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Domains controlling cell polarity and proliferation in the *Drosophila* tumor suppressor Scribble

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Cell polarity and cell proliferation can be coupled in animal tissues, but how they are coupled is not understood. In *Drosophila* imaginal discs, loss of the neoplastic tumor suppressor gene *scribble* (*scrib*), which encodes a multidomain scaffolding protein, disrupts epithelial organization and also causes unchecked proliferation. Using an allelic series of mutations along with rescuing transgenes, we have identified domain requirements for polarity, proliferation control, and other Scrib functions. The leucine-rich repeats (LRR) tether Scrib to the plasma membrane, are both necessary and sufficient to organize a polarized epithelial monolayer, and are required for all proliferation control. The PDZ domains, which recruit the LRR to the junctional complex, are dispensable for overall epithelial organization. PDZ domain absence leads to mild polarity defects accompanied by moderate overproliferation, but the PDZ domains alone are insufficient to provide any Scrib function in mutant discs. We suggest a model in which Scrib, via the activity of the LRR, governs proliferation primarily by regulating apicobasal polarity.

Introduction

The epithelial organization of animal tissues can have an acute influence on the control of cell proliferation. Disruption of epithelia by for instance wounding can stimulate proliferative activity, whereas epithelial cell–cell communication regulates cessation of proliferation when final organ size is achieved (Bryant and Simpson, 1984; Johnston and Gallant, 2002). In malignant carcinomas, loss of epithelial cytoarchitecture correlates with the development of aggressive tumors. These observations have raised the possibility that proliferation and polarity can be functionally coupled within cells. How these two functions are coupled is not understood.

Recent data has suggested that some proteins may coordinate polarity and proliferation control, integrating information about epithelial organization to regulate the mitotic state. In mammals, cells derived from mammary epithelial tumors can be reverted from neoplastic to normal phenotypes by inhibiting phosphatidylinositol 3-kinase signaling; interestingly, Rac1 and Akt are independently responsible for the polarity disruption and overproliferation conferred by ectopic phosphatidylinositol 3-kinase activity (Liu et al., 2004). The tumor suppressor Merlin, lost in *Neurofibromatosis type 2* patients, may function by linking cell–cell or cell–matrix contacts to growth inhibitory signaling mediated by the Pak kinase (Morrison et al., 2001; Lallemand et al., 2003). LKB1, the kinase mutated in Peutz-Jeghers syndrome, is homologous to Par-4, a regulator of cell polarity in invertebrates, and LKB1 polarizing activity may contribute to its role as a mammalian tumor suppressor (Baas et al., 2004).

A particularly intriguing case exists in *Drosophila*, where mutations in three genes cause simultaneous loss of polarity and overproliferation (for review see Bilder, 2004). These genes, *scribble* (*scrib*), *discs-large* (*dlg*), and *lethal giant larvae* (*lgl*), act as key regulators of apicobasal polarity by restricting apical proteins from the basolateral surface. Epithelia mutant for these genes mislocalize apical proteins and adherens junctions (AJs) to ectopic basolateral sites. Additionally, the mispolarized imaginal discs of mutant animals are dramatically overgrown, and assume a number of other characteristics reminiscent of malignant human tumors, including reduced differentiation capacity and susceptibility to oncogenic Ras activity (Gateff, 1978; Brumby and Richardson, 2003; Pagliarini and Xu, 2003). For this reason *scrib*, *dlg*, and *lgl* have been called *Drosophila* neoplastic tumor suppressor genes (nTSGs). Several studies have presented evidence that vertebrate homologues of the nTSGs also function in proliferation control (for review see Bilder, 2004). However, the mechanism by which *Drosophila* nTSGs or their vertebrate homologues influence proliferation remains unclear.

To clarify the relationship between cell polarity and proliferation in *Drosophila*, we have analyzed the nTSG *scrib*.

The online version of this article contains supplemental material.

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Abbreviations used in this paper: AJ, adherens junction; Cor, Coracle; Dlg, discs-large; GLC, germ line clone; LAP, LRR and PDZ domain-containing; Lgl, lethal giant larvae; LRR, leucine-rich repeats; Nrx, Neurexin IV; nTSG, neoplastic tumor suppressor gene; PDZ, PSD-95, Dlg, ZO-1 homology; Scrib, scribble; SJ, septate junction; WT, wild type.

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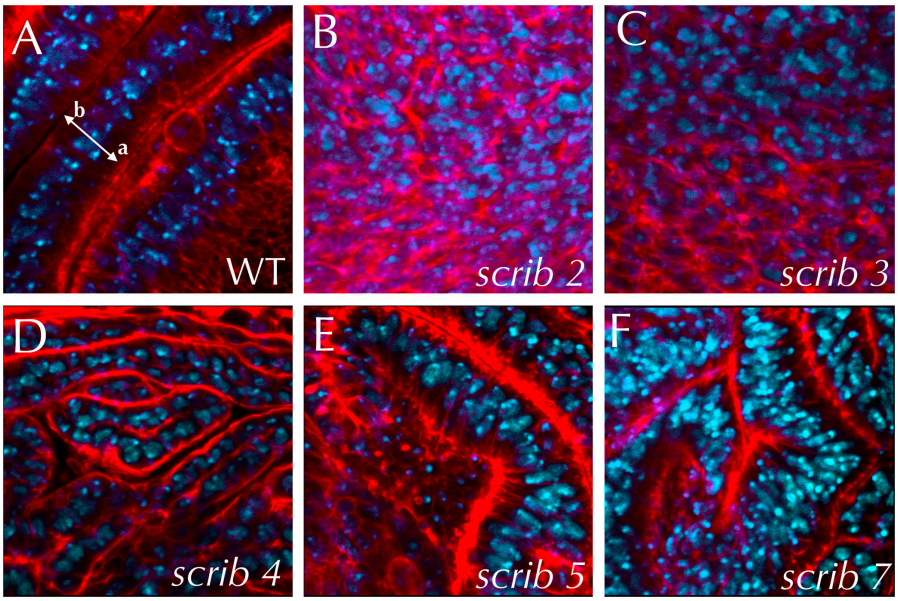

Figure 1. **Epithelial architecture of *scrib* wing discs.** Confocal images displaying multiple folds in the disc epithelium. WT disc cells (A) show apicobasal (a and b) polarity, revealed by apically enriched filamentous actin (phalloidin, red), and monolayered cellular organization, revealed by nuclear staining (DAPI, blue). Apical polarity and monolayered organization are absent in *scrib 2* (B) and *3* (C) discs, but present in *scrib 4* (D), *5* (E), and *7* (F) discs.

scrib encodes a cytoplasmic protein, found at the epithelial septate junction (SJ), that contains 16 NH_2-terminal leucine-rich repeats (LRR) as well as four PSD-95, Dlg, ZO-1 homology (PDZ) domains (Bilder and Perrimon, 2000). Proteins containing both LRR and PDZ domains are members of the LRR and PDZ domain-containing (LAP) family found throughout metazoan animals (for review see Santoni et al., 2002), and have been suggested to function as cellular scaffolds. LAP proteins are localized to the basolateral surface of epithelia, and appear to have a conserved function in polarity regulation, because loss of *C. elegans* Let-413, like loss of Scrib, causes an expansion of the apical epithelial surface (Legouis et al., 2000). LAP proteins have also been linked to signal transduction. ERBIN was first isolated as a binding partner for the cytoplasmic domain of the human EGF receptor family member ErbB2 (Borg et al., 2000), and ERBIN overexpression alters EGF receptor–mediated activation of the MAPK cascade (Huang et al., 2003). Additionally, Scrib and Dlg-related proteins present at mammalian synapses are thought to promote efficient signaling by assembling complexes of cytoplasmic and transmembrane signal transduction components (for review see Sheng, 2001). Loss of LAP proteins like Scrib could therefore affect the activity of signal transduction pathways as well as the polarized localization of cellular components.

The multidomain Scrib protein is required for multiple functions in imaginal discs, including polarity, proliferation control, and differentiation. Are these functions, in particular polarity and proliferation control, genetically coupled in the fly by independent and separable activities of Scrib? Or are polarity and proliferation control intrinsically linked, such that depolarization consequent to Scrib loss causes overproliferation? We have addressed this issue by determining the roles of the different protein–protein interaction domains of Scrib.

Results

A *scrib* allelic series

We began our analysis of Scrib functions by obtaining seven ethyl methanesulfonate–induced alleles (see Materials and methods). In an initial characterization, the lethal phases of each allele over a scrib deficiency were assayed (Table I). Two alleles (*scrib 2* and *3*) fulfill a criterion for genetic nulls; animals either hemizygous or transheterozygous between these alleles exhibit the same lethal phase, dying as giant larvae after an extended third instar (L3) stage. Though *scrib 1* hemizygotes also die as giant larvae, in trans to either *scrib 6* or *7* escapers are produced, suggesting that *scrib 1* retains activity in certain contexts. Three alleles (*scrib 4*, *5*, *6*) behave as hypomorphs, dying as early pupae; heteroallelic combinations suggest that *scrib 4* is

Table I. **Lethal phases of *scrib* transheterozygotes**

Alleles	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*Df (3R) Tl-x*
1		L3 (giant)	L3 (giant)	Early pupal	Mid-pupal	Pharate adults	Escapers	L3 (giant)
2			L3 (giant)	Early pupal	Mid-pupal	Mid-pupal	Mid-pupal	L3 (giant)
3				Early pupal	Mid-pupal	Mid-pupal	Mid-pupal	L3 (giant)
4					L1[a]	Late pupal	Mid-pupal	L1[a]
5						Escapers	Escapers	L1[a]
6							Complement	Mid-pupal
7								Mid-pupal

[a]The reason for this early lethality is not known; because these alleles were generated on a similar parental chromosome they may share an accessory lethal.

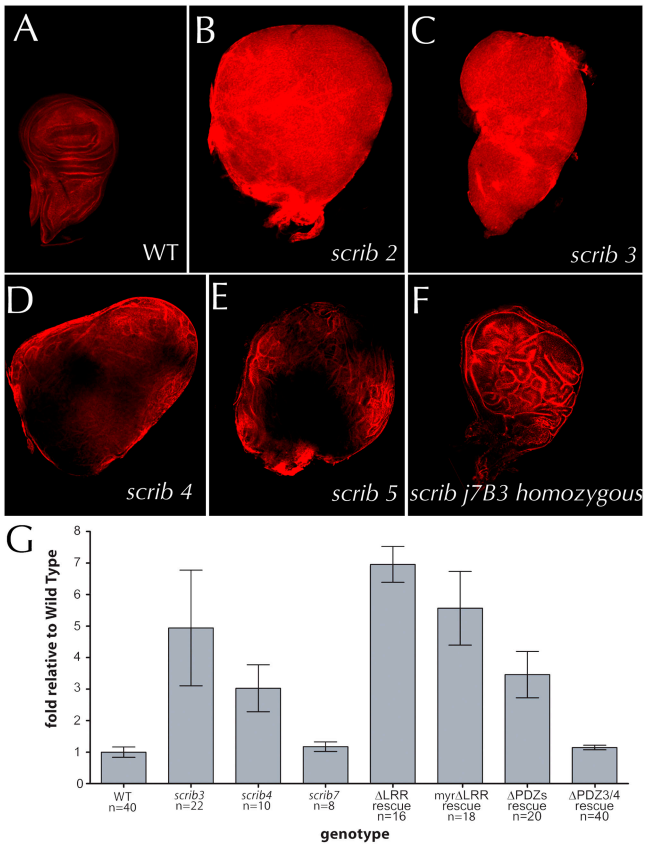

Figure 2. **Size control in *scrib* wing discs.** Single confocal sections of phalloidin stained wing discs are shown. When compared with WT (A), all *scrib* alleles show increased size (B, *scrib 2*; C, *scrib 3*; D, *scrib 4*; E, *scrib 5*; F, *scrib j7B3*). G quantitates the degree of wing disc cell overproliferation in the mutant alleles and transgene rescues, normalized to WT. Bars indicate SDs.

the most severe. Finally, *scrib 7* acts like a weak hypomorph, as hemizygotes die during mid-pupal stages, whereas homozygotes die as pharate adults with normal external morphology. These mutations thus form an allelic series, ranging from genetically null to weakly hypomorphic and nearly viable.

Functions of mutant Scrib proteins

To test the functions that each mutant Scrib protein is capable of providing, we analyzed the phenotypes of wing imaginal discs from hemizygous larvae. The wing disc includes a monolayer of highly elongated epithelial cells that attains a consistent size and organization. We first assayed epithelial architecture using rhodamine-phalloidin, which binds to apically enriched filamentous actin (Fig. 1 A). In discs from the null allele *scrib 2*, phalloidin staining reveals uniform actin localization throughout the cortex, and the cells themselves are round rather than elongated (Fig. 1 B). Moreover, these cells have a multilayered rather than monolayered organization, forming a solid spherical mass rather than a flat disc. These phenotypes are identical to those of *scrib 1* (Bilder et al., 2000) as well as *scrib 3* (Fig. 1 C). By contrast, discs from *scrib 4, 5, 6,* and *7* larvae contain cells arranged in epithelial monolayers, maintaining the distinctive folded structure of the tissue (Fig. 1, D–F). Interestingly, epithelial cells from *scrib 4* and *5* discs display altered cell shapes, with *scrib 4* in particular showing cuboidal rather than columnar morphology. Nevertheless, apical accumulation of actin is readily seen. These results demonstrate that the proteins produced by *scrib 4, 5, 6,* and *7* can provide polarity function in imaginal discs, whereas those produced by *scrib 1*, *2*, and *3* cannot.

We next assayed the ability of the mutant Scrib proteins to control cell proliferation in the wing disc. Confocal sections reveal an increase in both surface area and volume of mutant discs when compared with wild type (WT). The size difference is most dramatic for the multilayered discs of *scrib 1*, *2*, and *3* animals, intermediate for *scrib 4* and *5* and very slight for *scrib 6* and *7* (Fig. 2, A–E). To exclude differences in cell shape or tissue organization and quantitatively assay proliferation, cell counts on dissociated discs were performed (Fig. 2 G). These counts reveal that the unpolarized tissue of *scrib 3* animals contains ~500% as many cells as WT, an increase similar to *scrib 1* (Bilder et al., 2000). In the polarized discs of *scrib 4* animals, a ~300% increase is evident, indicating that *scrib* cells that form polarized epithelial monolayers can nevertheless be partially defective in proliferation control.

Overproliferation of animal cells can be associated with a failure to terminally differentiate and exit from the cell cycle. Therefore, we investigated the ability of *scrib* cells to differentiate and contribute to adult structures. In genetically mosaic imaginal discs, clones of cells mutant for strong *scrib* alleles grow slowly and are outcompeted by WT cells. We used the *EGUF/hid* system (Stowers and Schwarz, 1999) to generate eyes consisting exclusively of *scrib* mutant cells. Adult eye tissue homozygous for *scrib 4* was readily obtained, although it was rough, enlarged and folded (Fig. 3 C). However, animals with eyes homozygous for *scrib 1*, *2*, and *3* never eclose, instead dying as headless pharate adults (Fig. 3 B). Analysis of *scrib 2* eye discs reveals severe defects in the expression of the neuronal differentiation marker Elav, as well as a failure to organize ommatidial preclusters; these processes occur normally in *scrib 4* eye discs (Fig. 3, E and F). Differentiation capacity in *scrib* cells therefore correlates with the ability to organize an epithelial monolayer rather than the ability to control all cell proliferation.

Junctional defects in hypomorphic scrib epithelia

The phenotype of *scrib 4* discs indicates that a portion of Scrib-mediated proliferation control is independent of the ability to regulate formation of a polarized epithelial monolayer. However, the altered shape of cells in these discs raises the possibility that these overproliferating epithelia might have more subtle defects in protein localization. We tested this hypothesis by examining the distribution of polarized membrane and cytoplasmic proteins in *scrib 4* epithelia. In both the wing disc of mutant larvae and the ectoderm of germ line clone (GLC) mutant embryos, apical proteins such as atypical PKC and AJ markers such as E-Cadherin are properly localized (unpublished data). However, the basolateral proteins Coracle (Cor) and Neurexin IV (Nrx) show aberrant distributions (Fig. 3 H). In WT epithe-

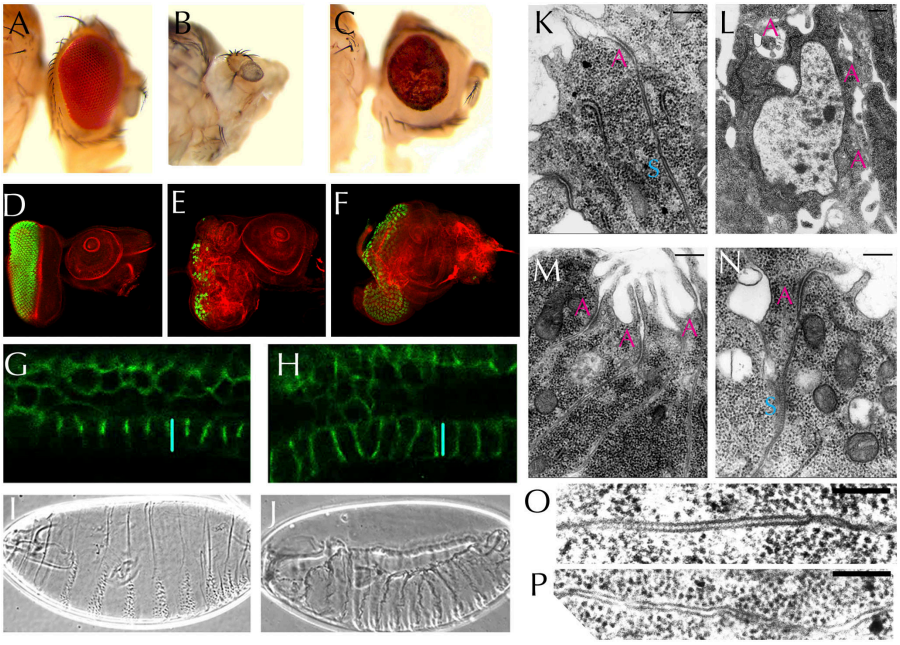

Figure 3. **Differentiation and junctions in *scrib* epithelia.** Eyes consisting of cells homozygous for WT (A), *scrib 2* (B), and *scrib 4* (C) were produced using the *EGUF/hid* system. *Scrib 4* cells can differentiate into adult eye tissue, whereas *scrib 2* cannot. The neuronal differentiation marker Elav (green, phalloidin in red) is severely reduced in *scrib 2* (E) but, like WT (D), present in *scrib 4* eye discs (F). (G–P) Junctional defects in *scrib 4* epithelia. Apicolateral polarization of Cor (green; blue bar shows the full extent of the lateral membrane) in the hindgut (G, WT embryos) is lost in *scrib 4* GLC embryos (H). *scrib 4* GLC embryos also have a large dorsal hole (J, compare with WT in I). K–P show TEM images of WT (K), *scrib 1* (L, note lower magnification), *scrib 4* (M) and *scrib 5* (N) wing disc cells, with AJs (magenta, A) and SJs (cyan, S) marked. Bars, 0.25 μm. AJs are mislocalized in *scrib 2* but found normally in the other mutants; SJs are absent in *scrib 2* and most *scrib 4* cells, although dispersed septa are rarely found in the latter (P, compare with WT in O).

lia, these markers are restricted to the apex of the basolateral membrane, at the site of the SJ where Scrib also localizes. In *scrib 4* epithelia, however, they are found throughout the basolateral membrane with no sign of enrichment. Interestingly, *scrib 4* GLC embryos display a consistent failure of dorsal closure, a phenotype also reported for *cor* and *Nrx* mutants (Fehon et al., 1994; Baumgartner et al., 1996; Fig. 3 J), as well as mildly penetrant anterior open defects that are not seen in *cor* or *Nrx*.

Because Cor and Nrx are components of the SJ, we tested whether *scrib 4* epithelia are defective in SJ formation. Indeed, transmission electron microscopy of *scrib 4* mutant discs reveals that although AJ formation is normal, SJs are severely disrupted; extensive arrays of septa are not assembled and only rarely can small groups of septa be seen (Fig. 3, M and P). This contrasts with *scrib 1* mutant discs, in which AJs are mislocalized and SJs are absent (Fig. 3 L). However, in *scrib 5* mutant discs both AJs and SJs are readily found (Fig. 3 N). These results indicate that Scrib is required for SJ formation independent of its role in apicobasal polarity, and that hyperproliferative *scrib 4* discs have compromised epithelial organization.

Molecular nature of the Scrib lesions

Our phenotypic analysis of the *scrib* allelic series indicates that many of the mutant proteins retain some aspects of WT *scrib* function, whereas being compromised in others. To identify the molecular lesions associated with each *scrib* allele, we isolated and sequenced genomic DNA from homozygous animals. Single base pair changes were identified in the coding region of each of the seven alleles (Fig. 4 A). Six of these changes create premature termination codons, and are predicted to cause truncations in the WT 1751–amino acid Scrib protein. *scrib 2* alters the third codon to an amber stop, whereas *scrib 3* terminates within the 12th LRR. *scrib 4* terminates in the midst of PDZ1, whereas *scrib 5* terminates between PDZ2 and PDZ3, and *scrib 6* in the midst of PDZ4. Because *scrib 4* and *6* delete residues in the PDZ ligand binding groove (Doyle et al., 1996), these

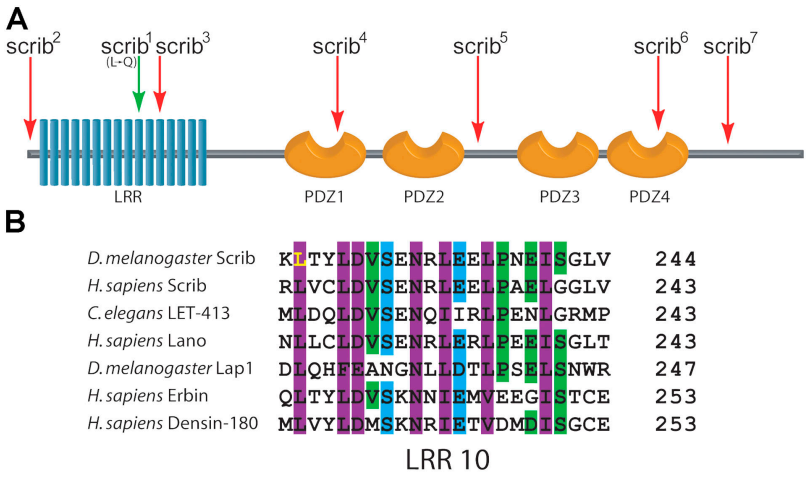

Figure 4. **Location of *scrib* mutations.** (A) Amino acid changes in *scrib* alleles, mapped onto a diagram of the Scrib protein. Red arrows indicate premature termination codons; the green arrow indicates a missense mutation. (B) Conservation of the leucine mutated in *scrib 1* (yellow) between LRR 10 of known LAP proteins. Coloring indicates residues with similar properties: purple =100% conservation, blue >85% conservation, green >70% conservation.

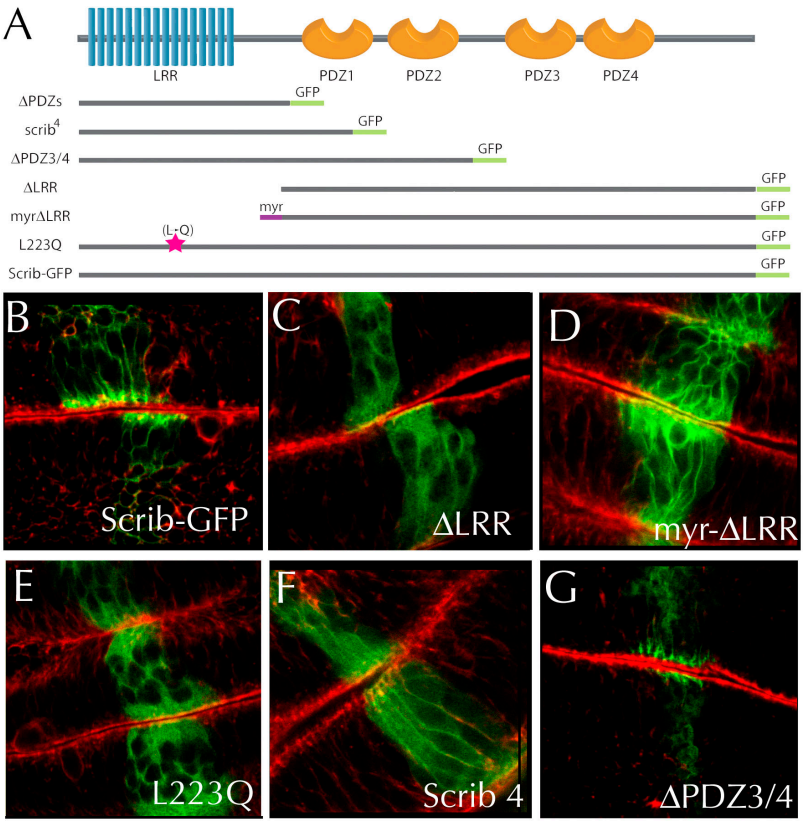

Figure 5. **Construction and localization of ScribGFP transgenes.** (A) Diagram of UAS-driven transgenes. (B–G) Localization of *ptcGal4*-driven ScribGFP proteins (green, phalloidin in red) in WT wing discs. Full-length Scrib (B) is basolaterally restricted and enriched at the SJ, whereas ΔLRR (C) and L223Q (E) are found in the cytoplasm. Membrane attachment with some SJ enrichment is seen with myr-ΔLRR (D). Scrib4 (F) is localized broadly along basolateral membranes without evident polarization. ΔPDZ3/4 (G) is found at SJs, similar to full-length Scrib.

truncated domains are not expected to be functional. *Scrib 7* removes the final 301 amino acids but deletes no conserved domains. In addition to these six premature stop codons, *scrib 1* alters leucine 223, in LRR 10, to a glutamine. This leucine is absolutely conserved between LAP protein LRRs (Fig. 4 B), and is predicted to lie in an α-helix on the external surface of the horseshoe-shaped LRR superstructure, opposite the putative binding pocket (Kobe and Kajava, 2001). The *scrib* alleles thus produce proteins with mutations in, or deletions of, many of the Scrib interaction domains.

Rescue with transgenic Scrib constructs

Comparison of the molecular and phenotypic studies of the *scrib* allelic series reveals a clear correlation with respect to polarity. Mutant proteins that retain the LRR are capable of forming an epithelial monolayer, whereas mutations that disrupt the LRR cannot. PDZ domains, by contrast, are not essential for the development of monolayered epithelia, but could be involved in full control of cell proliferation. However, it is also possible that reduced levels of mutant protein in these alleles (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407158/DC1) may contribute to the observed phenotypes. To distinguish between these possibilities we generated flies transgenically overexpressing mutant Scrib proteins and tested the functions of these proteins in vivo. This approach, which complements the analysis of the allelic series, also allows us to visualize protein localization as well as to test the functions of additional mutant Scrib proteins.

To establish a rescue assay, we expressed WT as well as GFP-tagged Scrib in null *scrib* wing discs using the UAS-GAL4 system. Both constructs provide full rescue, allowing a normally sized and folded disc with fully polarized epithelia to form (Fig. 6 A). In WT and rescued animals, Scrib-GFP localizes at the lateral membranes and is enriched at the SJ, identical to endogenous Scrib (Fig. 5 B). GFP-tagged transgenes can therefore be used to assay both proper localization and function of Scrib.

We initially asked whether the LRR is indeed critical for all Scrib functions. Expression of Scrib lacking the LRR but retaining all four PDZ domains (UAS-ΔLRR) in WT discs reveals cytoplasmic and unpolarized localization, with no membrane association apparent (Fig. 5 C). When expressed in *scrib* discs, this construct has no rescuing activity: cells remain unpolarized, and disc overgrowth is unaffected (Fig. 6 B and Fig. 2 G). Because the phenotypically strong *scrib 1* missense mutation (L223Q) also maps to the LRR, we assayed a transgene containing this mutation. Like UAS-ΔLRR, UAS-L223Q is predominantly cytoplasmic and mutant discs expressing this construct show no signs of rescue (Fig. 5 E and Fig. 6 D). Together, these experiments demonstrate the importance of the LRR for Scrib subcellular localization as well as both polarity and proliferation control activity.

The above constructs fail to associate with the plasma membrane, where Scrib is presumed to act. This raises the possibility that the absence of activity in LRR mutant Scrib is due solely to mislocalization. Therefore, we tested whether Scrib lacking the LRR but attached to the membrane by myristoylation (UAS-myr-ΔLRR) could provide any rescuing activity. Although indeed membrane localized, with some enhancement at the SJ evident, this protein also fails to rescue polarity or

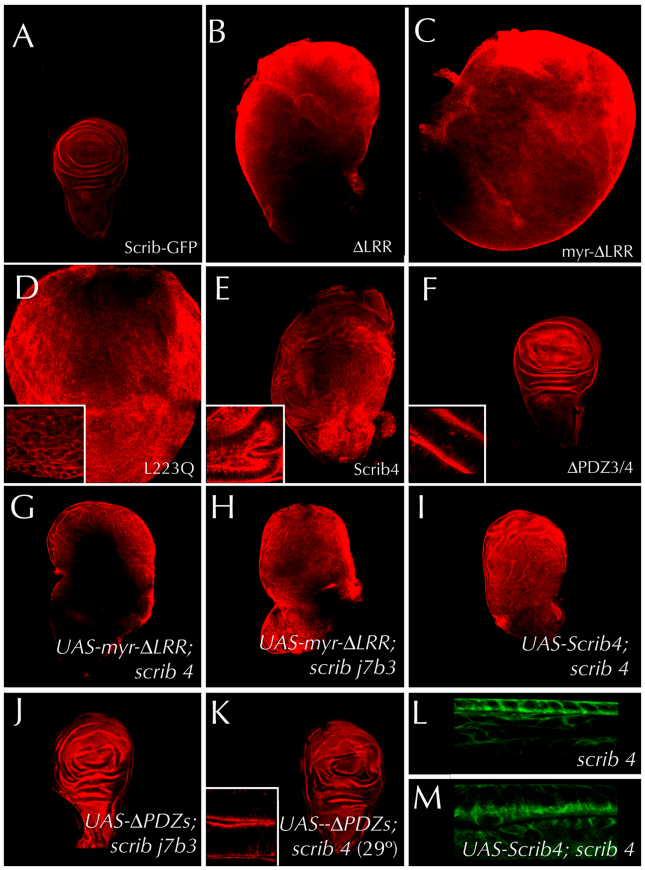

Figure 6. **Rescue of *scrib* discs by ScribGFP transgenes.** (A–F) Constructs are driven in null *scrib 3* discs. Full-length Scrib (A) restores both polarized organization and disc size. ΔLRR (B), myr-ΔLRR (C), and L223Q (D) rescue neither polarity nor proliferation control. Scrib4 (E) restores polarized and monolayered organization (inset, compare with D and F) but discs are overgrown. ΔPDZ3/4 (F) discs are indistinguishable from WT. (G–M) Constructs are driven in hypomorphic, polarized *scrib* discs. Myr-ΔLRR does not rescue *scrib 4* (G) or *scrib j7b3* (H) discs. Expression of Scrib4 or ΔPDZs in *scrib 4* (I) or *scrib j7b3* (J) provides more complete rescue than these transgenes in null discs. Rescue is further improved at 29° (K, ΔPDZs in *scrib 4*). Scrib4 overexpression also restores Cor localization (green) and columnar shape to *scrib 4* discs (M, compare disc without transgene in L).

growth control in the null mutant disc (Fig. 5 D; Fig. 6 C; Fig. 2 G). These results establish that the LRR is required for Scrib function in addition to regulating Scrib localization.

To test what functions the LRR alone is capable of providing, we generated constructs that delete the PDZ domains. A construct mimicking the PDZ1 truncation of *scrib 4* (UAS-Scrib4) shows nonpolarized localization at cell membranes (Fig. 5 F). A construct terminating shortly before PDZ1 (UAS-ΔPDZs) shows similar localization, as well as nuclear signal (unpublished data). Like *scrib 4* animals, null mutants expressing UAS-Scrib4 or UAS-ΔPDZs contain discs with polarized but overgrown epithelial monolayers (Fig. 6 E). Notably, this overgrowth was quantitatively intermediate between null mutant and WT discs, similar to that seen in *scrib 4* discs (Fig. 2 G). These data confirm the hypothesis that the Scrib LRR is both necessary and sufficient to form a polarized epithelial monolayer, and can alone provide significant control of disc growth.

To identify regions that assist in conferring full growth control, we added PDZ domains back to the LRR. Addition of PDZ1+2 (UAS-ΔPDZ3/4), which generates a protein similar to that found in *scrib 5*, allows Scrib to localize to basolateral membranes and become enriched at the SJ identical to Scrib-GFP (Fig. 5 G). Interestingly, expression of this protein in null mutant discs rescues all tested aspects of both epithelial organization and growth control (Fig. 6 F), and cell counts reveal no significant difference (Fig. 2 G). Rescue by UAS-ΔPDZ3/4 demonstrates that two Scrib PDZ domains in combination with the LRR can provide the growth control properties of the full-length protein.

Proliferation control is sensitive to Scrib levels

The observation that the LRR with PDZ1 and 2 can function equivalently to WT Scrib contrasts with the phenotype of animals mutant for *scrib 5* and *6*, which encode proteins that retain these domains yet show disc overproliferation. We hypothesized that reduced levels of Scrib might compromise growth regulation. To test this hypothesis, we examined discs from larvae homozygous for *scrib j7B3*, a P-element insertion in the first intron of *scrib* (Bilder and Perrimon, 2000). Such P element insertions often compromise expression of the affected genes. Indeed, Western blotting and immunohistochemistry shows reductions of Scrib protein in *scrib j7B3*as well as *scrib 5* and *6* animals, although the mutant protein is normally localized (Fig. S1, not depicted). Moreover, like *scrib 4*, *5*, and *6* discs, discs from larvae homozygous for *scrib j7B3* show normal epithelial organization but intermediate overproliferation (Fig. 2 F). We conclude from these experiments that growth control is sensitive to Scrib levels.

Scrib PDZ domains are nonfunctional when separated from the LRR

Scrib j7b3 discs, which have reduced levels of full-length protein, hyperproliferate in a manner similar to *scrib 4* discs, which lack PDZ domains. *Scrib 4* discs additionally show loss of SJs. The SJ loss and overproliferation observed in the polarized epithelia of *scrib 4* discs could reflect disruption of distinct, polarity-independent functions of the Scrib PDZ domains. Alternatively, these phenotypes could both reflect a partial defect in a single Scrib role in organizing apicobasal polarity, due to inefficient activity provided by the PDZ-less mutant protein. To distinguish between these possibilities we assessed the ability of transgenes expressing the LRR and the PDZ domains to independently rescue *scrib 4* discs, which already produce the LRR, or *scrib j7b3* discs, which produce reduced levels of full-length Scrib protein.

To test the functions that the PDZ domains can provide in polarized discs, we again used UAS-ΔLRR and UAS-Myr-ΔLRR. When expressed in *scrib 4* or *scrib j7b3* animals, neither ΔLRR or Myr-ΔLRR prevents disc overgrowth, nor does either cause Cor to polarize correctly (Fig. 6, G and H, not depicted). Similar results are seen when UAS-Myr-ΔLRR is coexpressed with UAS-ΔPDZ in *scrib* null discs, even when expression levels are increased via raising the larvae at 29°. These results indicate that even in a polarized disc the Scrib PDZ do-

mains alone are not sufficient to provide activity, and suggest that the PDZ domains function only when linked to the LRR.

By contrast, expression of the LRR domain alone in either *scrib 4* and *scrib j7b3* mutant discs enhances the rescue seen with the same construct in null discs. UAS-Scrib4 and UAS-ΔPDZ-expressing discs in these animals are consistently smaller than nonexpressing counterparts, with sizes approaching those of WT (Fig. 6, I and J). Increasing the level of transgene expression, via raising the animals at 29°, further enhances this rescue, as the discs attain nearly WT morphology (Fig. 6 K). Moreover, the apicolateral enhancement of SJ markers is restored in rescued *scrib 4* discs, and cell shape is columnar rather than cuboidal (Fig. 6, L and M). The fact that high levels of the LRR can largely rescue polarity and proliferation control in a disc already expressing the LRR demonstrates that this single domain alone can provide, albeit less efficiently, most functions of the full-length protein.

Discussion

We have analyzed domain requirements for Scrib function in order to untangle the relationship between epithelial polarity and proliferation in *Drosophila* imaginal discs. Our analysis demonstrates that the Scrib LRR is necessary for both cell polarity and control of cell proliferation and is also nearly sufficient for these two functions. The PDZ domains are dispensable for formation of an epithelial monolayer, but enhance the ability of the LRR to localize SJ proteins and to provide full proliferation control. Together, the data are consistent with a model in which proliferation control in imaginal discs requires the achievement, via LRR activity, of full Scrib-mediated polarization.

Role of the LRR

Our results highlight the central role of the LRR in Scrib function. Animals with absent or mutant LRR have phenotypes identical to those entirely lacking Scrib, with dramatic effects on both epithelial polarity and growth control. Of the five evolutionarily conserved protein–protein interaction domains in Scrib, only expression of the LRR can provide polarizing and proliferation-controlling activity, with high levels sufficient to effect nearly full rescue. The LRR is also sufficient to mediate membrane localization, whereas a protein lacking the LRR remains in the cytoplasm. Interestingly, alteration of a conserved leucine in the 10th LRR disrupts all Scrib functions and displaces the protein into the cytoplasm. A related alteration in the 13th LRR of *C. elegans* Let-413 also prevents membrane localization and function (Legouis et al., 2003). However, it is clear that the LRR functions as more than a membrane attachment domain because the Scrib PDZs alone are incapable of rescuing any aspect of the mutant phenotype, even when provided with an exogenous membrane targeting signal.

How can the LRR, which is broadly localized in the absence of PDZ domains, convey information to specifically polarize the apicobasal axis? We have previously suggested that a critical role of Scrib in epithelial polarity is the recruitment of Lgl to the lateral cell cortex (Bilder et al., 2000). Lgl itself is not highly polarized in its distribution (Strand et al., 1994), and while it is displaced from the cortex in *scrib* null GLC embryos, immunofluorescense reveals that Lgl is indeed cortically localized in LRR-expressing *scrib 4* GLC embryos, consistent with proper apicobasal polarization in these animals (unpublished data). Therefore, it appears that membrane-localized LRR can mediate interactions that effect cortical recruitment of Lgl, where Lgl can perform its still unknown activities in regulating protein trafficking.

Role of the PDZ domains

A surprising result of our experiments is that epithelia can achieve apicobasal organization in the absence of Scrib PDZ domains. Requirement of the PDZ domains for epithelial polarization was expected because of the frequent occurrence of these motifs in proteins that regulate cell polarity (Bilder, 2001). However, Let-413 PDZ domains are also not required to rescue polarity (Legouis et al., 2003) suggesting that LAP protein PDZ domains may be generally dispensable for organizing the apicobasal axis. This finding has implications for understanding the biological roles of LAP protein PDZ-binding partners (Borg et al., 2000; Audebert et al., 2004). In the case of Scrib, we find that high level expression of a protein lacking the PDZ domains can provide function similar to low levels of expression of full-length protein. Our data thus suggest that under physiological conditions PDZ domain interactions contribute to Scrib function in polarity and proliferation control quantitatively rather than qualitatively.

The quantitative contribution of the PDZ domains may involve their role in polarizing Scrib along the plasma membrane. Analysis of tagged transgenes suggests that Scrib is localized by a two-part mechanism. In the first step, interactions mediated by the LRR bring the protein to the plasma membrane. In the second step, PDZ domain interactions enrich membrane-bound Scrib at the future site of the SJ. This mechanism mirrors the gradual polarization of Scrib during embryonic development, where Scrib is initially localized homogeneously basolaterally, but becomes focused apicolaterally as the final junctional complex forms. Scrib localization to the SJ thus parallels the maturation of the epithelium. Cell junctions are known to be sites of polarized vesicle trafficking, and proteins that control polarity of the entire epithelial cell membrane are localized to the small perijunctional region (for review see Tepass et al., 2001). Efficient regulation of cell polarity may require high Scrib levels at this location, with the PDZ domains serving to increase the local concentration of the active LRR.

Scrib and SJs

Our data provide direct evidence for a role of Scrib in SJ formation. Although Scrib and Dlg both localize to the SJ (Woods and Bryant, 1991; Bilder and Perrimon, 2000) an unambiguous demonstration that either protein is an SJ component has proven difficult. Physical interactions of SJ components with Scrib or Dlg have not yet been found, and whereas *dlg* and *scrib* null mutant discs have no SJs (Woods et al., 1996; Fig. 3), the dramatic disorganization of these tissues raises the possibility that SJ loss might be secondary to the gross polarity disruption. In this work, we identify *scrib* mutant discs and em-

bryos that polarize and form normal AJs but nevertheless contain severely disrupted SJs. Interestingly, SJ proteins are zygotically produced and first become concentrated at the apical region of the lateral membrane during embryonic stage 14, when the SJ ultrastructurally appears (for review see Tepass et al., 2001). By contrast, Scrib and Dlg, which are maternally provided, are enriched in this membrane region at stage 9, long before SJs develop. Scrib may therefore prepattern the site of the future SJ to mediate the subsequent coalescence of other SJ components.

Relationship between proliferation and other Scrib functions

Loss of *scrib* from imaginal epithelia causes two major defects: mispolarization, reflected in the ectopic distribution of apical and AJ proteins, and overproliferation, resulting in an enormous increase in cell numbers. A key question is whether these effects, which are seen in all three *Drosophila* nTSG mutants, are independent or if they are causally interrelated. The independent model posits the existence of Scrib domains that control proliferation and cell polarity without influencing the other function. By contrast, the interdependent model posits that Scrib acts primarily to regulate apicobasal polarity, and that loss of polarity itself disrupts proliferation control.

Previous views on control of polarity and proliferation by the nTSGs have favored an independent model. These views are influenced by a study of Dlg functional domains (Hough et al., 1997), in which deletion of two PDZ domains caused overproliferation within a maintained epithelial monolayer. This finding led to the proposal that Dlg has separable functions in polarity and growth control, with the latter mediated by PDZ domain interactions. Using analogous methods here we have found that deletion of Scrib PDZ domains similarly causes overproliferation in the absence of gross epithelial disorganization. However, our quantitative analysis reveals that PDZ domain deletion does not entirely disrupt, but only partially compromises, proliferation control. Moreover, we show that this proliferation defect can also result from lower levels of full-length Scrib, and in fact can be rescued by expressing high levels of a single Scrib domain, the LRR. Our analyses do not rule out the possibility that the PDZ domains, when covalently linked to the LRR, directly contribute to cell proliferation signaling. Nevertheless, any such contribution is likely minor because high levels of LRR alone restore null mutant discs to nearly WT size.

A formal demonstration of independent functions requires the identification not only of mutant proteins that rescue polarity without restoring proliferation control but also of those that rescue proliferation control without restoring polarity. We have not found such proteins, using either random mutagenesis or rescue constructs engineered with a knowledge of conserved domains. Both polarity and proliferation control are simultaneously lost when the LRR is mutated, and expression of domains dispensable for polarity (ΔLRR, myr-ΔLRR) does not provide any proliferation control, even to a polarized disc. Although we cannot exclude the possibility that for instance specific LRR mutations could create a protein incapable of polarizing tissue but still allows the proper cessation of proliferation, our failure to identify such proteins encourages the consideration of alternative models.

Several of our data indicate that proliferation control by the Scrib LRR is intimately linked to its polarity-regulating activity. As with *C. elegans* Let-413 (Legouis et al., 2003), the LRR is both necessary and sufficient to polarize epithelial cells, including embryonic cells that do not overproliferate in *scrib* mutants. An LRR-specific missense mutation causes a phenotype equivalent to the protein-null condition. By contrast, in hypomorphic mutant animals moderate disc polarity defects are accompanied by moderate proliferation defects, and in rescue experiments improvements in epithelial architecture accompany reductions in overproliferation. Because hyperproliferation itself is not sufficient to induce mispolarization (for review see Watson et al., 1994), our data are consistent with a model in which the primary role of Scrib is to govern cell polarity, and overproliferation is a consequence of polarity disruption.

The finding that polarized but nevertheless hyperproliferative *scrib 4* cells contain specific mislocalized proteins points to a possible mechanism for how polarity disruption could alter proliferation control. Our survey revealed misdistributed SJ components, but the altered shapes of *scrib 4* (as well as *scrib 5* and *j7b3*) cells suggest that additional proteins may be aberrantly or inefficiently localized. Polarized proteins include growth factor receptors, which are often clustered near cell junctions, and mislocalization of these receptors can lead to altered activity (Simske et al., 1996). Although we have not yet identified a growth-regulatory protein mislocalized in *scrib 4* discs, the role of the PDZs in Scrib localization to and assembly of the SJ suggests that such a partner might require junctional localization for efficient signaling. A test of this model, and a mechanistic understanding of Scrib function, awaits the identification of Scrib-binding partners.

The coupling of proliferation control to cell polarity demonstrated in the fly indicates that polarization loss may contribute to human oncogenesis, and not only in neoplastic tumors. It is generally thought that polarity defects are amongst the last steps during the development of carcinoma in situ, promoting primarily invasion and metastasis. Our data suggest that loss of polarity-regulating proteins might also play a role at earlier steps in tumor development by disorganizing specific growth control pathways. Future work will identify, in both flies and mammals, the effectors of cancerous properties altered in depolarized tumors.

Materials and methods

Drosophila genetics

Scrib alleles provided by K. Anderson (Memorial Sloan-Kettering Cancer Center, New York, NY; *ird15, l(3)882*; Wu et al., 2001) and M. Bender (University of Georgia, Athens, GA; *dt6*, *dt12*, *dt14*; Kidd et al., 1999) were renamed *scrib 3*, *6*, *4*, *5*, and *7*, respectively. *Scrib* animals referred to in the text are transheterozygous to the null allele *scrib 2*. Mutant eyes were generated as in Stowers and Schwarz (1999). Rescue assays used the GAL4 driver *69B* in *scrib 2/scrib 3* animals at 25° unless otherwise noted; two independent inserts were tested for each transgene rescue assay. *ptcGAL4* was used to assess transgenic protein localization in WT wing discs; localization was identical when driven by *69B*.

Scrib transgenes

The following fragments of scrib were cloned into pUASP containing a COOH-terminal GFP tag, where numbers indicate Scrib amino acids: ScribGFP (1–1751); ΔLRR (531–1751); ΔPDZs (1–692); Scrib4 (1–797); ΔPDZ3/4 (1–1105). For Myr-ΔLRR, the Src42D myristoylation sequence was added via an NH_2-terminal oligo. L223Q was introduced using a Quikchange kit (Stratagene). Inserts with comparable expression levels, assessed by Western blotting using anti-GFP antibodies (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200407158/DC1), were selected for use in rescue assays.

Cell counts

Imaginal discs were dissected from L3 larvae displaying early signs of pupariation. This staging permits overproliferation in *scrib* mutants, which occurs during an extended L3 period (Woods and Bryant, 1989). Wing discs were dissociated and counted as in Martin (1982). WT values were 30,320 cells, similar to previously published values (Garcia-Bellido and Merriam, 1971; Woods and Bryant, 1989).

Allele sequencing

Nucleic acids were isolated from homozygous *scrib* larvae and parental strains. *Scrib* exons were amplified from RNA using RT-PCR and sequenced. Mutations were confirmed by sequencing PCR products amplified from genomic DNA; at least two independent reactions were sequenced for each allele.

Microscopy

For polarity and proliferation assays, wing discs were dissected from L3 instar larvae before pupariation. Tissue was stained with rhodamine-phalloidin (Molecular Probes) and the following primary antibodies: anti-Cor (R. Fehon, Duke University, Durham, NC), anti-Elav (Developmental Studies Hybridoma Bank), or anti-Scrib PDZ2-4 (this work). Secondary antibodies were coupled to FITC or Texas red (Jackson Immunochemicals). Nuclei were visualized with DAPI. All images are single confocal sections taken with a TCS microscope (Leica) using 16×/NA 0.5 or 40×/NA 1.25 oil lenses. Transmission electron microscopy of wing discs followed standard protocols (McDonald et al., 2000), photographed on a JEOL 1200. Images were assembled using Adobe Photoshop 7.0.

Online supplemental material

Fig. S1 shows WT and *scrib* hypomorphic wing discs stained with anti-Scrib, showing normal localization but reduced levels in *scrib 5, scrib 7,* and *scrib j7b3*. Fig. S2 shows Western blots demonstrating comparable expression levels of transgene inserts used for rescue assays. Online material is available at http://www.jcb.org/cgi/content/full/jcb.200407158/DC1.

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