A Novel Function for the PAR Complex in Subcellular Morphogenesis of Tracheal Terminal Cells in *Drosophila melanogaster*

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ABSTRACT The processes that generate cellular morphology are not well understood. To investigate this problem, we use *Drosophila melanogaster* tracheal terminal cells, which undergo two distinct morphogenetic processes: subcellular branching morphogenesis and subcellular apical lumen formation. Here we show these processes are regulated by components of the PAR-polarity complex. This complex, composed of the proteins Par-6, Bazooka (Par-3), aPKC, and Cdc42, is best known for roles in asymmetric cell division and apical/basal polarity. We find Par-6, Bazooka, and aPKC, as well as known interactions between them, are required for subcellular branch initiation, but not for branch outgrowth. By analysis of single and double mutants, and isolation of two novel alleles of Par-6, one of which specifically truncates the Par-6 PDZ domain, we conclude that dynamic interactions between apical PAR-complex members control the branching pattern of terminal cells. These data suggest that canonical apical PAR-complex activity is required for subcellular branching morphogenesis. In addition, we find the PAR proteins are downstream of the FGF pathway that controls terminal cell branching. In contrast, we find that while Par-6 and aPKC are both required for subcellular lumen formation, neither Bazooka nor a direct interaction between Par-6 and aPKC is needed for this process. Thus a novel, noncanonical role for the polarity proteins Par-6 and aPKC is used in formation of this subcellular apical compartment. Our results demonstrate that proteins from the PAR complex can be deployed independently within a single cell to control two different morphogenetic processes.

FOR most cell types, morphology is key to cell function. A dramatic example of this association is seen in cells that undergo subcellular branching morphogenesis. In this process, cells send out extensions from their plasma membranes, which grow and undergo bifurcation events to form complex, branched networks. Examples of subcellular branching morphogenesis are seen in glial oligodendrocytes (Bauer *et al.* 2009) and in dendritic cells of the mammalian immune system (Makala and Nagasawa 2002), but by far the best studied examples of this process are in neurons (reviewed by Gibson and Ma 2011; Jan and Jan 2010). Indeed, neurons are frequently categorized entirely by differences in their branching morphologies (see Puelles 2009). However, despite the importance of subcellular branching morphogenesis,

little is known about the molecular mechanisms that organize distinctive subcellular branching patterns.

We are studying the process of subcellular branching morphogenesis in *Drosophila* tracheal terminal cells, a component of the insect respiratory system. Terminal cells reside at the ends of a network of cellular tubes that functions in delivering air to internal tissues (Guillemin *et al.* 1996). The cells are specified during embryogenesis, primarily through a process of competitive FGF signaling and lateral inhibition among tracheal precursors (Llimargas 1999; Ghabrial and Krasnow 2006). At hatching, terminal cells occupy stereotypical positions within the larvae and have a simple morphology, typically consisting of a cell body, connected at its base to the rest of the tracheal system, with a single, subcellular cytoplasmic projection. During larval development, terminal cells undergo considerable growth and branching, such that in late larvae, the cells have an elaborate morphology composed of a branched network of cytoplasmic extensions (Figure 1A). Growth and branching are primarily under the control of the Branchless protein, an FGF growth factor, which is secreted by oxygen-starved target tissues

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(Jarecki *et al.* 1999). The mechanisms for outgrowth are not well understood, though likely involve cytoskeletal components, including actin (Levi *et al.* 2006; Gervais and Casanova 2010); how branch sites are selected is currently unknown.

In addition to the process of cytoplasmic extension and branching, each subcellular projection forms an internal membrane-lined tube. The mechanism for tube formation is not well understood, but may involve vesicle trafficking to the center of the cell followed by vesicle fusion (Jarecki *et al.* 1999). The mature terminal cell lumen is lined by an apical membrane, which is continuous with the apical domains of other tubes of the tracheal system, but is distinguished from these other apical domains in that it forms without cellular junctions (Noirot-Timothee and Noirot 1982), typically found in polarized epithelia (Plaza *et al.* 2010).

Terminal cell development epitomizes a number of important questions in cell biology. How does local receptor activation regulate directional cell growth and migration? How are subcellular domains specified and organized? How are branch points patterned and molecularly defined? A common player in the regulation of subcellular organization is the evolutionarily conserved PAR-polarity complex (referred to here as the PAR complex), consisting of the scaffolding proteins Par-6 and Bazooka (Baz, the *Drosophila* homolog of Par-3), atypical protein kinase C (aPKC), and the small GTP-binding protein Cdc42 (reviewed by Suzuki and Ohno 2006; Goldstein and Macara 2007). In many contexts, these proteins function together (Welchman *et al.* 2007) to effect biological roles such as asymmetric cell division (e.g., Kemphues *et al.* 1988; Prehoda 2009) and establishment and maintenance of apical/basal polarity in epithelial cells (reviewed by Martin-Belmonte and Mostov 2008). However, a role for the PAR complex in subcellular branching morphogenesis or subcellular lumenogenesis has not been directly assayed.

Here, we show that PAR-complex proteins are required for both subcellular branching morphogenesis and subcellular lumen formation in tracheal terminal cells. We find that all members of the complex, as well as known physical interactions among them, are required for subcellular branching, indicating that canonical complex activity contributes to this process. The defects we observe in branching suggest that interactions between PAR-complex proteins may regulate an iterative process that generates branch patterns in terminal cells. Surprisingly, although the PAR complex is well known to be required for apical/basal polarity in other epithelial cell types, we find that only a subset of the complex members is needed for subcellular lumen formation in terminal cells. Furthermore, the proteins that are required may be acting independently in this process. Therefore, we have identified both a novel role for the PAR complex in the control of subcellular branching morphogenesis and a novel mechanisms by which PAR-complex proteins participate in forming an apical domain.

Materials and Methods

Fly stocks and genetics

Flies were reared on standard cornmeal/dextrose media and larvae to be scored were raised at 25°. The control chromosomes used in experiments were *y w FRT^{19A}* (Xu and Rubin 1993) or *FRT^{G13}* (Chou and Perrimon 1992), unless otherwise stated. Alleles analyzed were *baz^{EH171}* (Eberl and Hilliker 1988), *baz^{FA50}* [(Simoes *et al.* 2010) a gift from T. Schüpbach (Princeton University, Princeton, New Jersey) via J. Zallen (Sloan-Kettering Institute, New York, New York)], *par-6^{Δ226}* (Petronczki and Knoblich 2001), *par-6^{f05334}* (Bellen *et al.* 2004), *par-6^{29VV}* and *par-6^{15N}* (this work), *aPKC^{k06403}* (Wodarz *et al.* 2000), *aPKC^{psu69}* and *aPKC^{psu265}* (Kim *et al.* 2009), and *Cdc42⁴* (Fehon *et al.* 1997). For construction of the *baz par-6* double-mutant chromosome, see [Supporting Information, File S1](#). For mosaic analysis we used the tracheal specific *breathless* (*btl*) promoter (Shiga *et al.* 1996) in the stocks *y w P{w⁺, btl-Gal80} FRT^{19A}, hsFLP¹²²; btl-Gal4 UAS-GFP* (M. M. Metzstein, unpublished data) and *y w hsFLP¹²²; FRT^{G13} P{w⁺, tub-Gal80}; btl-Gal4 UAS-GFP* [gift from S. Luschig (University of Zurich, Zurich, Switzerland)]. The *par-6* genomic rescue transgene has been described previously (Petronczki and Knoblich 2001). To perform mosaic analysis, *par-6^{Δ226}*, *baz^{EH171}*, and *Cdc42⁴* were recombined onto *FRT^{19A}* and *aPKC^{k06403}* was recombined onto *FRT^{G13}* using standard methods. *UAS-baz* RNAi lines (5055R-1 and 5055R-2) were obtained from National Institute of Genetics Fly Stock Center, Japan (NIG-Fly) and *UAS-par-6* RNAi lines (108560 and 19730) were obtained from the Vienna *Drosophila* RNAi Center (Dietzl *et al.* 2007). Homozygous mutant cells were generated using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo 1999). We also used this technique to express λ Btl, using *UAS- λ Btl* (Lee *et al.* 1996), in GFP-marked terminal cells that were simultaneously mutant or wild type for PAR-polarity genes. To generate the mosaics, 0- to 6-hr embryos were collected in fly food vials at 25° and treated to a 45-min heat shock at 38° in a circulating water bath before being returned to 25° for development. For light microscopy, tracheal terminal cells were scored at wandering third instar.

Tracheal terminal cell screen

par-6^{29VV} and *par-6^{15N}* were isolated in a mosaic screen for mutations affecting terminal cell development, the details of which are to be published elsewhere (M. M. Metzstein and M. A. Krasnow, unpublished results). Briefly, mutations were induced on a *y w FRT^{19A}* chromosome using 25 mM EMS (Lewis and Bacher 1968). We made MARCM mosaics in ~900 lines carrying X-linked lethal mutations and scored for defects in terminal cells, using GFP expression to assess branching and brightfield microscopy to assess lumen formation. The lethality associated with *par-6^{29VV}* was mapped with respect to visible X-linked markers using standard methods. For basic characterization of *par-6^{15N}* obtained from this screen see [File S1](#) and [Table S1](#).

Immunofluorescence analysis

Wandering third instar larvae were dissected in 1× PBS to make fillets exposing the tracheal system. Fillets were fixed for 30 min in 4% paraformaldehyde in 1× PBS, rinsed three times for 15 min in 1× PBST (1× PBS + 0.1% TX100), blocked for 30 min at room temperature in PBSTB (1× PBST + 0.02% BSA), and then incubated with primary antibody overnight at 4°. Fillets were then rinsed three times for 15 min in 1× PBSTB and incubated with secondary antibody for 2 hr at room temperature. Fillets were then rinsed and mounted on glass slides in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Antibodies were used in the following concentrations: guinea pig anti-Baz, 1:500 (Wodarz *et al.* 2000); rabbit anti-Par-6, 1:500 (Petronczki and Knoblich 2001); goat anti-aPKC, 1:200 (Santa Cruz Biotechnology; sc-15727); and mouse anti-GFP, 1:1000 (Clontech; 632375). Secondary antibodies, conjugated to Alexa-488 or Alexa-568 (Molecular Probes, Eugene, OR), were used at 1:1000. Images were taken on a Zeiss (Carl Zeiss, Thornwood, NY) AxioImager M1 equipped with an AxioCam MRm.

Terminal cell branching and lumen quantification

For determination of the number of terminal cell branches and lumens in homozygous wild-type and mutant terminal cells, we collected fluorescent and brightfield images of lateral group branches [LF, LG, and LH terminal cells (Ruhle 1932)] in mosaic animals. Terminal cell branches and lumens from these images were traced manually using Neurolucida (Meijering *et al.* 2004). Branch order was assigned on the basis of the following criteria: each tracheal terminal cell has a single central branch that contains the cell body, class I terminal branches arise directly from the central branch, and class II terminal branches arise directly from class I branches. We extended this scheme for further orders of branches, if present. Lumens were quantified as a ratio of total lumen length to total branch length, and different orders of lumens were not separated. For statistical comparisons we used the two-tailed Mann–Whitney *U*-test (<http://elegans.swmed.edu/~leon/stats/utest.cgi>).

Results

par-6 is required for branching and lumenogenesis in Drosophila tracheal terminal cells

In a genetic mosaic screen, we identified a lethal mutation, designated 29VV, that showed distinct branching defects in *Drosophila* tracheal terminal cells. Wild-type cells possess a single central branch containing the cell nucleus and a set of side branches (class I branches) sprouting from the central branch (Figure 1A). In wild-type cells, class I branches bifurcate to produce class II, class III, and so forth, branches (Figure 1, A and A'). Homozygous 29VV cells have normal class I branching, but subsequent branching is much reduced, so that cells contain many fewer higher-order branches (Figure 1, B and B', quantitated in Figure

2A). In addition, wild-type cells contain a gas-filled lumen running through each branch (Figure 1A'), but 29VV homozygous cells lack gas-filled lumens (Figure 1B', quantitated in Figure 2B), apart from a region at the proximal end of the cell near the nucleus. At this level of analysis, we cannot distinguish whether mutant cells generate a lumen that does not subsequently gas fill or whether no lumen is generated at all. Finally, in contrast to wild-type cells where branches continually reduce in diameter, leading to smooth, tapered branch tips (Figure 1D), 29VV terminal cell tips often appear bulbous (Figure 1E). The morphology of these abnormal tip structures is quite variable; some appear to contain internal membranous structures, while others appear to be simple accumulations of cytoplasm (Figure 1E).

We mapped the lethality associated with 29VV to a region ~2 map units to the right of the gene *forked*. This region contained a candidate for causing the observed tracheal cell defects: the PAR-complex gene *par-6*. We sequenced the coding region for *par-6* in 29VV and found a single, non-conservative change altering the initiation codon (ATG → ATA, Figure S1A), suggesting that 29VV leads to a severe loss or complete absence of *par-6* function. Consistent with this, we found that 29VV fails to complement the *par-6* alleles $\Delta 226$ (Petronczki and Knoblich 2001) and *f05534* (Bellen *et al.* 2004) for lethality. Furthermore, a genomic construct containing wild-type *par-6* (Petronczki and Knoblich 2001) rescued the lethality associated with 29VV (data not shown). Importantly, all observed defects (branching, lumen formation, and tip abnormalities) in 29VV terminal cells were rescued by the *par-6*⁺ genomic construct (Figure S2, A and B) or by trachea-specific expression of a *par-6* cDNA (Doerflinger *et al.* 2010) under the control of the GAL4/UAS system (data not shown).

To compare the *par-6*^{29VV} terminal cell phenotype to a known null allele of *par-6*, we generated *par-6* ^{$\Delta 226$} mosaics. $\Delta 226$ is an N-terminal deletion of *par-6* that lacks detectable Par-6 protein expression (Petronczki and Knoblich 2001). We found *par-6* ^{$\Delta 226$} mutant terminal cells had defects similar to *par-6*^{29VV} in branching (Figure 1C), lumen formation (Figure 1C'), and branch tip morphology (Figure 1F). The extent of these defects was quantitatively similar between 29VV and $\Delta 226$ cells in branching (Figure 2A, *P* > 0.7) and lumen formation (Figure 2B, *P* = 0.029). *par-6* is known to be required for proper development of the embryonic cuticle (Petronczki and Knoblich 2001), and cuticular phenotypes of *par-6* ^{$\Delta 226$} and *par-6*^{29VV} were identical, either as zygotic mutants or in germline clones (data not shown). From these data, we conclude that *par-6*^{29VV} is a null allele and shows that Par-6 is required for diverse aspects of tracheal terminal cell morphology.

The canonical PAR complex is required for terminal cell branching but not all components are required for lumen formation

Our results with *par-6* mutants led us to test whether other PAR-complex members also function in tracheal terminal

Figure 1 *par-6* is required for subcellular branching and lumen formation. Mosaic L3 larvae were generated using the MARCM system, such that only homozygous tracheal cells express GFP under the control of the tracheal-specific *breathless* promoter. Expression of GFP was used to identify homozygous cells and characterize the cellular branching pattern (A–C) and branch tips (D–F). The gas-filled intracellular lumen was examined using brightfield microscopy (A'–C'). Wild-type terminal cells have extensive outgrowth and subcellular branching (A), a single gas-filled lumen within each branch (A'), and normal tapered tip morphology (D). Terminal cells homozygous for *par-6*^{29VV} or *par-6*^{Δ226} have branching defects (B and C), very little gas-filled lumen (B' and C'), and abnormal tip morphology (E and F). Note that in B' and C' other non GFP-labeled (thus wild-type) terminal cells in the fields of view have normal, darkly contrasting, gas-filled lumens. (A''–C'') Tracing of the branching pattern observed in A–C. Branch hierarchy is indicated by color: green, central branch; red, class I terminal branches; blue, class II terminal branches; yellow, class III terminal branches; orange, tip abnormality. Dashed white lines demarcate the proximal end of GFP-labeled cells; arrows highlight gas-filled lumens. Bars: A–C, 75 μ m; D–F, 25 μ m.

cell development. First, we made mosaics of the *aPKC* null allele *k06403* (Wodarz *et al.* 2000; Rolls *et al.* 2003). We found that *aPKC*^{*k06403*} mutant terminal cells have branching (Figure 3A), lumenogenesis (Figure 3A'), and tip morphogenesis defects (Figure 3B) similar to *par-6* null alleles. Also,

like *par-6*, loss of *aPKC* primarily affects class II and later-order branches (Figure 2A).

Mosaics of the null *baz* alleles *FA50* (Simoes *et al.* 2010) and *EH171* (Eberl and Hilliker 1988; Cox *et al.* 2001) display a similar branching defect to *par-6*, both qualitatively

Figure 2 Quantification of terminal cell defects. (A) Quantification of mutant terminal cell branches, using tracings as shown in Figure 1. Gray bars, total number of branches per terminal cell; red bars, number of class I branches per cell; and blue bars, number of class II branches per cell. (B) Quantification of gas-filled lumens for terminal cells mutant for the indicated polarity gene calculated as a ratio of gas-filled lumen length to total branch length and normalized to wild-type cells. Error bars represent ± 2 SEM ($n = 10$).

Figure 3 The PAR complex is required for subcellular branching, but not all components are required for lumen formation. As in Figure 1, homozygous terminal cell branches and tips were visualized by GFP expression in mosaic L3 larvae (A–F) and gas-filled lumens visualized by brightfield microscopy (A', C', and E'). Terminal cells homozygous for *aPKC^{k06403}* have branching defects (A), no gas-filled lumen (A'), and abnormal tip morphology (B). Terminal cells homozygous for *baz^{FA50}* have branching defects (C), but do contain gas-filled lumens (C') and have normal tip morphologies (D). Terminal cells homozygous for *baz^{FA50} par-6^{Δ226}* have branching defects (E), no gas-filled lumen (E'), and abnormal tip morphologies (F). (A'', C'', and E'') Tracing of the branching pattern observed in A, C, and E. Branch hierarchy colors and other labels are as in Figure 1. Bars: A, C, and E, 75 μ m; B, D, and F, 25 μ m.

and quantitatively (Figures 3C and 2A and data not shown; $P > 0.6$ for total branches). Surprisingly, terminal cells mutant for either allele of *baz* appeared to have normal gas-filled lumens (Figures 3C' and 2B and data not shown). In addition, tip morphology in *baz* mutant cells was similar to that of wild type, with a smooth tapered appearance (Figure 3D).

Mosaics of the *Cdc42* allele, *Cdc42⁴* (Fehon *et al.* 1997) had very strong branching (Figure S3A) and lumen formation (Figure S3A') defects. However, the cells also had a number of other morphological abnormalities (Figure S3A), confounding our analysis of tracheal defects. We have not characterized the role of *Cdc42* in tracheal terminal cells further.

In summary, we found that all components of the PAR complex are required for normal tracheal terminal cell branching and that the branching defect observed in each of the mutants consists primarily of a failure in higher-order bifurcation events. However, not all the components are required for subcellular lumen formation.

***par-6* and *baz* are partially redundant for branching in tracheal terminal cells**

When we compared terminal cells mutant for various members of the PAR complex, we noted a difference among

them in the severity of branching defects. In particular, terminal cells mutant for the *aPKC* null allele had a significantly more severe branching defect than either *par-6* or *baz* null mutants (Figure 2A, $P < 0.01$). One interpretation of this result is that *baz* and *par-6* are partially redundant in regulating *aPKC*. To test this, we examined *baz par-6* double-mutant cells and found that they had severe branching defects (Figure 3E), quantitatively more similar to those observed in the *aPKC* null single mutant than in either *par-6* or *baz* single mutants (Figure 2A). These data are consistent with the idea that *par-6* and *baz* are partially redundant in terminal cell branching morphogenesis.

PAR-polarity proteins show distinct localization in tracheal terminal cells

We used immunocytochemistry to determine the localization of PAR-complex proteins within tracheal terminal cells in late L3 larvae (Figure 4). In all cases, no specific staining was observed in cells mutant for the corresponding gene, demonstrating the specificity of the antibodies used (Figure S4).

We found that in wild-type terminal cells, Par-6 protein is enriched adjacent to the intracellular lumen, with little staining observed in the rest of the cytoplasm (Figure 4A). The apical localization of Par-6 is lost in *baz* mutant cells, and instead staining is found throughout the cytoplasm (Figure

Figure 4 Localization of PAR-complex proteins in wild-type and mutant terminal cells. Individual homozygous terminal cells in L3 larvae were visualized with cytoplasmic GFP (A'–G'; green channel in A''–G'') and stained for the indicated protein (A–G; red channel in A''–G''). In wild-type cells, Par-6 is enriched at the lumen (A, arrowhead), distinct from the cytoplasmic GFP (A'). In *baz^{FA50}* homozygous mutant cells, Par-6 loses luminal enrichment and is instead found throughout the cytoplasm (B), as seen by colocalization with GFP (B'). *aPKC^{k06430}* homozygous mutant cells have patches of gas-filled lumen proximal to the cell body. In these regions, Par-6 is enriched around the lumen (C, arrowhead), but its domain is expanded compared to wild-type cells. Some regions in *aPKC^{k06430}* mutant cells contain reduced GFP expression, possibly indicative of a non-gas-filled lumen (outlined with dashed white lines in C' and C''). Par-6 is not enriched around such lumens. In wild-type cells, Baz is cytoplasmically localized (D) and overlaps with cytoplasmic GFP (D''). In *aPKC^{psu265}* cells, which lack aPKC kinase activity, Baz is found enriched around the lumen (E, arrowhead). In wild-type cells, aPKC shows punctate cytoplasmic and luminal localization (F). This localization remains the same in *baz^{FA50}* mutant cells (G). Bars: 2 μm.

4B). This result is consistent with multiple reports showing that Baz is at the top of a PAR-complex localization hierarchy (reviewed in Harris and Peifer 2005). *aPKC* mutant cells mostly lack a gas-filled lumen, having this structure only in the proximal part of the cell. In these cells, Par-6 is found localized around this residual lumen, but in a broader domain than is found in wild-type cells (Figure 4C).

Baz shows no enrichment around the lumen in terminal cells that we examined, but is instead localized entirely in the cytoplasm (Figure 4D). This lack of luminal localization (and thus non-colocalization with Par-6) was surprising given our result that Par-6 accumulation at the lumen is dependent on Baz. However, this result is consistent with reports that Par-6 and Baz do not colocalize perfectly in

other mature epithelia (Harris and Peifer 2005; Morais-de-Sa *et al.* 2010). The final localization of Baz is thought to occur by a two-step process: first, apically localized Baz recruits Par-6/aPKC; second, aPKC phosphorylates Baz, causing its relocalization to subapical junctions (Nagai-Tamai *et al.* 2002; Morais-de-Sa *et al.* 2010). We wanted to know whether such a mechanism might be displacing Baz from mature subcellular lumens, and since terminal cell branches lack cellular junctions, Baz relocates to the cytoplasm. To test this idea, we examined terminal cells mutant for the kinase-dead *aPKC* allele, *psu265* (Kim *et al.* 2009). We found that *aPKC^{psu265}* mutant cells have branching defects similar to aPKC null mutant cells, but contain gas-filled lumens (data not shown). In these cells, we found that Baz was

now localized to the lumen (Figure 4E), suggesting that aPKC-dependent phosphorylation indeed relocalizes Baz from the luminal membrane to the cytoplasm. Our results also indicate that kinase activity of aPKC is required for branching, but not for lumen formation. Finally, in *par-6* or *aPKC* null mutants, which lack lumens, Baz is found in the cytoplasm (Figure S5, A and B). Therefore, apical localization of Par-6 and Baz appears to occur by mechanisms similar to those occurring in other epithelia.

aPKC shows enrichment to the lumen, but rather than having a continuous domain of localization, is present in distinct puncta (Figure 4F). aPKC is also found in dispersed puncta within the cytoplasm. Unlike Par-6, aPKC luminal localization is unaffected by loss of *baz* (Figure 4G). In *par-6* mutant cells, aPKC shows punctate staining around the residual lumen, but expression levels appear to be reduced (Figure S5C). Thus, each of the three proteins examined showed distinct localization behavior within terminal cells.

Finally, we noted neither enrichment nor depletion of any PAR-complex protein around branch sites. Thus we conclude that it is the localized activity of the polarity complex, for instance by regulated interaction with downstream effectors, that mediates branching. Alternatively, the polarity complex may function within the whole cell to control branching, independent of specific sites.

***par-6* and aPKC function independently in lumen formation**

We have shown that Par-6 and aPKC are both required for branching and lumen formation in terminal cells. These proteins are known to have a direct interaction mediated through their respective PB1 domains (Lin *et al.* 2000; Hirano *et al.* 2005). To test whether this interaction is required for branching and/or lumen formation, we examined an allele of *aPKC*, *psu69*, that contains a single point mutation located just outside the PB1 domain. This mutation completely abolishes the interaction between aPKC and Par-6 (Kim *et al.* 2009). We found that tracheal terminal cells mutant for *aPKC^{psu69}* have branching defects (Figure 5A) similar to those observed in *par-6* or *baz* null mutants and significantly less severe than those observed in the *aPKC* null (compare Figures 3A and 5A, quantitated in Figure 5D, $P < 0.01$). Interestingly, we observed that *aPKC^{psu69}* mutant terminal cells have a normal gas-filled lumen running through each branch (Figure 5A'), suggesting that the physical interaction between aPKC and Par-6 is not required for normal lumen formation. *aPKC^{psu69}* mutant terminal cells also differed from *aPKC* null cells in that they show normal branch tip morphology (data not shown). Finally, we found the defects observed in *aPKC^{psu69}* mutant cells are independent of *baz* (Figure S6).

A further line of evidence suggesting *par-6* and *aPKC* function independently in lumen formation comes from experiments in which we used RNAi to reduce the activity of *par-6*. When expressed in the tracheal system, an RNAi

transgene directed against *par-6* resulted in branching defects similar to those in *par-6*-null mutants (Figure 5B, quantitated in Figure 5D). However, we observed only weak defects in lumen formation (Figure 5B', quantitated in Figure 5E), suggesting the knockdown is only partial. When we performed this *par-6* knockdown in an *aPKC^{psu69}* mutant background, we found no difference in branching defects (Figure 5, C and D), but the lumen formation defects were partially ameliorated (Figure 5, C' and E, $P < 0.05$). An explanation for this is that Par-6 functions in two pools, and disruption of binding to aPKC releases Par-6 into the lumen formation pool (Figure 5F).

The PDZ domain of Par-6 is required for branching and lumen formation

In our screen, we identified a second *par-6* allele, designated *15N* (see *Materials and Methods*). DNA sequence analysis revealed *15N* contains a 592-bp deletion in the *par-6* gene. This mutation is predicted to truncate the Par-6 protein within its single, C-terminally located PDZ domain (Figure S1, A and B). Similar to null mutations in *par-6*, terminal cells homozygous for *par-6^{15N}* have defects in branching (Figure 6A), lumen formation (Figure 6A'), and tip morphology (Figure 6B). However, unlike null mutations, *15N* would not be expected to completely eliminate expression of Par-6 protein. In particular, while the deletion removes the C-terminal coding regions of *par-6*, the 3'-UTR is mostly left intact, missing only the first 97 (of 1677) bases. On the basis of this, we would predict that *15N* would not cause transcript instability, and while the PDZ domain is disrupted, the PB1 and semi-CRIB domains, which are required for interactions with aPKC and Cdc42, respectively (Lin *et al.* 2000; Yamanaka *et al.* 2001; Li *et al.* 2010) are left intact (Figure S1A).

To test residual activity in *par-6^{15N}* we examined the cuticle phenotype of *par-6* zygotic mutants. Embryos hemizygous for null alleles of *par-6* are known to contain large cuticular holes, indicative of epithelial polarity defects (Petronczki and Knoblich 2001). We observed this defect in the *par-6* null allele $\Delta 226$ (Figure 6D) and our new allele *29VV* (data not shown). However, we found that *par-6^{15N}* mutants do not show large cuticular holes (Figure 6E). *Trans*-heterozygotes between *15N* and null alleles of *par-6* show occasional small holes (Figure 6F), suggesting that *15N* is hypomorphic for the regulation of embryonic epithelial polarity.

In contrast to this relatively mild embryonic cuticular defect, *par-6^{15N}* homozygous terminal cells were quantitatively more severe than null alleles of *par-6* (Figure 6, G and H, $P < 0.001$) and quantitatively similar to *aPKC* null alleles. Furthermore, while *par-6^{29VV}* homozygous cells contain a small portion of gas-filled lumen proximal in the cell, *par-6^{15N}* cells have almost no observable gas-filled lumen (Figure 6A'). *par-6^{15N}* homozygous terminal cell-tip abnormalities are extensive and include large varicosities and membrane-filled cytoplasmic swellings (Figure 6B). It is

Figure 5 A direct interaction between Par-6 and aPKC is required for subcellular branching, but not for lumen formation. As in Figure 1, homozygous terminal cell branches were visualized by GFP expression in mosaic L3 larvae (A–C) and gas-filled lumens visualized by brightfield microscopy (A'–C'). Terminal cells homozygous for *aPKC^{psu69}* have branching defects similar to other polarity complex mutants (A), but have gas-filled lumens (A'). Expression of *UAS-par-6* RNAi leads to branching defects (B) similar to those seen in *par-6* null cells. These terminal cells also have lumen defects (B'), but these are not as severe as the defects observed in *par-6* null cells. Terminal cells homozygous for *aPKC^{psu69}* and also expressing the *par-6* RNAi show branching defects (C), but show more extensive gas-filled lumens than that seen in *aPKC⁺ par-6* RNAi cells (C', compare to B'). (A''–C'') Tracing of the branching pattern observed in A–C. Quantitation of branching (D) and lumen formation (E) in cells was performed as in Figure 2. (F) Model for how *aPKC^{psu69}* ameliorates the lumen defects observed in *par-6* partial RNAi knockdown cells. In wild-type cells, Par-6 is present in two pools. One pool is complexed with aPKC and functions in branching, but not lumen formation. A second pool of Par-6 functions independently of aPKC and is required for lumen formation. In the *par-6* partial knockdown there are limited amounts of Par-6 available for both branching and lumen formation, and both processes are defective. In *aPKC^{psu69}* mutant cells, since aPKC and Par-6 can no longer interact, more of the limiting amount of Par-6 is now available for lumen formation, resulting in a weaker lumen formation defect. Bar: 75 μ m. Branch hierarchy colors and other labels are as in Figure 1. Error bars represent ± 2 SEM ($n = 10$ for *aPKC^{psu69}*; $n = 5$ for *par-6* RNAi and *aPKC^{psu69} par-6* RNAi).

important to note that *par-6^{15N/+}* heterozygotes have completely normal terminal cells, indicating *15N* is fully recessive (data not shown).

Thus, *15N* appears to have complex properties, showing weaker phenotypes in some contexts, but stronger phenotypes—even stronger than null alleles—in other contexts. Since the Par-6 PDZ domain is known to be required for its physical interaction with Baz (Lin *et al.* 2000), our data suggest that a direct protein–protein interaction between Par-6 and Baz is not required for embryonic epithelial polarity, but is required for branching morphogenesis and lumen formation in tracheal terminal cells.

PAR-polarity proteins function downstream of the FGF signaling pathway to regulate subcellular branching

Directional growth and branching in *Drosophila* tracheal terminal cells are known to be controlled by an FGF extracellular signal (Jarecki *et al.* 1999), potentiated by detection of intracellular oxygen tension (Centanin *et al.* 2008). Increase in FGF, either by reducing oxygen levels or by using a transgene to directly increase expression, results in increased growth and branching of terminal cells (Jarecki *et al.* 1999). We wanted to determine whether this increase

in branching was dependent on PAR proteins. To test this, we made use of an activated form of the FGF Receptor, λ Btl (Lee *et al.* 1996). We expressed λ Btl in individual terminal cells and found that this leads to an increase in branch number, as well as cell growth, particularly obvious in the cell body (Figure 7A). While branch numbers were hard to determine precisely in this genetic background (due to the overall disorganized pattern and large number of overlapping branches), they were clearly significantly higher (>40) than those observed in wild-type cells (32 ± 2). However, when we expressed λ Btl in cells that were also mutant for *par-6^{29VV}*, we found these cells did not show increased branching (Figure 7B). Indeed, the number of branches in these *par-6^{29VV}; λ Btl* cells (10 ± 0.5) was very similar to that in *par-6^{29VV}* mutant cells not expressing λ Btl (13 ± 2). In contrast, λ Btl-stimulated cell growth, as determined by the increase in cell body size, was unaffected by *par-6^{29VV}* (Figure 7, A and B). We observed similar results for *baz*: *baz* mutant cells expressing λ Btl show reduced branching compared to wild-type cells expressing λ Btl (data not shown). From these data we conclude that Par-6 and Baz are downstream of FGF signaling for branching, but not for cell growth.

Figure 6 Analysis of *par-6^{15N}*. As in Figure 1, homozygous terminal cell branches and tips were visualized by GFP expression in mosaic L3 larvae (A and B) and gas-filled lumens visualized by brightfield microscopy (A'). Terminal cells homozygous for *par-6^{15N}* show severe branching defects (A), have no gas-filled lumen (A'), and have strong tip morphology defects (B). (A'') Tracing of the branching pattern observed in A. (C–F) Darkfield image of embryonic cuticle preparations. (C) Wild-type embryo. (D) *par-6^{Δ226}* hemizygote has a large cuticle hole (arrowhead), indicative of a polarity defect. (E) *par-6^{15N}* hemizygote has no cuticle hole. Arrow indicates a head involution defect in this genotype. (F) *par-6^{Δ226/15N}* trans-heterozygote has a small cuticle hole (arrowhead). (G and H) Quantification of terminal cell branches (G) and lumen formation (H) in *par-6^{15N}* mutant cells. Wild-type and *par-6^{29VV}* data from Figure 2 are shown for comparison. Bars: A, 75 μ m; B, 25 μ m. Branch hierarchy colors and other labels are as in Figure 1. Error bars represent ± 2 SEM ($n = 10$).

Discussion

The shape of branched networks is controlled by two parameters: the directional growth of extensions and the location of branching points. When combined in different patterns, these two processes can result in a great diversity of branched structures (Turcotte *et al.* 1998). Here, we have shown that mutations in PAR complex genes uncouple FGF-mediated growth from branching in tracheal terminal cells. We find that in PAR-complex mutants, terminal branches typically extend as far as they do in wild type and average branch length is not affected by mutations in any of the PAR-complex genes (data not shown), suggesting the PAR complex is not required for branch outgrowth, but is required for branch initiation. Furthermore, increase in branch numbers

caused by overactivation of the FGF signaling pathway requires *par-6* and *baz*, while FGF-induced cell growth is independent of these genes. We propose that the FGF signal goes through two independent pathways; one controls growth and is independent of the PAR complex, while a second, PAR-complex-dependent mechanism, controls branching (Figure 7C).

Little is known about the cellular mechanisms that initiate subcellular branching, either for terminal cells or for other cells, such as neurons. There is considerable evidence that the PAR complex regulates different aspects of cytoskeletal organization (Nance and Zallen 2011) and it has been suggested that actin may play a role in outgrowth of at least the initial terminal cell branch (Gervais and Casanova 2010), although we find development of this branch

Figure 7 Par-6 is required for FGF-induced cell branching, but not cell growth. As in Figure 1, homozygous terminal cell branches were visualized by GFP expression in mosaic L3 larvae (A and B). *par-6⁺* terminal cells expressing activated FGF receptor (λ Btl) show an increase in branch number (A) and cell growth, most obvious in the cell body (arrowhead). *par-6^{29VV}* terminal cells expressing λ Btl do not

show increased branch numbers (B), but still have increased cell growth (arrowhead). (C) Model for PAR complex in FGF-mediated branching in terminal cells. FGF stimulates outgrowth, independently of the PAR complex, and branching, dependent on the PAR complex. Bar: 75 μ m.

is apparently not affected by PAR complex mutations. Since branch outgrowth occurs from the basal cell surface, one interesting possibility is that outgrowth is controlled by the counterpart to apical PAR proteins, basal polarity proteins that include Par-1 and Lethal (2) giant larvae (Lgl) (reviewed by Goldstein and Macara 2007). These apical and basal proteins are known to negatively regulate each other (Benton and St. Johnston 2003; Hao *et al.* 2006), and this cross-regulation is critical for establishing and maintaining stable apical/basal domains (reviewed by Prehoda 2009). One possibility is that basal proteins keep the basal surface in a nonbranching configuration until it is locally downregulated by the apical PAR complex, thus triggering branching events. Characterization of targets of both the apical and the basal PAR complexes should thus shed light on mechanisms of subcellular branching.

We have found that some, but not all, of the PAR-complex components are required for subcellular lumen formation. Specifically, both Par-6 and aPKC are required, while we cannot detect any role for Bazooka in this process. In other epithelia, it is well known that disruption of any of the four complex members generally leads to loss of epithelial integrity (Goldstein and Macara 2007). However, in these epithelia, the PAR complex is invariably associated with apical junctions that form between cells. Tracheal terminal branches do not possess such junctions, being seamless, intracellular tubes (Noirot-Timothee and Noirot 1982), so perhaps canonical complex function is required only for the formation and maintenance of junctions, rather than apical determination *per se*. Consistent with this, mutations in *crumbs*, a key apical junctional component that is generally required for stable epithelia, have no effect on terminal cell lumen formation (S. Luschnig, personal communication). Furthermore, we propose that Par-6 localization to the apical surface is a consequence of lumen formation, rather than a cause. One model suggests that the localization of PAR-complex proteins starts with a difference in lipid composition of the apical membrane. This composition allows binding by Baz (Gallardo *et al.* 2010; Krahn *et al.* 2010), which then functions to recruit Par-6/aPKC (Harris and Peifer 2005). We propose a similar mechanism for the terminal cell subcellular lumen: the lumen forms with a lipid composition similar to that of typical apical membranes, causing Baz localization, which in turn localizes Par-6.

We have multiple lines of evidence that Par-6 and aPKC may function independently of each other in the lumenogenic process. First, loss of interaction between Par-6 and aPKC, as in the *aPKC^{psu69}* allele, has no effect on lumen formation, even in the absence of a potential bridging interaction through Baz. Second, the kinase activity of aPKC, which is regulated by Par-6, is not required for lumen formation. Finally, the localization of aPKC and Par-6 differs in terminal cells, with aPKC showing punctate, Baz-independent luminal localization, while Par-6 has a continuous, Baz-dependent luminal enrichment. These data suggest that Par-6 and aPKC may affect different steps in a lumen formation

pathway. Other studies have identified Par-6- and aPKC-dependent, but Baz-independent cellular processes. Specifically, cell junction formation in imaginal epithelia is thought to be regulated by a Par-6- and aPKC-dependent endocytic pathway that regulates levels of E-Cadherin at cellular contacts (Georgiou *et al.* 2008; Leibfried *et al.* 2008). The phenotypes of *par-6* and *aPKC* mutant cells in these studies were similar, leading to the proposal that Par-6 and aPKC function together at a specific, but as yet unidentified, endocytic step. However, interfering with endocytosis even at biochemically distinct steps can lead to similar phenotypes (Babst *et al.* 2002). Hence, Par-6 and aPKC may function independently of each other in this cell junctional regulation, as we have proposed here for lumen formation.

The membranes that line intracellular lumens are thought to be generated by a process of vesicle biogenesis, trafficking of these vesicles to the center of the branch, and fusion (Jarecki *et al.* 1999; Ghabrial *et al.* 2003; but see Gervais and Casanova 2010, for an alternative model). One additional phenotype present in *par-6* and *aPKC* mutant terminal cells suggests a role in membrane trafficking. These terminal cells not only lack a subcellular lumen, but also have abnormal morphology at branch tips, showing swelling of their plasma membranes and sometimes the appearance of abnormal internal membranous structures. Both these defects are suggestive of ectopic membrane at branch tips. This defect is correlated with the lack of lumen formation. Mutants such as *baz* or *aPKC^{psu69}* with abnormal branching, but no lumen defects, never show tip abnormalities. We propose that this ectopic membrane is material that normally contributes to the membrane surrounding the intracellular lumen. In this model, Par-6 and aPKC function to partition membranes between growing tips and intracellular lumens. In their absence, membrane intended for the lumen is trafficked to the tips and this excess membrane leads to the morphological defects observed.

Our genetic analysis of the PAR complex suggests that not all its components are required equally for branching. Specifically, aPKC mutant cells show a significantly more severe defect than either *par-6* or *baz* mutants. Also, we have found that *baz par-6* double mutants have a stronger defect than either of the single mutants. These data suggest the active form in branching is not the ternary Par-6/aPKC/Baz complex, since loss of any one of the components should give the same defect, and loss of any two should not give a stronger defect. Rather, we propose Par-6 and Baz act in parallel to regulate aPKC. Both the Par-6/aPKC and the Baz/aPKC complexes are required for branching, but either one has some activity on its own. Our multicomplex model may also explain why *par-6^{15N}* leads to such strong branching defects, which are comparable to those of *aPKC* single mutants, but stronger than even null alleles of *par-6*. We propose that aPKC switching between the active Baz and Par-6 bound forms goes through a ternary complex, but this complex is not active for branching. Transition from this complex to one of the active binary complexes requires the

Par-6 PDZ domain, such that in *15N* mutants the components become locked into the inactive ternary complex and are thus unable to regulate branching. This model also explains why Par-6^{15N}, lacking the interaction between Par-6 and Baz, has defects in lumen formation, even though Baz itself is not required in this process: the locked ternary complex sequesters both Par-6 and aPKC from their function in lumen formation.

Finally, we suggest that the dynamic switching of partners within the PAR complex is not unique to subcellular branching. Rather, this may be a common phenomenon in cases in which the PAR complex must be remodeled during dynamic processes, such as asymmetric cell division, but it may be less critical for the complex to function in static systems, such as in apical/basal polarity. This is evident from the cuticles of *par-6^{15N}* zygotic mutant embryos that lack large holes, which are indicative of polarity defects. We further predict from this model that *par-6^{15N}* would lead to severe defects in other dynamic processes, such as asymmetric division of neuroblasts.

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GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.111.130351/DC1>

A Novel Function for the PAR Complex in Subcellular Morphogenesis of Tracheal Terminal Cells in *Drosophila melanogaster*

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Supporting Material

Construction of *baz par-6* double mutant

Because *baz* and *par-6* are located close to each other on the *Drosophila* X chromosome (*baz* is ~1.3 m.u. to the left of *par-6*), we used a two step recombination process to construct the *baz par-6* double mutant. First, we recombined the visible marker, *scalloped* (*sd*¹), located about 7 m.u. to the left of *par-6* (thus ~5.7 m.u. from *baz*) onto *par-6*^{Δ226}. *par-6*^{Δ226} *FRT*^{19A}/*FM7c* females were crossed to *sd*¹/*Y* males, non-balancer female progeny were collected and crossed to *Dp(1;Y)W73*, *y B f*⁺ bearing males. *Dp(1;Y)W73* is a Y-linked duplication that covers *par-6*, *baz*, but not *sd*. The progeny from this cross were selected for G418 resistance (*neo*^R) encoded by the *FRT*^{19A} transgene, and individual *sd* males were used to establish lines balanced with *FM7c*. Lethal lines, presumably carrying *par-6*^{Δ226}, were chosen and complementation tests, using two independent alleles of *par-6*, were performed to confirm the presence of *par-6*. Terminal cell defects were also evaluated in mosaic animals and found to be identical to those observed in the *par-6* null, also confirming the presence of *FRT*^{19A}. These *sd*¹ *par-6*^{Δ226} *FRT*^{19A}/*FM7c* females were then crossed to *baz*^{FA50} *FRT*^{19A}/*Dp(1;Y)W73*, *y B f*⁺ males, non-balancer female offspring were collected and crossed to *Dp(1;Y)W73*, *y B f*⁺ bearing males. Single *sd*⁺ males were selected, and were crossed to *baz* and *par-6* carrying females. Animals that failed to complement both *par-6* and *baz* were used to establish lines.

Characterization of *par-6*^{15N}

When first isolated, we found that lethality associated with *15N* mapped ~2 m.u. to the right of the visible marker *forked* (*f*), located at ~57 m.u. on the X chromosome and our most rightward visible marker. We then mapped *15N* with respect to *FRT*^{19A}, using G418 resistance as a marker. We found 0 recombinants among ~10,000 flies scored. The inferred map distance was inconsistent with the previous mapped lethal location near *f*, since *FRT*^{19A} is at ~64 m.u., thus ~8 m.u. from *f*, and suggested the existence of an additional lethal very closely linked to *FRT*^{19A}. We have named these two putative lethals *let1* (~2 m.u. from *f*) and *let2* (close to *FRT*^{19A}).

To determine which, if either, of these two lethals corresponded to the *15N* tracheal cell defect, we performed experiments to separate and test the terminal cell phenotype of the two lethals independently of each other. Since *let2* maps very close to *FRT*^{19A}, we predicted that *Df(1)mal3*, an X terminal deficiency that removes chromosome segments 19A2--h26 (SCHALET and LEFEVRE 1973) would fail to complement *let2*, but would complement *let1*. We generated females of genotype *f*⁺ *let1 let2 FRT*^{19A}/*f let1⁺ let2⁺ FRT*^{19A} and crossed them to males of genotype *f*¹ *Df(1)mal3/Dp(1;Y)y⁺ mal*¹⁰⁶, and collected *f*⁺ female progeny of putative genotypes *f*⁺ *let1 let2⁺ FRT*^{19A}/*f*¹ *let1⁺ Df(1)mal3* or, less frequently, *f*⁺ *let1⁺ let2⁺ FRT*^{19A}/*f*¹ *let1⁺ Df(1)mal3*, which result from recombination between *let1* and *let2* (~6 m.u.) or recombination between *let1* and *f* (~2 m.u.), respectively. We then isolated and balanced the recombinant chromosome, confirmed lethality (thus is *f*⁺ *let1 let2⁺ FRT*^{19A}) and tested the chromosome in tracheal terminal cell mosaics. This chromosome showed identical terminal cell defects as did the original *15N* mutant isolate. Subsequent mapping experiments showed a single lethal locus,

mapping ~6 m.u. from FRT^{19A} consistent with the map distance from f . All subsequent analysis of $15N$ was carried out using this recombinant chromosome.

To confirm the second putative lethal locus ($let2$), which is close to FRT^{19A} , did not contribute to the terminal cell phenotype, we generated females of genotype $f^+ let1 let2 FRT^{19A} / f let1^+ let2^+$ and crossed them to $FM0, f/Y$ males. A number of resulting $f neo^R$ progeny were selected (putative genotypes $f let1^+ let2 FRT^{19A} / FM0, f$ or $f let1 let2 FRT^{19A} / FM0, f$) and tested for terminal cell defects. One line that was tested was found to be lethal, but terminal cells appeared completely wild-type in mosaic animals. This line thus contains the recombinant chromosome $f let1^+ let2 FRT^{19A}$ and $let2$ does not contribute to a terminal cell defect. We have not characterized $let2$ any further.

The following data showed that $15N$ is an allele of $par-6$. First, we found the lethality associated with $15N$ (after removal of the irrelevant lethal mutation) mapped to a very small (59 kb) interval that contains $par-6$. Second, $15N$ failed to complement known alleles of $par-6$ for lethality (data not shown). Finally, $15N$ lethality (data not shown) and terminal cell defects (Figures S2C and S2D) were rescued by the $par-6^+$ containing genomic transgene (PETRONCZKI and KNOBLICH 2001).

SNP mapping

To map the $15N$ mutation, we used meiotic recombination with visible and single nucleotide polymorphism markers (SNPs). The mutations were induced on a $y w FRT^{19A}$ chromosome. To map with respect with visible markers, we used the mapping chromosome $sc cv ct v g f$. We found that this chromosome is also highly polymorphic on the sequence level with the $y w FRT^{19A}$ chromosome (M.M.M. and M.A.Krasnow, unpublished data), with a SNP variant approximately every 250 bp. To isolate recombination events, we took females of genotype $f^+ 15N FRT^{19A} / f 15N^+$ (Since we found $15N$ was to the right of f , this is the only visible marker relevant for recombination analysis) and crossed them to $FM7c/Y$ males. To isolate recombination events to the left of $15N$, we selected for viable (i.e. $15N^+$) male offspring that were also f^+ , thus had a recombination event between f and $15N$. To isolate recombination events to the right of $15N$, we selected for viable (i.e. $15N^+$) G418 resistant males. Individual males were then typed for SNPs in the $f-FRT^{19A}$ interval. SNPs were typed by performing PCR to amplify a 500-1000 bp fragment around the SNP site followed by a diagnostic restriction enzyme digest or direct sequencing of the PCR product. Location of the SNPs and their relative position to $15N$ are described in supplemental table 1. The smallest interval we determined to contain $15N$ was ~59 kb (XE10-XE01).

PETRONCZKI, M., and J. A. KNOBLICH, 2001 DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat Cell Biol 3: 43-49.

SCHALET, A., and G. LEFEVRE, JR., 1973 The localization of "ordinary" sex-linked genes in section 20 of the polytene X chromosome of *Drosophila melanogaster*. Chromosoma 44: 183-202.

Figure S1 Molecular analysis of new *par-6* alleles. (A) Genomic structure of *par-6* showing location of the 29VV mutation and the extent of the 15N deletion. Filled boxes represent coding sequence, open boxes represent 5' and 3' UTRs, the open arrow indicates the direction of transcription. Colors represent different domains of Par-6: PB1 (green); semi-CRIB (blue); PDZ (magenta). Ticks above sequence show out-of-frame (red) and first in-frame (black) start codons after the usual start. The extent of the 15N deletion is bracketed under the structure. (B) Partial sequence of the final exon of *par-6*. The dark line represents the sequence deleted in 15N. The first asterisk represents the normal *par-6* stop codon and the second asterisk represents the inferred 15N stop codon. 15N is predicted to remove 64 amino acids of Par-6 and add 10 amino acids that are not normally coding before translation termination.

Figure S2 *par-6* rescue experiments. Homozygous terminal cell branches and tips were visualized by GFP expression in mosaic L3 larvae (A-D) and gas-filled lumens visualized by bright-field microscopy (A' and C'). Terminal cells homozygous for *par-6*^{29VV} (A, B) or *par-6*^{15N} (C, D) carrying one copy of a genomic *par-6*⁺ containing transgene have normal branching (A, C), a complete gas-filled lumen (A', C') and normal tapered tip morphology (B, D). Dashed white lines demarks the proximal end of the cell; arrows point to gas-filled lumens. Scale bar; 75μm.

Figure S3 Terminal cell defects in *Cdc42* mutants. Tracheal terminal cells homozygous for *Cdc42*⁴ have branching defects (A) and no gas-filled lumen (A'). Dashed white lines demarcate where the homozygous GFP-labeled cell starts relative to other (wild-type) tracheal cells. Additional defects are observed in *Cdc42* mutants, including fine filopodial-like extensions (arrowheads) and regions of branches in which the cell membranes appear to spread out on the substrate, leading to a wider and thinner appearance (arrow). Scale bar; 75μm.

Figure S4 Staining of mutant cells showing specificity of antibody staining. Individual terminal cells in L3 larvae stained for the indicated protein (A-C). Par-6 is localized to the lumen in *par-6*^{29VV} heterozygous cells (A), but completely absent in terminal cells homozygous for *par-6*^{29VV} (A'). aPKC is detected in terminal cells heterozygous for *aPKC*^{k06403} (B), but absent in *aPKC*^{k06403} homozygous terminal cells (B'). Baz is cytoplasmically localized in terminal cells heterozygous for *baz*^{FA50} (C), but absent in *baz*^{FA50} homozygous terminal cells (C'). Heterozygous and homozygous mutant cells were scored within the same mosaic animals in each case. Scale bar; 2μm.

Figure S5 Localization of Baz and aPKC in mutant backgrounds. Individual homozygous terminal cells in L3 larvae visualized with cytoplasmic GFP (A'-C'; green channel in A''-C'') and stained for the indicated protein (A-C; red channel in A''-C''). Baz is cytoplasmically localized and overlaps with cytoplasmic GFP in *aPKC*^{k06403} (A-A''), and *par-6*^{29VV} (B-B'') terminal cells. In *par-6*^{29VV} mutant cells, aPKC shows punctate cytoplasmic and luminal localization (C-C''), similar to that seen in wild-type cells. Scale bar; 2µm.

Figure S6 Baz does not function to bridge interactions between Par-6 and aPKC^{psu69} in lumen formation. Baz has protein-protein interactions with both aPKC and Par-6, and one possibility we considered is that Baz may act to bridge the interaction between aPKC and Par-6, missing in *psu69*, to allow lumen formation to occur. One prediction of this model is that baz, while not normally required for lumen formation, would be required in *psu69* mutant terminal cells. To test this, we made mosaics of *aPKCpsu69* in animals also expressing an RNAi transgene directed against *baz*. We first confirmed that terminal cells expressing the *baz* RNAi transgene, under the control of the tracheal-specific *breathless* promoter, show branching defects (A), but no lumen defects (A'), akin to *baz* mutant terminal cells. Next, we examined terminal cells mutant for *aPKC^{psu69}* that also expressed the *baz* RNAi transgene. We found that these cells still had a gas-filled lumen running through each branch (B'). These results indicate that the *aPKC^{psu69}* mutant terminal cells generate lumens independent of Baz, and therefore Baz does not bridge the interaction between aPKC and Par-6 in *psu69* mutant cells. Dashed white lines demarks the proximal end of the cell; arrows point to gas-filled lumens. Scale bar; 75μm.

TABLE S1 15N SNP mapping data

SNP ID	SNP position ¹	SNP sequence ²	Detection method ³	Position relative to 15N
XA70	17405759	C -> G	<i>ScrFI</i> polymorphism	Left
XB78	17503989	A -> T	Sequencing	Left
XE07	17515033	T -> C	<i>HaeIII</i> polymorphism	Left
XE10	17546733	A -> G	Sequencing	Left
XE01	17605985	G -> T	<i>MslI</i> polymorphism	Right
XB92	17707066	15 bp insertion	Sequencing	Right
XB93	17766184	C -> T	<i>AccI</i> polymorphism	Right

Notes:

1. Based on *D. melanogaster* genome release 5.30 (www.flybase.org). The XB92 insertion is after the base listed.
2. Difference between the $y\ w\ FRT^{19A}$ (listed first) and *sc cv ct v gf* (listed second) chromosomes. For XB92, the insertion is present on *sc cv ct v gf*.
3. SNP changes a restriction enzyme recognition site or is scored by direct sequencing of PCR products.