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Scribble protein domain mapping reveals a multistep localization mechanism and domains necessary for establishing cortical polarity

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Summary

The *Drosophila* tumor suppressor protein Scribble is required for epithelial polarity, neuroblast polarity, neuroblast spindle asymmetry and limiting cell proliferation. It is a member of the newly described LAP protein family, containing 16 leucine rich repeats (LRRs), four PDZ domains and an extensive carboxyl-terminal (CT) domain. LRR and PDZ domains mediate protein-protein interactions, but little is know about their function within LAP family proteins. We have determined the role of the LRR, PDZ and CT domains for Scribble localization in neuroblasts and epithelia, and for Scribble function in neuroblasts. We found that the LRR and PDZ domains are both required for proper targeting of Scribble to septate junctions in epithelia; that the LRR domain is necessary and sufficient for cortical localization in mitotic neuroblasts, and that the PDZ2 domain is required for efficient cortical and apical localization of Scribble in neuroblasts. In addition, we show that the LRR domain is sufficient to target Miranda protein to the neuroblast cortex, but that LRR+PDZ will exclude Miranda from the cortex. Our results highlight the importance of both LRR and PDZ domains for the proper localization and function of Scribble in neuroblasts.

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Key words: Scribble, Polarity, Asymmetry, Neuroblast, *Drosophila melanogaster*, LAP

Introduction

The establishment of polarized cortical protein domains is essential for both asymmetric cell division and proper function of many cell types, such as epithelia, neurons and muscle. Studies in *Drosophila*, *C. elegans* and mammalian epithelial cells have demonstrated that apical/basal cell polarity is created through membrane domains and junctional complexes with characteristic positions along the apical/basal axis (Bryant, 1997). In vertebrates, these are the tight junctions, adherens junctions and a basolateral domain, from apical to basal, respectively. Tight junctions promote cortical protein polarity by providing a boundary that restricts apical and basolateral proteins; arthropods may employ septate junctions for an analogous role. In *Drosophila*, epithelial cells have a similar apical/basal polarity that is reflected in the establishment of an apical membrane domain, apical adherens junctions, slightly more basal septate junctions and a basolateral membrane domain (Tepass et al., 2001). The evolutionarily conserved Par3-Par6-aPKC protein complex is targeted to the apical membrane domain and is enriched at the adherens junctions (Bachmann et al., 2001; Hong et al., 2001; Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Tepass et al., 1990; Wodarz et al., 2000). Scribble (Scrib), Lethal giant larvae (Lgl) and Discs large (Dlg) are localized to the basolateral membrane with enrichment at septate junctions, and are required for the formation of septate junctions (Bilder and Perrimon, 2000). Loss of Scrib, Lgl or Dlg leads to a disruption of septate junctions causing apical proteins such as Par3-Par6-aPKC to diffuse from the apical cortex to the basolateral cortex, resulting in defects in junctional complexes, cell morphology, and growth control in *Drosophila* epithelia (Bilder and Perrimon, 2000; Bilder et al., 2003).

A modified form of cell polarity is present in *Drosophila* mitotic neuroblasts, where it is used to generate cellular diversity by asymmetric cell division. Neuroblasts exhibit multiple aspects of asymmetry: they have distinct apical/basal cortical protein domains, an asymmetric mitotic spindle (the apical pole contains a larger centrosome and more extensive astral microtubule network) and divide asymmetrically along an apical/basal axis to regenerate a large neuroblast and bud off a small ganglion mother cell (GMC) (Albertson and Doe, 2003; Kaltschmidt et al., 2000). The GMC undergoes one subsequent cell division to generate neurons or glia (Goodman and Doe, 1993). The neuroblast apical domain includes the Par3-Par6-aPKC protein complex; yet unlike epithelial cells, also contains the protein Inscuteable (Insc) which is required to orient the mitotic spindle along the apical/basal axis (Petronczki and Knoblich, 2001; Schober et al., 1999; Wodarz et al., 2000; Wodarz et al., 1999). Neuroblasts localize several sets of proteins and mRNA to the basal cortex during mitosis:

the coiled-coil Miranda (Mira) protein, the transcription factor Prospero (Pros), the RNA-binding protein Staufen, and its cargo *pros* mRNA form one complex (Hirata et al., 1995; Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Shen et al., 1997; Spana and Doe, 1995) and Partner of numb (Pon) and Numb form another complex (Lu et al., 1998; Rhyu et al., 1994). Formation of the apical cortical domain is necessary for proper basal targeting; loss of Par3-Par6-aPKC components results in a uniform cortical distribution of Pros and Mira at metaphase (Petronczki and Knoblich, 2001; Schober et al., 1999; Rolls et al., 2003; Wodarz et al., 1999).

Over the last few years it has become clear that the *Drosophila* tumor suppressor genes – *dlg*, *scrib* and *lgl* – regulate several aspects of neuroblast cell asymmetry (Albertson and Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000). In mitotic neuroblasts, Dlg, Scrib and Lgl proteins all show cortical localization with apical enrichment. Dlg is required for Scrib localization (Albertson and Doe, 2003), but probably binds Scrib indirectly, via the linker protein Gukholder (Gukh). Gukh interacts with both the Dlg GUK domain and the Scrib second PDZ domain (Mathew et al., 2002), and is co-localized with Dlg and Scrib in mitotic neuroblasts (Albertson and Doe, 2003). Loss of Dlg, Lgl or Scrib causes a mislocalization of Pros and Mira away from the basal cortex and into the cytoplasm and onto the mitotic spindle (Albertson and Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000). Thus, Dlg/Lgl/Scrib are essential to target Mira to the cell cortex. Scrib also has important roles in promoting spindle asymmetry in neuroblasts and growth control of CNS and imaginal disc tissue (Albertson and Doe, 2003; Humbert et al., 2003).

Despite the importance of Dlg, Scrib and Lgl in regulating cellular asymmetry and growth control, little is known about how the proteins operate mechanistically. We have characterized the functional domains of the Scrib protein. Scrib protein has 16 leucine-rich repeats (LRRs) and four Psd95-Dlg-Zo1 (PDZ) domains, and is one of the founding members of the LRR and PDZ (LAP) protein family that includes *C. elegans* Let-413, rat and human Densin180, and human Erbin and hScrib (Apperson et al., 1996; Bilder and Perrimon, 2000; Borg et al., 2000; Legouis et al., 2000; Nakagawa and Huibregtse, 2000). In addition, all known LAP proteins contain a LAP-specific domain (LAPSD) containing a 38 amino acid LAPSDa that is similar to LRRs, and a 24 amino acid LAPSDb that is unrelated to LRRs (Santoni et al., 2002). Both LRR and PDZ domains function as protein-protein interaction motifs (Kobe and Kajava, 2001; Sheng and Sala, 2001). In *C. elegans*, the LRRs are necessary for cortical targeting of the Let-413 protein in epithelia (Legouis et al., 2003), and in mammals it has been shown that βPIX binds the PDZ domains of hScrib but not the closely related LAP protein Lano (Audebert et al., 2004); otherwise very little is known about the function of each domain of any LAP protein.

Materials and Methods

Constructs for germline transformation

The *scrib* deletion constructs were generated through PCR amplification of specific *scrib* regions; fragments were ligated and inserted into the pUAST vector, modified with six Myc tags, and sequenced to confirm the absence of PCR-induced mutations in the coding region. The deletion constructs encode the following Scrib protein amino acids: FL, 1-1756; ΔCT, 1-1349; LRR, 1-467; ΔPDZ, 1-730 and 1333-1756; ΔPDZ2, 1-928 and 1017-1756; PDZ, 672-1400; ΔLRR, 385-1756; CT, 1348-1756. Primer sequences for all transgenes are available upon request.

Drosophila genetics

Each *UAS-scrib:myc* deletion transgene (see above) was crossed into the *scrib*^{1} mutant background by standard genetic methods. Flies carrying *scabrous-gal4* (Bloomington stock #6479) or *worniu-gal4* (the 8 kb upstream sequence of the *worniu* gene driving expression of *gal4*) were also crossed into the *scrib*^{1} mutant background. *scrib*^{1} is reported to be a null allele, and homozygous *scrib*^{1} stage 15, or older, embryos show no protein staining with our C-terminal Scrib antisera. Expression of each *UAS-scrib:myc* transgene was assayed in progeny from *UAS-scrib:myc*; *scrib*^{1}*/TM3, ftzlacZ* crossed to *scabrous-* or *worniu-gal4; scrib*^{1}*/TM3, ftzlacZ* crosses. *scrib* mutant embryos were identified by the lack of anti-β-galactosidase staining, while Scrib:myc proteins were detected by anti-Myc staining.

Immunohistochemistry, western blots, and quantification

Embryos were collected and fixed according to the method of Albertson and Doe (Albertson and Doe, 2003). Primary antibodies were used at 1:1000, except where indicated: rabbit and mouse anti-Scrib C-terminal polyclonal antibody (1:2500) (Albertson and Doe, 2003), rabbit and mouse anti-Myc (Santa Cruz), rabbit anti-phosphohistone H3 (1:1500; Upstate), mouse anti-α-tubulin (1:3000), rat anti-Mira (1:500), rabbit and rat anti-β-galactosidase (1:1500; Cappel), rat anti-aPKC (1:500), and mouse anti-Dlg (1:500). Western blot analysis followed the method of Srinivasan et al. (Srinivasan et al., 1998) using *sca-gal4, UAS-scrib:myc* embryos grown at 29°C. Cell size quantitation was done by averaging the longest axis and the orthogonal axis of the apical or basal cortical domain in telophase neuroblasts.

Results

Generation and characterization of Scrib domain constructs

The *scrib* gene encodes a 1766 amino acid protein (hereafter referred to as Scrib) that contains 16 LRRs, LAPSDa/b domains and four PDZ domains; this is the form of the protein we have worked with in this investigation. There is also a shorter 1247 amino acid Scrib2 protein that has the 16 LRRs, LAPSDa/b and the first two PDZ domains (Li et al., 2001), which we did not analyze. We made *scrib* deletion transgenes with a Myc epitope tag (Fig. 1; see Materials and Methods) and expressed them using the Gal4/UAS system (Brand and Perrimon, 1993). We used *scabrous-gal4* to drive expression in both epithelia and neuroblasts, or *worniu-gal4* to drive expression in neuroblasts but not epithelia. Importantly, both *sca-gal4* and *worniu-gal4* drive low to moderate levels of Scrib:myc protein: enough to rescue the *scrib* mutant phenotype using full length Scrib but not so much as to cause dominant phenotypes in otherwise wild-type cells. In each case the level of the Scrib:myc transgene appeared roughly equal to the level of endogenous Scrib protein (compare Fig. 2A with 2B-H, collected at the same confocal settings) and when embryos were double-labeled with an anti-Scrib antibody directed against the Scrib C-terminal (CT) domain and an anti-myc antibody that recognized the Scrib:myc fusion protein (data not shown; this comparison was only done for Scrib:myc proteins lacking the C-terminal domain).

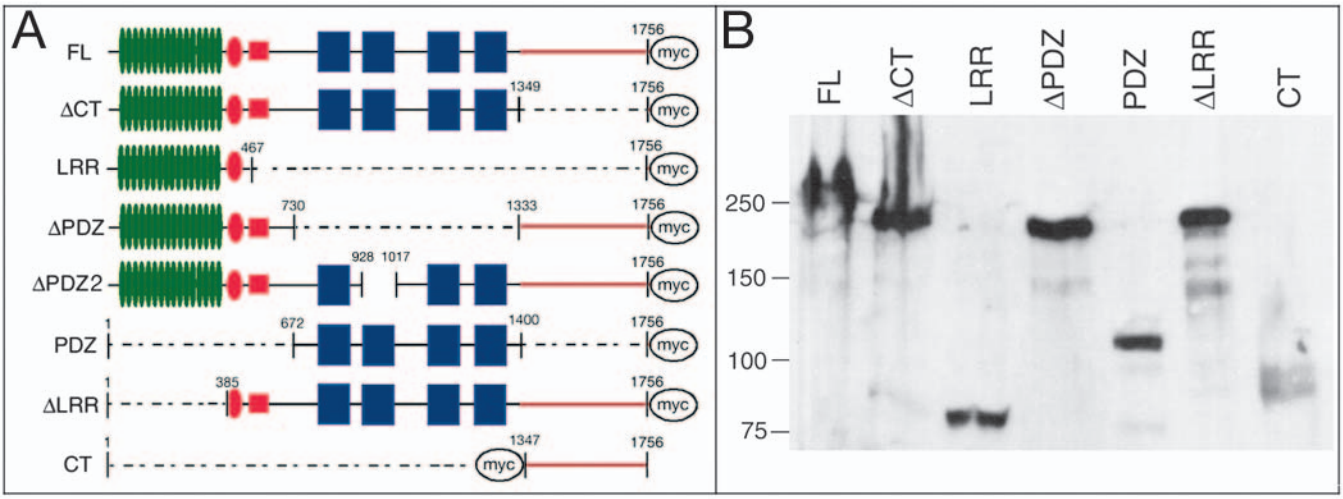


Fig. 1. Generation and characterization Scrib domain constructs. (A) Schematic representation of the seven different Scrib:myc proteins used in this study. LRR repeats (green ovals), LAPSDa (red oval), LAPSDb (red square), PDZ domains (blue boxes) and C-terminal region (red line) are indicated. FL is the full-length Scrib; ΔCT lacks the C-terminal region; LRR contains only the N-terminal LRR region; ΔPDZ lacks the central PDZ region; PDZ contains only the central PDZ region; ΔLRR lacks the N-terminal LRR region; CT contains only the C-terminal region. The numbers above each line represent the amino acids present at the beginning or end of each domain. All constructs carry six myc tags (white circle). (B) Western blot showing the size and stability of Scrib:myc proteins in vivo. Embryos expressing each Scrib:myc proteins (progeny of *Sca-Gal4* × *UAS-scrib:myc* crosses) were homogenized, run in the indicated lanes and the western blot was probed with a Myc antibody.

We analyzed the localization and function of all Scrib:myc deletion transgenes in a $scrib^1/scrib^1$ homozygous mutant background (Figs 2-4), with the exception of the ΔPDZ2 protein which was analyzed in a wild-type background (see Fig. S1 in supplementary material). Maternally provided Scrib protein is detectable in $scrib^1/scrib^1$ embryos up to embryonic stage 14, but not at stage 15 (Albertson and Doe, 2003), so we only assay Scrib:myc localization and function in $scrib^1/scrib^1$ embryos at stage 15 or later.

Distinct Scrib domains regulate cortical and apical protein targeting in neuroblasts

Endogenous Scrib protein is cortical in neuroblasts, with apical enrichment from late interphase into metaphase (Fig. 2A) (Albertson and Doe, 2003). To determine the domains of Scrib that regulate cortical and apical targeting, we assayed localization of each Scrib:myc protein in *scrib* mutant neuroblasts at embryonic stage 15 (Fig. 2A-H, quantified in Fig. 2I). Full length Scrib (FL) and Scrib lacking the carboxyl-terminal domain (ΔCT) showed normal localization in metaphase and interphase neuroblasts, in both *scrib* mutants (Fig. 2B,C) and wild type (data not shown). This indicates that the LRR and PDZ domains are sufficient for normal Scrib localization in neuroblasts and that the extensive C-terminal domain is not required for Scrib localization.

The two Scrib proteins that lack the PDZ domains (LRR and ΔPDZ proteins) showed uniform cortical association with occasional weak apical enrichment in metaphase neuroblasts, in both *scrib* mutant neuroblasts (Fig. 2D,E; left panels) and wild-type neuroblasts (data not shown). However, they showed different localization in interphase neuroblasts: the ΔPDZ protein is cortical and cytoplasmic, whereas the LRR protein is cortical and nuclear (Fig. 2D,E; center panels). The difference in nuclear/cytoplasmic localization may be regulated by the LAPSDb domain, which is present in the ΔPDZ protein but not the LRR protein (see Discussion).

Next we wanted to test the role of PDZ2 in Scrib protein localization, because Scrib PDZ2 binds the Gukh protein (Mathew et al., 2002) and Gukh and Scrib are co-localized at the apical cortex of neuroblasts (Albertson and Doe, 2003). We made a *scrib* transgene lacking only the second PDZ (ΔPDZ2) and found that this Scrib protein shows weak apical enrichment and cytoplasmic localization in mitotic neuroblasts, similar to the Scrib protein lacking all four PDZ domains (see Fig. S1 in supplementary material). Thus, the Scrib PDZ2 is essential for proper cortical and apical localization of the Scrib protein.

The three Scrib proteins that lack the LRRs are cytoplasmic at all stages of the cell cycle in *scrib* mutant neuroblasts (Fig. 2F-H) and wild-type neuroblasts (data not shown). Thus, the LRR domain is necessary and sufficient for cortical localization. It may also play a minor role in apical targeting, because Scrib proteins containing the LRR only (LRR, ΔPDZ, ΔPDZ2) show weak apical enrichment in a minority of neuroblasts.

We have been unable to generate a Scrib transgenic line that specifically lacks the LAPSDa or LAPSDb, but we can draw several conclusions from our existing Scrib deletion transgenic lines. First, the Scrib LRR protein lacks the LAPSDb domain and yet is still targeted to the cortex in mitotic neuroblasts; thus, the LAPSDb is not required for Scrib cortical targeting in neuroblasts. Second, LAPSDb domain may have a function in regulating nuclear localization of Scrib: the LRR protein missing LAPSDb is nuclear, whereas the closely related ΔPDZ protein containing LAPSDb is excluded from the nucleus; thus the LAPSDb may provide a nuclear export signal or mask a nuclear import signal within the Scrib LRR protein (see Discussion). Third, the Scrib ΔPDZ and ΔPDZ2 proteins contain both LAPSDa and LAPSDb domains and localize to the neuroblast cortex, yet they fail to be apically enriched in

mitotic neuroblasts; thus, the paired LAPSDa/b domains are not sufficient for proper apical Scrib localization.

The LRR and PDZ2 domains are both required for Scrib localization in epithelia

Endogenous Scrib is targeted to the septate junction and lateral membrane domain in epithelia (Fig. 2A) (Bilder and Perrimon, 2000). We assayed the localization of Scrib:myc proteins in *scrib* mutant epithelia (Fig. 2; right column). The same patterns were seen in wild-type epithelia containing endogenous Scrib protein (data not shown). We found that Scrib proteins that contain both LRR and PDZ domains (i.e. FL and ΔCT) show normal localization to the lateral membrane and enrichment at the septate junction (Fig. 2B,C), indicating the C-terminal region is dispensable for proper Scrib localization in epithelia.

In contrast, Scrib proteins that lack PDZ domains (LRR, ΔPDZ, ΔPDZ2 proteins) or lack the LRR domain (PDZ, ΔLRR) showed only weak localization to septate junctions (Fig. 2D-G; see Fig. S1 in supplementary material). The LRR protein was detected in the nucleus (Fig. 2D), while the ΔPDZ and ΔPDZ2 proteins were cytoplasmic (Fig. 2E; see Fig. S1 in supplementary material), similar to the localization of each protein in interphase neuroblasts. Finally, the Scrib CT protein that lacks both LRR and PDZ domains was completely cytoplasmic (Fig. 2H). Taken together, we conclude that both LRR and PDZ2 domains are required for efficient targeting of Scrib to the lateral membrane and septate junctions of epithelial cells. We can also conclude that the LAPSDa and LAPSDb domains, which are both present in the ΔPDZ and ΔPDZ2 proteins (and each present in the LRR and ΔLRR proteins, respectively), are insufficient for normal Scrib targeting in epithelia.

Mira requires Scrib LRRs for cortical targeting and Scrib PDZ domains for efficient basal localization

Scrib is required to target the Mira cell fate scaffolding protein to the basal cortex of mitotic neuroblasts; in *scrib* mutants Mira accumulates in the cytoplasm, on the mitotic spindle and uniformly around the cortex (Fig. 3A,B) (Albertson and Doe, 2003). We expressed different Scrib domains in *scrib* mutant neuroblasts and scored for rescue of proper Mira localization (Fig. 3C-I). We found that only the Scrib proteins containing both LRR and PDZ domains (i.e. FL and ΔCT, both apically enriched; Fig. 2) have the

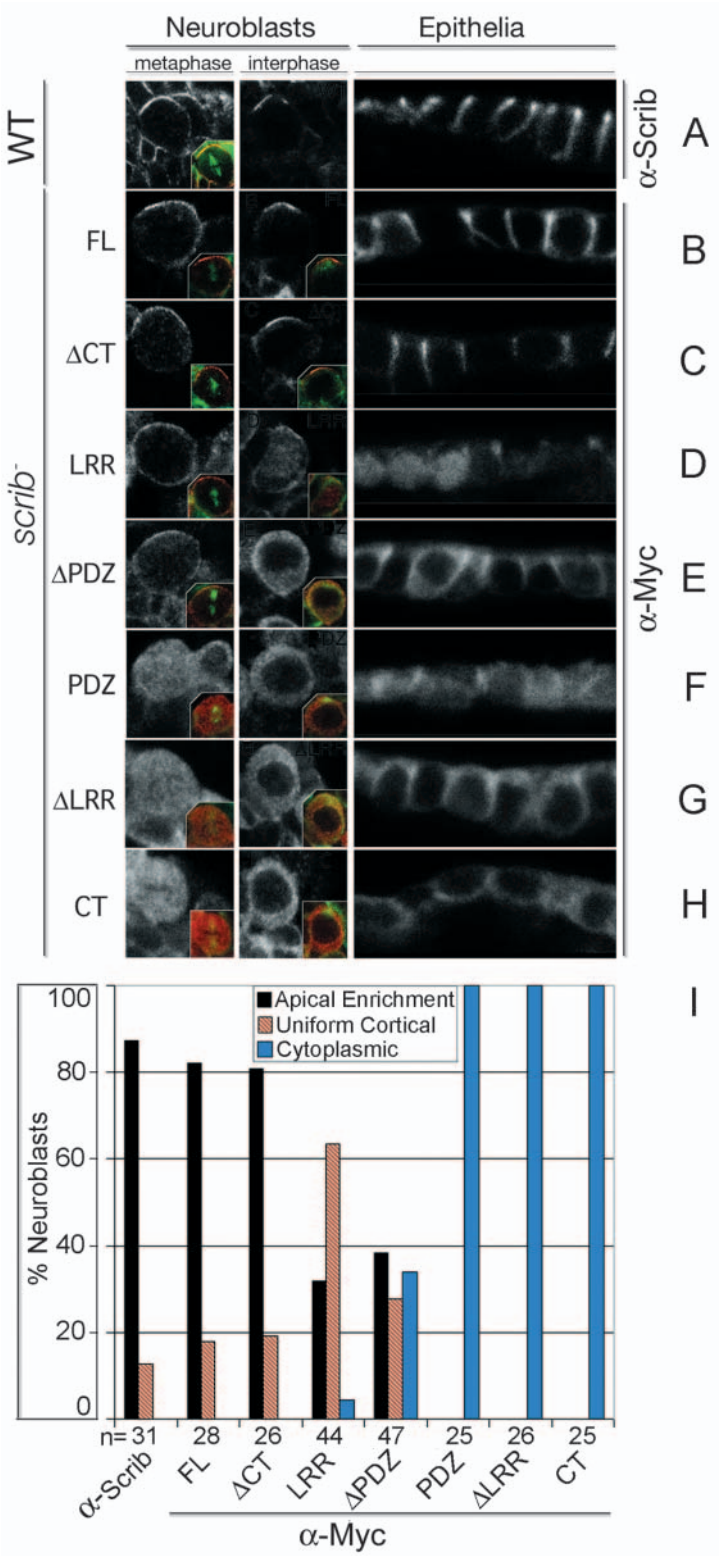


Fig. 2. Scrib domain localization in *scrib* mutant neuroblasts and epithelia of stage 15 embryos. Left column, metaphase neuroblasts; middle column, interphase neuroblasts; right column, interphase epithelial cells; apical up for all images. The genotype is indicated to the left of each row; antibodies used to detect endogenous Scrib (A) or Scrib:myc (B-H) are indicated to the right of each row. (A) Wild-type embryos showing endogenous Scrib protein localization. (B-H) $scrib^{1}/scrib^{1}$ embryos showing localization of the indicated Scrib:myc proteins detected with Myc antibody. Each *UAS-scrib:myc* transgene was expressed using *worniu-gal4* (for the neuroblasts) or *scabrous-gal4* (for the epithelial). Insets show the same neuroblast stained for α-tubulin (green) as a cell cycle indicator and Scrib (A) or Scrib:myc (B-H) protein (red). Scrib domain abbreviations are given in Fig. 1. (I) Quantification of endogenous Scrib and Scrib domain localization in stage 15 metaphase neuroblasts. Apical enrichment, black bar; uniform cortical localization, crosshatched red bar; cytoplasmic localization, blue bar. Scrib domain abbreviations are given in Fig. 1.

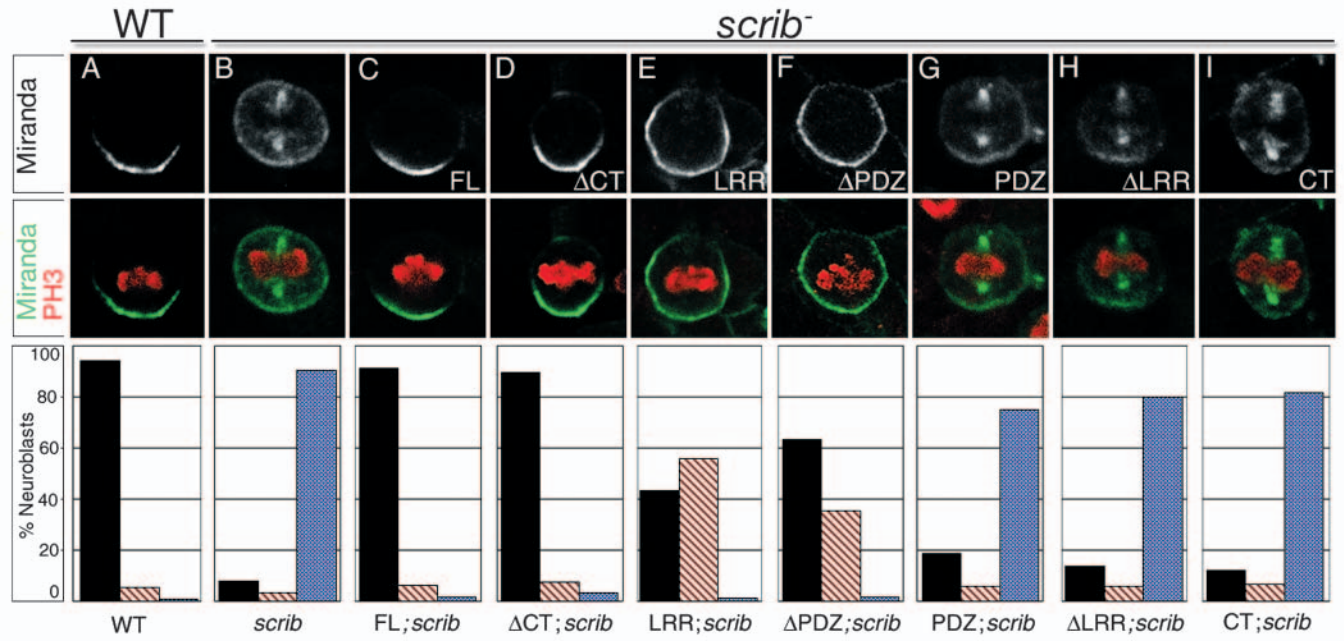


Fig. 3. Scrib domain regulation of Mira basal localization in stage 15 neuroblasts. Top row, Mira localization in metaphase neuroblasts; middle row, the same neuroblasts showing both Mira (green) and the mitotic DNA marker phosphohistone H3 (red); lower row, quantification of the Mira localization phenotype: normal basal crescent, black bar; uniform cortical localization, crosshatched bar; uniform cortical plus cytoplasmic and spindle-associated, blue bar; apical up for all images. Scrib domain abbreviations are given in Fig. 1. (A) Wild-type neuroblast showing normal Mira basal crescent. (B) *scrib*^{1}/*scrib*^{1} neuroblast showing cytoplasmic and spindle-associated Mira. (C-I) *scrib*^{1}/*scrib*^{1} embryos expressing the indicated Scrib:myc transgene (progeny from *UAS-scrib:myc* x *worniu-gal4*). (C,D) FL and ΔCT proteins provide full rescue of basal Mira localization. (E,F) LRR and ΔPDZ result in predominantly uniform cortical Mira localization or cortical with weak basal enrichment. (G-I) PDZ, ΔLRR and CT proteins give no rescue of Mira cortical or basal localization.

ability to fully rescue Mira basal localization (Fig. 3C,D). Thus, the extensive C-terminal Scrib domain is unnecessary for proper Mira localization.

Scrib proteins containing LRR but not PDZ domains (i.e. LRR or ΔPDZ; both cortical with weak apical enrichment; Fig. 2) can fully restore Mira cortical localization but only partially rescue basal localization (Fig. 3E,F). Mira was either uniformly cortical (58% and 38% in LRR and ΔPDZ proteins, respectively) or formed an expanded basal crescent that is not properly excluded from the apical cortical domain (Fig. 3E,F). This shows that the LRR domain alone is sufficient to promote Mira cortical localization, but is inefficient in restricting Mira to the basal cortex. This could be due to the lower levels of the Scrib LRR protein at the apical cortex or because it lacks a specific function provided by the PDZ domains (see Discussion). Furthermore, the LAPSDa/b domains, which are both present in the ΔPDZ protein, are not sufficient for basal targeting of Mira; and deletion of the LAPSDb domain (in the LRR protein) does not block Mira cortical localization.

All Scrib proteins lacking the LRR domain are cytoplasmic (Fig. 2) and are non-functional for Mira localization (Fig. 3G-I), even if they contain most of the LAPSDa/b domains. We conclude that the LRR domain is necessary and sufficient for cortical Mira targeting; that both LRR and PDZ domains are necessary for efficient basal Mira targeting; and that the LAPSDa/b domains are not sufficient for Mira basal targeting.

We also tested the effect of each Scrib domain on the localization of several other neuroblast cell polarity markers. Wild-type neuroblasts show apical localization of the Bazooka-Par6-aPKC-Insc-Pins complex, and apical enrichment of the Dlg-Gukh-Scrib-Lgl proteins. We assayed aPKC as a representative of the first complex and Dlg as the most 'upstream' component of the second group (Fig. 4). In *scrib* zygotic mutant stage 15 neuroblasts we found that aPKC and Dlg are normally localized to the apical cortex (Albertson and Doe, 2003; Kaltschmidt et al., 2000) (data not shown); expression of each Scrib domain has no effect on the apical localization of either aPKC or Dlg (Fig. 4). We conclude all tested Scrib domains had no deleterious effect on apical cortical polarity in neuroblasts.

Scrib LRR and PDZ domains cooperate to establish cell size asymmetry and spindle asymmetry

In *scrib* mutants only 65% of the neuroblasts have normal mitotic spindle and cell size asymmetry at telophase, compared to over 95% of wild-type neuroblasts (Fig. 5) (Albertson and Doe, 2003).We assessed the ability of each Scrib domain to rescue the *scrib* mutant cell size and spindle asymmetry defects. Expression of FL and ΔCT proteins significantly restores normal cell size asymmetry (91% and 85% normal; Fig. 5), indicating the C-terminal region is not required for this aspect of Scrib function. In contrast, proteins lacking the LRR or PDZ domains were unable to re-establish proper cell size asymmetry: we observed a similar percentage of symmetric and inverted telophase neuroblasts as compared to *scrib* mutant embryos (66%-73% normal divisions verses 65% normal in *scrib* embryos; Fig. 5). The presence of the LAPSDa/b

domains (e.g. in the ΔPDZ protein) were unable to rescue this phenotype. We conclude that both the LRR and PDZ domains are required for establishing mitotic spindle and cell size asymmetry in neuroblasts, and that LRRs, PDZs or LAPSDa/b domains individually are not sufficient to confer cell size and mitotic spindle asymmetry.

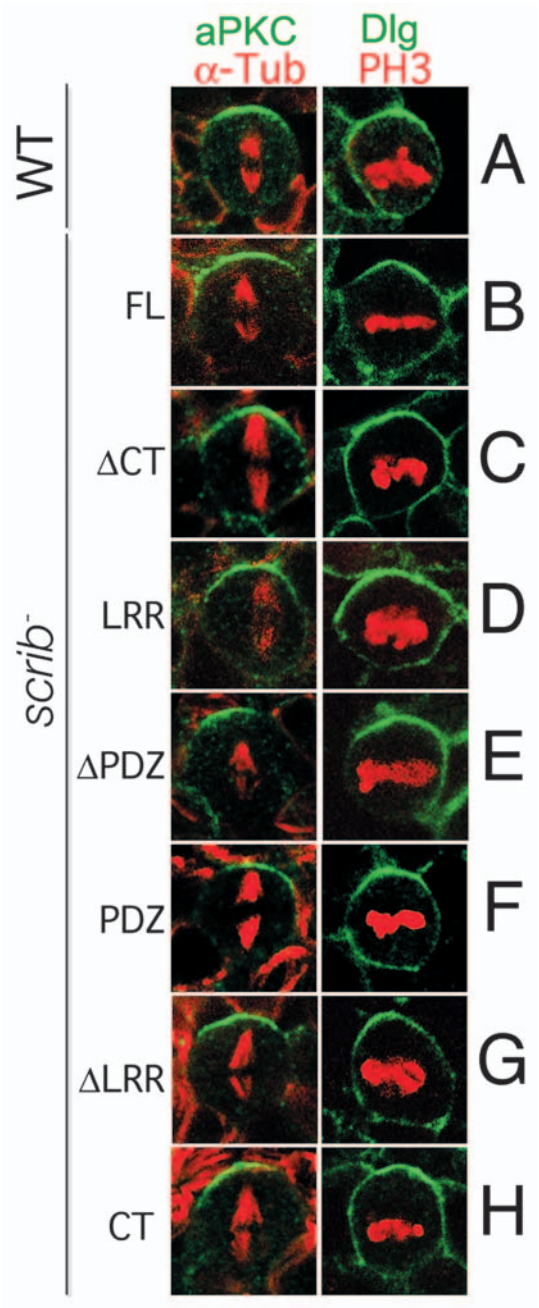


Fig. 4. Scrib domain regulation of apical protein localization in stage 15 neuroblasts. Embryonic neuroblasts stained for the apical proteins aPKC or Dlg (green) and mitotic markers α-tubulin or PhosphohistoneH3 (PH3; red). Stage 15 embryos, apical up for all images. Scrib domain abbreviations given in Fig. 1. (A) Wild-type metaphase neuroblast showing apical localization of aPKC and Dlg. (B-H) *scrib*^{1}/*scrib*^{1} metaphase neuroblasts expressing the indicated Scrib:myc transgene (progeny from *UAS-scrib:myc* x *worniu-gal4*). All show normal localization of aPKC and Dlg.

Discussion

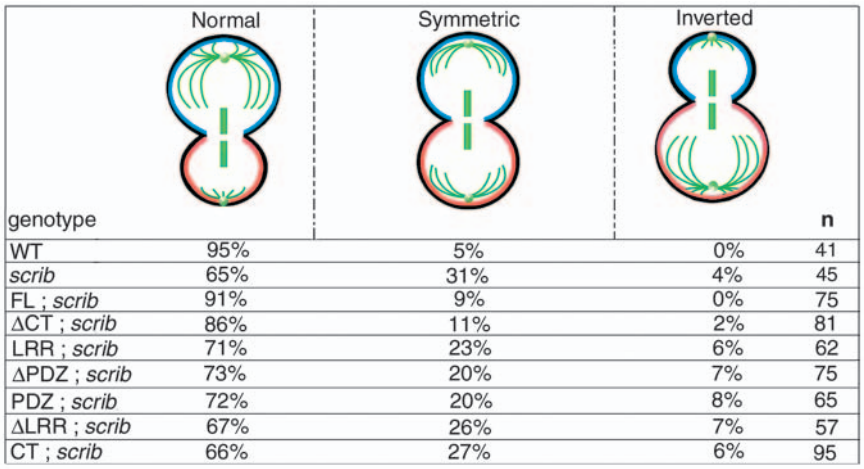
Scrib LRRs regulate cortical protein localization

Our results demonstrate that the Scrib LRRs are absolutely required for Scrib cortical localization in neuroblasts and epithelial cells. How might the LRRs direct Scrib to the plasma membrane? Little is known about binding specificities of LRRs in LAP proteins, yet LRRs in other cytoplasmic, transmembrane and extracellular proteins have been extensively studied. One LRR subfamily has an LRR-containing extracellular domain which is responsible for high-affinity binding to ligands (Battye et al., 2001; el Tayar, 1996; Hocking et al., 1998). Another LRR protein subfamily binds small cytoplasmic GTPases, such as Ras and Ran (Haberland and Gerke, 1999; Sieburth et al., 1998; Suzuki et al., 1990). Based on these reports, activated small G proteins (Cdc42, Rac1, Ran) are candidates for cortical targeting of the Scrib LRR domain, although the distribution of these proteins in neuroblasts has not been determined, nor have they been tested for physical interactions with Scrib LRRs.

Scrib PDZ2 is required for efficient apical targeting in neuroblasts

Scrib proteins lacking all four PDZ domains, or just PDZ2, are detected in the cytoplasm and uniformly around the neuroblast cortex, with only occasional weak apical enrichment. Scrib PDZ2 is likely to target Scrib to the neuroblast cortex, and more specifically to the apical neuroblast cortex, by associating with Gukh and Dlg proteins. Gukh binds to both Scrib PDZ2 and Dlg (Albertson and Doe, 2003; Mathew et al., 2002); Scrib, Gukh and Dlg are all co-localized at the neuroblast cortex (Albertson and Doe, 2003); *dlg* mutants have cytoplasmic Scrib protein (Albertson and Doe, 2003). One simple model consistent with these data is that Dlg binds Gukh, which binds Scrib PDZ2, resulting in the observed localization of Scrib within neuroblasts. Nevertheless, the PDZ domains are not sufficient for proper Scrib localization: Scrib proteins that contain the PDZs but lack the LRRs (ΔLRR or PDZ) are completely cytoplasmic. We propose a two-step model for Scrib localization in which Scrib LRR-dependent cortical localization precedes and is a prerequisite for Scrib PDZ2-dependent apical enrichment. We note that proteins lacking all four PDZ domains still show weak apical enrichment in some neuroblasts, however, indicating a minor role for the LRR domain in apical Scrib targeting.

Our data suggest a mechanism in which Scrib neuroblast and epithelial localization involves distinct steps, moving from cytoplasm to cortex then apically. Other proteins targeted to specific cortical domains within neuroblasts show a similar cortical-to-asymmetric localization mechanism. The Insc protein contains a 158 aa region that directs Insc to the neuroblast cortex; the addition of a separate 100 aa domain confers apical localization (Knoblich et al., 1999; Yu et al., 2002). Similarly, the Pins protein contains four C-terminal GoLoco repeats that are sufficient for cortical localization, but addition of the first three of seven N-terminal tetratricopeptide repeats (TPRs) is required for asymmetric apical targeting (Yu et al., 2002). Stepwise targeting of Dlg has also been observed in epithelia and neuromuscular junctions. In epithelia, the Hook domain targets Dlg to the cell cortex while PDZ2 is required to restrict Dlg to the septate junction (Hough et al.,



genotype	Normal	Symmetric	Inverted	n
WT	95%	5%	0%	41
scrib	65%	31%	4%	45
FL ; *scrib*	91%	9%	0%	75
ΔCT ; *scrib*	86%	11%	2%	81
LRR ; *scrib*	71%	23%	6%	62
ΔPDZ ; *scrib*	73%	20%	7%	75
PDZ ; *scrib*	72%	20%	8%	65
ΔLRR ; *scrib*	67%	26%	7%	57
CT ; *scrib*	66%	27%	6%	95

Fig. 5. Scrib domain regulation of mitotic spindle and cell size asymmetry in neuroblasts. Stage 15 telophase neuroblasts (identified as Pins$^+$ and Mira$^+$) were scored for cortical markers, spindle morphology, and cell size. Schematic of the phenotypic categories is shown at the top: apical cortex, blue (Pins); basal cortex, red (Mira); mitotic spindle and centrosomes, green (α-tubulin). Cell size ratios are apical/basal and are defined as: normal, >1.25; symmetrical, 0.8-1.25; inverted, <0.8. Wild-type neuroblasts divide asymmetrically. *scrib^1/scrib^1* neuroblasts show only 65% asymmetric divisions. *scrib^1/scrib^1* neuroblasts expressing FL or ΔCT proteins rescue normal asymmetric divisions. *scrib^1/scrib^1* neuroblasts expressing LRR, ΔPDZ, PDZ, ΔLRR, or CT proteins fail to rescue asymmetric cell division.

1997). At neuromuscular junctions, the Hook domain is necessary to localize Dlg to the plasma membrane and both PDZ1 and PDZ2 domains promote transport to the synapse (Thomas et al., 2000). These observations suggest cortical localization may be a prerequisite for subsequent targeting to specific membrane domains and raise the possibility that similar transport mechanisms are shared among protein complexes in neuroblasts, epithelia and synapses.

Despite the fact that Scrib has a plethora of protein-protein interaction motifs, only one binding partner for *Drosophila* Scrib has been identified, Gukh, which binds to Scrib PDZ2 (Mathew et al., 2002). Binding assays such as yeast two-hybrid assays and mass spectrometry will be essential to identify additional *Drosophila* Scrib binding partners, as has been shown for mammalian LAP proteins (Audebert et al., 2004). Such discoveries will enable us to determine upstream and downstream players in Scrib-mediated pathways and will further our knowledge of many cellular functions, including establishment of epithelial cell polarity, stem cell division, mitotic spindle biology, cell cycle progression and synapse formation and homeostasis.

Scrib LRR and PDZ domains are required for efficient septate junction targeting in epithelia

Scrib proteins lacking LRRs fail to be properly targeted to the lateral membrane and septate junctions of mature epithelial cells, whereas Scrib proteins lacking all four PDZ domains, or just PDZ2, still show some septate junction enrichment with an elevated level of cytoplasmic protein. Thus, the LRR domains are the primary determinant of cortical/septate junction targeting, and PDZ2 increases the efficiency or stability of this localization. Scrib localization in epithelia also requires Dlg (Bilder and Perrimon, 2000), but the role of the Dlg and Scrib PDZ2-binding protein Gukh have not been examined in epithelia. We could not test the ability of each Scrib domain to establish or maintain epithelial polarity in this system, however, because zygotic *scrib* mutant embryos develop normal epithelial polarity because of maternally derived Scrib protein (even though there is no detectable maternal Scrib protein in stage 15 or later embryos).

The proteins that interact with the Scrib LRRs to mediate cortical association in epithelia are unknown. More is known about the role of PDZ domains in junctional targeting of LAP proteins. The mammalian LAP proteins Erbin and Densin180 show PDZ-mediated interactions with p120-catenins (Izawa et al., 2002a; Izawa et al., 2002b; Jaulin-Bastard et al., 2002; Laura et al., 2002), and Erbin-catenin p0071 colocalize to adherens junctions and desmosomes in cultured epithelial cells (Izawa et al., 2002b; Jaulin-Bastard et al., 2002). Disruption of Erbin-p0071 interactions leads to aberrant cell morphology and disruption of cell-cell contacts (Jaulin-Bastard et al., 2002). Similarly, *Drosophila* Scrib is required for proper morphology and formation of septate junctions in epithelia (Bilder and Perrimon, 2000), and we find the PDZ domains are necessary for efficient Scrib localization to the epithelial cell septate junctions. Thus, *Drosophila* catenins are excellent candidates for recruiting Scrib to the septate junction.

Distinct Scrib domains regulate cortical and basal targeting of Mira

In the absence of all Scrib function, Mira is predominantly localized to the cytoplasm and mitotic spindle of neuroblasts. Expression of just the Scrib LRR domain results in uniform cortical Scrib LRR distribution and the restoration of uniform cortical Mira localization. Conversely, all Scrib proteins that lack the LRR domain (PDZ, ΔLRR and CT) fail to efficiently target Mira to the cortex. These results reveal a positive role for the Scrib LRR domain in targeting Mira to all regions of the neuroblast cortex. A similar ‘uniform cortical Mira’ phenotype is also observed in certain *aPKC* and *lgl* genetic backgrounds. Neuroblasts lacking aPKC show uniform cortical Mira (Rolls et al., 2003) and neuroblasts misexpressing a dephospho-Lgl protein also show uniform cortical Mira (Betschinger et al., 2003). In addition, loss of *lgl* leads to cytoplasmic Mira localization in neuroblasts (Albertson and Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000). This has led to a model in which the apically-localized aPKC phosphorylates Lgl to inactivate it, thus restricting active dephospho-Lgl to the basal cortex, where it promotes cortical localization of Mira (Betschinger et al., 2003). The Scrib LRRs could act upstream of aPKC and Lgl, perhaps by blocking aPKC/Lgl interactions, and thus allowing activated Lgl to target Mira to the entire cortex. Alternatively, the Scrib LRRs could act downstream of aPKC and Lgl, perhaps by allowing

Table 1. Summary of Scrib domain localization and function

Gentotype	Scrib localization			Neuroblast phenotype			
	Epidermis (inter)	Neuroblast (inter)	Neuroblast (meta)	Mira	aPKC	Dlg	Spindle, cell size
wild type	sj/lat	apical	apical	basal	apical	apical	asym
scrib^{-}	–	–	–	cyto	apical	apical	asym/sym
scrib^{-}+FL	sj/lat	apical	apical	basal	apical	apical	asym
scrib^{-}+ΔCT	sj/lat	apical	apical	basal	apical	apical	asym
scrib^{-}+LRR	sj/cyto	nucl	uni	uni	apical	apical	asym/sym
scrib^{-}+ΔPDZ	sj/cyto	cyto	cyto/uni	uni	apical	apical	asym/sym
scrib^{-}+ΔPDZ2	sj/cyto	cyto	cyto/uni	na	na	na	na
scrib^{-}+PDZ	sj/cyto	cyto	cyto	cyto	apical	apical	asym/sym
scrib^{-}+ΔLRR	sj/cyto	cyto	cyto	cyto	apical	apical	asym/sym
scrib^{-}+CT	cyto	cyto	cyto	cyto	apical	apical	asym/sym

All genotypes (except wild type) are *scrib*^{1}/*scrib*^{1} plus expression of the indicated Scrib transgenes (abbreviations as in Fig. 1) expressed by the *worniu-gal4* transgene (for neuroblasts) or the *sca-gal4* transgene (for epithelia). Scrib localization scored in stage 15 neuroblasts and epithelia; neuroblast phenotype scored in stage 15 mitotic neuroblasts. inter, interphase; meta, metaphase; sj, septate junction; lat, lateral membrane; apical, apical cortical; basal, basal cortical; uni, uniform cortical; cyto, cytoplasmic; nucl, nuclear; asym, asymmetric; sym, symmetric; na, not assayed.

both dephospho- and phospho-Lgl to target Mira to the cortex. In addition, loss of *jaguar* (myosin VI) leads to cytoplasmic localization of Mira (Petritsch et al., 2003), raising the possibility that the Scrib LRRs could stimulate myosin VI activity around the neuroblast cortex to promote uniform cortical Mira localization. The identification of Scrib LRR-binding proteins will help distinguish between these models.

Addition of the PDZ domains back to the Scrib LRR protein dramatically alters the function of the protein. Whereas the Scrib LRR protein is uniformly cortical and promotes Mira cortical localization, Scrib LRR+PDZ proteins (FL, ΔCT) are apically enriched and exclude Mira from the apical cortex. Thus, addition of the PDZ domains switches Scrib from promoting cortical Mira localization to excluding cortical Mira localization. The PDZ domains could carry out this function of excluding Mira from the apical cortex in at least three different ways. (1) The Scrib PDZ domains could promote aPKC-Lgl interactions, thereby leading to the phosphorylation and inactivation of apical Lgl (Betschinger et al., 2003); this would restrict active Lgl to the basal cortex, where it promotes cortical Mira localization. (2) The Scrib PDZ domains could promote myosin II (zipper) activity at the apical cortex; myosin II is a known inhibitor of Lgl (Ohshiro et al., 2000; Peng et al., 2000b), and thus this would restrict active Lgl to the basal cortex where it could promote cortical Mira localization. (3) The Scrib PDZ domains could provide directionality to the actin-myosin VI cytoskeleton, which could transport Mira specifically to the basal cortex (Petritsch et al., 2003). Identification of proteins that interact with the Scrib PDZ domains would help distinguish between these models.

Scrib LRR and PDZ domains are both required to establish mitotic spindle and cell size asymmetry

Little is known about how Dlg, Scrib and Lgl regulate cell size asymmetry and spindle asymmetry. We show both LRR and PDZ domains of Scrib are necessary for this function. How might Scrib regulate cell size and spindle asymmetry? Two good candidate effectors are Ran GTPase and Pins. LRRs are known to physically interact with Ran, which promotes spindle assembly through several target proteins (Dasso, 2001; Haberland et al., 1997; Haberland and Gerke, 1999). For example, Ran stimulates the activity of NuMA (a microtubule motor accessory protein that promotes spindle assembly) by destabilizing inhibitory complexes associated with NuMA (Dasso, 2001; Dasso, 2002; Nachury et al., 2001; Wiese et al., 2001). LGN (a mammalian Pins homolog) is essential for mitotic spindle assembly and binds NuMA; release from LGN is an important event in the activation of mitotic NuMA (Dasso, 2002; Du et al., 2001; Du et al., 2002). In *Drosophila*, Pins physically interacts with Dlg and is asymmetrically localized to the apical cortex of mitotic neuroblasts, where it promotes spindle asymmetry (Bellaiche et al., 2001; Cai et al., 2003; Parmentier et al., 2000; Schaefer et al., 2000). These data suggest possible links between Dlg, Scrib, Ran and Pins and establishment of mitotic spindle asymmetry. Genetic and biochemical studies investigating interactions between Scrib, Ran and Pins may further our understanding of spindle asymmetry establishment in *Drosophila* neuroblasts.

The role of the LAPSDa/b and CT domains

Deletion of the CT domain has no effect on Scrib localization or its ability to rescue all tested *scrib* mutant phenotypes in neuroblasts; the CT domain alone is cytoplasmic and has no rescuing ability in any assay that we performed. We conclude that the CT domain is not essential for any aspect of Scrib localization or function that we have tested (summarized in Table 1).

We have not assayed a *scrib* transgenic line that specifically lacks the LAPSDa/b domains, however, we can draw some conclusions based on our existing Scrib domain analysis. The Scrib ΔPDZ protein contains both LAPSDa/b domains is membrane targeted but not enriched apically, it fails to promote basal Mira targeting, and it is defective for asymmetric mitotic spindle and cell size asymmetry. Thus, the LAPSD domains are insufficient for apical enrichment of Scrib and all of its tested functions in neuroblasts. The Scrib LRR protein lacking the LAPSDb domain is still membrane-associated, showing that the LAPSDb domain is not required for Scrib membrane targeting. We did find some evidence that the LAPSDb domain regulates nuclear import/export of the Scrib protein. The LRR protein contains just the LRRs and the LAPSDa domain and is targeted to the nucleus; this shows that there is a nuclear import signal or binding site for a nuclear protein within the LRR/LAPSDa domains, although a predicted nuclear

localization signal is not detectable within these domains. In contrast, the ΔPDZ protein contains the same LRR/LAPSDa domains plus the LAPSDb and CT domains, and it is excluded from the nucleus. This shows that the LAPSDb or CT domains can prevent nuclear import of the LRR/LAPSDa protein; is highly likely that this function is provided by the LAPSDb domain, because deletion of the CT domain from an otherwise wild-type Scrib protein (i.e. ΔCT) does not result in nuclear localization. Our results are in contrast to the role of the LAPSDa/b domains in the related *C. elegans* Let-413 protein, where the LAPSDa/b domains are required for establishing epithelial polarity but not Let-413 protein localization (Legouis et al., 2003). It will be interesting to determine whether the Scrib LAPSDa/b domains play a similar role in Scrib epithelial localization.

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