to give rise to the basolateral domain.

Cell polarity is particularly evident at the plasma membrane, and most polarity regulators act at the cell cortex. Therefore, a key question in the field has concerned the mechanisms that allow cortical localization of the Scrib module and Par complex proteins, which exhibit no classic membrane-association domains (57). We found a simple linear hierarchy for cortical localization in the follicle that places Dlg most upstream, and contrasts with that recently described in the adult midgut, where Scrib appears to be most upstream (27, 29). Our work highlights the requirement of Dlg for Scrib localization, and provides insight into the mechanism, in part by ruling out previous models. One model involves a direct physical interaction, mediated by the Scrib PDZ domains and Dlg GUK domain (23, 24, 41). However, our in vivo analyses show that follicle cells mutant for alleles lacking either of these domains have normal polarity; these results are supported by data from imaginal discs (21, 33, 58). In contrast, we show that the SH3 domain is critical for Scrib cortical localization as well as polarity (58). The Dlg SH3 and GUK domains engage in an intramolecular "autoinhibitory" interaction that negatively regulates binding of partners, such as Gukh and CASK (40, 59–64). The dispensability of the GUK

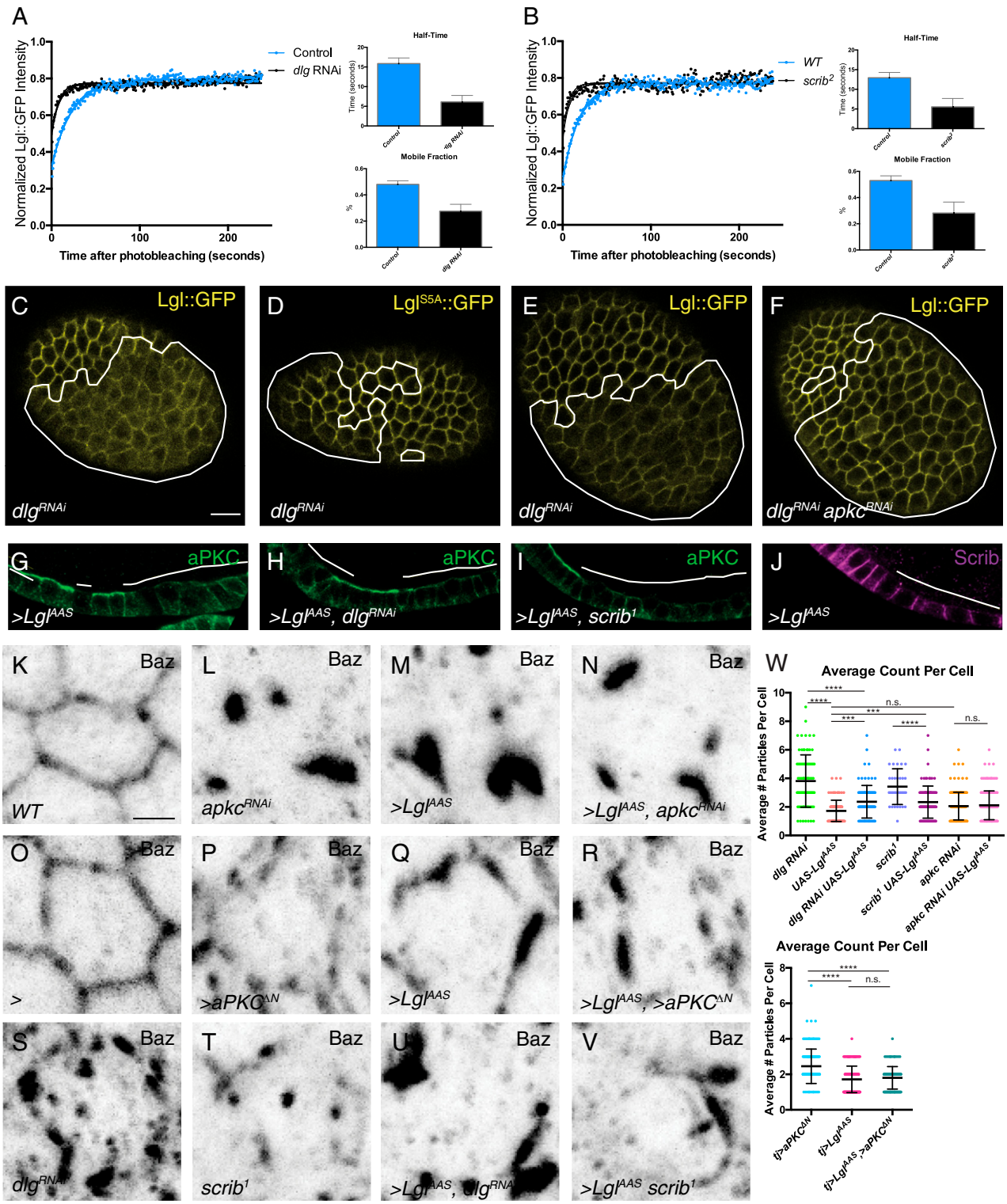

Fig. 5. Scrib and Dlg support basolateral Lgl activity. (*A* and *B*) In both *scrib* and *dlg* mutant follicle cells, Lgl::GFP shows increased FRAP kinetics compared to WT. (*C* and *D*) In *dlg* RNAi cells, Lgl::GFP is displaced to the cytoplasm but nonphosphorylatable Lgl^{S5A}::GFP remains cortical. (*E* and *F*) Codepletion of *apkc* rescues Lgl localization in *dlg*-depleted cells. (*G*) Lgl^{AAS} expression causes loss of apical aPKC. (*H* and *I*) Apical aPKC depletion by Lgl^{AAS} persists in the absence of Dlg and Scrib. (*J*) Lgl^{AAS} is not sufficient to create an ectopic basolateral membrane, as it fails to recruit Scrib apically. (*K*–*M*) Baz forms several aggregates in each Lgl^{AAS}-expressing cell, similar to *apkc*-depleted cells. (*M* and *N*) Depletion of *apkc* in Lgl^{AAS}-expressing cells does not modify the Baz phenotype. Compared to WT (*O*), expression of a constitutively active aPKC (*P*) causes a Baz phenotype similar to *dlg* (*S*) or *scrib* loss-of-function (*T*). (*Q* and *R*) Coexpression of Lgl^{AAS} causes a phenotype that resembles Lgl^{AAS} alone. In *dlg*-depleted (*S*) or *scrib* mutant (*T*) cells, Baz localizes to more frequent, fragmented puncta. Expression of Lgl^{AAS} in *dlg*-depleted cells (*U*) or *scrib* mutant cells (*V*) reduces Baz particle number. (*W*) Quantification of Baz phenotypes in *K*–*V*. One-way ANOVA with Tukey's multiple comparisons test. *K*–*V* show maximum-intensity projections centered on the nuclei of single cells. (Scale bars, 10 μm in *C*–*J* and 2 μm in *K*–*V*.) Error bars in *A* and *B* represent 95% confidence intervals; error bars in *W* represent SD. Data points in *W* are measurements from single cells. *D* and *F*–*I* are stage 7, *C* is stage 6, *E* and *J* are stage 8. n.s. (not significant), $P > 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

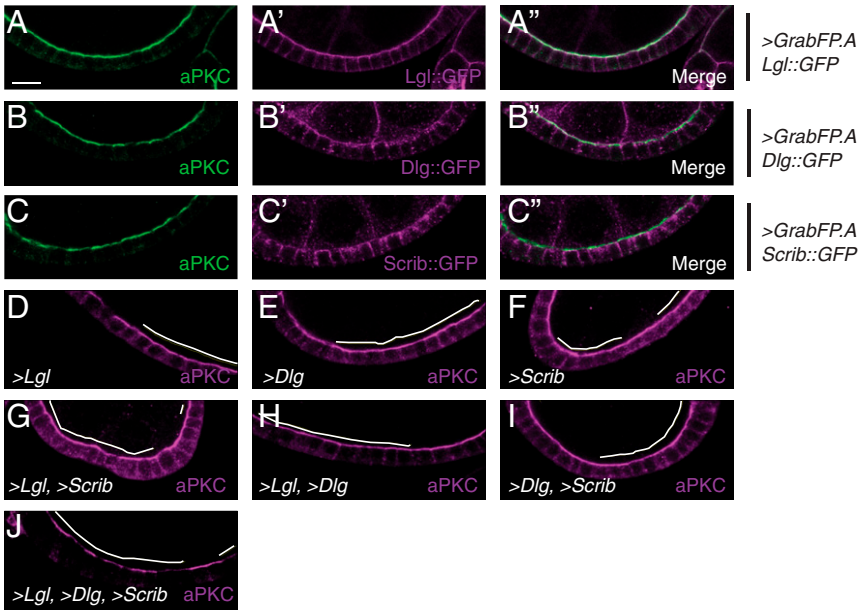

Fig. 6. The entire Scrib module is not sufficient to establish basolateral polarity. Ectopic apical localization of Lgl (*A*), Dlg (*B*), or Scrib (*C*) using apical GrabFP has no effect on epithelial polarity. WT Scrib, Dlg, and Lgl are not sufficient to disrupt the apical domain when overexpressed singly (*D–F*), in pairs (*G–I*), or as a holo-module (*J*). (Scale bars, 10 μm.) *A–C*, *F*, and *I* are stage 6; *G* is stage 5; *D*, *E*, and *J* are stage 7; *H* is stage 8.

domain provides evidence against an essential role for this mode of regulation in epithelial polarity, and highlights the value of investigating the GUK-independent function of the Dlg SH3.

We also exclude a second mechanism of Scrib cortical association. Mammalian Scrib is S-palmitoylated and this modification is required for both cortical localization and function (46). As *Drosophila* Scrib was also recently shown to be palmitoylated, an appealing model would involve Dlg regulating this post-translational modification (45). However, we could detect no changes to Scrib palmitoylation in a *dlg* mutant, and chemically or genetically inhibiting *Drosophila* palmitoyltransferases also had no effect on Scrib localization, although we cannot discount the possibility that Scrib palmitoylation may be part of a multi-part localization mechanism. Surprisingly, palmitoylated Scrib is incapable of reaching the cortex in *dlg* mutants. While a constitutively myristoylated Scrib can bypass this requirement for localization, it is nevertheless insufficient to support polarity in the absence of Dlg. These results indicate that Dlg regulates additional basolateral activities beyond localizing Scrib.

Lgl's role as an aPKC inhibitor is well-characterized, but how Scrib and Dlg influence this antagonism is not understood (12, 16, 17). Our data show that Scrib and Dlg maintain cortical Lgl by regulating its phosphorylation by aPKC, rather than by direct physical recruitment to the membrane. A contemporaneous study by Ventura et al. (31) supports this finding, further showing that the major factor in Lgl cortical stabilization is PIP2. Our data also suggest that the basolateral-promoting activities of Scrib and Dlg are not via direct inhibition of aPKC kinase activity or intrinsic antagonism of aPKC localization. Instead, they are consistent with models in which Scrib and Dlg regulate the three specific aPKC-targeted residues in Lgl. Previous work has demonstrated that these phosphorylated serines (656, 660, 664) are neither functionally nor kinetically equivalent, and a recent model proposes that S664 is required for basolateral polarization by mediating a phosphorylation-dependent interaction with the Dlg GUK domain (34, 43, 65, 66). Beyond the dispensability of the GUK domain, the enhanced ability of Lgl^{AAS} to inhibit aPKC and its ability to do so largely independently of Scrib and Dlg, argues against this model. Moreover, only Lgl^{AAS} among the phospho-mutants can dominantly affect aPKC activity, while WT Lgl can do the same only if Scrib and Dlg are present (66). Together, these results suggest that S656 is the critical inhibitory residue whose phosphorylation must be limited to enable Lgl's activity.

The mechanism by which Lgl^{AAS} can suppress even constitutively active $aPKC^{\Delta N}$ remains unclear. aPKC substrates can act as competitive inhibitors; either an increased substrate affinity for aPKC or reduced ability to be inhibited by virtue of having fewer phosphorylation sites could make Lgl^{AAS} a more effective inhibitor than WT Lgl (65, 67, 68). Supporting this idea, it was previously shown that S664, the only residue still available in Lgl^{AAS}, is phosphorylated with higher kinetic preference than S656 or S660 (65). It is also possible that some Lgl^{AAS} phenotypes may be due to aPKC-independent effects resulting from reduced phosphorylation on S656 and S660. Nevertheless, we propose a model in which Scrib and Dlg "protect" Lgl by limiting phosphorylation of S656, thus tipping the inhibitory balance to allow Lgl to inhibit aPKC and establish the basolateral domain.

What mechanism could underlie Scrib and Dlg protection of Lgl? One mechanism could involve generating a high phospholipid charge density at the basolateral membrane, which has been shown to desensitize Lgl to aPKC phosphorylation in vitro (69). However, our data do not find evidence for regulation of phosphoinositides by Scrib and Dlg. A second possibility is that Scrib and Dlg could scaffold an additional factor, such as protein phosphatase 1, which counteracts aPKC phosphorylation of Lgl (70). Alternative mechanisms include those suggested by recent work on PAR-1 and PAR-2 in *Caenorhabditis elegans* zygotes, a circuit with several parallels to the Scrib module (71–73). In this system, PAR-2 protects PAR-1 at the cortex by shielding it from aPKC phosphorylation through physical interaction-dependent and -independent mechanisms (71). By analogy, binding with Scrib or Dlg could allosterically regulate Lgl to prevent phosphorylation, although we have ruled out the Lgl–Dlg interaction documented in the literature (43). Scrib or Dlg might also act as a "decoy substrate" for aPKC, as PAR-2 does in PAR-1 protection (71). Indeed, Scrib is phosphorylated on at least 13 residues in *Drosophila* embryos, although the functional relevance of this is not yet known (74).

Overall, our work highlights the multifaceted nature of Scrib module function. The failure to bypass Scrib module mutants by transgenic supply of any single or double combination of other module components, including several that were constitutively membrane-tethered, suggests that every member contributes a specific activity to polarity. Nevertheless, even the simultaneous ectopic localization of all three Scrib module proteins was insufficient to disrupt the apical domain. This insufficiency in basolateral specification may reflect an inability of apical Scrib and Dlg to protect Lgl from aPKC phosphorylation, perhaps due to the distinct molecular composition of the apical and basolateral domains. This supports the idea that in addition to intrinsic activity via Lgl, the Scrib module must recruit or activate additional, as yet unidentified effectors in basolateral polarity establishment. The independent as well as cooperative activities of the Scrib module delineated here demonstrate previously unappreciated complexity in the determination of basolateral polarity and set the stage for future mechanistic studies of Scrib module function.

Materials and Methods

Mutant and overexpression analyses in follicle cells employed *hsFLP*, *GR1-GAL4 UAS-FLP*, or *traffic jam-GAL4. UAS-myr-Scrib::V5* was generated by appending the N-terminal myristoylation signal from Src42A and C-terminal V5 tag to the Scrib A2 cDNA, and *UASp-Scrib^{C4AC11A}::GFP* was generated via site-directed mutagenesis. ABE was performed by modifying published protocols (44, 75), using anterior L3 larval lysates; biotinylated protein was purified using magnetic beads and analyzed by Western blot. Images were acquired using Zeiss LSM700 or LSM780 laser-scanning confocal microscopes with LD C-Apochromat 40×/NA1.1 W or Plan Apochromat 63×/NA1.4 oil objectives. Image processing and quantification was performed using Fiji software (76); for significance in statistical tests: n.s. (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$. FRAP experiments were performed as previously described, and processed and analyzed using Fiji and Graphpad Prism (77). Baz particles were quantified by the Analyze Particles function and the FeatureJ plugin. Details are provided in *SI Appendix, SI Materials and Methods*.

Data Availability Statement. All data supporting the findings of the study are included in the main text and *SI Appendix*. All materials and reagents will be made available to readers upon request.

ACKNOWLEDGMENTS. We thank E. Morais de Sá, D. Bergstralh, B. Thompson, V. Budnik, T. Schupbach, R. Davis, Y. Hong, F. Matsuzaki, M. Metzstein, A. Wodarz, D. St Johnston, and I. Hariharan for generously providing fly stocks and reagents; C. Brownlee and K. Strassburger for providing technical expertise for the acyl-biotin exchange experiments; Laura Mathies for cloning the modified Scribble constructs; and G. de Vreede, M. Peifer, T. Bonello, K. Prehoda, M. Kitaoka, and the D.B. laboratory for helpful discussions. Imaging on the LSM780 was performed at the University of California, Berkeley, Cancer Research Lab Molecular Imaging Center, supported by National Science Foundation Grant DBI-1041078. This work was supported by NIH Grants GM068675, GM090150, and GM130388 (to D.B.) and American Heart Association Predoctoral Fellowship 20PRE35120150 (to M.J.K.).

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