The apical/basal-polarity determinant Scribble cooperates with the PCP core factor Stbm/Vang and functions as one of its effectors

Jean-Remy Courbard, Alexandre Djiane 1, Jun Wu, Marek Mlodzik *

Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, Annenberg Bldg 18-92, One Gustave L. Levy Place, New York, NY 10029, USA

ARTICLE INFO

Article history:
Received for publication 2 February 2009
Accepted 21 June 2009
Available online 27 June 2009

Keywords:
Apical-basal polarity
Epithelial patterning
Planar cell polarity
Van Gogh
Drosophila

ABSTRACT

Most tissues display several features of cellular polarization. Besides the ubiquitous epithelial polarization in the Apical–Basal (A/B) axis, many epithelia (and associated organs) display a Planar Cell Polarization (PCP). Recently, a crosstalk between the PCP and A/B polarity determinants has been suggested, i.e. the activity or stability of the PCP factor Frizzled is regulated by the A/B determinants aPKC and Bazooka in the Drosophila eye. We have systematically investigated genetic and physical interactions between the Drosophila A/B factors and the core PCP component Strabismus (Stbm)/Van Gogh (Vang). The A/B determinant Scribble was found to interact both genetically and physically with Stbm/Vang. We demonstrate that Scribble binds Stbm/Vang through its PDZ domain and that it cooperates with Stbm/Vang in PCP establishment. Our data indicate that Scribble, in addition to its role in A/B polarity, has a distinct requirement in PCP establishment in the Drosophila eye and wing. We define a scribble allele that is largely PCP specific. Our data show that Scribble is part of the Stbm/Vang PCP complex and further suggest that it might act as an effector of Stbm/Vang during PCP establishment.

© 2009 Elsevier Inc. All rights reserved.

Introduction

In metazoans, epithelial tissues exhibit two types of polarity: (1) the Apical/Basal (A/B) polarity perpendicular to the epithelial plane, and (2) Planar Cell Polarity (PCP; often also referred to as tissue polarity) within the plane of the epithelium. Both types of polarity are required for the development, morphogenesis, and function of most (if not all) tissues and organs. Defects in both types of polarity are associated with several genetic diseases (reviewed in Simons and Mlodzik, 2008).

Genetic screens in Drosophila and C. elegans have identified 3 protein complexes playing a major role in A/B polarity establishment and maintenance (see also Supplemental Fig. S1): (1) the Crumbs (Crb) complex, constituted of Crb, Stardust (Sdt) and Patj; (2) the Par3/Bazooka (Baz) complex, comprised of Baz, Par6 and atypical protein kinase C (aPKC); and (3) the Scribble (Scrib) complex, comprising Scrib, Lethal giant larvae (Lgl), and Discs large (Dlg) (for reviews see Bilder, 2004; Macara, 2004; Nelson, 2003). In Drosophila, the Crumbs complex is the most apical, followed by the Baz/Par3 complex, which is associated with the apical junctional areas, and the Scrib complex is localized to the septate junctions just basal to the adherens junction (for reviews see Bilder, 2004; Macara, 2004; Nelson, 2003). Many of the proteins belonging to these 3 complexes contain PDZ protein–protein interaction domains. In particular Sdt, dPatj, Baz, Scrib and Dlg all contain multiple PDZ domains.

Planar Cell Polarity (PCP) was first described in Drosophila and is manifest in almost all external structures derived from imaginal disc epithelia (Adler, 2002; Klein and Mlodzik, 2005; Strutt, 2003). This is most evident in the distal orientation of wing hairs, the posterior orientation of cellular hairs and sensory bristles on the thorax and abdomen, and the very ordered arrangement of ommatidia in the eye. A number of genes have been defined as core PCP genes, as their mutations affect PCP features in most if not all tissues. These include genes encoding for the membrane associated factors such as the 7-pass transmembrane (TM) protein Frizzled (Fz), the atypical cadherin Flamingo/Starry Night (Fmi/Stan), and the 4-TM protein Strabismus/Van Gogh (Stbm/Vang), as well as for the cytoplasmic factors Disheveled (Dsh) and Diego (Dgo), which associate with Fz, and Prickle (Pk), which binds Stbm/Vang (reviewed in Adler, 2002; Klein and Mlodzik, 2005; Lawrence et al., 2007; Strutt, 2003). This first set of core PCP factors is referred to as the Frizzled group (or also Fz/Stan-group) (Lawrence et al., 2007). A second core group of PCP factors centered around the proto-cadherins Fat and Dachsous acts in parallel to the Fz/Stan group (Lawrence et al., 2007).

In the Drosophila eye, the PCP genes control not only the correct cell fate choice within the R3/R4 equivalence group, but also the orientation of the entire ommatidial units with respect to their neighbors and the entire eye field. In the adult eye, this is translated into the chiral arrangement of ommatidia, forming a mirror image across the dorso-ventral (D/V) midline, the equator (for reviews see Klein and Mlodzik, 2005; Strutt, 2003; see also Fig. 1A). Cell
specification of the photoreceptor R3 vs. R4 subtype is the first and most critical step during PCP establishment in the third instar eye imaginal disc. Each ommatidium initially emerges as a symmetrical precluster posterior to the morphogenetic furrow (MF), where it then becomes “asymmetric” following the action of the PCP genes, with the precursor cell closer to the equator becoming specified as R3 and its polar neighbor as R4 (Klein and Mlodzik, 2005; Strutt, 2003) (also Fig. 1A). The specification of the R3/R4 photoreceptor pair is subsequently followed by a 90° rotation of the ommatidial precursors towards the equator, eventually causing the dorsal and ventral halves of the eye to form a mirror image across the equator (for reviews see Klein and Mlodzik, 2005; Strutt, 2003; also Fig. 1B). PCP establishment in the fly wing results in the asymmetric formation of a single actin-rich prehair on the distal vertex of each wing cell, reflecting an orientation in the proximal–distal (P/D) axis. This is preceded by the asymmetric localization of the core PCP factors within the P/D axis (Mlodzik, 2002; Strutt, 2003).

As a prerequisite to PCP establishment and the regulatory interactions among the PCP core factors, all PCP factors are apically localized in epithelial cells (Adler, 2002; Klein and Mlodzik, 2005; Strutt, 2003). Their ensuing interactions lead to the first detectable asymmetric localization, either within the P/D axis in the wing, the D/V axis in the eye, or the A/P axis in the body wall. In the eye, this is most evident across the R3/R4 membrane border: Fz, Dsh and Dgo localize to the R3 side, whereas Stbm and Pk localize to the R4 side of that border. This is also mirrored in the genetic requirements of the core PCP factors, with Fz, Dsh and Dgo being required in R3 for its cell fate induction, and Stbm and Pk acting in the R4 precursor (Fant and Mlodzik, 1999; Tomlinson and Struhl, 1999; Wolff and Rubin, 1998; Zheng et al., 1995).

Recent work has provided evidence for a crosstalk between the PCP and the A/B polarity determinants. For example, Fz activity or stability is regulated by phosphorylation mediated by aPKC and antagonized by Bazooka (Djiane et al., 2005) and Dsh has been shown to regulate Lgl to participate in A/B polarity (Dollar et al., 2005). Finally, Dlg and Scrib interact with Stbm/Vang respectively during Drosophila sensory organ asymmetric cell divisions, and during the alignment of mouse inner ear cochlear cilial cells (Bellaiche et al., 2004; Lee et al., 2003; Montcouquiol et al., 2003).

We systematically investigated the Drosophila A/B factors whether they can interact with Stbm/Vang and found that Scrib interacts both genetically and physically with Stbm/Vang. Here, we demonstrate that Scrib, in parallel to its major role in A/B polarity, is required genetically and physically with Stbm/Vang. Here, we demonstrate that Scrib interacts both genetically and physically with Stbm/Vang and found that Scrib interacts both genetically and physically with Stbm/Vang respectively during PCP establishment in the Drosophila eye and wing. We define a scrib allele that is largely PCP specific, which interestingly has very similar molecular defects to the PCP specific mouse Scrib1 allele Circletail. Our data suggest that Scrib is part of a Stbm/Vang protein complex and that it might act as an effector of this complex during PCP establishment.

Results

*sbtm* interacts genetically with *scrib*

Based on recent evidence for a crosstalk between A/B and PCP factors (Bellaiche et al., 2004; Djiane et al., 2005; Dollar et al., 2005; Montcouquiol et al., 2003), we systematically tested whether *sbtm* can interact genetically with any of the A/B polarity factors. We used the hypomorphic *stbm* mutant allele, which displays mild PCP phenotypes covering a wide range of defects, including chirality defects, misorientations, and rare symmetrical ommatidia in the eye (this allele is wrongly described as a null in Flybase; it is a phenotypically defined hypomorphic allele; Rawls and Wolff, 2003). With a total of 38% +/- 6.9 affected ommatidia (Figs. 1C, F; compare to wild-type, Fig. 1B) this allele appeared well suited for genetic modification studies (Rawls and Wolff, 2003). The homozygous *sbtm* background was combined with heterozygous conditions for alleles of the A/B determinants containing PDZ domains Lgl (see Supplemental Fig. S1 for schematic representation of A/B polarity factors).

Strikingly, homozygous *sbtm* in combination with heterozygous mutant alleles *scrib* or *scrib* (both strong loss-of-function/LOF alleles) showed an enhancement of the *stbm* associated PCP defects (Figs. 1D–F). In contrast, none of the other A/B determinants tested for dominant modification of *sbtm* did show an interaction. In particular strong LOF alleles for *dlg* (*dlg*; *dlg*; *dlg*; *dlg*; *dlg*; *dlg*), and *lgl* (*lgl*; *lgl*), the partners of scrib during A/B polarity establishment, did not modify the PCP defects of *stbm* (Fig. 1F), suggesting that the interaction between *scrib* and *stbm/Vang* is specific. Similarly, none of the other A/B polarity genes tested showed a modification of the *stbm* PCP phenotype (Fig. 1F and not shown).

In parallel to the genetic approach, we tested in a yeast two-hybrid assay whether the intracellular carboxy-tail (C-tail) of Stbm would display a protein–protein interaction with these A/B polarity determinants, using their PDZ domains as prey (see Supplemental Fig. S1 for schematics of the A/B factors). Interestingly, the Stbm C-tail interacted with the PDZ domains of Scrib and Dlg, whereas no interaction was observed between the Stbm C-tail and the Baz, Par6, Sdt or DPath PDZ domains (Fig. 2A). A Stbm–Dlg interaction has been reported in another context and thus this interaction could be used as a positive control (Bellaiche et al., 2004; Lee et al., 2003). Similarly, mouse Stbm (Vangl2/Looptail) has been shown to bind mouse Scrib1 (Montcouquiol et al., 2003; Montcouquiol et al., 2006), supporting our results.

Taken together, these experiments suggested a specific interaction between Stbm/Vang and Scrib in the PCP context. We thus focused our efforts on dissecting the potential role of *scrib* in PCP establishment.

*Stbm interacts with the Scrib PDZ 3 domain independent of its PDZ-domain binding motif*

In order to confirm the two-hybrid results and also define which PDZ domain(s) of Scrib interacts with Stbm, we performed GST-pull-down assays using a GST-Stbm C-tail, and radiolabeled in vitro translated PDZ domains of Scribble. As the Stbm C-tail is known to interact with itself (Jenny et al., 2003), it served as a positive control. The Stbm C-tail pulled down PDZ domains 3–4 as well as itself, but it did not interact with PDZ domains 1–2 (Fig. 2B). When individual PDZ domains of Scrib were tested, Stbm C-tail pulled down PDZ 3 efficiently and only a weak interaction was detected with PDZ 4 (Fig. 2C; again no interaction was detected with PDZ 1
or 2). These results are consistent with what has been observed with the mouse proteins (Montcouquiol et al., 2006). We conclude that PDZ 3 domain is the domain of Drosophila Scrib that specifically binds to the Stbm C-tail.

A conserved PDZ binding motif (PBM) has been identified for the last 4 amino acids in the C-tail of Stbm/Vang (Wolf and Rubin, 1998). We thus next tested whether the PBM was necessary for the interaction between Stbm and the Scrib PDZ 3 domain and performed the equivalent pull-down assays using a GST-Stbm C-tail with its last 4 amino acids deleted (StbmΔPBM). Unexpectedly, StbmΔPBM still effectively bound the Scrib PDZ 3 domain (albeit slightly less efficiently as compared to wild-type Stbm) (Fig. 2B, middle panel). This was confirmed using GST-PDZ3 of Scrib and testing its binding to either radiolabeled Stbm-C-tail or Stbm-C-tailΔPBM (Fig. 2C). Again the binding was not significantly affected by the presence or absence of the PBM and thus we conclude that the PBM is not necessary for the interaction.

In order to analyze the functional requirements of the Stbm PBM in vivo, we generated transgenic flies expressing stbmΔPBM. To avoid the caveats of exogenous (or ectopic) expression of stbm, we expressed stbmΔPBM under the control of its endogenous promoter and control sequences of stbm (stbm-stbmΔPBM; see Methods). Several independent insertions on the third chromosome, rescued the stbm mutant phenotype to differing degrees with up to 90% (Fig. 2D and not shown; the differences in rescue efficiency resulted most likely from position effects, as similar variations were observed with the equivalent stbm-stbmΔmwt transgenics). These data indicated that the PBM is not essential for stbm function in vivo and are also consistent with the observation that stbmΔPBM expressed under the control of an actin promoter was also able to rescue stbm−/−PCP phenotypes (Bastock et al., 2003) (although a different group has suggested that the PBM is functionally important; T. Wolff, personal communication). Taken together with the molecular interaction data, these results indicated that the Stbm PBM is neither necessary for the physical Stbm/Vang-Scrib association in vitro nor the stbm/Vang function in vivo.

scribble is required for PCP establishment

Based on the interactions described above, we next tested whether scribble was required for PCP establishment. Scribble is a critical factor of apical/basal polarity establishment and maintenance, and thus scribble mutant epithelial cells lose A/B polarity leading to multi-layered cell sheets with rounded cells (Bilder et al., 2000; Bilder and Perrimon, 2000) (see also Supplemental Fig. S2). It is therefore not possible to study PCP in the null allele condition. We therefore analyzed a hypomorphic scribble allele, scribble. This allele was useful for our study as A/B polarity appears to be largely unaffected (Zeitler et al., 2004) and, importantly, it encodes for a protein lacking the last two PDZs domains, PDZ 3 and 4 (Fig. 3A), which bind Stbm physically (see above; Fig. 2). Using this allele, we performed clonal analyses in developing eyes and wings using the FRT/FLP system (Golic and Lindquist, 1989). Clones of the scribble allele show normal A/B polarity in imaginal discs, with apical and basal factors being correctly localized (Supplemental Fig. S2C and data not shown). In adult eye sections we could however only recover small scribble clones. In such clones, we noticed occasional R3/R3 symmetrical ommatidia as well as rotation and chirality defects (Fig. 3B; highlighted by yellow arrowheads, and data not shown.). In very rare larger clones, the overall eye morphology showed significant abnormalities in ommatidial architecture with fused photoreceptors, malformed rhabdomeres, and loss of photoreceptors (Fig. 3B'), making it impossible to quantitate and analyze the eye PCP phenotype in detail (the morphology defects are likely due to a potential requirement of full length Scrib during the formation of the rhabdomeres and morphogenesis of photoreceptors in pupal eye development, which requires changes in the apical/basal polarization of these specialized neurons; Pellikka et al., 2002; Tepass and Harris, 2007).

To bypass the late photoreceptor morphogenesis requirement of scribble in the eye, we performed a clonal analysis in eye imaginal discs using the molecular markers for the R3/R4 precursors: mi0.5-lacZ, which is expressed predominantly in R4 (Cooper and Bray, 1999), and psq-Gal4, UAS-GFP (psq-GFP, Weber et al., 2008), which is expressed...
differentially in both cells of the pair with high levels in R3 and low levels in R4. Analysis of scrib5 clones using the mδ0.5-lacZ marker, showed typical PCP defects with misrotations, chirality flips, and a fairly high frequency of loss of the R4 marker (~15%), suggesting that these clusters were R3/R3 symmetrical clusters (Figs. 4A–A'). Interestingly, we never observed a symmetrical expression of mδ0.5-lacZ in both R3/R4 precursor cells (reflecting R4/R4 type clusters), which can be observed in for example f2 mutant clones (not shown, Cooper and Bray, 1999; Zheng et al., 1995). As expected, in clones of stronger alleles, like the null allele scrib1, severe defects in A/B polarity were observed (e.g. the apical markers dPatj and DE-Cad were mislocalized; Supplemental Fig. S2B; Bilder and Perrimon, 2000) and none of the neuronal markers (ELAV and mδ0.5-lacZ) were expressed (Supplemental Fig. S2A). It was thus not possible to assess PCP in the scrib null allele.

Analysis of scrib5 mutant clones with the psq-GFP marker confirmed and refined the phenotypic analysis with respect to PCP defects. Strikingly, ~25% of R3/R4 precursor pairs displayed identically high levels of GFP expression, reflecting R3/R3-type clusters (Figs. 4B–B'). Taken together with the mδ0.5-lacZ R4 marker results (where no R4/R4-type clusters were detected), we conclude that these clusters represent indeed R3/R3-type symmetrical clusters. Similar to the mδ0.5-lacZ marker, we observed ~15% of chirality flips (Figs. 4B–B'; example highlighted by red arrowhead) and frequent misrotations. This latter defect was best visualized with DE-Cad as marker for the overall orientation of the preclusters (Figs. 4C–C').
summary, this scrib5 clonal analysis in the eye with the two independent and complementary markers mo9.5-lacZ and psq-GFP revealed a range of defects very similar to and characteristic of core PCP determinants (∼40–45% of all clusters; similar to strong hypomorphic alleles of fz or stbm/Vang; e.g. Rawls and Wolff, 2003).

Similar to the eye, marked scrib5 clones in wings also displayed characteristic PCP defects. These were manifest in cellular misorientations as apparent in “waves” and “whorls” (Figs. 3C, D), typical for mutations in PCP factors. Multiple wing hair defects were also observed (Fig. 3E). These defects are already apparent during development in pupal wings as reflected in the misorientation of actin hairs (Figs. 5A–A’; note that some non-autonomous PCP defects in wild-type cells neighboring mutant clones can be observed, examples marked by yellow arrowheads; see Discussion). In addition, PCP defects are also apparent in pupal wings in scrib5 clones by their failure to resolve the PCP factor localization to the membranes in the proximal-distal (P/D) axis (Figs. 5B–C and quantified in Fig. 5D). The Fmi staining pattern in scrib5 clones resembles in part the defect seen in stbm6 clones, in that in both genotypes Fmi accumulates at the border between wild-type and mutant cells (such cells are highlighted by yellow dots in Figs. 5B–C’ and C–C’; compare to stbm6 clones in Figs. 5E–E’). The main difference between scrib5 and stbm6 clones is that mutant scrib5 cells still display Fmi around the apical cortex, albeit without enrichment in the P/D axis (quantified in Fig. 5D), whereas Fmi is reduced apically in stbm6 clones (Figs. 5E–E’; as was also shown earlier).

Taken together, these data indicate that in the wing, like in the eye, scrib5 mutant tissue displays characteristic PCP factor like phenotypes, suggesting that scrib, besides its critical role in A/B polarity establishment, shares a distinct PCP specific function with similarity to stbm/Vang (see below and Discussion).
As Scrib can bind to Stbm we also tested whether Scrib localization is affected in PCP mutants. Surprisingly, we did not see detectable Scrib localization defects in either \textit{stbm}^6 (Figs. 5F'–F) or \textit{fzP21} clones (not shown). This can be explained with the vast majority of Scrib being associated with the A/B determinant complexes and thus a small change in localization due to a PCP association of Scrib appears undetectable.

**Fig. 5.** 
\textit{scribble}^5 clones display typical PCP defects in pupal wings. All panels show clones in pupal wings: A–C' are \textit{scrib}^5 clones; E–F' are \textit{stbm}^6 clones. Proximal is to the left, anterior is up. (A–A') \textit{scrib}^5 clones in pupal wings (marked by absence of \textit{arm-lacZ}, green) stained for F-actin (phalloidin in red) highlighting cell orientation, note that some cells outside the clones also show defects (non-autonomous; examples marked by yellow arrows). (B–C') Higher magnification \textit{scrib}^5 clones (marked by absence of \textit{arm-lacZ}/\textit{Arm-Z} in green) stained for Fmi (magenta in A and C, monochrome in A' and C'); mutant cells at border of clone are marked with yellow dots, note that Fmi is enriched at the border between mutant and wild-type cells and that inside mutant clone Fmi is not enriched at proximal–distal membranes but also found on membranes along anterior–posterior axis. (D) Quantification of the Fmi staining ratio between the P/D and A/P axes. Whereas in wild-type cells Fmi is ~3 fold enriched on P/D membranes relative to A/P membranes, in \textit{scrib}^5 mutant cells the distribution is even with a ratio of 1.14. This effect is highly significant with \( p = 4.27E-14 \) in the student t-test; examples where Fmi is barely detected in the A/P axis in wild-type cells are indicated by yellow arrowheads in B'. (E–F') \textit{stbm}^6 clones (marked by absence of GFP, green) stained for Fmi (magenta in E and monochrome in E'; and red in F) and Scrib (blue in F, monochrome in F'). In E' mutant cells abutting wild-type areas are marked by yellow dots; note that Fmi is enriched at the border between \textit{stbm}^6 and wild-type cells; this is similar to the effect of \textit{scrib}^5 clones. In addition, Fmi is reduced in \textit{stbm}^6 clones, which is not seen in \textit{scrib}^5 mutant tissue. (F, F') Scrib localization is not affected in a detectable manner in \textit{stbm}-mutant clones.
scribble is required in R4 precursors for the R4 cell fate

Next we wished to determine the cellular requirement of scrib in the context of the R3/R4 pair, in analogy to the data established for example for the core PCP factors fz and stbm/Vang (Weber et al., 2008; Wolff and Rubin, 1998; Zheng et al., 1995). In order to do this, we performed a mosaic analysis using the psq-GFP marker, a reliable marker for differential R3/R4 specification used in previous PCP studies (Weber et al., 2008). We have analyzed 121 clusters mosaic within the R3/R4 pair. Strikingly, when precursor for R3 was mutant and the R4 precursor wild-type, the large majority of such clusters showed wild-type R3/R4 marker expression (Figs. 6A–C). In contrast, when the precursor for R3 was wild-type and R4 mutant, the majority of such mosaic clusters (73%) displayed chirality defects (31.7% showed inverted chirality, and 41.3% were symmetrical; Figs. 6B–D). Our results thus indicate that scrib is required in the R4 precursors for

![Fig. 6.](image)

Mosaic analysis of scrib5 clones in developing eye disc. Mosaic analysis was performed using the psq-GAL4, UAS-GFP (psq-GFP) marker, which is expressed at high levels in R3 and at low levels in R4. (A–A’) Wild-type eye imaginal disc, stained for pan-neuronal marker Elav (in blue) and psq-GFP (in green). Photoreceptors 3 and 4 are labelled in a few examples. (B, B’ and C, C’) Two independent eye discs displaying scrib5 mutant clones (marked by absence of βGal staining; red). Photoreceptors are labeled with anti-Elav (blue) and R3/R4 cells by psq-GFP (green). At the border of mutant tissue in mosaic clusters, chirality flips (marked by green and yellow asterisks representing R3 and R4 cells respectively) and symmetrical R3–R3 clusters (e.g. green double asterisks) are often observed; their appearance correlates with the presence of a scrib5 mutant R4 precursor. (D) Quantification of the mosaic analyses of ommatidial defects when either R3 or R4 were mutants: when R4 was a wild-type the ommatidial clusters displayed mostly normal/correct orientation and chirality (R3−/R4+; left side of panel); in contrast, chirality inversions and symmetrical clusters were frequently observed when R3 was a wild-type and R4 a mutant (R3+/R4−; right side of graph: note that only about 20% of such clusters show wild-type orientation, whereas all others are either symmetrical or display inverted chirality).
R4 cell specification. Taken together with the genetic and physical interactions, our data suggest that *scrib* cooperates with *stbm/Vang* in R4 precursors and together they contribute to R4 cell fate specification.

To further address the potential interaction of *scrib* and *stbm/Vang*, we used a gain-of-function non-autonomous signaling assay in the wing ([Wu and Mlodzik, 2008]). Unlike Fz, which readily causes non-autonomous re-orientation of hairs outside the expression domain, *Stbm/Vang* does so only poorly. For example, *Stbm* driven with *dppGal4* (at 25 °C) in a stripe along the A/P compartment boundary does only very rarely lead to cell orientation defects (Fig. 3G). Similarly, *dppGal4* driven *Scrib* causes only very subtle defects (Fig. 3I). When *Stbm* and *Scrib* are co-expressed, however, robust cell re-orientation of cells outside the expression domain is observed in all wings (Fig. 3H). The direction of re-orientation is towards the expression domain, opposite to the effect of Frizzled ([Wu and Mlodzik, 2008]), which is what one would predict for a strong *Stbm* effect. These data further support the notion that *Scrib* cooperates with *Stbm/Vang* in PCP signaling (see Discussion).

*Scrib does not control Stbm localization*

All core PCP factors are apically localized in *Drosophila* epithelia, which is a prerequisite for their function in PCP ([e.g. Wu et al., 2004]) and subsequently as a consequence of their molecular interactions they segregate into asymmetrically localized patterns in the R3/R4 precursors and in pupal wing cells ([reviewed in Strutt, 2003; Klein and Mlodzik, 2005; Seifert and Mlodzik, 2007]). *Stbm* for instance is found on the R4 side at the R3/R4 border and on the proximal side pupal wing cells.

In A/B polarity establishment, the main role of *scrib* is to control apical localization of apical factors like Crb ([Bilder and Perrimon, 2000]). We therefore asked whether the role of *scrib* in the PCP context could be to properly localize *Stbm* to the apical junctional regions. Comparing *Stbm/Vang* localization between wild-type and *scrib* mutants in eye imaginal discs, we observed no apparent differences: *Stbm/Vang* was apically localized in mutant cells indistinguishably from neighboring wild-type cells (Supplemental Fig. S3). These data indicate that the role of *Scrib* in PCP is not primarily to localize *Stbm/Vang* to apical junctional complexes and suggest a distinct and specific function of *scrib* in PCP establishment (see Discussion).

**Discussion**

We have analyzed the requirements of the Apical–Basal (A/B) determinant Scribble (*Scrib*) during PCP establishment in *Drosophila*. Our data indicate that *scrib* acts generally in PCP (as shown by classical PCP defects in the wing and eye) with phenotypes comparable to *stbm/Vang* mutants (or also other members of the Fz-group of core PCP factors). Genetic and molecular data suggest that *scrib* cooperates with *stbm/Vang* and might possibly function as an effector of the core PCP factor *Stbm/Vang*.

**Specificity of the Stbm–Scrib interaction in PCP establishment**

Due to the subapical cortical localization requirement of the core PCP factors ([Wu et al., 2004]), we were interested to test for interactions between the PCP proteins and the A/B determinants. Earlier work has identified dPatj as a specific binding partner of Fz, required for restriction of Fz activity ([Djiane et al., 2005]). Among the major A/B polarity factors tested for interaction with *Stbm*, only *Scrib* interacted both physically and genetically. As shown earlier for the other members of the Scrib complex (*Scrib*, *Dlg*, *Lgl*; [Lee et al., 2003]), we also found *Dlg* to physically bind to the *Stbm* C-term. However, we did not detect any genetic interactions between *stbm* and *dlg* in PCP contexts. These data suggest that the interaction between *Stbm* and *Scrib* is specific and restricted to PCP establishment, which is also supported by genetic interactions of the respective genes in the mouse ([Montcouquiol et al., 2003]; see below).

A structure/function analysis of *Scrib* in the context A/B polarity in *C. elegans* and *Drosophila* has established that the LRR domains are necessary for targeting *Scrib* to the basolateral membrane, while PDZ domains 1 and 2 play an important role by concentrating *Scrib* proteins at the septate junctions ([Albertson et al., 2004; Legouis et al., 2003; Zeitler et al., 2004]). A specific role for the PDZ 3 and 4 domains was not observed in this context. Our data indicate that the *Scrib* PDZ 3 domain is necessary and sufficient for its physical interaction with *Stbm*. The *in vivo* data with the *scrib*-allele, in which the PDZ domains 3 and 4 are deleted, demonstrate that these domains are critical for PCP function (our work, see below).

Taken together our data suggest that *scrib* plays not only a major role in A/B polarity establishment through its LRR and PDZ 1 and 2 domains, but that it also has important and distinct requirements in PCP establishment through the PDZ 3 and 4 domains and their interaction with *Stbm*. How does this relate to the reported *Scrib–Stbm* interaction in vertebrates? Analyses with mammalian *Scrib* proteins have suggested that PDZ domains 2 and 3 of m*Scrib*1 can bind Vangl2, although, like in our studies, the PDZ domain 3 had the strongest affinity for the Vangl2 (*Stbm*) C-tail ([Kallay et al., 2006; Montcouquiol et al., 2006]).

**Is the PBM of *Stbm/Vang* important?**

The physical binding studies indicate that the PBM of *Stbm/Vang* is not necessary for *Scrib* PDZ 3 binding to *Stbm* (although the binding between *Stbm* C-tail and PDZ 3 is somewhat stronger in the presence of the PBM: Fig. 2C). However, analyses of the mammalian orthologs of *scrib* and *stbm* (mouse *scrib1* and *vangl2*, respectively) suggested that the Vangl2 PBM is required for its interaction with m*Scrib1* ([Kallay et al., 2006; Montcouquiol et al., 2006]). In functional assays *in vivo*, the PBM of *Stbm/Vang* appears dispensable and *StbmAPBM* rescues the *stbm/Vang* mutant as well as *StbmWT* does. This is observed in both our rescue experiments in the eye using the endogenous promoter of *stbm/Vang*, as well as in experiments using heterologous expression cassettes (the *actin* promoter) assaying rescue in the wing ([Bastock et al., 2003]). We conclude that although the *Stbm/Vang* PBM can make its interaction with *Scrib* more efficient in *Drosophila* it is largely dispensable here.

In vertebrates, the role of the PBM of *Stbm/Vangl* family members is controversial. In convergent extension (CE) in vertebrates, a process also regulated by the core Fz-group PCP factors ([Seifert and Mlodzik, 2007; Wang and Nathans, 2007]), it has been shown in *Xenopus* that increasing amounts of StbmAPBM inhibit the cell movement indistinguishable from xStbm full length and that the Stbm/Vang PBM is therefore not required in CE ([Darken et al., 2002]). However, other studies suggest that the PBM is necessary for Stbm function in modulating CE in *zebrafish* and *Xenopus*, and that StbmAPBM might antagonize StbmWT ([Goto and Keller, 2002; Park and Moon, 2002]). Without any functional rescue experiments in vertebrates this issue cannot be conclusively answered.

**Parallels of the PCP specific function of *Scrib* between mice and *Drosophila***

We have shown that *scrib* and *stbm* interact physically and genetically during PCP establishment in *Drosophila*. Similar observations were reported for PCP generation in the mouse cochlea ([Montcouquiol et al., 2003; Montcouquiol et al., 2006]). In the mouse, the *scrib* and *stbm* homologs, *Circletail* (*Crc/mScrib1*) and Vangl2 (originally named *Looptail/Lp*) also interact during neural tube closure ([Kibar et al., 2001; Murdoch et al., 2001; Murdoch et al., 2007]).
Moreover, only the double homozygous Crc and Vang12/Lp mutants have been so far shown to display craniorachischisis, the most severe form of neural tube defects (NTD) (Murdoch et al., 2003). NTDs are a consequence of morphogenetic movement defects during late stages of gastrulation and CE, highlighting the importance of the stbm and scrib cooperation in PCP associated processes. Most interestingly, the molecular defect in the mScrib1C1247 mutation has been identified as a single base insertion in codon 947, causing a frame shift and resulting in a premature stop codon and truncation of the protein before the PDZ 3 and 4 domains (Murdoch et al., 2003). Thus the molecular lesion in the mScrib1C1247 mice largely mimics the molecular mutation in the scrib5 allele in Drosophila, where also the protein is truncated before the PDZ 3 and 4 domains. This observation confirms the requirement of the PDZ domains 3 and 4 in PCP signaling and thus the scrib5 allele in Drosophila and the mScrib1C1247 allele in mouse are likely to also share functional defects in the PCP context in both the mouse and Drosophila.

It is worth noting that scrib is expressed in two isoforms during Drosophila embryogenesis, one giving rise the full length 1756 aa protein (called Scrib1) and a shorter form of 1247 residues (Scrib2), which is lacking the PDZ domains 3–4 (Li et al., 2001). Both isoforms are expressed in vivo during embryogenesis and thus one can speculate that it is functionally important to have the 2 different isoforms. Possibly, the Scrib2 isoform, missing PDZ domains 3 and 4 has a distinct function than the Scrib1 isoform (Bilder and Perrimon, 2000; Zeitler et al., 2004). In summary, we conclude that Scrib, through its interaction with Stbm/Vang, is an important PCP factor, and this function is evolutionarily conserved, requiring the PDZ domains 3–4.

**Scrib requirement(s) as a PCP factor**

The core PCP factors Stbm and Fz play opposing roles during PCP establishment, mutually antagonizing each other (Klein and Mlodzik, 2005; Seifert and Mlodzik, 2007; Strutt, 2003). In the R3/R4 PCP decision in the Drosophila eye fz is required in R3 (Zheng et al., 1995) and stbm/Vang is required in R4 (Wolff and Rubin, 1998). Mosaic analysis with scrib5 demonstrated that scrib is also required in R4 for its proper specification, very similar to stbm/Vang. In the wing, the LOF PCP phenotype of scrib5 clones looks also similar to the core PCP factors, in particular to stbm/Vang, with misoriented wing hairs and occasional “multiple wing hair” defects.

What is the role of Scrib in PCP? Localization studies of Stbm/Vang in scrib5 clones do not reveal appreciable defects in the A/B axis in the tissues analyzed, indicating that Scrib is not required for proper apical Stbm/Vang localization. Scrib protein localization is consistent with its role on A/B polarity throughout development. In addition, it appears to become transiently enriched at the subapical junctions within the proximal-distal axis in pupal wings (not shown), although it largely detected all around the subapical cortex. Thus, this effect is transient and very difficult to visualize due to the PCP independent “ubiquitous” Scrib localization at the junctional levels. Taken together with the physical binding data, the genetic interactions, and the cooperation (synergy) between Scrib and Stbm/Vang in the gain-of-function assay, we hypothesize that Scrib could function as a downstream effector of Stbm/Vang in PCP generation. In such a model, we would propose that the “PCP-activity” of Scrib is locally controlled by Stbm/Vang, either through local modification or recruitment to other factors or unknown means. Scrib has been implicated in the control of localized exocytosis through a complex with PIX (a Guanine nucleotide Exchange Factor/GFP for Arf; Audebert et al., 2004). One could imagine a model in which Stbm by recruiting Scrib would control exocytosis locally which could in turn affect the outcome of the PCP core factor interactions and or stability thus accounting for the effects on PCP in a general manner.

As discussed above the phenotypic features of scrib5 are similar to stbm/Vang mutant alleles. However, the defects in scrib5 are milder. In this regard scrib is similar to pk mutants, with Pk also being part of the Stbm/Vang complex displaying phenotypic defects that are milder than stbm/Vang mutant alleles. For example, Pk has no apparent function in the non-autonomous signaling role of Stbm/Vang (Adler et al., 2000). Interestingly, scrib5 mutant clones show some non-autonomous defects in wing tissue and Scrib cooperates with Stbm/Vang in non-autonomous signaling assay, which could suggest that Scrib mediates (in part) the non-autonomous function of Stbm/Vang (whereas Pk is strictly autonomous). A detailed dissection of the relationship of Scrib and Pk will be needed to address this further. Due to the general requirements and localization of Scrib in A/B polarity this is technically a very challenging task. Possibly, dissecting the functional requirement(s) of Stbm that are shared with Scrib will help to clarify this issue.

**Methods**

**Drosophila stocks**

flies were grown on standard fly medium at 25 °C unless otherwise indicated. The following mutant stocks were used:

- stbm153, stbmΔ1, stbmΔ2, stbm5
- lgl4w3
- par6δ2z
- baz4
- σd4pp16

**Immunofluorescence and histology**

Imaginal discs were dissected and stained as described (Fanto and Milostzik, 1999). The following primary antibodies were used:

- mouse α-Elav (Developmental Studies Hybridoma Bank, DSHB, http://www.uiowa.edu/~dshbwww),
- rat α-Elav (DSHB),
- rabbit α-jGal (Cappel, Promega),
- rabbit α-GFP (A-11222, Molecular Probes),
- mouse α-GFP (A-11120, Molecular Probes),
- rat α-DE Cadherin (DSHB),
- rabbit α-Stbm (a generous gift from T. Wolff, Wolff and Rubin, 1998),
- rabbit α-Scrib (a generous gift from C. Doe, Albertson et al., 2004),
- rabbit α-dPatj (a generous gift from M. Bhat).

All secondary antibodies were from Jackson Laboratories. Imaginal discs were mounted in Mowiol and viewed with a Zeiss LSM510 Meta Confocal microscope. Single optical sections or stacks of several sections processed using Image J and Photoshop are shown.
Molecular interaction experiments

Sbbr C-term (aa 294–584) was used as bait against the PDZ domain-containing Apical/Basal (A/B) determinants as preys. The A/B determinants were cloned as described (Djiane et al., 2005). Yeast cells were transfected with bait and prey plasmids using Match Maker kit (Clontech, according to the manufacturer’s protocol). Transfected cells were plated on selective media lacking Leu and Trp for 3 days to select for double transformants, and were then streaked on media lacking Leu, Trp and Ade in order to test for potential interactions.

GST::Sbbr_Cterm and GST::Sbbr_CtermPWM were cloned and expressed as described (Jenny et al., 2003). GST::Scrib_PDZ3–4 (aa 1151–1333) was cloned into pGEX4-T1. S35-radiolabeled PDZ domains of Scribble (aa 738–818; aa 947–1019; aa 738–1019; aa 1151–1234; aa 1264–1333; aa 1151–1333) and Sbbr C-term and Sbbr C-termPWM (Jenny et al., 2003) were generated by in vitro translation using the TNT Coupled Reticulocyte Lysate System (Promega, according to manufacturer’s protocols). Truncated isoforms of Scrib were cloned by PCR into a modified version of pGLOBin vector and GST-pull-downs were performed as described (Jenny et al., 2003).

Acknowledgments

We are grateful to Drs. David Bilder, Tanya Wolff, Chris Doe, and Manzoor Bhat, and the Bloomington stock center for Drosophila strains and antibodies. We thank S. Okello and A. Blitzer for technical help, W. Gault for careful reading and comments on the manuscript, and all members of the Modrzej lab for helpful discussions and input. This work has been supported by NIH grants EY13256 and GM62917 to MM. Confocal microscopy was performed at the MSSM-Microscopy Shared Resource Facility.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2009.06.024.

References


