RESEARCH REPORT

The F-box protein Slmb restricts the activity of aPKC to polarize epithelial cells

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ABSTRACT

The Par-3/Par-6/aPKC complex is the primary determinant of apical polarity in epithelia across animal species, but how the activity of this complex is restricted to allow polarization of the basolateral domain is less well understood. In Drosophila, several multiprotein modules antagonize the Par complex through a variety of means. Here we identify a new mechanism involving regulated protein degradation. Strong mutations in supernumerary limbs (slmb), which encodes the substrate adaptor of an SCF-class E3 ubiquitin ligase, cause dramatic loss of polarity in imaginal discs accompanied by tumorous proliferation defects. Slmb function is required to restrain apical aPKC activity in a manner that is independent of endolysosomal trafficking and parallel to the Scribble module of junctional scaffolding proteins. The involvement of the Slmb E3 ligase in epithelial polarity, specifically limiting Par complex activity to distinguish the basolateral domain, points to parallels with polarization of the C. elegans zygote.

KEY WORDS: Drosophila, Par, Epithelia, Polarity, Tumor, Ubiquitin ligase

INTRODUCTION

Polarization is a fundamental feature of animal cells, from newly fertilized zygotes to dividing stem cells to homeostatic epithelia. This common feature is controlled by a conserved set of regulators, which segregate the single plasma membrane into several discrete domains. The most broadly used polarity regulators comprise the Par complex, consisting of the PDZ-containing scaffolds Par-3 and Par-6, which associate with Cdc42-GTP and the atypical protein kinase (aPKC) (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010; Zonies et al., 2010). In the C. elegans zygote and the Drosophila oocyte, these proteins localize to and specify the anterior cortex. In most epithelial cells and neural stem cells, they localize to and specify the apical plasma membrane, and in migrating cells they define and act at the leading edge. The Par complex thus serves as a ‘master regulator’ for many types of cell polarity.

To achieve and maintain polarity, the Par complex must be restrained to distinguish a complementary membrane domain. In contrast to the pre-eminent role of the Par complex, multiple protein modules that limit Par activity have been identified in different contexts (Tepass, 2012). In the C. elegans zygote, the protein kinases PAR-1 and PAR-4 act downstream of the RING finger protein PAR-2 to antagonize Par localization and define the posterior cortex (St Johnston and Ahringer, 2010; Zonies et al., 2010). Par-1 and Par-4 (Lkb1 – FlyBase) are also key regulators of fly oocyte polarization, but often have less central roles in other polarized cell types (Haack et al., 2013; Partanen et al., 2013). Instead, in many of these tissues a second group of proteins, the Scribble (Scrib) module, acts to restrict the Par complex. In the Scrib module, Scrib and Dig are basolateral localized PDZ-containing scaffolds that regulate Lgl, a syntaxin- and myosin-binding protein that can directly antagonize aPKC (Bilder, 2004; Elsum et al., 2012). Yet another module, the Yurt/Coracle (Cor) complex, specifies the basolateral domain in mid-stage Drosophila embryos and zebrafish photoreceptors (Laprise and Tepass, 2011). Rac and PI3 kinase also play a role at this stage (Chartier et al., 2011). Further, in fly epithelia but not neuroblasts, AP-2-mediated endocytosis restricts apical polarity regulators to their appropriate surface; endocytosis also plays a crucial role in polarization of the worm zygote (Halbsgut et al., 2011; Shivas et al., 2010). The mechanisms by which this diverse set of proteins – which we will call Par or apical antagonists – negatively regulate the Par complex is an active field of investigation. In none of these cases is the mechanism well understood, nor how they coordinate with each other.

Our incomplete knowledge of the mechanisms of Par antagonists raises the possibility that additional regulators of basolateral polarity remain to be identified. Here we report that strong mutations in the F-box protein Slmb, a substrate adaptor for SCF E3 ubiquitin ligases, result in excess Par complex activity in Drosophila imaginal discs, thereby expanding the apical membrane domain. Our results indicate that Slmb-mediated protein degradation acts in parallel to the Scrib module to oppose aPKC activity and thus specify the epithelial basolateral membrane.

RESULTS AND DISCUSSION

Slmb is a novel Drosophila neoplastic tumor suppressor gene

To identify new regulators of basolateral polarity, we analyzed mutants isolated in a genetic screen for Drosophila tumor suppressor genes (TSGs). The screen utilized mitotic recombination to generate imaginal discs predominantly populated by homozygous mutant cells growing in an otherwise heterozygous larva. Mutations in a small set of genes cause larval or pupal lethality in this context; many of these show a set of tumor-like phenotypes collectively called ‘neoplastic’ (Menut et al., 2007). Discs mutant for one uncharacterized complementation group, MENE(3R)-B, show multiple hallmarks of neoplastic transformation. Monolayered organization is lost, disc size is dysregulated, F-actin levels are elevated and differentiation is prevented (Fig. 1A-F). In addition, Matrix metalloproteinase 1 (Mmp1), a mediator of tissue invasion, is upregulated (Fig. 1A,B).

These phenotypes closely resemble those of the Scrib module, suggesting that MENE(3R)-B identifies a gene with similar function. MENE(3R)-B alleles fail to complement mutants in slmb, which encodes an F-box and WD40 repeat protein homologous to vertebrate β-TrCP (Btrc) that functions as a specificity factor in a Skp–Cullin–F-box (SCF) E3 ubiquitin ligase complex (Frescas and Pagano, 2008;
Jiang and Struhl, 1998; Theodosiou et al., 1998). Sequencing identified coding region lesions, including early and late truncations as well as missense mutations, in all six alleles (Fig. 1G), and a slmb transgene rescues the neoplastic phenotype (supplementary material Fig. S1). The existing alleles slmb1 and slmb2 have been widely used, and epithelial organization phenotypes have not been reported. Sequencing revealed that these contain missense mutations in the fifth and seventh WD40 domains (Fig. 1G), indicating that both may be hypomorphic. We confirmed that slmb1 and slmb2 mutant discs show no and only a limited degree of neoplastic transformation, respectively (supplementary material Fig. S1). However, discs predominantly mutant for the deletion allele slmb8 (Milétich and Limbourg-Bouchon, 2000) show neoplasia, confirming that this phenotype is induced only by strong alleles. Null mutations in Roc1a, which encodes a frequent component of the SCFSlmb complex (Noureddine et al., 2002), also show neoplasia (supplementary material Fig. S1). These data demonstrate that slmb functions as a new neoplastic TSG, and suggest that it does so via its role in the SCFSlmb E3 ligase.

Slmb restricts apical polarity

Known neoplastic TSGs regulate epithelial polarity. We therefore analyzed slmb tissue with markers for polarized membrane domains. In wild-type (WT) imaginal epithelia, the transmembrane proteins Cadherin 87 (Cad87) and Fasciclin III (FasIII) occupy complementary apical and basolateral membrane domains. In slmb cells, Cad87 is distributed ectopically around the cell circumference in discontinuous domains that sometimes overlap with FasIII (Fig. 2A,B). Similar effects are seen with the polarized peripheral membrane proteins Bazooka (Baz; Drosophila Par-3) and Cor (Fig. 2C,D). Expanded apical domains of slmb tissue resemble those of Scrib module mutants (Fig. 2E), and indicate that Slmb also acts as an apical antagonist.

To explore the breadth of Slmb function in cell polarity, we attempted to generate clones of strong alleles in other tissues. We were generally unable to recover clones in the follicle cell epithelium, and females carrying germline clones failed to produce eggs, preventing analysis of embryonic epithelia (data not shown). Clones in the larval central nervous system showed defective optic lobe organization. Intriguingly, clones derived from type I neuroblasts frequently contained multiple neuroblast-like cells, suggesting that Slmb might also be required for asymmetric cell division (supplementary material Fig. S2) (Li et al., 2014).

Slmb does not regulate endolysosomal trafficking

Of the known Par antagonists, only the Scrib module and endocytic components have been shown to strongly regulate imaginal basolateral polarity, raising the possibility that slmb might act primarily via one of these pathways. We first asked whether either Scrib or endocytic components promote Slmb-mediated protein degradation. However, Armadillo (Arm) levels (Jiang and Struhl, 1998) are not increased in scrib or AP-2α depleted tissue (Fig. 3A-C), nor was there evidence for misregulation of other Slmb targets

Fig. 1. slmb is a novel neoplastic tumor suppressor gene. (A-D) slmb wing discs show upregulation of Mmp1 (green) and loss of epithelial organization. DNA (DAPI), blue; F-actin (Phalloidin stain) is red in all figures. (E,F) slmb eye discs fail to differentiate. Elav, cyan. (G) Domain structure of Slmb and identified lesions. MENE(3R)-8 alleles in violet text. Scale bars: 50 μm in A,E; 10 μm in C.
**Misregulation of known substrates cannot account for the slmb null phenotype**

The strong neoplastic phenotype seen in slmb tissue points to the existence of a polarity-regulating substrate, the levels of which must be controlled. We therefore examined known Slmb substrates to see whether any could account for this phenotype (supplementary material Fig. S4). Both Arm and Cubitus interruptus (Ci) are subject to proteolytic regulation by Slmb. Cells individually expressing or co-expressing active, non-degradable forms of Arm and Ci display a degree of hyperplastic overgrowth, consistent with known roles in the imaginal disc, but retain normal polarity and tissue architecture and do not upregulate Mmp1 (supplementary material Fig. S4A).

Overexpression of stabilized Plk4 (Sak kinase – FlyBase) and Cap-H2 (Buster et al., 2013; Rogers et al., 2009) caused no growth or polarity phenotypes (supplementary material Fig. S4B,C). Overexpression of a stabilized form of the polarity kinase Par-1, recently shown to be an Slmb substrate at California Berkeley, 2010. Thus, despite the many phenotypic similarities, the failure to control Scrib and Dlg membrane recruitment and the lack of genetic interaction suggest that Slmb regulates polarity in parallel to the Scrib module.

**aPKC is required for slmb-mediated neoplastic transformation**

An attractive candidate target of Slmb-mediated polarity regulation is the Par complex component aPKC. Overexpression of activated aPKC is sufficient to expand the apical domain and confer neoplastic phenotypes similar to those of slmb tissue (Fig. 4O) (Eder et al., 2005). Intriguingly, one predicted isoform of aPKC (aPKC-G) contains two Slmb-binding degrons and, when expressed in S2 cells, is degraded in an slmb-dependent manner (supplementary material Fig. S4). However, aPKC-G transcripts are present at very low levels in L3 discs, and expression of a degron-lacking aPKC-G had no effect on disc polarity or growth (supplementary material Fig. S4). We then used an antibody directed against a shared protein region to analyze total aPKC and found that it is mispolarized in slmb tissue and more widely distributed around the plasma membrane (Fig. 4A,B). However, aPKC levels are not obviously elevated, as assessed by immunohistochemistry, when compared with neighboring WT cells, in contrast to the evident elevation seen with Arm (Fig. 3A). We quantitated western blots of disc lysates which indicated a modest but not significant elevation of aPKC in slmb versus WT (Fig. 4C). These data suggest that, although aPKC is mislocalized in slmb cells, it is unlikely to be a target of slmb-mediated degradation.

Despite the absence of elevated levels of aPKC, we found multiple signs of increased aPKC activity in slmb discs. Excessive aPKC activity drives neoplastic overgrowth in part through upregulation of JAK-STAT pathway ligands, mediated by the Yorkie (Yki) transcription factor (Doggett et al., 2011; Robinson and Moberg, 2011; Sun and Irvine, 2011). slmb discs show robust activation of a STAT signaling reporter, and their neoplastic phenotype is sensitive to the dosage of yki (Fig. 4D-G). A second aPKC-regulated process is seen upon overexpression of the Crb intracellular domain in photoreceptors (Fig. 4H,I) (Tanentzapf and Morrison, 2011; Sun and Irvine, 2011).

**Slmb and Scrib regulate polarity via distinct but parallel pathways**

To test whether Slmb might directly influence Scrib module activity, we examined protein localization. A distinctive feature of scrib and dlg mutants is that, although many proteins are mislocalized, Dlg is specifically lost from the plasma membrane of scrib mutants, as is Scrib in dlg mutants (Bilder et al., 2000). By contrast, examination of slmb mutant cells reveals that both Scrib and Dlg retain tight, albeit dysregulated, cortical localization (Fig. 3H-M). Additionally, heterozygosity for slmb does not enhance weak lgl-RNAi nor lgl or dlg hypomorphic phenotypes, all of which are sensitive to the dosage of Scrib module components (H. A. Morrison and B. D. Bunker, PhD theses, University of California Berkeley, 2010). Thus, despite the many phenotypic similarities, the failure to control Scrib and Dlg membrane recruitment and the lack of genetic interaction suggest that Slmb regulates polarity in parallel to the Scrib module.
Tepass, 2003); the resulting morphogenetic defects are dependent on aPKC (Fig. 4J). Although heterozygosity for slmb does not enhance endocytic or Scrib module phenotypes, it does robustly enhance Crb overexpression (Fig. 4K). Finally, elevated aPKC activity is sufficient to induce trafficking defects of the retromer-dependent transmembrane cargo Wntless (Eaton, 2008), leading to a distinctive subcortical trapping phenotype (Fig. 4L,N; plasma membrane localization, 73±12% for WT versus 52±13% for aPKCAct) (de Vreede et al., 2014); slmb mutant tissue phenocopies this trapping (Fig. 4M; plasma membrane localization, 44±25%; P<2×10^-5 versus WT, P=0.14 versus aPKCAct).

To directly test the functional involvement of hyperactive aPKC, we reduced aPKC activity in slmb cells using a weak dominant-negative construct (aPKCDN) that has no effect on WT cells but can suppress phenotypes driven by elevated aPKC activity (Sotillos et al., 2004). Strikingly, expression of aPKCDN in slmb clones strongly suppressed tumorous growth (Fig. 4P-S). The resultant clones were smaller than WT clones, suggesting that aPKC activity also promotes slmb survival in this context, perhaps because tumorous slmb cells are ‘addicted’ to oncogenic aPKC, the excess activity of which allows them to survive in the presence of other misregulated slmb substrates. This reliance demonstrates a specific requirement for aPKC in slmb tissue and, along with the above results, reveal that Slmb acts as a negative regulator of aPKC activity.

Conclusions

Here, we extend the mechanisms involved in epithelial polarity to include a new function: targeted protein degradation. Targeted degradation can create spatial asymmetries in protein distributions, and there is precedent for roles of E3 ubiquitin ligases, including SCFSlmb (Li et al., 2014; Morais-de-Sá et al., 2013), in polarizing different aspects of cells. The involvement of Slmb in Drosophila apicobasal polarity has gone unnoticed due to the previous use of hypomorphic alleles. The strong alleles described here display potent expansion of the apical pole of imaginal epithelia, demonstrating that Slmb is a new polarity regulator that functions to restrict the apical domain.

Loss of Slmb phenocopies the polarity defects associated with mutations in two classes of ‘apical antagonists’: the Scrib module of core polarity regulators, and endocytic regulators that control trafficking through the early endosome. Despite the similar polarity defects, slmb mutations do not alter endolysosomal cargo traffic, nor do they display protein recruitment defects characteristic of Scrib module mutants; furthermore, no genetic interactions are seen with either pathway. Nevertheless, the downstream consequences of polarity misregulation – including tumor-like transformation and the upregulation of specific target genes – are again shared between slmb and the other apical antagonists, and, moreover, slmb and Scrib module mutant cells share a distinctive trafficking defect associated...
with elevated aPKC activity. We therefore suggest that Slmb acts in parallel to the Scrib module to antagonize the Par complex and other apical regulators.

The role for Slmb defined here points to the existence of an apical polarity-regulating protein substrate, the levels of which must be controlled. We have ruled out a number of validated Slmb substrates as the relevant target. Bioinformatic scans of Drosophila proteins for Slmb degron sequences suggest other candidates, including Expanded (Ex), but overexpression of Ex is not sufficient to induce polarity defects resembling those of slmb (Blaumueller and Mlodzik, 2000; Fernández et al., 2011). Although we cannot rule out a contribution from the elevation of multiple substrates, slmb-like polarity phenotypes can be induced by the elevated activity of individual proteins, including Crb or aPKC. Despite evidence that aPKC undergoes ubiquitin-mediated degradation in embryos (Colosimo et al., 2009), neither aPKC nor Crb levels appear to be controlled by Slmb-mediated degradation in imaginal discs. Nevertheless, our data together suggest that the substrate of Slmb in polarity regulation will function as a positive regulator of aPKC-driven outcomes.

Our demonstration that Slmb limits aPKC activity to distinguish the epithelial basolateral domain reveals intriguing parallels to polarization of the worm zygote. In this context, Par-2 is the primary antagonist that restricts aPKC/Par activity, while Lgl homologs function in a parallel, redundant role. Par-2 contains a RING finger domain that is characteristic of single-subunit E3 ligases, but Par-2 homologs have not been identified outside of nematodes, Par-2 does not affect aPKC/Par levels, and a degraded substrate in polarity regulation has yet to be identified (St Johnston and Ahringer, 2010; Zonies et al., 2010). The discovery of a Drosophila E3 ligase with a similar function to Par-2 raises the possibility of a conserved molecular logic to polarity in these two paradigmatic systems; determination of the relevant substrate will shed further light on this question.

MATERIALS AND METHODS
Fly stocks and genetics
Predominantly mutant imaginal discs were generated as described previously (Menut et al., 2007). slmb mutants represent slmbU11 unless otherwise specified. For further details of fly stocks and genetics and the generation of mutants and clones, see supplementary Materials and Methods.

Immunohistochemistry and microscopy
Western blots loaded with equivalent protein concentrations were probed with anti-β-tubulin (E7, Developmental Studies Hybridoma Bank) and anti-aPKC (sc-216, Santa Cruz Biotechnology). Two biological replicates for Western blots loaded with equivalent protein concentrations were probed

Author contributions
The authors declare no competing financial interests.

Funding
This project was supported by grants from the National Institutes of Health (GM090150 and GM068675), the American Cancer Society [RSG-07-040-01-CSM] and a Burroughs Wellcome Fund Career Development Award to D.B. G.C.R. is grateful for support from the National Cancer Institute [P30 CA23074] and the Arizona Biomedical Research Commission [1210]. L.C.S. was the Robert Black Family Fellow of the Damon-Runyon Cancer Research Foundation [DRG2057-10]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.109694/-/DC1

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