

RESEARCH ARTICLE

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

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ABSTRACT

Scribble (Scrib) module proteins are major regulators of cell polarity, but how they influence membrane traffic is not known. Endocytosis is also a key 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.

KEY WORDS: *Drosophila*, Endocytosis, Epithelia, Polarity, Retromer

INTRODUCTION

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

Two polarity modules, called the Par and Crumbs (Crb) modules, specify the apical membrane domain. In the *Drosophila* Par module, Bazooka (Baz; also known as 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 Crb, which can specify apical identity via a poorly characterized aPKC-dependent pathway (Bulgakova and Knust, 2009).

A third module, called the Scribble (Scrib) module, is a major regulator of the basolateral domain, where it serves to exclude apical protein localization. In *Drosophila* this module 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 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 the 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). Currently there is thus much 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 by Shivas et al., 2010). For instance, loss of Rab5 or endosomal sorting complex required for transport (ESCRT) components results in disorganized overgrowth of imaginal epithelia, whereas 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 by 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.

RESULTS

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

We recently reported the isolation of null mutations in *Drosophila* genes encoding regulators of endocytosis from the cell surface. These include subunits of the AP-2 adaptor complex, the Dynamin ortholog Shibire (Shi) 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 Matrix metalloprotease 1 (Mmp1) (Windler and Bilder, 2010). Mutant eye discs are also larger than their wild-type (WT) counterparts, lose neuronal differentiation and epithelial monolayering, and display disrupted cell shapes (Fig. 1A-D; supplementary material Fig. S1A-D). Mutant clones in the adult follicle epithelium also lose epithelial organization (supplementary material Fig. S1I-L). These phenotypes confirm that AP-2 subunits, shi and Chc act as neoplastic TSGs (Windler and Bilder, 2010).

Similar cortical polarity defects in endocytic and Scrib module mutant cells

We analyzed PM polarity in these endocytic mutants, first assessing 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 WT epithelial cells (Fig. 1E,I). In AP-2 follicle cells, aPKC is mislocalized around the

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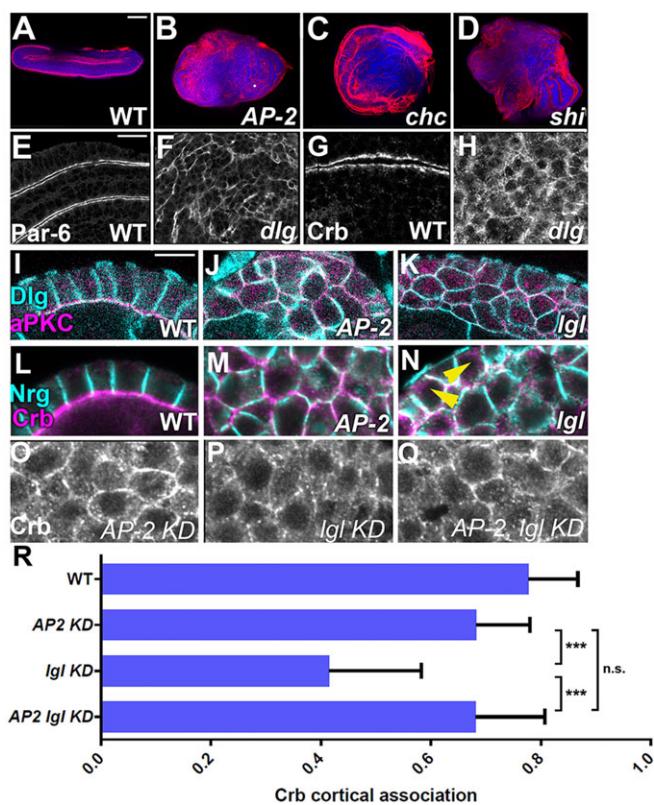


Fig. 1. Comparison of Scrib module and endocytic polarity phenotypes. Compared with WT (A), eye discs mutant for *AP-2α* (B), *Chc* (C) or *shi* (D) are overgrown, disorganized and multilayered [x-z sections; F-actin, red; nuclei, blue (DAPI)]. *Par-6* (E) and *Crb* (G) are apical in WT. In *dlg*, both are mislocalized, but *Par-6* remains at the plasma membrane (PM) (F) whereas *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). Separate domains of apical *Crb* (magenta) and basolateral *Nrg* (cyan) in WT (L) are lost in *AP-2* (M) and *lgl* (N), but *lgl* displays a subcortical haze of *Crb* (arrowheads). *Crb* localization is shown in follicle cells knocked down for *AP-2* (O), *lgl* (P) and for *AP-2* and *lgl* (Q). (R) Quantitation of *Crb* cortical association; 1.0 reflects full association. $n \geq 200$ cells from at least five samples for each. *** $P < 0.001$; n.s., not significant; individual P-values are given in supplementary material Table S1. Error bars indicate s.d. Scale bars: 100 μm in A; 25 μm in E; 10 μm in I.

cortex (Fig. 1J), both interspersed and overlapping with Dlg. This phenotype reflects a severe perturbation of apicobasal polarity. A similar distribution is seen in *lgl* follicle cells (Fig. 1K) and with *Par-6* in *dlg* and *scrib* imaginal discs (Fig. 1F). Follicle cells and discs behave similarly, and *dlg*, *lgl* and *scrib* genotypes were indistinguishable (data not shown). When we compared Scrib module and *AP-2* mutant discs with cortical markers, no consistent differences were detected. 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 similarly by both the endocytic regulators and the Scrib module.

The Scrib module regulates *Crb* trafficking after endocytic internalization

Because endocytosis acts primarily on integral membrane proteins, we then analyzed the transmembrane proteins *Crb* and *Neurogian* (*Nrg*), which are restricted to apical and basolateral domains, respectively, in WT cells (Fig. 1G,L). In *AP-2* cells, *Crb* shows 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, whereas *Nrg* was exclusively PM localized and indistinguishable between the two genotypes, *Crb* showed significantly reduced PM localization in *lgl* as compared with *AP-2* tissue, accompanied by a hazy, subcortical distribution (Fig. 1N,P,R). Subcortical *Crb* was also seen in *dlg* discs (Fig. 1H and Fig. 2D). Therefore, while Scrib module and *AP-2* mutants phenocopy each other in most respects, they show a specific difference in *Crb* subcellular localization.

Subcortical *Crb* could result from defects in exocytic delivery to the PM, or 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 dual depleted cells show levels of *Crb* cortical association comparable to cells depleted of *AP-2* alone (Fig. 1O-R). The epistasis suggests that, whereas *AP-2* is required for *Crb* internalization, Scrib module mutants are defective in post-internalization trafficking of *Crb*.

Scrib module mutations 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 WT discs, Notch is internalized by *AP-2* and degraded after 5 h (supplementary material Fig. S2A) (Lu and Bilder, 2005). This process is intact in discs mutant for *dlg*, *scrib* or *lgl* (supplementary material Fig. S2B-D), in contrast to discs mutant for *AP-2* (supplementary material Fig. S2F) or *Rab5* (Lu and Bilder, 2005). We found no evidence of a decreased rate of endocytosis (supplementary material Fig. S2G-I), and the endocytic tracer Dextran was also internalized and degraded (supplementary material Fig. S2K). Because Notch internalization and degradation, like epithelial polarity and proliferation control, require *AP-2* (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 altered endocytic traffic of *Crb* (Fig. 1) with the normal degradative traffic of Notch (supplementary material Fig. S2) in Scrib module mutant cells, we considered whether these cells might be defective in a distinct post-internalization route. An alternative to endolysosomal transport is traffic through the retromer pathway from endosomes to Golgi. *Crb* transits 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, whereas the canonical retromer cargo Wntless (Wls) (Eaton, 2008) is found at steady state at the PM of WT discs, in *dlg* discs it shows an additional, substantial subcortical distribution (Fig. 2A,B,K). By contrast, E-cadherin (Ecad; Shotgun – FlyBase) and transgenic CD8, as well as other transmembrane proteins, remain PM associated in *dlg* discs as in WT (Fig. 2E-H,K; supplementary material Fig. S3), 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* (Fig. 2C,D,K), which colocalized poorly with the vesicular markers examined (supplementary material Fig. S4). Moreover, treatment with lysosomal inhibitors revealed increased lysosomal accumulation of both Wls and *Crb*,

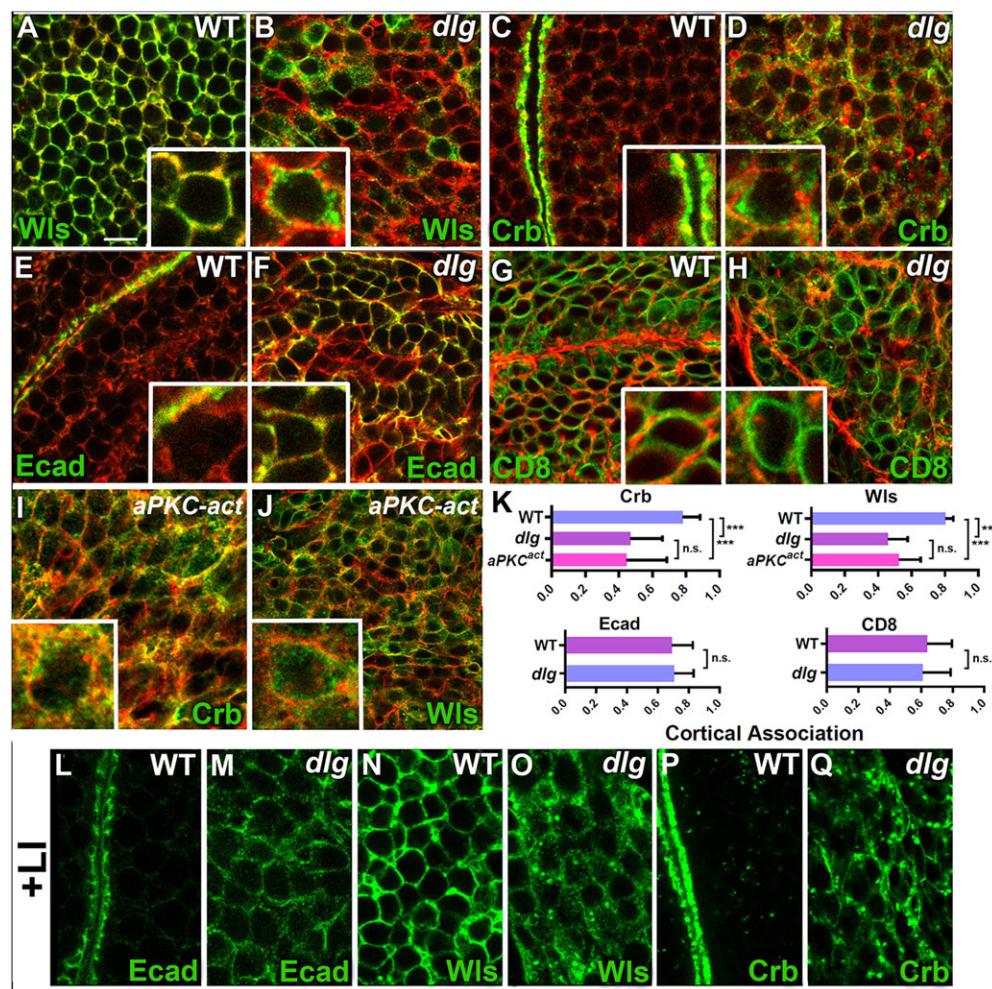


Fig. 2. Defective traffic of retromer cargo in Scrib module mutant discs. (A–J) Wing imaginal discs were stained with phalloidin (red). (A–D) Transgenic WIs and endogenous Crb are enriched subcortically in *drg* but not WT. (E–H) Endogenous Ecad and transgenic CD8 localize to both WT and *drg* PMs. (I,J) Activated aPKC is sufficient to induce Crb and WIs subcortical localization. (K) Quantitation of cortical association; 1.0 reflects full association. *** $P < 0.001$; individual P -values are given in supplementary material Table S1. Error bars indicate s.d., $n \geq 200$ cells from at least five samples for each. (L–Q) In discs cultured with lysosomal inhibitors (Li), Ecad accumulation in *drg* does not differ from WT, whereas the accumulation of Crb and WIs is increased. Scale bar: 10 μ m.

but not Ecad, specifically in *drg* tissue (Fig. 2L–Q; supplementary material Fig. S5). The demonstration that WIs, like Crb, is defectively trafficked in *drg* 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* in the dorsal wing disc leads to ruffling of the adult wing (Fig. 3A,B). This phenotype is enhanced when flies are heterozygous for *scrib* (Fig. 3C), but not *shi* 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 effect on WT wings (Fig. 3E,F), dramatically enhanced the effect of mild *lgl* knockdown, resulting in a lethal ‘giant larvae’ phenotype with mispolarized and tumorous discs when *Vps26* and mild *lgl* knockdown are combined (100%, $n=43$; Fig. 3G,H). These genetic interactions are consistent with a model in which Scrib module proteins regulate polarity by influencing endocytic sorting into retromer pathways.

The Scrib module influences retromer-dependent sorting

We sought further evidence for Scrib module involvement by examining vesicular trafficking compartments. Antibodies and

tagged transgenes showed that, although polarized distribution is lost, the overall morphology of exocytic and endolysosomal compartments in *drg* discs is similar to WT (supplementary material Fig. S6). A marker for the recycling endosome, Rab11, is also not obviously changed. By contrast, *drg* 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 *drg* mutant cells (Fig. 3I–N). Vps29 and Rab9 colocalize with endosomal and Golgi markers in WT cells (Burgess et al., 2012; Dong et al., 2013), but as these compartments are unaltered in *drg* tissue (supplementary material Fig. S6) the data suggest that the Scrib module specifically controls the enrichment of retromer at sites of endocytic sorting.

We further investigated the relationship between the regulation of retromer sorting and the Scrib module by carrying out double-depletion experiments. WIs traffics via retromer (Eaton, 2008), and strong RNAi-mediated knockdown of *Vps26* in otherwise WT cells reduces steady-state PM levels (Fig. 3O,P). When compared with *drg* knockdown alone (Fig. 3Q), simultaneous depletion of *Vps26* with *drg* prevents WIs from reaching the PM and achieving a subcortical distribution, and WIs is found instead in endosomal puncta (Fig. 3R). These data, showing that the *drg* trafficking phenotype requires retromer activity, are consistent with the genetic interactions uncovered above and suggest that the Scrib module normally regulates trafficking via retromer.

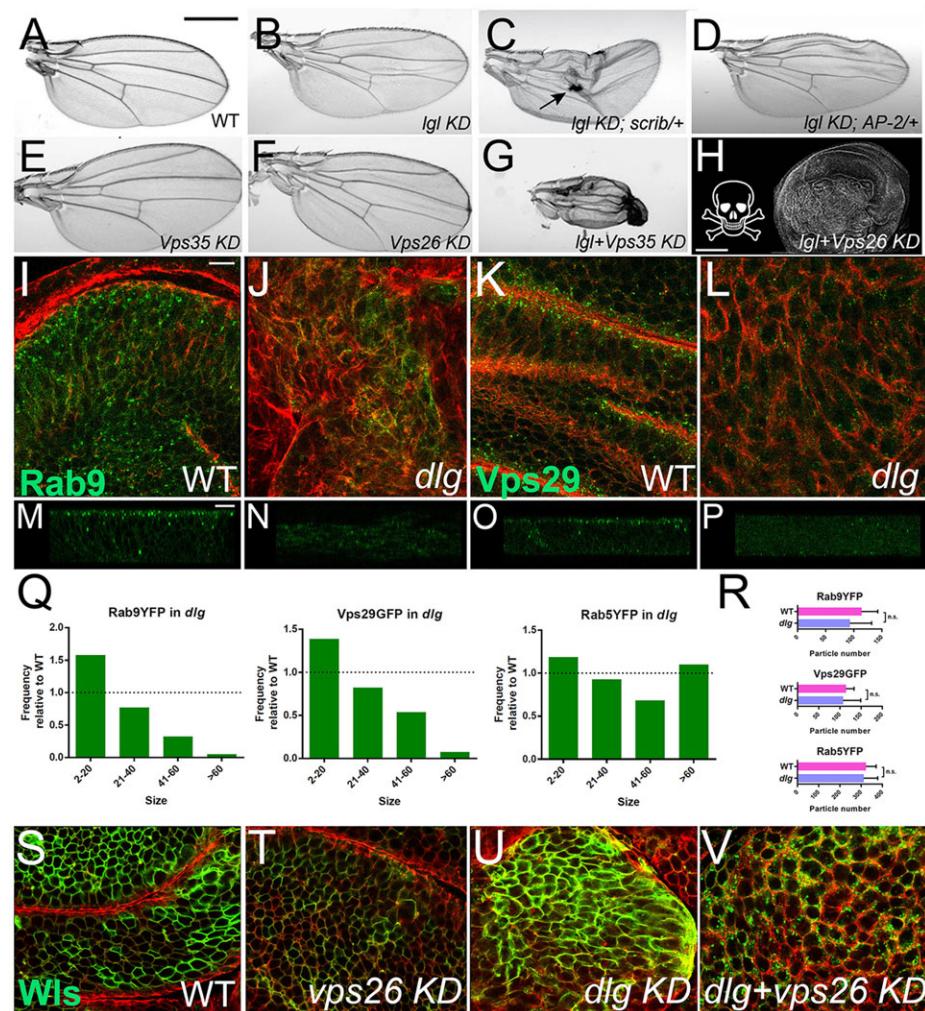


Fig. 3. Scrib module proteins control polarity via retromer. The WT wing (A) wrinkles upon mild *lgf* knockdown in the dorsal compartment (B). Heterozygosity for *scrib* (C) but not *AP-2* (D) enhances this phenotype. Mild *Vps35* (E) or *Vps26* (F) knockdown has little effect alone, but strongly enhances mild *lgf* knockdown (G), including neoplastic transformation and pupal death (H). WT punctate localization of *Rab9*-YFP and *Vps29*-GFP (I,K) is dispersed in *dlg* discs (J,L). Quantitation (M) reveals that large *Rab9*-YFP and *Vps29*-GFP puncta are strongly depleted, whereas *Rab5*-YFP puncta are much less affected. Puncta numbers (N) do not change significantly. *P*-values are given in supplementary material Table S1. Error bars indicate s.d. $n \geq 200$ cells from at least five samples for each. PM levels of *Wls*-V5 in WT (O) are reduced upon *Vps26* knockdown (P). Compared with *dlg* knockdown alone (Q), *Vps26* *dlg* double knockdown shows reduced cortical and subcortical localization and enhanced punctate trapping (R) of *Wls*-V5. (I-L,O-R) Phalloidin staining is in red. Scale bars: 400 μ m in A; 100 μ m in H; 10 μ m in I.

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

The above data indicating specific and functionally relevant retromer defects raise the question of exactly which cargo is mistrafficked to alter apicobasal polarity. Crb is mistrafficked in Scrib module mutant cells (Fig. 1H,N and Fig. 2D) and is 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 to 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. However, discs and follicle cells completely lacking Crb and the Scrib module, or Crb and an endocytic regulator, remained mispolarized and neoplastic (supplementary material Fig. S1Q-X) (Leong et al., 2009). 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. Baz, 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 mutants. 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. In these cells, the subcortical haze of Crb is eliminated (Fig. 4A-F) and cells almost completely lack an apical domain (Fig. 4G-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 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.

DISCUSSION

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 and instead identify a role for the

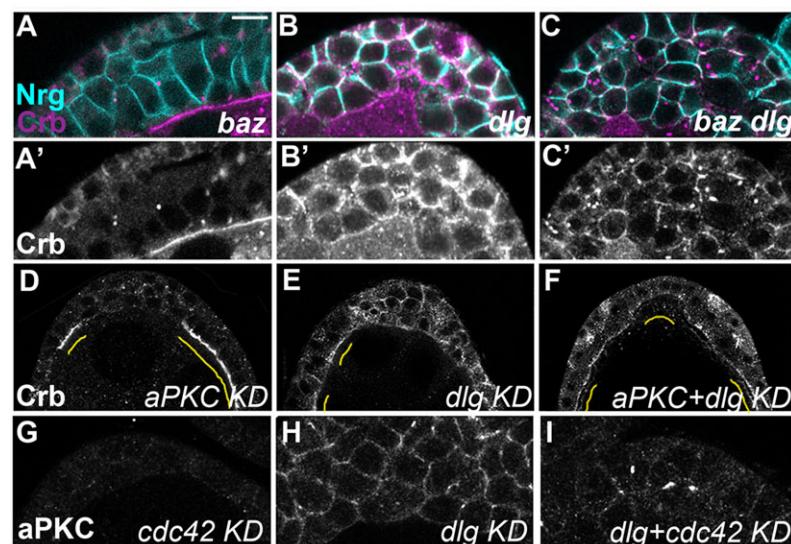


Fig. 4. Scrib module trafficking regulation requires Par activity. (A–C') Mutating *baz* in *dlg* mutant follicle cells largely prevents the inappropriate localization of Crb (magenta, with Nrg in cyan; A'–C' show the Crb channel alone) and causes enhanced endosomal trapping. (D–F) Knocking down *aPKC* 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 (as revealed by *aPKC* in white). Scale bar: 10 μ m.

Scrib module in sorting cargo that passes through the retromer. 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.

Our data 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 the loss of 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 does not simply negatively regulate retromer sorting, since reducing retromer function potently enhances Scrib module hypomorphic phenotypes, and the Scrib module null phenotype induces defects in retromer component localization and retromer-dependent trafficking. A recent paper describes a role for mammalian Scrib in 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 neither a wholesale gain nor loss of retromer activity, but rather inappropriate sorting that results in cargo ectopically returning 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 is also involved in biosynthetic transport (Ang and Fölsch, 2012), rendering its inhibition toxic, and Rab11-dependent recycling cargoes are not well-validated in fly epithelia, we are currently unable to test this model. 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; Vergés et al., 2004), would be consistent with many of the results reported here. The Scrib module could influence transcytotic sorting by regulating cargo modifications at the basolateral surface in a manner distinct from 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 points 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, studies of double mutants show that Crb is not the sole cargo responsible for polarity control. The Scrib module phenotypes show a strong requirement for the Par module, consistent with previous data pointing to Cdc42/Par endosomal sorting activity (reviewed by 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; the identification of this cargo will open the door to defining the precise molecular mechanisms by which Scrib controls its trafficking.

MATERIALS AND METHODS

Fly stocks and genetics

Mutant eye discs and follicle cell clones were generated as described (Lu and Bilder, 2005). Follicle cell knockdown employed *traffic jam-Gal4* (Tanentzapf et al., 2007) to drive expression of RNAi stocks, except for Fig. 4D–G, which used *hsFLP; act>STOP>GAL4 UASGFP* with 5' induction. WT control flies were *w* or isogenized *FRT* stocks. Owing to the similar mutant phenotypes of AP-2 complex subunits, representative experiments carried out with the *AP-2 α^{40-31}* allele (Windler and Bilder, 2010) are labeled *AP-2*. Additional alleles used include *shi^{FL54}*, *Chc³*, *lgl⁴*, *baz^{XII06}*, *fabI²¹*, *Vps25^A*, *scrib¹*, *dlg^{40.2}*, *Vps45^{GG11}*, *crb^{11A22}*. Other transgenes included *tub-Rab5-YFP*, *tub-Rab7-YFP*, *tub-Vps29-GFP*, *en-Gal4*, *Ms1096-Gal4*, *UAS-CD8-GFP*, *UAS-aPKC^{WTCAA}*, *UAS-wls-V5*, *UAS-Rab-YFP*, *UAS-Vps26-myc*, *UAS-cdc42-V5*. RNAi constructs were created by the Transgenic RNAi Resource Project (TRiP) (*Cdc42-IR*, *Vps26-IR*), Vienna Drosophila Resource Center (VDRC) (*AP-2a-IR* 15566, *dlg-IR* 41134), the D.B. lab (*lgl-IR* 'weak') or provided by X. Lin (Cincinnati Children's Hospital, OH, USA) [*Vps35-IR* (III vp2), *Vps26-IR* 'mild']. Mutant eye discs were generated as described (Menut et al., 2007).

Descriptions of *Drosophila* stocks can be found on FlyBase. The *crb* coding region was PCR amplified and sequenced from heterozygous *crb*^{11A22} adults; on the mutant chromosome, the nucleotide change C902T replaces amino acid Q950 within the EGF repeats to create a stop codon.

Immunohistochemistry and microscopy

Ovaries and L3 larvae were dissected in PBS, fixed in 4% formaldehyde in PBS for 20 min at room temperature, and stained using standard procedures (Bilder and Perrimon, 2000). The following primary antibodies were used: rat anti-Elav (7E8A10, 1:50), mouse anti-Notch^{ECD} (C458.2H, 1:25), mouse anti-Dlg (4F3, 1:100), rat anti-Ecad (DECAD2, 1:25), mouse anti-MMP1 (5H7B11, 3B8D12 and 3A6B4, 1:100) (all obtained from Developmental Studies Hybridoma Bank), rabbit anti-PKC ζ (1:200; sc216, Santa Cruz Biotechnology), rat anti-Crb (1:1000; U. Tepass, University of Toronto, Canada and E. Knust, MPI-CBG, Dresden, Germany), rabbit anti-Cdc42 (1:1000; U. Tepass), mouse anti-Nrg (1:200; 1B7, M. Hortsch, University of Michigan, Ann Arbor, USA), rabbit anti-Lva (J. Sisson, UT Austin, TX, USA), guinea pig anti-Scrib (1:500). TRITC-phalloidin (1:500, Sigma) was used to visualize the cell cortex. Secondary antibodies (1:250) were from Molecular Probes. Fluorescent images are confocal sections acquired on a Leica TCS or Zeiss 700 confocal microscope. 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. Eye discs imaged in transverse section were mounted in 2-hydroxyethylagarose (Sigma); other tissues were mounted in SlowFade (Molecular Probes). Adult female wings were mounted dorsal side up in 3:1 Canada balsam:methyl salicylate and imaged using a Z16 APO microscope (Leica), with a Planapo 2.0 \times lens, fitted with a DFC300 FX camera. Images were assembled with Adobe Photoshop CS5.

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 quantified using Analyze Particles in Fiji. Data were analyzed using a two-sample t-test assuming unequal variances. For lysosomal inhibition, discs were cultured for 5 h in Drosophila cell medium (M3, Sigma) containing 200 μ m Leupeptin and 50 mM NH₄Cl. Notch and Dextran trafficking experiments were carried out as described previously (Lu and Bilder, 2005), and endocytic puncta per cell volume were counted in confocal stacks analyzed in ImageJ (Schindelin et al., 2012) using the LOCI Bio-Formats Importer and Image 5D plugins.

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Competing interests

The authors declare no competing financial interests.

Author contributions

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

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105403/-DC1>

References

- Ang, S. F. and Fölsch, H. (2012). The role of secretory and endocytic pathways in the maintenance of cell polarity. *Essays Biochem.* **53**, 29-39.
- Bilder, D. (2004). Epithelial polarity and proliferation control: links from the *Drosophila* neoplastic tumor suppressors. *Genes Dev.* **18**, 1909-1925.
- Bilder, D. and Perrimon, N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676-680.
- Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat. Cell Biol.* **5**, 53-58.
- Blankenship, J. T., Fuller, M. T. and Zallen, J. A. (2007). The *Drosophila* homolog of the Exo84 exocyst subunit promotes apical epithelial identity. *J. Cell Sci.* **120**, 3099-3110.
- Bulgakova, N. A. and Knust, E. (2009). The Crumbs complex: from epithelial-cell polarity to retinal degeneration. *J. Cell Sci.* **122**, 2587-2596.
- Burgess, J., Del Bel, L. M., Ma, C.-I. J., Barylko, B., Polevoy, G., Rollins, J., Albanesi, J. P., Kramer, H. and Brill, J. A. (2012). Type II phosphatidylinositol 4-kinase regulates trafficking of secretory granule proteins in *Drosophila*. *Development* **139**, 3040-3050.
- Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., Inui, M., Montagner, M., Parenti, A. R., Poletti, A. et al. (2011). The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell* **147**, 759-772.
- Dong, B., Kakihara, K., Otani, T., Wada, H. and Hayashi, S. (2013). Rab9 and retromer regulate retrograde trafficking of luminal protein required for epithelial tube length control. *Nat. Commun.* **4**, 1358.
- Dukes, J. D., Fish, L., Richardson, J. D., Blaikley, E., Burns, S., Caunt, C. J., Chalmers, A. D. and Whitley, P. (2011). Functional ESCRT machinery is required for constitutive recycling of claudin-1 and maintenance of polarity in vertebrate epithelial cells. *Mol. Biol. Cell* **22**, 3192-3205.
- Eaton, S. (2008). Retromer retrieves wntless. *Dev. Cell* **14**, 4-6.
- Fletcher, G. C., Lucas, E. P., Brain, R., Tournier, A. and Thompson, B. J. (2012). Positive feedback and mutual antagonism combine to polarize crumbs in the *Drosophila* follicle cell epithelium. *Curr. Biol.* **22**, 1116-1122.
- Goldstein, B. and Macara, I. G. (2007). The PAR proteins: fundamental players in animal cell polarization. *Dev. Cell* **13**, 609-622.
- Grant, B. D. and Donaldson, J. G. (2009). Pathways and mechanisms of endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **10**, 597-608.
- Harris, K. P. and Tepass, U. (2008). Cdc42 and Par proteins stabilize dynamic adherens junctions in the *Drosophila* neuroectoderm through regulation of apical endocytosis. *J. Cell Biol.* **183**, 1129-1143.
- Harris, K. P. and Tepass, U. (2010). Cdc42 and vesicle trafficking in polarized cells. *Traffic* **11**, 1272-1279.
- Johannes, L. and Popoff, V. (2008). Tracing the retrograde route in protein trafficking. *Cell* **135**, 1175-1187.
- Leong, G. R., Goulding, K. R., Amin, N., Richardson, H. E. and Brunby, A. M. (2009). Scribble mutants promote aPKC and JNK-dependent epithelial neoplasia independently of Crumbs. *BMC Biol.* **7**, 62.
- Lohia, M., Qin, Y. and Macara, I. G. (2012). The Scribble polarity protein stabilizes E-cadherin/p120-catenin binding and blocks retrieval of E-cadherin to the Golgi. *PLoS ONE* **7**, e51130.
- Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in *Drosophila*. *Nat. Cell Biol.* **7**, 1232-1239.
- Martin-Belmonte, F. and Perez-Moreno, M. (2011). Epithelial cell polarity, stem cells and cancer. *Nat. Rev. Cancer* **12**, 23-38.
- Menut, L., Vaccari, T., Dionne, H., Hill, J., Wu, G. and Bilder, D. (2007). A mosaic genetic screen for *Drosophila* neoplastic tumor suppressor genes based on defective pupation. *Genetics* **177**, 1667-1677.
- Moberg, K. H., Schelble, S., Burdick, S. K. and Hariharan, I. K. (2005). Mutations in *erupted*, the *Drosophila* ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. *Dev. Cell* **9**, 699-710.
- Pearson, H. B., Perez-Mancera, P. A., Dow, L. E., Ryan, A., Tennstedt, P., Bogani, D., Elsum, I., Greenfield, A., Tuveson, D. A., Simon, R. et al. (2011). SCRIB expression is deregulated in human prostate cancer, and its deficiency in mice promotes prostate neoplasia. *J. Clin. Invest.* **121**, 4257-4267.
- Pocha, S. M., Wassmer, T., Niehage, C., Hoflack, B. and Knust, E. (2011). Retromer controls epithelial cell polarity by trafficking the apical determinant Crumbs. *Curr. Biol.* **21**, 1111-1117.
- Roeth, J. F., Sawyer, J. K., Wilner, D. A. and Peifer, M. (2009). Rab11 helps maintain apical crumb and adherens junctions in the *Drosophila* embryonic ectoderm. *PLoS ONE* **4**, e7634.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676-682.
- Shivas, J. M., Morrison, H. A., Bilder, D. and Skop, A. R. (2010). Polarity and endocytosis: reciprocal regulation. *Trends Cell Biol.* **20**, 445-452.
- Sommer, C., Straehle, C., Kothe, U. and Hamprecht, F. A. (2011). Ilastik: interactive learning and segmentation toolkit. *8th IEEE International Symposium on Biomedical Imaging*, 230-233.

- St Johnston, D. and Ahringer, J.** (2010). Cell polarity in eggs and epithelia: parallels and diversity. *Cell* **141**, 757-774.
- Su, T., Bryant, D. M., Luton, F., Vergés, M., Ulrich, S. M., Hansen, K. C., Datta, A., Eastburn, D. J., Burlingame, A. L., Shokat, K. M. et al.** (2010). A kinase cascade leading to Rab11-FIP5 controls transcytosis of the polymeric immunoglobulin receptor. *Nat. Cell Biol.* **12**, 1143-1153.
- Tanentzapf, G. and Tepass, U.** (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat. Cell Biol.* **5**, 46-52.
- Tanentzapf, G., Devenport, D., Godt, D. and Brown, N. H.** (2007). Integrin-dependent anchoring of a stem-cell niche. *Nat. Cell Biol.* **9**, 1413-1418.
- Vergés, M., Luton, F., Gruber, C., Tiemann, F., Reinders, L. G., Huang, L., Burlingame, A. L., Haft, C. R. and Mostov, K. E.** (2004). The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. *Nat. Cell Biol.* **6**, 763-769.
- Windler, S. L. and Bilder, D.** (2010). Endocytic internalization routes required for delta/notch signaling. *Curr. Biol.* **20**, 538-543.
- Wodarz, A., Hinz, U., Engelbert, M. and Knust, E.** (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67-76.
- Yamanaka, T. and Ohno, S.** (2008). Role of Lgl/Dlg/Scribble in the regulation of epithelial junction, polarity and growth. *Front. Biosci.* **13**, 6693-6707.
- Zeigerer, A., Gilleron, J., Bogorad, R. L., Marsico, G., Nonaka, H., Seifert, S., Epstein-Barash, H., Kuchimanchi, S., Peng, C. G., Ruda, V. M. et al.** (2012). Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. *Nature* **485**, 465-470.
- Zhou, B., Wu, Y. and Lin, X.** (2011). Retromer regulates apical-basal polarity through recycling Crumbs. *Dev. Biol.* **360**, 87-95.