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The Scribble module regulates retromer-dependent endocytic trafficking during epithelial polarization

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**Equal contributions

Running title: Scribble, polarity and retromer

ABSTRACT
Scribble (Scrib) module proteins are major regulators of cell polarity, but how they influence membrane traffic has been mysterious. Endocytosis is also a critical regulator of polarity through roles that remain unclear. Here we link Scrib to a specific arm of the endocytic trafficking system. Drosophila mutants that block AP-2-dependent endocytosis share many phenotypes with Scrib module mutants, but Scrib module mutants show intact internalization and endolysosomal transport. However, defective traffic of retromer pathway cargo is seen, and retromer components show strong genetic interactions with the Scrib module. The Scrib module is required for proper retromer localization to endosomes, and promotes appropriate cargo sorting into the retromer pathway, via both aPKC-dependent and –independent mechanisms. We propose that the Scrib module regulates epithelial polarity by influencing endocytic itineraries of Crumbs and other retromer-dependent cargo.

INTRODUCTION

The polarized distribution of proteins along a cellular axis is central to biological function. Foundational work has identified several multiprotein modules that act as key polarity regulators throughout both vertebrate and invertebrate animals (St Johnston and Ahringer, 2010). Polarity control must ultimately impact vesicular trafficking pathways to achieve a restricted protein distribution at the plasma membrane (PM), but how specific polarity-controlling modules influence the general process of membrane trafficking is a longstanding mystery.

Two polarity modules, called the Par and Crumbs (Crb) modules, specify the apical membrane domain. In the Drosophila Par module, Bazooka (Baz/Par-3) and Par-6 serve as scaffolding proteins that direct aPKC kinase activity to appropriate targets in response to a Cdc42-GTP-mediated cue (Goldstein and Macara, 2007). One potential target is the transmembrane protein Crumbs, which can specify apical identity via a poorly characterized but aPKC-dependent pathway (Bulgakova and Knust, 2009).

A third module, called the Scribble (Scrib) module, is a major regulator of the basolateral domain, serving there to exclude apical protein localization. In Drosophila it consists of Scrib, Discs-large (Dlg), and Lethal giant larvae (Lgl) (Yamanaka and Ohno, 2008), which are 'junctional scaffolds' that contain multiple protein-protein interaction motifs. Lgl shows reciprocal negative regulation with aPKC, but how it and other Scrib module proteins interface with the polarize membrane trafficking machinery is not known. Polarity control by the Scrib module is also required to prevent malignant overgrowth in several fly tissues (Bilder, 2004), leading Scrib module genes to be described as 'neoplastic' tumor suppressor genes (TSGs). Evidence suggests conservation of a tumor suppressive role in mammals (Martin-Belmonte and Perez-Moreno, 2011; Pearson et al., 2011) as well as an important role in influencing the Hippo pathway (Cordenonsi et al., 2011). There is thus high current interest in understanding the fundamental activity of the Scrib module.

An intriguing hint comes from recent work revealing that certain canonical regulators of endocytic trafficking also act as fly neoplastic TSGs (reviewed in Shivas et al., 2010). For instance, loss of Rab5 or ESCRT components results in disorganized overgrowth of imaginal epithelia; mutations that disrupt subsequent stages of endocytic trafficking do not. Rab5 and ESCRT also regulate apical polarity in mammalian epithelia (Dukes et al., 2011; Zeigerer et al., 2012), while Par mutations in several systems can cause defects in cargo internalization and endolysosomal traffic (reviewed in Shivas et al., 2010). Here we investigate the hypothesis that Scrib mediates polarity through influencing endocytic itineraries. We show that the Scrib module regulates retromer-dependent sorting events that can return internalized cargo to the cell surface, thereby linking this conserved polarity-regulating module to a specific, bona fide vesicle trafficking pathway.

MATERIALS AND METHODS

For stocks and antibodies used, see **Supplementary Information**.

Genetics

Mutant eye discs and follicle cell clones were generated as in (Lu and Bilder, 2005). Follicle cell knockdown employed *Traffic jam-Gal4* (Tanentzapf et al., 2007) to drive expression of RNAi stocks except Fig. 4D-G, which used *hsFLP; act>STOP>GAL4 UASGFP* with 5' induction.

Microscopy

All fluorescent images are single confocal sections acquired on a Leica TCS or Zeiss 700 confocal microscope using 10×/NA 0.3 or 40×/NA 1.25 oil lenses. Follicle sections at stages 6-7 are taken at the equator; eye and wing disc sections are taken below the peripodium. Note that mispolarized disc cells do not show a clear apical domain; images presented are representative cross-sections.

Quantitation and Disc Culture

For cortical association analysis, pixel intensity profiles were generated in Fiji (Schindelin et al., 2012). Correlation coefficients between transmembrane protein immunoreactivity and F-actin were calculated to determine the degree of cortical association. For particle analysis of endocytic markers, Ilastik software (Sommer et al., 2011) was used for thresholding, segmentation and generating binary images. Particle size and number were then quantified using Analyse Particles in Fiji. For lysosomal inhibition, discs were cultured for 5 hours in Drosophila cell medium (M3, Sigma) containing 200um Leupeptin and 50mM NH_4Cl.

RESULTS AND DISCUSSION

AP-2-dependent endocytosis is required for epithelial organization and proliferation control

We recently reported the isolation, in Drosophila, of null mutations in genes encoding regulators of cargo internalization from the cell surface. These genes include subunits of the AP-2 adaptor complex, the Dynamin ortholog Shibire, and the Clathrin heavy chain (Chc). When imaginal discs consist predominantly of cells mutant for these genes, the tissues are severely disorganized and show upregulation of the pro-invasive JNK target Matrix Metalloprotease 1 (Mmp1) (Windler and Bilder, 2010). Mutant eye discs are also larger than their WT counterparts, lose neuronal differentiation, and display disrupted cell shapes and an absence of epithelial monolayering **(Figs. 1A-D; S1A-D)**. Mutant clones in the follicle epithelium of the adult ovary also lose epithelial organization **(Fig. S1I-L)**. These phenotypes confirm that *AP-2* subunits, *shibire*, and *chc* act as neoplastic TSGs (Windler and Bilder, 2010).

Similar cortical polarity defects in endocytic and Scrib module mutant cells

The neoplastic phenotypes of these endocytic mutants prompted an analysis of PM polarity. We first assessed polarization of proteins that are peripherally associated with the cell cortex. The apical markers aPKC and Par-6 and the basolateral marker Dlg are found in separate but contiguous domains in all WT epithelial cells (**Figs. 1E, I**). In *AP-2* mutant follicle cells, aPKC is mislocalized around the cortex **(Fig. 1J)**, in some regions interspersed with Dlg while in other regions overlapping in a manner never seen in WT. This phenotype reflects a severe perturbation of apicobasal polarity. A similar distribution is seen in follicle cells mutant for *lgl* **(Fig. 1K)** and with Par-6 in imaginal discs mutant for *dlg* and *scrib* **(Fig. 1F)**. Here and below, follicle cells and imaginal discs behave similarly, and *dlg, lgl* and *scrib* genotypes were indistinguishable (data not shown). Interestingly, when we compared the Scrib module mutant and *AP-2* mutant discs with cortical markers, no consistent differences could be detected between them. These results demonstrate that proper restriction of the apical membrane domain requires AP-2-dependent endocytosis, and further suggest that polarization of the cell cortex might be controlled in a related manner by both the endocytic and Scrib module classes of polarity regulators.

The Scrib module regulates Crb trafficking after endocytic internalization

Because endocytosis acts primarily on integral membrane proteins, we then analyzed Crb and Neuroglian (Nrg), transmembrane proteins which are restricted to apical and basolateral domains respectively in WT cells (**Figs. 1G,L**). In *AP-2* cells, Crb is found in a fragmented distribution around the PM (**Fig. 1M**), with regions of overlap as well as complementary distribution with Nrg. Mislocalization, exclusion and overlap between Crb and Nrg are also seen in *lgl* cells. Strikingly, while Nrg was exclusively PM-localized and indistinguishable between the two genotypes, Crb showed significantly reduced PM localization in *lgl* as compared to *AP-2* mutant tissue, accompanied by a hazy, sub-cortical distribution (**Fig. 1N, P, R**). Subcortical Crb, distinct from peripheral apical markers, was also seen in *dlg* imaginal discs (**Figs. 1F, H, 2D**). This indicates a specific difference in Scrib module mutants that along other

parameters are phenotypically similar to *AP-2* mutants. The subcortical distribution of Crb could result from defects in exocytic delivery to the PM, or alternatively from defects in endocytic traffic. To distinguish between these possibilities, we examined cells depleted simultaneously of *AP-2* and *lgl*. In contrast to *lgl*-depleted cells, these cells show levels of Crb cortical association comparable to cells depleted of *AP-2* alone (**Fig. 1O-R**). This epistasis suggests that, while AP-2 is required for Crb internalization, Scrib module mutants are defective in post-internalization trafficking of Crb.

Scrib module mutants do not alter AP-2 dependent internalization or lysosomal trafficking

The evidence for endocytic trafficking defects in Scrib module mutant cells, as well as the polarity phenotypes shared with endocytic mutant cells, raised the possibility that the Scrib module controls epithelial polarity by regulating general endocytic traffic. We directly analyzed endocytosis using the cargo Notch in imaginal discs. In WT discs, Notch is internalized in an AP-2 dependent manner and degraded after 5 hours (**Fig. S2A**) (Lu and Bilder, 2005). This process is intact in discs mutant for *dlg, scrib,* or *lgl*: Notch is internalized into endocytic puncta and transported for lysosomal degradation **(Fig. S2B-D)**, in contrast to discs mutant for *AP-2* (**Fig. S2F**) or *Rab5* (Lu and Bilder, 2005). We found no evidence of a decreased rate of endocytosis (**Fig. S2G-I**), and the endocytic tracer Dextran is also internalized and degraded readily in *scrib* discs (**Fig. S2K**). Because Notch internalization and degradation, like epithelial polarity and proliferation control, require AP-2 function (Windler and Bilder, 2010), we conclude that the Scrib module does not regulate polarity via general control of AP-2 dependent internalization or endolysosomal traffic.

Altered trafficking of retromer cargo in Scrib module mutants

To reconcile the evidence for altered endocytic traffic of Crb (**Fig. 1**) with the normal degradative traffic of Notch (**Fig. S2**) in Scrib module mutant cells, we considered whether these cells might be defective in a distinct post-internalization route that Crb follows. An alternative to endolysosomal transport is traffic through the retromer-dependent pathway that takes cargo from endosomes to Golgi. Crb has been recently found to transit this pathway, which promotes its recycling to the PM (Pocha et al., 2011; Zhou et al., 2011), as the retromer does with other cargo (Grant and Donaldson, 2009; Johannes and Popoff, 2008). Strikingly, while the canonical retromer cargo Wntless (Wls) (Eaton, 2008) expressed in WT discs is found at steady-state at the PM, in *dlg* discs Wls shows an additional, substantial subcortical distribution (**Figs. 2A, B, K**). By contrast, endogenous FasIII and E-cadherin, as well as transgenic CD8 and several other transmembrane proteins remain PM-associated in *dlg* discs as in WT (**Figs. 2E-H, K**), demonstrating that subcortical trapping is seen only with specific cargo, is not due to general exocytic defects, and is not an artifact of overexpression. Altered localization of Wls resembled that of Crb (**Figs. 2C, D, K**), which colocalized poorly with any vesicular markers examined. In addition to subcortical trapping, treatment with lysosomal inhibitors revealed increased vesicular accumulation of both Wls and Crb, but not Ecad, specifically in *dlg* tissue (**Figs. 2L-Q**). The demonstration that Wls, like Crb,

is defectively trafficked in *dlg* discs suggests that the Scrib module is required for proper sorting into and/or transit of endocytic cargo through the retromer pathway.

Disrupting retromer trafficking enhances Scrib module phenotypes

If endocytic sorting into the retromer pathway is functionally involved in polarization by the Scrib module, then genes regulating the two processes should genetically interact. Mild knockdown of *lgl* via transgenic RNAi in the dorsal compartment of the wing disc leads to ventral curving of the adult wing, which presents as a ruffle when the wing is mounted flat (**Fig. 3A,B)**. This phenotype is enhanced when flies are heterozygous for *scrib* (**Fig. 3C)**, but not *shibire* or *AP-2* subunits (**Fig. 3D**), demonstrating that it represents a specifically sensitized background. Strikingly, mild knockdown of the retromer subunits *vps35* and *vps26,* which have little to no effect on WT wings (**Fig. 3E, F**), dramatically enhanced mild *lgl* knockdown, resulting in a lethal 'giant larvae' phenotype with mispolarized and tumorous discs for the latter (100%, n=43, **Figs. 3G, H)**. These genetic interactions are consistent with a model in which Scrib module proteins regulate polarity via influencing endocytic sorting into retromer pathways.

The Scrib module influences retromer-dependent sorting

We sought further evidence for such a role by examining vesicular trafficking compartments. Antibodies and tagged transgenes showed that, although polarized distribution of all compartments is lost in *dlg* discs as expected, the overall morphology of compartments along the exocytic and endolysosomal routes are similar to those in WT discs (**Fig. S3**). A marker for the recycling endosome, Rab11, is also not obviously changed. In contrast, *dlg* tissue shows clear alterations of two markers associated with retromer sorting compartments: Rab9 and Vps29 (Burgess et al., 2012; Dong et al., 2013). The restricted and punctate localization of these markers seen in WT is replaced by widespread and diffuse staining in mutant cells (**Fig. 3I-N**). Vps29 and Rab9 colcoalize with endosomal and Golgi markers in WT cells (Burgess et al., 2012; Dong et al., 2013), but as these compartments are unaltered in *dlg* tissue (**Fig. S3**), the data suggest that the Scrib module specifically controls the enrichment of retromer at sites of endocytic sorting.

We further investigated the relationship between sorting regulation by retromer and the Scrib module by carrying out double-depletion experiments. Wls traffics via retromer (Eaton, 2008), and strong RNAi-mediated knockdown of the retromer component *vps26* in otherwise WT cells reduces steady-state PM levels (**Fig. 3O, P**). When compared to *dlg* knockdown alone **(Fig. 3Q)**, simultaneous depletion of *vps26* with *dlg* prevents Wls from reaching both the PM and the subcortical distributions, and Wls is found instead in endosomal puncta (**Fig. 3R**). These data, showing that that the *dlg* trafficking phenotype requires retromer activity, are consistent with the genetic interactions and suggest that the Scrib module normally regulates trafficking via retromer.

aPKC-dependent and –independent trafficking regulation by the Scrib module

The above data, indicating specific and functionally relevant retromer defects in Scrib module mutant cells, raise the question of exactly which cargo are mistrafficked to alter apicobasal polarity. Crb is mistrafficked in Scrib module mutant cells (**Figs. 1H, 1N, 2D)** and basolaterally mislocalized when endosomal entry is blocked (Lu and Bilder, 2005). Mislocalization of Crb is also sufficient to specify apical character on PMs (Wodarz et al., 1995) and induce neoplastic growth (Lu and Bilder, 2005). We tested whether Crb was the single relevant cargo by completely removing it from Scrib module mutant cells using a null allele (see Supplemental Methods). However, discs and follicle cells completely lacking Crb and the Scrib module remained mispolarized and neoplastic; a similar result was found when Crb was removed from endocytosis-defective discs **(Fig. S1Q-X)**. These data rule out Crb as the sole polarity-regulating cargo that requires Scrib module-dependent trafficking.

We considered whether other apical regulators might be controlled by Scrib-influenced trafficking. Par-3, Par-6 and aPKC remained at the PM in Scrib module mutant cells, and, unlike Crb (Moberg et al., 2005), they were not trapped in endocytic compartments in ESCRT mutant cells (**Fig. 1F**, data not shown). Antibodies and a tagged transgene (Fletcher et al., 2012; Harris and Tepass, 2008) revealed a significant cytosolic population of Cdc42 in WT cells, preventing an assessment of altered distribution in Scrib module mutant cells. We then asked whether Par module activity was involved in Scrib-mediated trafficking. To test sufficiency, we expressed an activated form of aPKC and found that it induces subcortical trapping of Crb and Wls in imaginal discs **(Fig. 2I, J**). To test necessity, we analyzed mutant follicle cells in which both the Par and Scrib modules are inactivated. When both modules are mutated or depleted by RNAi, the subcortical haze of Crb is eliminated (**Fig. 4A-F**), and cells almost completely lack an apical domain (**Fig. G-I**). However, double mutant and depleted cells do not show the extensive degradation of Crb seen when the Par module alone is inactivated (**Fig. 4A, D**). Instead, Crb accumulates in frequent internal puncta, and some residual PM localization is evident (**Fig**. **4C, F)**. This incomplete epistasis with respect to cargo localization contrasts with the strong epistasis with respect to polarity (Bilder et al., 2003; Tanentzapf and Tepass, 2003), revealing that the Scrib module regulates endosomal trafficking in part through an aPKC-independent mechanism. However, the necessity and sufficiency experiments together indicate that excess Par module activity in Scrib module mutant cells is a major contributor to defective trafficking, suggesting that the Scrib module influences trafficking of aPKC-regulating cargo in addition to Crb.

Our data identify a specific trafficking role for the Scrib module, a core player in the conserved polarization machinery. The evidence that the Scrib module has an endocytic mechanism integrates two major pathways that control cell polarity. Our results rule out AP-2 dependent endolysosomal transport as the arm of the endocytic pathway through which Scrib controls epithelial organization. They instead identify a role in sorting cargo that pass through the retromer route. The data further indicate that this relationship is direct and specific, given the requirement of the Scrib module for retromer organization on endosomes and the strong genetic interaction seen with

retromer subunits, which stand in contrast to the lack of effect on other trafficking compartments and components.

Our data overall point to complexity in the action of the Scrib module. It is clearly not a positive regulator of retromer activity, as the depletion of PM Crb and Wls, their shunting to the lysosome, and lost apical polarity seen in retromer mutants (Pocha et al., 2011; Zhou et al., 2011) are largely opposite to the Crb misdistribution seen in Scrib module mutants. However, the Scrib module also does not simply negatively regulate retromer sorting, since reducing retromer function potently enhances Scrib module hypomorphic phenotypes, and the Scrib module null phenotype alters both retromer component localization and induces defects in retromer-dependent cargo trafficking. A recent paper describes a role for mammalian Scrib in both stabilizing the Ecad-p120 interaction and in preventing retromer sorting of lysosomally-destined Ecad (Lohia et al., 2012); however, our data, which show that Scrib module mutant cells display PM-localized Ecad, lysosomal Wls and Crb, and no evident Golgi trapping, demonstrate that a different mechanism is at work in the fly.

One possibility is that Scrib module mutants cause not a wholesale gain or block within the retromer pathway, but rather inappropriate sorting that results in a portion of cargo becoming ectopically returned to an incorrect PM domain. In addition to retromer-dependent retrograde transport and ESCRT-dependent lysosomal targeting, cargo can also exit the sorting endosome via Rab11 recycling, and Crb is known to pass through Rab11 compartments (Blankenship et al., 2007; Fletcher et al., 2012; Roeth et al., 2009); cargo could be aberrantly shunted into this route when Scrib module loss alters retromer activity. Because Rab11 also plays an important role in initial biosynthetic transport to the PM (Ang and Folsch, 2012), rendering its inhibition toxic, and Rab11-dependent recycling cargo are not well-validated in fly epithelia, we are currently unable to test this model conclusively. An activity of Scrib module proteins in influencing the sorting and subsequent destination of transcytotic cargo, which can involve retromer activity (Su et al., 2010; Verges et al., 2004), would be consistent with many of the results reported here. The Scrib module could influence sorting of transcytotic cargo by regulating cargo modifications at the basolateral surface in a manner distinct than those that occur at the apical surface (for instance, via Lgl-mediated inhibition of aPKC (Yamanaka and Ohno, 2008)). Alternatively, the requirement for proper Rab9 and Vps29 localization on endosomes point to Scrib affecting more general aspects of retromer function. Overall, a model consistent with our data is that Scrib regulates polarity by influencing sensitive sorting steps within endosomes, specifically the itinerary of apically-destined proteins that can transit the retromer pathways.

As data demonstrate that polarity regulators can influence endocytic trafficking of distinct cargo in different ways (Shivas et al., 2010), strict tests of these hypotheses must await identification of the specific polarity-regulating cargoes involved. Crb is one of these, and our data build on recent advances in understanding Crb trafficking (Fletcher et al., 2012; Pocha et al., 2011; Roeth et al., 2009; Zhou et al., 2011). However, double mutant studies show that Crb is not the sole cargo responsible for polarity control. By contrast, the Scrib module phenotypes show a strong requirement for the Par module, consistent with previous data pointing to Cdc42/Par endosomal sorting activity (reviewed in Harris and Tepass, 2010), although the data also reveal a

Par-independent role. Overall, our findings point to an additional Par-regulating cargo that undergoes AP-2 dependent, retromer-mediated recycling to specify the apical surface; identification of this cargo will open the door to defining the precise molecular mechanisms by which Scrib controls its trafficking.

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AUTHOR CONTRIBUTIONS
G. deV. J.S. and D.B. designed the research; G. deV. J.S., S.W., H.M, and H.L. performed experiments; G. deV. J.S. and D.B. analyzed the data; G. deV. J.S. and D.B. wrote the manuscript.

CONFLICT OF INTEREST
The authors declare no competing financial interests.

SUPPLEMENTARY MATERIAL is available online.

FIGURE LEGENDS

Figure 1: Comparison of Scrib module and endocytic polarity phenotypes
Compared to WT eye imaginal discs (**A**), those mutant for *AP-2α* (**B**), *chc* (**C**), or *shi* (**D**) are overgrown, disorganized and multilayered (X-Z sections; F-actin: red; nuclei: blue). Par-6 (**E**) and Crb (**G**) are apical in WT discs. In *dlg*, both are mislocalized, but Par-6 remains at the PM (**F**) while Crb also shows a hazy subcortical distribution (**H**). Separate domains of apical aPKC (magenta) and basolateral Dlg (cyan) in WT follicle cells (**I**) are lost in *AP-2* (**J**) and *lgl* (**K**) (note that Dlg staining adjacent to apical aPKC is from the germline)., Separate domains of apical Crb (magenta) and basolateral Nrg (cyan) in WT (**L**) are lost in *AP-2* (**M**) and *lgl* (**N**), but in *lgl* an additional subcortical haze of Crb is evident (arrowheads). Crb localization in follicle cells knocked down for *AP-2* (**O**), *lgl* (**P**), and *AP-2* and *lgl* (**Q**)*;* phalloidin channel marking cell cortex is not shown. (**R**) quantitates Crb cortical association. 1.0 would reflect that all TM protein immunoreactivity is cortically associated. n>=200 cells from >5 samples for each. ***=P<0.0005; P values are in **Table S1**. Scale bars: A 100 μm, E 25 μm, I 10 μm.

Figure 2: Defective traffic of retromer cargo in Scrib module mutant discs
Confocal images of wing imaginal discs; phalloidin staining is red. **A-D**: Transgenic Wls and endogenous Crb are enriched subcortically specifically in *dlg* mutant cells as compared to WT. **E-H**: Endogenous Ecad and transgenic CD8 are found primarily at the PM of both WT and *dlg* mutant cells. **I, J:** Ectopic expression of activated aPKC is sufficient to induce Crb and Wls subcortical localization **K:** Quantitation of TM protein association with the cell cortex. 1.0 would reflect that all TM protein immunoreactivity is cortically associated. ***=P<0.0005; P values are in **Table S1. L-Q**: In discs cultured with lysosomal inhibitors (LI), Ecad accumulation in *dlg* does not differ from WT, while Crb and Wls lysosomal accumulation is increased. Scale bar: 10 μm

Figure 3: Scrib module proteins control polarity via retromer
The flat WT wing (**A**) becomes curved when *lgl* is mildly reduced by RNAi in the dorsal compartment (**B**). This phenotype is enhanced by heterozygosity for *scrib* (**C**) but not AP-2 subunits (**D**). Mild knockdown of *Vps35* (**E**) or *Vps26* (**F)** have little to no effect on otherwise WT wing discs, but these strongly enhance the *lgl* mild knockdown phenotype (**G**), even leading to neoplastic transformation of wing discs and pupal death (**H**). The punctate localization of retromer-associated GTPase Rab9-YFP and retromer component Vps29-GFP in WT wing discs (**I, K**) is dispersed in *dlg* discs (**J, L**). Quantitation (**M**) reveals that large puncta of Rab9-YFP and Vps29-GFP are strongly depleted in *dlg* tissue, while Rab5-YFP puncta are much less affected. Numbers of puncta (**N**) do not significantly change. ***=P<0.0005; P values are in **Table S1**. PM levels of Wls-V5 in WT discs (**O**) are reduced when *Vps26* is knocked down (**P**). Compared to *dlg* knockdown alone (**Q**), *Vps26 dlg* double knockdown discs show reduced PM and diffuse subcortical localization and enhanced punctate trapping (**R**). Scale bars: A 400 μm, H 100 μm, I 10 μm.

Figure 4: Scrib module trafficking regulation requires Par activity

A-C: Mutating *baz* in *dlg* mutant follicle cells prevents most inappropriate localization of Crb (magenta with Nrg in cyan; A'-C' show Crb channel alone) and causes enhanced endosomal trapping. **D-F**: Knocking down *aPKC* in *dlg* knockdown cells has a similar effect. Yellow lines mark WT cells. **G-I**: Knocking down *Cdc42* in *dlg* cells causes loss of most apical PM identity (revealed by aPKC in white). Scale bar: 10 μm.

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