There was an error published in the first e-press version of the article printed in *J. Cell Sci.* 123, 1089-1098.

The authors regret the following erroneous statement in the Discussion, p. 1096, second column:

“However, in line with Zhang and Macara (Zhang and Macara, 2006) but not Nishimura and co-workers and Mertens and colleagues (Mertens et al., 2005; Nishimura et al., 2005), our analysis reveals an antagonistic relationship between Sif/TIAM1 and Baz/Par3 in protrusion formation.”

In fact, the authors’ data support the conclusions of Zhang and Macara that Baz/Par3 mediates Rac inhibition.

The authors apologise for this mistake.
Research Article

Polarity proteins and Rho GTPases cooperate to spatially organise epithelial actin-based protrusions

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Summary
Different actin-filament-based structures co-exist in many cells. Here, we characterise dynamic actin-based protrusions that form at distinct positions within columnar epithelial cells, focusing on basal filopodia and sheet-like intermediate-level protrusions that extend between surrounding epithelial cells. Using a genetic analysis, we found that the form and distribution of these actin-filament-based structures depends on the activities of apical polarity determinants, not on basal integrin signalling. Bazooka/Par3 acts upstream of the RacGEF Sif/TIAM1 to limit filopodia to the basal domain, whereas Cdc42, aPKC and Par6 are required for normal protrusion morphology and dynamics. Downstream of these polarity regulators, Sif/TIAM1, Rac, SCAR and Arp2/3 complexes catalyse actin nucleation to generate lamellipodia and filopodia, whose form depends on the level of Rac activation. Taken together, these data reveal a role for Baz/Par3 in the establishment of an intercellular gradient of Rac inhibition, from apical to basal, and an intimate association between different apically concentrated Par proteins and Rho-family GTPases in the regulation of the distribution and structure of the polarised epithelial actin cytoskeleton.

Key words: Par, Polarity, Epithelia, Rho-GTPases, Protrusions, Actin

Introduction
Animal cells are able to form a number of distinct dynamic actin-filament-based structures. These include microvilli, filopodia and lamellipodia, each of which has a distinct dynamic form that depends on the action of overlapping sets of actin regulators (Ridley, 2006). Although these different structures frequently coexist in the context of a polarised animal cell, it is not understood in any detail how this type of polarised organisation is achieved. Epithelia are an ideal system in which to study such a problem, because adjacent cells use apically localised cell-cell junctions (Gumbiner, 2005; Knust and Bossinger, 2002), and interactions between integrins and the underlying basal lamina (Deng et al., 2003; Yu et al., 2005), to align their apical-basal axes. The result is a coordinated polarisation of a wide-range of intracellular structures and activities across an entire tissue, which includes apically localised cell-cell junctions, distinct cortical membrane compartments (Goldstein and Macara, 2007; Hutterer et al., 2004), polarised vesicle trafficking (Lu and Bilder, 2005), and a polarised actin cytoskeleton (Baum and Perrimon, 2001; Blankenship et al., 2006; Vasioukhin et al., 2000).

Through the use of Drosophila genetic techniques it is possible to study the mechanisms underlying the generation of a polarised epithelial cell architecture (Baum and Perrimon, 2001). For example, using the Flp-FRT system (Xu and Rubin, 1993) it is possible to observe the behaviour of the epithelium as a whole when introducing small clones of mutant tissue. Alternatively, the MARCM technique (Lee and Luo, 2001) allows the specific labelling of individual mutant cells, allowing for a detailed analysis of cell morphology.

This type of analysis has been used to show that apically localised polarity proteins have important roles in the regulation of cell polarity and cytoskeletal organisation in many systems. In particular, Par6 and its binding partners Cdc42 and aPKC act together with Baz/Par3 in the establishment and maintenance of polarised cortical domains in a wide variety of epithelia (Muller and Wieschaus, 1996; Horikoshi et al., 2009; Macara, 2004; Suzuki and Ohno, 2006), and function with CIP4, WASp and Arp2/3 in the regulation of adherens junction endocytosis (Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008). Moreover, recent studies indicate that interactions between Par complex proteins and RhoGTPases are essential for the proper regulation of cell and cytoskeletal polarity in an even wider variety of polarised cell types (Anderson et al., 2008; Mertens et al., 2005; Nakayama et al., 2008; Nishimura et al., 2005; Pegtel et al., 2007; Schwamborn and Puschel, 2004; Zhang and Macara, 2006; Zhang and Macara, 2008). Thus, for example, Baz/Par3 interacts with the RacGEF Tiam1 to regulate actin protrusions in mammalian fibroblasts and neuronal cells (Nishimura et al., 2005; Zhang and Macara, 2006), and tight junction formation in epithelial cells (Chen and Macara, 2005; Gopalakrishnan et al., 2007; Mertens et al., 2005). Since Rho-family GTPases are known to control the formation of distinct actin filament based structures (Ridley, 2006), this coupling between Par complex components and downstream GTPases suggests a mechanism by which different structures could be formed in distinct regions of a polarised cell.

In this study, we used the developing Drosophila pupal notum as a model system in which to explore the molecular mechanisms responsible for the generation of a polarised distribution of different actin filament-based structures within an in vivo tissue context. By focusing this combined genetic and cell biological analysis on the formation and positioning of basal actin filament based structures we found that: (1) dynamic filopodia and lamellipodia are formed at distinct positions within the basolateral domain of epithelial cells; (2) Baz/Par3 activity acts in opposition to Sif (the Drosophila homologue of Tiam1) and Rac to limit filopodia to the basal domain of the cell, in a process that is independent of basal integrin-signal; (3) Cdc42, aPKC and Par6 are required for the correct morphology and dynamics of these protrusions; and (4) the formation of both lamellipodia and filopodia in this system is
dependent on actin filaments nucleated downstream of Rac by the SCAR and the Arp2/3 complexes, with high levels of Rac generating filopodia and intermediate levels generating lamellipodia. These data support the idea that Par complex components act in concert with Rho-family GTPases to generate a graded inhibition of protrusive activity that polarises the form and dynamics of these epithelial cells.

**Results**

**Epithelial cells possess three classes of dynamic actin-based protrusions**

Epithelial cells are known to have distinct apical, junctional and basal domains, each with its own characteristic actin-based structures (Pilot and Lecuit, 2005). Here, to study the molecular machinery governing the formation of epithelial actin-filament-based protrusions, we have taken advantage of the Neuralized-Gal4 driver (Venugopala Reddy et al., 1999) to label actin filaments (UAS-GFP:Moe) or the plasma membrane (UAS-Pon:GFP) in isolated columnar epithelial cells within the dorsal thorax of developing *Drosophila* pupae. Neuralized-Gal4 drives expression specifically within the precursor cells for the external sensory organ. Importantly, at the time of our analysis [12-15 hours after puparium formation (APF)], these epithelial cells are not yet fully committed to a precursor fate (Cohen et al., unpublished). Thus, these well-spaced GFP positive cells are representative of all cells within the epithelium.

When Neuralized-Gal4:UAS-GFP nota were imaged using a confocal microscope between ~12-15 hours APF, we identified three types of characteristically positioned actin-rich protrusions (Fig. 1A-F). GFP:Moe targets GFP to filamentous actin and was seen to preferentially localise to all classes of protrusion, co-localising with phalloidin, a marker for F-actin (Fig. 1F-F'). Apically, when using the GFP:Moe reporter, short dynamic actin-rich microvillus-like structures were observed (Fig. 1F,K-M). Then, approximately 4-5 μm below the cell apex at the level of epithelial nuclei (Fig. 1G-I), sheet-like protrusions were seen extending between surrounding epithelial cells, as previously described (Demontis and Dahmann, 2007). These intermediate-level protrusions lay beneath adherens junctions and could be labelled with Dlg (Fig. 1A-F). In addition, they crossed multiple confocal planes (Fig. 1J, Fig. 4A) and were dynamic (Fig. 1N-N'), undergoing cycles of extension and retraction at a rate of 0.8±0.3 μm/minute (n=40 protrusions). Finally, at the basal end of cells, a distinct population of filopodial processes were observed embedded in lamellipodia in the region that contacts the underlying basement membrane (Fig. 1A-F). These basal filopodia had an average extension or retraction rate three times that of intermediate-level protrusions (3±1.4 μm/minute, n=40 protrusions, Fig. 1O-O' and supplementary material Movie 1). To test whether these distinct classes of protrusion were common to all cells in the epithelium we then carried out two additional experiments. First, we used a UV laser pulse to visualise
ubiquitously expressed photoactivatable GFP:Moe (PA-GFP:Moe) in limited numbers of epithelial cells (Fig. 1K-K’). Second, we used a patchy Daughterless Gal4 driver to label a subset of epithelial cells with GFP:Moe (Fig. 1L-L’). In both experiments, similar apical, intermediate and basal-level protrusions rich in actin filaments were evident in labelled cells irrespective of the GFP fusion protein used (GFP:Moe, Pon:GFP, PA-GFP:Moe), the level of expression (Neutralized GAL4, Daughterless Gal4, direct fusion) or the position of the cell within the pupal notum.

Having identified two distinct populations of dynamic protrusions within the basolateral domain of epithelial cells of the notum, we used actin inhibitors to test whether they were formed on a foundation of actin filaments (Fig. 2A-B). To do this, tissue was cut from Neu-Gal4; GFP:Moe animals 14 hours APF and incubated in M3 medium containing 1 μg/ml of the actin-monomer-binding drug latrunculin B. After 20 minutes, explants were fixed and imaged for GFP. Although GFP:Moe-rich protrusions could be observed in control explants incubated in drug-free M3 medium (Fig. 2A,A’), all actin-rich protrusions were lost after a 20 minute treatment with latrunculin B (Fig. 2B,B’). This demonstrates that actin-filament dynamics are required for the formation of apical, intermediate and basal protrusions. By contrast, parallel experiments using microtubule inhibitors failed to prevent protrusion formation (Fig. 2C,C’). Next, to identify the actin regulators underlying the formation of these actin-dependent protrusions, we analysed the function of the SCAR/WAVE complex in this system, because previous work identified a crucial role for the SCAR complex in the formation of actin-based protrusions in *Drosophila* cells in culture (Biyasheva et al., 2004; Kunda et al., 2003; Rogers et al., 2003). We used the MARCM technique (Lee and Luo, 2001) to positively label protrusions in cells mutant for two putative components of the SCAR/WAVE complex: SCAR (Fig. 2D,D’; supplementary material Fig. S1A) and HSPC300 (Qurashi et al., 2007) (Fig. 2E,E’). In both cases, the loss of SCAR complex activity resulted in the specific loss of all basolateral protrusions. This demonstrates a role for SCAR-complex activity in the formation of both lamellipodia and filopodia in the context of a three-dimensional tissue, even though most work in cell culture implicates SCAR/WAVE in the formation of lamellipodia rather than filopodia (Hahn et al., 2001; Macheský and Insall, 1998; Miki et al., 1998). By contrast, apical microvillus-like structures were unaffected by the loss of SCAR or HSPC300 (Chhabra and Higgs, 2007) (Fig. 2E-E; supplementary material Fig. S1A). Importantly, the loss of SCAR complex function seems to specifically affect protrusion formation, because columnar cell shape (supplementary material Fig. S1A,B) and apical-basal polarity (supplementary material Fig. S1C-D) were unaffected in *scar* loss-of-function clones.

Additionally, we used a Sanpodo (Spdo) antibody to label the cortex of sensory organ precursors (O’Connor-Giles and Skeath, 2003) to compare protrusions in wild-type and mutant cells. Although basal level protrusions proved difficult to observe using this method (data not shown), 83% of GFP-positive intermediate-level protrusions were co-labelled with Spdo antibody (n=20 cells) in tissue expressing a cortical Neuralized-Gal4;UAS-GFP reporter. Using this assay, we observed a significant loss of intermediate-level protrusions in *scar* mutant cells (Fig. 2G; supplementary material Fig. S2). As controls for this analysis, we used the Spdo antibody to image intermediate protrusions in *arp66b* (a component of the Arp2/3 complex) and *wasp* mutant tissue. Intermediate protrusions were lost in both *scar* and *arp66b* mutant cells, but were unaffected by the loss of WASp, which is required for Arp2/3-dependent endocytosis (Georgiou et al., 2008) (Fig. 2G; supplementary material Fig. S2).

**Cdc42-Par6-aPKC complex controls the dynamic form of basal protrusions**

Having established a role for actin, SCAR and HSPC300 in the formation of intermediate and basal-level protrusions, we next sought to identify the molecular machinery responsible for the characteristic distribution of these protrusions along the epithelial apical-basal axis. As the basal-most protrusions appeared to contact the extracellular matrix (ECM), we used the MARCM technique to positively label cells mutant for *myospheroid* (*mys*) the major *Drosophila* β-integrin protein. Integrins are cell-surface transmembrane receptors that recognise and bind to ECM proteins, thereby linking the ECM with the actin cytoskeleton (Legate et al., 2009). However, the loss of *mys* function did not alter the position (Fig. 2F-F”) or dynamics (Fig. 2F’-F’”) of protrusions in the basolateral domain, even though it caused basal filopodia to branch (8.9±2.5 branched protrusions per cell; wild type, 0.2±0.5 branched protrusions per cell; n=20 cells per genotype, Fig. 2F’’). This argues against the underlying extracellular matrix having an instructive role in protrusion positioning. Therefore in our search for cues that guide polarisation of the actin cytoskeleton, we next explored the roles of apical polarity...
determinants. We focused this analysis on the Par complex proteins, Par6, aPKC and Baz/Par3, and the associated Rho-family GTPase Cdc42 (Atwood et al., 2007; Georgiou et al., 2008; Welchman et al., 2007), because these proteins have been previously implicated in the regulation of cell polarity in a wide variety of systems, (Suzuki and Ohno, 2006) and in the regulation of actin cytoskeleton dynamics (Nishimura et al., 2005; Pegtel et al., 2007; Schwamborn and Puschel, 2004; Zhang and Macara, 2006; Zhang and Macara, 2008). Cdc42 in particular seemed to be a good candidate for a regulator of the polarised organisation of the epithelial actin cytoskeleton, because it has been implicated in SCAR-dependent actin filament formation (Biyasheva et al., 2004; Kunda et al., 2003). As previously observed (Georgiou et al., 2008), the apical area is reduced and adherens junctions destabilised in cdc42 mutant epithelial cells as a result of defects in actin-dependent endocytosis (Fig. 3C). However, the loss of Cdc42 from cells positively labelled using a GFP:Moe reporter had no effect on the small apical microvillus-like protrusions (Fig. 3C), despite the physical proximity of these protrusions to apical junctions and the loss of apical surface as the result of apical constriction (Georgiou et al., 2008). The loss of Cdc42 caused a profound reduction in basal actin filament levels (Fig. 3C, C′). A similar analysis carried out using positively labelled clones that were mutant for components of the Par complex, par6 and apkc, phenocopied the cdc42 mutant in every respect, with: (1) a reduced cell apex, as previously reported (Georgiou et al., 2008); (2) dramatically reduced levels of the GFP:Moe reporter, indicative of a loss of actin filaments; (3) a loss of basolateral filopodia and lamellipodia; and (4) defects in cell shape (Fig. 3D,E and supplementary material Fig. S3). Significantly, however, the loss of Baz/Par3 led to an opposite phenotype. Thus, positively labelled baz/par3 mutant cells exhibited excessive numbers of dynamic, actin-rich basolateral filopodia (Fig. 3F–F′), many of which appeared excessively large and branched.

To quantify the defects in basal protrusion dynamics and cell morphology, we used Pon:GFP to label the plasma membrane in positively labelled clones (Fig. 4). Unlike GFP:Moe, this labels protrusions irrespective of the number of actin filaments present. Using this approach, Pon:GFP-labelled cdc42 mutant cells exhibited severe defects in the structure of basal protrusions when compared with the wild type (Fig. 4A–C), as would be expected for a positive regulator of actin-filament formation (Kunda et al., 2003). The affect on protrusion morphology and number was variable. Mildly affected cdc42 mutant cells (representing 36% of cdc42 mutant cells, n=50) possessed abnormal protrusions at both intermediate and basal levels (Fig. 4B), whereas severely affected cells (representing 64% of cdc42 mutant cells, n=50) exhibited a complete loss of intermediate-level protrusions and thick cellular extensions at basal levels (Fig. 4C). All abnormal protrusions exhibited severely limited dynamics, being unable to extend and retract as well as wild-type protrusions (supplementary material Fig. S4 and Movie 2). By contrast, baz/par3 mutant cells possessed numerous dynamic protrusions along their entire length (Fig. 4D). Moreover, these basolateral protrusions did not fall into the two distinct classes of protrusion characteristic of the wild type. In the place of intermediate-level sheet-like protrusions, filopodia-like protrusions formed throughout the basolateral domain (Fig. 4D, quantified in Fig. 5A). These protrusions not only adopted a filopodial-like morphology but additionally exhibited filopodia-like dynamics irrespective of their location within the basolateral domain (supplementary material Fig. S5). Moreover, whereas cdc42, par6 and apkc mutants had an elongated cell morphology, with an average cell length of 14.88 μm, 14.3 μm and 13.82 μm, respectively (supplementary material Fig. S3), baz mutant cells were significantly shorter than the wild type (baz/par3 mutant cell length 8.39 μm, n=33; wild-type cell length 10.97 μm, n=33; supplementary material Fig. S3). These data suggest that Baz/Par3 acts in opposition to Cdc42, Par6 and aPKC in the generation and positioning of basal protrusions in this system. Further evidence that Baz/Par3 is required to shape protrusion formation within the basolateral domain was obtained by overexpressing Baz within labelled cells. The overexpression of Baz led to a measurable reduction in the numbers of both intermediate and basal-level protrusions (Fig. 5B).

Although these changes in cell shape seen following the loss of Baz/Par3 could be attributed to changes in cell polarity, we have previously shown that E-cadherin remains localised at adherens
In other polarised cell systems, Baz/Par3 has been shown to bind and regulate the activity of TIAM1, which is called Sif in Drosophila (Chen and Macara, 2005; Gopalakrishnan et al., 2007; Mertens et al., 2005). Sif is a nucleotide-exchange factor that is able to activate the small GTPase Rac (Mertens et al., 2003). Since Rac is thought to be the primary regulator of SCAR-dependent actin filament formation in many systems (Blagg et al., 2003; Eden et al., 2002; Kunda et al., 2003), we explored the possibility that Baz/Par3 acts on Sif in a similar way in Drosophila epithelial cells to control basal protrusion formation. As expected if this were the case, the overexpression of Sif within individual labelled cells within the epithelium phenocopied the loss of Baz/Par3, leading to the formation of filopodia-like protrusions along the entire length of the cell at the expense of the population of morphologically distinct intermediate-level protrusions (Fig. 5C; supplementary material Fig. S6). These data provide evidence to suggest that the effect of loss of Baz on protrusion number and morphology is not an indirect effect of defects in the maintenance of cell-cell adhesion in this tissue.

Rac activity is required for basolateral protrusion formation downstream of Cdc42

To determine whether apical polarity determinants and the Rac-GEF Sif act through the Rho-family GTPase Rac to regulate the form and positioning of protrusions, we tested the effects of expressing the dominant-negative and activated forms of Rac in Neu-Gal4 cells. RacV12 expression induced the transformation of lamellipodial intermediate-level protrusions into filopodia-like protrusions along the entire length of the cell (Fig. 6A, quantified in Fig. 6C and supplementary material Fig. S5). In this, RacV12 expression phenocopied the loss of Baz/Par3 and the overexpression of Sif/TIAM1, cells expressing RacV12 were also significantly shorter than wild-type cells (cell length in RacV12-expressing cells, 7.54 μm, n=33; wild-type cell length 10.97 μm, n=33; supplementary material Fig. S3). These data suggest that Rac regulates the shape, dynamics and location of protrusions within the basolateral domain of epithelial cells, with high levels of Rac promoting a shift from lamellipodia-like to filopodia-type protrusions.

To test whether Rac acts downstream of Cdc42 in this system, as previously reported for Rac in Drosophila cells in culture (Kunda et al., 2003), we tested the effects of expressing activated Rac in cdc42 mutant epithelial cells. As described earlier, cdc42 mutant cells possess protrusions of abnormal morphology (Fig. 4B,C) with limited dynamics (supplementary material Fig. S4 and Movie 2) within the basolateral domain. However, where present, the position of these abnormal protrusions appeared similar to wild-type basal protrusion formation. As expected if this were the case, the overexpression of Sif within individual labelled cells within the epithelium phenocopied the loss of Baz/Par3, leading to the formation of filopodia-like protrusions along the entire length of the cell at the expense of the population of morphologically distinct intermediate-level protrusions (Fig. 5C; supplementary material Fig. S6). These data provide evidence to suggest that the effect of loss of Baz on protrusion number and morphology is not an indirect effect of defects in the maintenance of cell-cell adhesion in this tissue.

![Fig. 4. Regulation of basal protrusion positioning by Baz.](Image)

(A-D) Live imaging of Pon:GFP-labelled epithelial cells, showing frequent confocal slices from the apex to the base of the cell. Cells shown are: (A) wild type, (B-C) cdc42 mutant, (D) baz mutant. Arrows show novel protrusions. Asterisks highlight protrusions that were previously seen at a more apical plane. Note how intermediate level protrusions in the wild type span a number of confocal planes (A). Scale bars: 10 μm.

junctions in the pupal notum in baz/par3 mutant clones (Georgiou et al., 2008). Conversely, Baz localisation is unaffected in negatively marked shg (Ecad) mutant clones (supplementary material Fig. S6A-A’). Additionally, using the MARCM system, we observed that positively marked shg mutant cells differ from baz/par3 mutant cells in that they have both shape and polarity defects; shg cells appear rounded with relatively few poorly positioned protrusions (supplementary material Fig. S6B). These data provide evidence to suggest that the effect of loss of Baz on protrusion number and morphology is not an indirect effect of defects in the maintenance of cell-cell adhesion in this tissue.
and dynamics (Fig. 6B,D). In addition, RacV12 expression in cdc42 mutant cells resulted in the formation of filopodia along the entire length of the basolateral domain (Fig. 6D), as observed when we overexpressed RacV12 or Sif in wild-type cells, or in baz mutant cells. This was the case even though RacV12 did not rescue the effect of loss of Cdc42 on apical adherens junctions or on columnar cell shape; effects that are probably mediated via a Rac-independent pathway. Additionally, as expected following this result, overexpressing Sif in cdc42 mutant epithelial cells could rescue filopodial dynamic protrusions, which were again positioned throughout the basolateral domain (supplementary material Fig. S7B-C).

The overexpression of a dominant-negative version of Rac, RacN17, from the Neu-Gal4 driver, phenocopied scar mutant cells by inhibiting the formation of both intermediate and basal-level protrusions (Fig. 7A), without affecting apical microvillus-like structures (data not shown). We noted during this analysis that RacN17 expression induced a preferential loss of filopodia. Thus, cells expressing the dominant-negative construct frequently retained basal broad lamellipodia even though they lacked filopodia, implying that filopodial formation is dependent upon relatively high levels of Rac activity. To further investigate this observation, we looked at protrusion number and morphology in flies with different genetic backgrounds (Fig. 7B,C). We found that cells with reduced Rac activity [i.e. cells heterozygous for Rac (Rac1/J1 Rac2/H1) or sif, or cells overexpressing RacN17] had significantly fewer protrusions per cell when compared with the wild type (Fig. 7B) and a significantly greater proportion of sheet-like protrusions (Fig. 7C-E). By contrast, cells likely to have higher activated Rac levels (cells overexpressing RacV12 or Sif, or baz/par3 mutant cells) had significantly more protrusions per cell and a greater proportion of filopodia-like protrusions (Fig. 7B,C,G). Additionally, the loss of one copy of sif/TIAM1 reduced protrusion number in baz/par3 mutant cells to levels similar to those seen in the heterozygous sif/TIAM1 mutants alone. Thus, Baz/Par3 is only required to limit protrusion formation in animals with wild-type levels of Sif/TIAM1, showing that sif/TIAM1 functions genetically downstream of baz/par3 (Fig. 7B,C). Finally, when using the GFP:Moe reporter to label RacN17-expressing cells, we found that cells with low levels of GFP signal were more likely to possess sheet-like protrusions with fewer finger-like protrusions (Fig. 7H-J). This series of experiments, showing the effects of increasing or decreasing Rac levels on protrusion number and shape suggests that high levels of Rac activity lead to filopodia-like structures, whereas lower levels of Rac activity lead to lamellipodia-type protrusions. Thus, the gradation in the wild type from apical lamellipodia to basal filopodia might correspond to a gradient of Rac activity, from low to high.

To confirm that Rac acts via SCAR to promote dynamic protrusions in the basolateral domain as shown in other systems (Kunda et al., 2003; Rogers et al., 2003), we used an antibody to Abi, a member of the SCAR complex and carried out immunostaining in animals with different genetic backgrounds. In wild-type cells Abi immunolabelling was weak and did not colocalise with basal protrusions (supplementary material Fig. S8A-A'). However, the level of Abi signal increased and was found to be concentrated within basal protrusions in cells overexpressing RacV12 or in baz/par3 mutant tissue (supplementary material Fig. S8B-C), confirming that Rac activation functions to recruit Abi and the SCAR complex to the membrane to induce actin filament nucleation (Steffen et al., 2004).

Taken together, these results suggest that the apically concentrated polarity regulators, Cdc42, Par6, aPKC and Baz/Par3 act together with the RacGEF Sif to define the positioning and dynamic form of protrusions within the basolateral domain of epithelial cells by delimiting the regions in which Rac is active. Active Rac-GTP then functions locally to activate the SCAR and Arp2/3 complexes to nucleate actin filaments to generate both lamellipodia and/or filopodia, depending on the level of Rac activation.

**Discussion**

Epithelial cells have a polarised morphology, with distinct apical, intermediate and basolateral actin-based protrusions

Although many studies have used the segregation of apical, junctional and basolateral markers as a model of epithelial polarity, and a number of studies have reported the existence of cell protrusions in the notum (De Joussineau et al., 2003; Renaud and Simpson, 2001) and other epithelia (Demontis and Dahlmann, 2007; Hsiung et al., 2005), these structures and the genes regulating their formation have not been characterised in detail. Here, we have used Neuralized-Gal4 (Venugopala Reddy et al., 1999) to express GFP-fusion proteins in isolated epithelial cells to reveal the dynamic shape of cells within the dorsal thorax of the fly during pupal development. Using this method, we characterised distinct populations of protrusions based on their form, dynamics and location within the basolateral domain of columnar epithelial cells. Our analysis reveals dynamic protrusions at three distinct locations within the epithelial cell: apical microvillus-like structures, intermediate-level sheet-like protrusions and basal-level lamellipodia and filopodia. Importantly, although these are all
Polar epithelial actin organisation dependent on continued actin filament dynamics, these populations of protrusions rely on different gene activities for their formation.

Cdc42, Rac, SCAR/WAVE and the Arp2/3 complex are required for the formation of basal lamellipodia and filopodia, but not for the formation of the apical microvillus-like structures. This analysis also confirms that HSPC300 should be considered to be a functional component of the SCAR complex (Qurashi et al., 2007). Moreover, the SCAR and Arp2/3 complexes are required to induce the formation of both lamellipodia and filopodia in this system. Although many studies have suggested that Rac activates the SCAR complex to induce branched Arp2/3-dependent actin nucleation that underlies lamellipodial formation, whereas Cdc42 is required to induce filopodial formation (Ladwein and Rottner, 2008; Nobes and Hall, 1995; Ridley et al., 1992), our analysis suggests that the macroscopic form of the protrusion in a tissue context is not dictated by the nucleator used. In this, our results are in line with several recent studies in cell culture (Kunda et al., 2003; Borisy and Svitkina, 2000). Instead, the macroscopic structure generated depends on the local level of Rac activity, with high levels of Rac driving filopodial formation and low levels leading to lamellipodial formation. Since the forces required to distort the membrane to generate finger-like protrusions are likely to be greater than those required to generate the equivalent section of a sheet-like protrusion, protrusion morphology might be a product of a force balance between membrane tension, extracellular confinement and local actin-filament formation. Since wild-type cells have a graded distribution of protrusions, with lamellipodia predominating apically and filopodia basally, wild-type cell morphology might reflect a gradient in the level of Rac activation, from high basal levels to low apical levels.

Components of the apically localised Par complex act in concert to regulate the position, form and dynamics of basolateral epithelial cell protrusions

Within this system, Cdc42-Par6-aPKC and Baz/Par3 appear to have antagonistic roles in the formation of basolateral protrusions. Cdc42-Par6-aPKC is required for actin filament formation and protrusion dynamics, whereas Baz/Par3 ensures the separation of basal and intermediate protrusions by limiting the extent of basal filopodia along the apical-basal axis. In this, our analysis adds to
the growing body of evidence that Baz/Par3 and Par6-aPKC have distinct molecular targets. Moreover, our data confirm that Par6-aPKC act together with the Rho-family GTPase Cdc42 (Georgiou et al., 2008; Nakayama et al., 2008; Nam and Choi, 2003). Significantly, the loss of Baz/Par3 phenocopies gain-of-function mutations in Rac and the overexpression of the Rac-GEF Sif/TIAM1, a Par3-interacting protein (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005). Baz/Par3 might therefore serve as a cell-intrinsic cue to polarise the dynamic actin cytoskeleton along the epithelial apical-basal axis, giving epithelial cells their characteristic polarised morphology.

Baz/Par3 has previously been implicated in the restriction of actin polymerisation to specific subcompartments within a cell, allowing for the formation of distinct populations of protrusions (Nishimura et al., 2005; Schwamborn and Puschel, 2004; Zhang and Macara, 2006; Zhang and Macara, 2008). This has been studied most extensively in hippocampal neurons, in which Par3 was shown to interact with Tiam1 to regulate the activation of Rac within distinct domains of the cell during axon specification and dendritic spine morphogenesis (Nishimura et al., 2005; Schwamborn and Puschel, 2004; Zhang and Macara, 2006; Zhang and Macara, 2008). Indeed, it has been suggested that the formation of a Cdc42-Par6-Par3-Tiam1-Rac1 complex is required to establish neuronal polarity (Nishimura et al., 2005). Our work suggests that Baz/Par3 acts in a similar fashion to the morphogenesis and positioning of dynamic protrusions in epithelia. However, in line with Zhang and Macara (Zhang and Macara, 2006) but not Nishimura and colleagues and Mertens and colleagues (Mertens et al., 2005; Nishimura et al., 2005), our analysis reveals an antagonistic relationship between Sif/Tiam1 and Baz/Par3 in protrusion formation. Baz/Par3 might sequester Sif/Tiam1 to prevent its association with Rac (Gopalakrishnan et al., 2007). Furthermore, because the loss of Cdc42, Par6 or aPKC results in the loss of basolateral protrusions and a marked reduction in the GFP:Moe reporter (a phenotype that can be rescued by the coexpression of RacV12 or Sif) Cdc42, Par6 and aPKC are probably required for the basal activation of Rac in epithelial cells in the Drosophila notum. Thus, signals from apically concentrated polarity determinants appear to be communicated and translated into local protrusion formation within the basolateral domain. Whether this occurs through the diffusion of an apically localised regulator (Sawin, 2009) or via long-range transmission of polarity information e.g. via microtubules, will be an important area of future research. An intriguing correlation is the largely apical localisation of Baz and its proximity to intermediate-level sheet-
like protrusions. This would suggest a possible gradient of Baz/Par3-mediated Rac inhibition, allowing sheet-like protrusions at an intermediate level and restricting filopodial protrusions to the very base of the cell. Since Baz/Par3 has been shown to localise PTEN to apical junctions (Pinal et al., 2006), it is possible that Baz recruits PTEN, which acts on PtdIns(3,4,5)P3 to generate a PtdIns(3,4,5)P3 gradient from high levels basally to low levels apically. PIP3 could then act to aid in the recruitment and activation of Rac at the membrane (Innocenti et al., 2003; Lengsford and Kirschner, 2009).

Taken together, these data demonstrate that different components of the apical determinants of cell polarity act in conjunction with the Rho-family GTPases Cdc42 and Rac to regulate the positioning of lamellipodial and filopodial protrusions over the entire span of the apical-basal cell axis. Significantly, in this tissue context, Rac, SCAR and Arp2/3 complexes promote the formation of both lamellipodia and filopodia, whose structure appears to depend on the level of Rac activation.

**Materials and Methods**

**Fly Stocks**

Mutant clones were generated using a FRT-FRT or Ubx-FRT system for the following mutants: cdc421 (Fenton et al., 1997), paxillin (Petrucoski and Koblischke, 2001), pPKC-30b (Wodarz et al., 2000), baz1 (Wodarz et al., 2000), scr(2) (Zallen et al., 2002), HSPC300(11a2) (Quarishi et al., 2007), myo5 (Bunch et al., 1992). For the genetic analysis of Rac, Sif, and the Arp2/3 complex, we studied the phenotypes of sif(23) Df3;64D-F (Sone et al., 2000), wasp(2) Df3;3R3450 (Ben-Yaacov et al., 2001) and arp66b (Hudson and Cooley, 2002) homozygous mutant pupa and Rac1(2) Rac2(2) Mt4 (Hakeda-Suzuki et al., 2002) heterozygous pupae. Overexpression studies were carried out using the following transgenes: UAS Sif (Sone et al., 2000), UAS Baz-GFP (Benton and St Johnston, 2003), UAS DRac1N17 and UAS DRac1V12 (Luo et al., 1994).

**Dissections and live imaging**

Note from pupae 12-16 hours APF were dissected in PBS for direct fixation or serum-free M3 medium (Sigma) at room temperature for drug-treatment experiments. For immunofluorescent analysis, tissue was fixed in 4% formaldehyde for 20 minutes, or SP5 microscopes. For both fixed and live imaging, a minimum of five animals were used for each experiment.

For live imaging, animals expressing the appropriate reporter were prepared by cutting and Lippincott-Schwartz, 2002). Positively labelled clones were induced with either aPKC-K06403 or SP5 microscopes. For both fixed and live imaging, a minimum of five animals were used for each experiment.

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**Supplementary material available online at**

http://jcs.biologists.org/cgi/content/full/123/7/1089/DC1

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