

The positioning and segregation of apical cues during epithelial polarity establishment in *Drosophila*

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Cell polarity is critical for epithelial structure and function. Adherens junctions (AJs) often direct this polarity, but we previously found that Bazooka (Baz) acts upstream of AJs as epithelial polarity is first established in *Drosophila*. This prompted us to ask how Baz is positioned and how downstream polarity is elaborated. Surprisingly, we found that Baz localizes to an apical domain below its typical binding partners atypical protein kinase C (aPKC) and partitioning defective (PAR)-6 as the *Drosophila* epithelium first forms. In fact, Baz positioning

is independent of aPKC and PAR-6 relying instead on cytoskeletal cues, including an apical scaffold and dynein-mediated basal-to-apical transport. AJ assembly is closely coupled to Baz positioning, whereas aPKC and PAR-6 are positioned separately. This forms a stratified apical domain with Baz and AJs localizing basal to aPKC and PAR-6, and we identify specific mechanisms that keep these proteins apart. These results reveal key steps in the assembly of the apical domain in *Drosophila*.

Introduction

Cell polarity is fundamental to all cells, from bacteria positioning their septa to neurons forming axons and dendrites. Cells use landmarks to establish and elaborate polarity. An initial cortical landmark organizes other cell components (some are recruited and others repulsed). In this way, specific proteins are positioned to different cortical sites to control cell fate, differentiation, and function. This basic mechanism appears to underlie all cell polarization, but its molecular bases vary and in many cases remain unclear (for review see Tepass et al., 2001; Doe and Bowerman, 2001; Ohno, 2001; Knust and Bossinger, 2002; Nelson, 2003; Macara, 2004).

An excellent model of polarity establishment emerged from studies of the partitioning defective (PAR) genes in *Caenorhabditis elegans*. Here, sperm entry likely creates the initial polarity landmark at the posterior of the one-cell embryo (Goldstein and Hird, 1996). Cytoskeletal flow then carries PAR-3 (homologue of fly Bazooka [Baz]), PAR-6, and atypical protein kinase C (aPKC) to the anterior cortex (Cheeks et al., 2004; Munro et al., 2004). These proteins develop mutual

antagonism with posterior PAR-2 and the resulting polarity directs the partitioning of cell fate determinants. Remarkably, key elements of this polarity-generating system are highly conserved, acting in neuronal and epithelial polarity from flies to humans (Doe and Bowerman, 2001; Macara 2004).

Epithelia (the most common tissue architecture) are sheets of adherent cells that separate body compartments. Each epithelial cell is polarized with an apical domain facing either luminal space or the animal exterior, and a basolateral domain facing the extracellular matrix. This polarity is critical for epithelial structure and function, regulating, for example, nutrient uptake in the gut. Establishing and maintaining this polarity involves cell junctions, other cortical landmarks (including PAR proteins), cytoskeletal cues, and membrane trafficking.

Adherens junctions (AJs) are often considered the primary epithelial polarity landmark. AJs are adhesion complexes composed of cadherin receptors linked to cytoplasmic β -catenin (*Drosophila* Armadillo [Arm]), α -catenin, and actin (reviewed in Tepass et al., 2001). They assemble at the boundary of the apical and basolateral domains and are often required for epithelial polarity. However, other landmarks may act upstream or in parallel to AJs to establish epithelial polarity. For example, activation of LKB1 (PAR-4) can polarize single intestinal epithelial cells in the absence of AJs (Baas et al., 2004), and AJs act semiredundantly with apical and basal cues in *Drosophila* follicle cells (Tanentzapf et al., 2000).

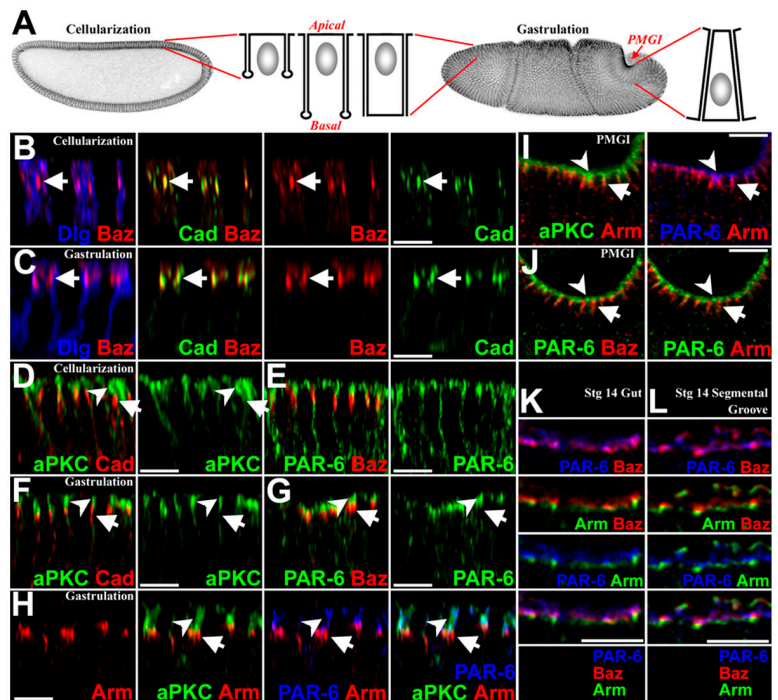
We examined epithelial polarity as it is first established during *Drosophila* cellularization. *Drosophila* development

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Abbreviations used in this paper: AJ, adherens junction; aPKC, atypical protein kinase C; Arm, Armadillo; Baz, Bazooka; CD, cytochalasin D; Crb, Crumbs; DE-Cad, *Drosophila* E-cadherin; Dhc, dynein heavy chain; DIC, dynein intermediate chain; Dlg, Discs large; IP, immunoprecipitation; Lgl, lethal giant larvae; MT, microtubule; m/z, maternal/zygotic; PAR, partitioning defective; PMGI, posterior midgut invagination; WT, wild type.

The online version of this article contains supplemental material.

Figure 1. Baz and AJs localize below aPKC and PAR-6 during WT epithelial development. (A) Early epithelial development (Dlg-stained embryos and schematics). At cellularization (left), membrane furrows separate each cell. At gastrulation (right), cells form the ectoderm and body compartments, e.g., the PMGI. (B) Cellularization. Baz (red) and DE-Cad (green) colocalize in apical puncta below the top of furrows (Dlg; blue). (C) Gastrulation. Baz (red) and DE-Cad (green) colocalize in apical puncta below the top of lateral membranes (Dlg; blue). (D and E) Cellularization. (D) aPKC (green) enriched above DE-Cad (red). (E) PAR-6 (green) cytoplasmic and along the entire furrow with slight enrichment above Baz (red). (F–H) Gastrulation. (F) aPKC (green) above DE-Cad (red). (G) PAR-6 (green) above Baz (red). (H) aPKC (green) and PAR-6 (blue) colocalize above Arm (red). (I and J) Stages 7 and 8 PMGI. (I) aPKC (green) and PAR-6 (blue) colocalize above Arm (red). (J) PAR-6 (green) above both Baz (red) and Arm (red, right). (K) Stage 14 gut. Baz (red) below PAR-6 (blue), but above Arm (green). (L) Segmental grooves, stage 14 epidermis. Baz (red) below PAR-6 (blue), but above Arm (green). Bars, 5 μ m.



begins in a syncytium. After 13 nuclear divisions, furrows form synchronously from the overlying plasma membrane compartmentalizing \sim 6,000 nuclei into individual columnar cells (Nelson, 2003; see Fig. 1 A). Cellularization thus forms the first embryonic epithelium, which is then remodeled to drive gastrulation and further morphogenesis (see Fig. 1 A).

To our surprise, we found that the apical cue Baz (PAR-3) acts as a landmark upstream of AJs as epithelial polarity is first established (Harris and Peifer, 2004). During cellularization, Baz accumulates apically without AJs while AJs fail to form without Baz. Thus, Baz must be positioned by other cues present in each cellularization compartment—what are these cues? Moreover, if Baz is atop the polarity hierarchy, how does it direct downstream polarity?

The cues that position Baz must be polarized during cellularization. Obvious candidates emerged; the syncytial and cellularizing embryo has clear cytoskeletal polarity. Actin forms specific apical and basal networks and microtubules (MTs) nucleated from apical centrosomes form inverted baskets over each nucleus. We hypothesized that these cytoskeletal cues might position Baz.

Baz and AJs act with other cortical cues to elaborate downstream polarity. Baz can interact physically with PAR-6 and aPKC (Wodarz et al., 2000; Hutterer et al., 2004), and these proteins recruit other apical cues. Baz and PAR-6 recruit apical Crumbs (Crb) and Patj, respectively (Bilder et al., 2003; Hutterer et al., 2004), and aPKC stabilizes apical Crb (Sotillos et al., 2004). In turn, Crb stabilizes apical AJs and Baz (Grawe et al., 1996; Tepass, 1996; Harris and Peifer, 2004). Mutually antagonistic interactions between apical and basolateral cues further segregate the apical and basolateral domains (Bilder et al., 2003; Tanentzapf and Tepass, 2003; Benton and St Johnston, 2003a; Hutterer et al., 2004). These interactions are

critical for establishing apical–basal polarity, but many questions remain. We were interested in how the Baz–PAR-6–aPKC complex is assembled and how these proteins interact with the cytoskeleton and other cortical complexes.

Here, we show that Baz and AJs colocalize and that AJ positioning is closely coupled to Baz. Surprisingly, we find that most Baz is positioned basal to its proposed binding partners aPKC and PAR-6. We next show that even though Baz and aPKC are both positioned apically early in cellularization, Baz acts upstream of aPKC. Baz itself is positioned by both an apical scaffold and by dynein-mediated basal-to-apical transport during cellularization. Distinct pathways position aPKC and PAR-6 apical to Baz and AJs, and specific mechanisms keep these proteins apart. These and other results reveal key mechanisms that establish a stratified apical domain during early epithelial development in *Drosophila*.

Results

Baz does not colocalize with aPKC and PAR-6

In most models, Baz, aPKC, and PAR-6 form a complex to regulate epithelial polarity. In mammalian cells, this complex localizes above AJs at tight junctions (Nelson, 2003). Thus, it was surprising that Baz colocalizes with AJs during *Drosophila* cellularization (Harris and Peifer, 2004; Fig. 1 B, arrow). Considering aPKC and PAR-6 can bind Baz (Wodarz et al., 2000; Hutterer et al., 2004), we hypothesized that they might also localize to AJs in this context. During cellularization, aPKC is apically enriched with low levels along the furrows, as shown by Wodarz et al. (2000), but this enrichment (Fig. 1 D, arrowhead) is above the forming AJs (Fig. 1 D, arrow, DE-Cad [*Drosophila* E-cadherin] marks AJs). PAR-6 localizes cyto-

plasmically and cortically, with only slight apical enrichment, as shown by Petronczki and Knoblich (2001), and is not enriched in apical Baz puncta (Fig. 1 E). Thus, Baz colocalizes with AJs rather than aPKC or PAR-6 during cellularization.

To address whether Baz remains segregated from aPKC and PAR-6 in later epithelia, we examined gastrulating (stages 7 and 8) embryos (Fig. 1 A, right). Baz continues to colocalize with DE-Cad in the epidermis (Harris and Peifer, 2004; Fig. 1 C, arrow) and in the posterior midgut invagination (PMGI; a body compartment formed by invaginating posterior cells; Fig. 1 J, arrow). aPKC remains apical to AJs in both tissues (Fig. 1, F and I, arrowheads; AJs marked by Arm) whereas PAR-6 becomes enriched above Baz (Fig. 1, G, H, and J, arrowheads), colocalizing with aPKC in the extreme apical domain (Fig. 1, H and I, arrowheads). Thus, most cortical Baz remains segregated from aPKC and PAR-6 during gastrulation, and retains close AJ association.

Baz was previously found to localize above AJs at stage 14 when the epithelium is fully polarized (Wodarz et al., 2000). To determine when Baz segregates from AJs, we examined embryos over development. We detected some segregation at stages 11 and 12 (unpublished data) that became more pronounced at stage 14 and later. At stage 14, segregation was most evident in the gut (Fig. 1 K) and in segmental furrows of the epidermis (Fig. 1 L). In each case, Baz appears to localize just apical to AJs. However, PAR-6 continues to localize just apical to Baz (Fig. 1, K and L). Thus, in late stage epithelia, the apical domain is stratified into three regions.

Baz acts upstream of aPKC as polarity is first established

Our previous work implicated Baz as a primary apical landmark during cellularization (Harris and Peifer, 2004). Considering both Baz and aPKC localize apically as polarity is established, we wondered whether one functions upstream to position the other. To address this, we first analyzed aPKC localization in cellularizing *baz* maternal/zygotic (*m/z*) mutants. In *baz^{m/z}* mutants, aPKC is mislocalized along the full furrow length (Fig. 2 B, bracket), in contrast to its apical wild-type (WT) localization (Figs. 1 D and 2 A, arrow). Thus, Baz is required for aPKC positioning during cellularization. PAR-6 has a nonpolarized cytoplasmic and cortical distribution in *baz^{m/z}* mutants (Fig. 2 B), as in WT (Fig. 1 E). As *baz^{m/z}* mutants begin gastrulation, aPKC remains basally mislocalized (Fig. 2 C, bracket), but PAR-6 shows some apical enrichment (Fig. 2 C, arrows). Thus, although Baz is required for apical aPKC positioning, later PAR-6 positioning may be partially Baz independent.

We next asked if Baz positioning requires aPKC, by analyzing *apkc^{m/z}* mutants. As these mutants cellularize, Baz has a normal apical distribution (Fig. 2 D, arrow) in structures resembling WT spot junctions (Fig. 2 E, arrow; inset, WT). Baz colocalizes with DE-Cad (Fig. 2, D and E, arrows) and Arm (unpublished data) at these sites, although AJ proteins also localize over the cortex at lower levels. In contrast, PAR-6 has a diffuse cytoplasmic distribution in *apkc^{m/z}* mutants with little cortical association (Fig. 2 F) and no enrichment in apical Baz puncta (Fig. 2 F, arrows). Thus, apical Baz and DE-Cad are positioned

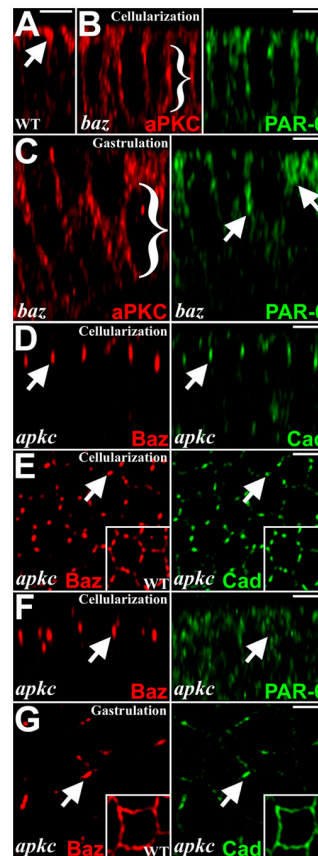


Figure 2. Baz acts upstream of aPKC as epithelial polarity is established. (A) WT cellularization. aPKC (red) apical. (B and C) *baz^{m/z}* mutants. (B) Cellularization. aPKC (red) mislocalized basally. PAR-6 (green) cortical and cytoplasmic. (C) Gastrulation. aPKC (red) still basally mislocalized. PAR-6 (green) has some apical enrichment. (D-F) *apkc^{m/z}* mutants, cellularization. (D) Cross section. Baz (red) and DE-Cad (green) are apical. (E) Surface view. Baz (red) and DE-Cad (green) colocalize in spot junctions, similar to WT (insets). (F) PAR-6 (green) is cytoplasmic. (G) *apkc^{m/z}* mutant, stage 8 (late gastrulation). Surface view. Baz (red) and DE-Cad (green) colocalize in discontinuous cortical patches (WT junctions are continuous; insets). Bars, 5 μ m.

independently of aPKC during cellularization. However, as *apkc^{m/z}* mutants gastrulate (stage 8), Baz and AJs fail to form belt junctions (both mislocalize to cortical patches and cells lose polarity; Fig. 2 G, arrow; compare with WT in insets). Similarly, analyses of *par-6^{m/z}* mutants showed that PAR-6 becomes required for Baz and AJ positioning at gastrulation (Hutterer et al., 2004). The correct apical positioning of Baz and AJs with largely nonpolarized PAR-6 during WT cellularization, and with noncortical PAR-6 during *apkc^{m/z}* mutant cellularization, further indicates that PAR-6 is a later positional cue. Thus, Baz appears to be positioned independently of aPKC and PAR-6 as polarity is first established during cellularization.

Baz localization and preexisting cytoskeletal cues

Baz is required for positioning AJs and aPKC as polarity is established during cellularization (Harris and Peifer, 2004; Fig. 2), but what upstream cues position Baz? Considering cellularization requires preexisting cytoskeletal polarity, we hypothesized

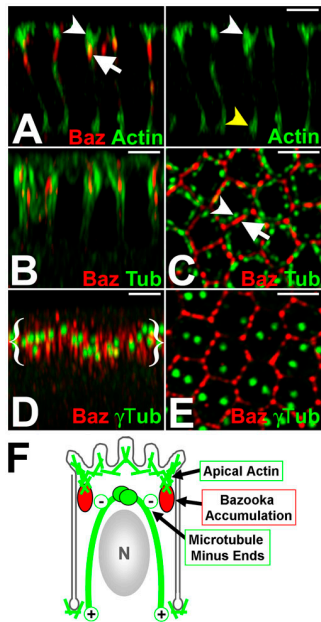


Figure 3. Baz is in proximity to the actin and MT cytoskeletons during WT cellularization. (A) Cross section. Baz (red) at the base of the apical actin meshwork (green; actin decorated with the actin binding domain of moesin fused to GFP). (B) Cross section. Apical Baz (red) next to longitudinal MTs (green). (C) Surface view. Apical Baz (red) has minimal overlap with MTs (green). (D) 10 μm deep cross section. Apical Baz (red) next to centrosomes (γ -tubulin, green). (E) Surface view. Apical Baz (red) next to centrosomes (γ -tubulin, green). (F) Schematic of apical Baz in proximity to cytoskeletal cues. Bars, 5 μm .

that cytoskeletal cues might position apical Baz. To test this, we first examined Baz localization relative to actin and MTs during WT cellularization. Actin is enriched in furrow canals at the base of invaginating furrows (Fig. 3 A, yellow arrowhead), and in an apical meshwork (Fig. 3 A arrowhead; lower actin levels are along the full furrow length). Baz is not enriched at basal furrow canals, but does overlap with the apical actin meshwork, although only at its basal edge (Fig. 3 A, arrow).

We next assessed Baz positioning relative to MTs. During cellularization, centrosomes localize above each nucleus and MTs project down the lateral membrane, with their minus ends at the apical centrosomes, forming inverted baskets over the nuclei (Fig. 3, B–F). Apical Baz accumulates in proximity to MTs, but with little or no colocalization (Fig. 3, B and C, arrow versus arrowhead). More strikingly, Baz localizes at the same position along the apical–basal axis as the apical centrosomes (Fig. 3, D [bracketed] and E), suggesting Baz is in close proximity to MT minus ends. We hypothesized that Baz might be transported along MTs to the apical domain, and then anchored there (Fig. 3 F).

Baz positioning requires a saturable apical scaffold

To assess how Baz is positioned during cellularization, we first performed time lapse imaging of embryos expressing UAS-driven BazGFP under the control of a maternal GAL4 driver. BazGFP is functional as it rescues *baz* mutant follicle cells and embryos (Benton and St Johnston, 2003a). We imaged the

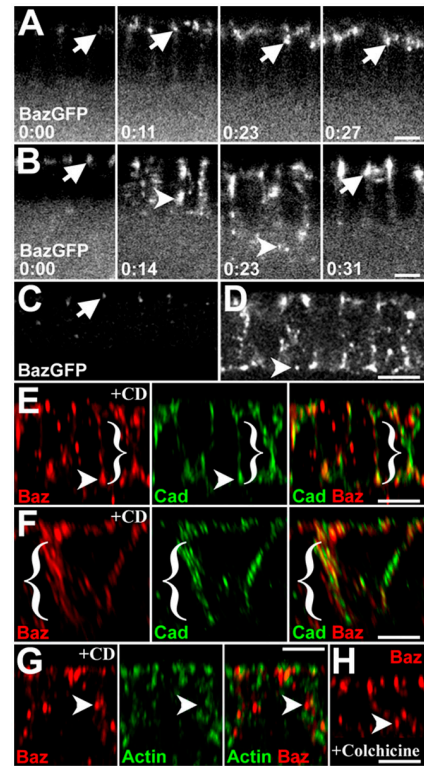


Figure 4. Baz positioning involves a saturable, apical scaffold, and actin. (A) In 6/22 embryos, BazGFP forms apical puncta at early cellularization (0:00 min, arrow) and stays apical as furrows pass the base of the nuclei (0:11, arrow), reach full length (0:23, arrow) and as gastrulation begins (0:27, arrow; Video S1). (B) In 16/22 embryos, BazGFP accumulates apically (0:00, arrow), but many BazGFP puncta move basally with the furrows (0:14, arrowhead; furrows at base of nuclei; Video S2). Some BazGFP puncta remain basal at full furrow length (0:23, arrowhead), but in 14/16 embryos BazGFP is repositioned apically by gastrulation (0:31, arrow; Video S3). (C and D) Fixed mid-cellularization, BazGFP-expressing embryos imaged with the same settings. 6/6 embryos with the lowest BazGFP levels had mainly apical BazGFP (C, arrow). 10/11 embryos with higher BazGFP levels had ectopic basal BazGFP (D, arrowhead). (E–G) CD treatment. (E) Endogenous Baz (red) shifts basally but largely remains furrow associated (DE-Cad; green). Note relatively normal furrows and colocalization of Baz and DE-Cad (arrowhead). (F) With severe furrow defects (note wide furrow spacing), basal Baz is largely cortical. (G) Some actin is detected (green; actin decorated with the actin binding domain of moesin fused to GFP) but without specific enrichment at Baz accumulations (red, arrowhead). (H) Colchicine also leads to basal endogenous Baz. Bars, 5 μm .

apical–basal axis of the epithelium in embryo cross sections. At early cellularization, BazGFP shows low level, even accumulation on nascent furrows (Fig. 4 A, B, 0:00, arrows). BazGFP then coalesces into punctate accumulations (Fig. 4 A, 0:11, arrow), closely resembling endogenous Baz (Harris and Peifer, 2004). This confirms that Baz is recruited apically during the earliest stages of polarity establishment.

As cellularization proceeds, however, BazGFP displays two different distributions that depend on its expression level. Staining for Baz revealed a wide range of BazGFP accumulation levels, all higher than endogenous Baz (unpublished data). In embryos with the lowest levels, BazGFP remained mainly apical (6/6 embryos; Fig. 4 C, arrow), as does endogenous Baz (Harris and Peifer, 2004). In contrast, in embryos with higher BazGFP levels BazGFP became mislocalized basally along the lateral

membrane, often accumulating at the furrow base (10/11 embryos; Fig. 4 D [arrowhead] and Fig. 7, A–C). These differences were also obvious in live imaging. In 6/22 live embryos, BazGFP was retained apically throughout cellularization (Fig. 4 A, 0:23 and 0:27, arrows; Video S1 available at <http://www.jcb.org/cgi/content/full/jcb.200505127/DC1>). However, in 16/22 embryos, many BazGFP puncta spread basally along the invaginating furrows (Fig. 4 B, 0:14, arrowhead; Video S2 available at <http://www.jcb.org/cgi/content/full/jcb.200505127/DC1>). This suggests that Baz is normally anchored by an apical scaffold, but at high levels, Baz may saturate the scaffold and excess Baz moves basally with the invaginating furrows.

We hypothesized that the apical Baz-binding scaffold might require actin. To test this, we exposed WT embryos to cytochalasin D (CD) for 30 min to disrupt actin and fixed them immediately to assess Baz. In treated embryos, endogenous Baz becomes basally mislocalized all along the lateral membrane (Fig. 4 E, bracket; the DMSO carrier had no effect [not depicted]). Although some Baz dissociates from the cortex, most remains cortical, as marked by DE-Cad (Fig. 4 E, bracket) or Discs Large (Dlg; unpublished data). This is true in embryos with minimal disruption of cell arrangement (Fig. 4 E), and in embryos with more extreme phenotypes (Fig. 4 F, brackets). Many ectopic Baz puncta colocalize with DE-Cad (Fig. 4, E [arrowhead] and F), but Baz also localizes to other cortical sites. We noted that residual actin remains after the CD treatment, but it is not enriched at sites of Baz accumulation (often it shows depletion from these sites; Fig. 4 G). These data suggest that actin is involved in directly or indirectly positioning apical Baz but it may not be essential for Baz cortical association. Baz also mislocalizes basally after MT disruption with colchicine (Fig. 4 H). Thus, apical Baz positioning involves both the actin and MT cytoskeletons.

Baz positioning also requires dynein-mediated basal-to-apical transport

A later Baz-positioning mechanism was revealed when we analyzed the ectopic BazGFP puncta at the end of cellularization. In 14/16 embryos with ectopic BazGFP puncta during cellularization (Fig. 4 B, 0:14 and 0:23, arrowheads), the basal BazGFP was cleared by gastrulation onset, restoring nearly normal localization (Fig. 4 B, 0:31, arrow). Moreover, 70% of BazGFP embryos complete embryogenesis and the 30% that die have head holes but an otherwise normal embryonic cuticle, indicative of normal epithelial polarity (unpublished data). By examining cellularizing embryos, we observed basal BazGFP puncta undergoing basal-to-apical translocations as the furrows pass the base of the nucleus (Fig. 5 A, arrows; Video S3 available at <http://www.jcb.org/cgi/content/full/jcb.200505127/DC1>). Particles move at 183 ± 60 nm/s ($n = 42$; Fig. 5 A), progressively clearing ectopic BazGFP from basal regions. Thus, endogenous Baz positioning may also involve basal-to-apical transport.

Considering endogenous Baz is positioned near the apical MT minus-ends, we hypothesized that Baz might be transported apically by a minus-end–directed MT motor such as dynein. To test this, we first assessed whether dynein colocalizes with the

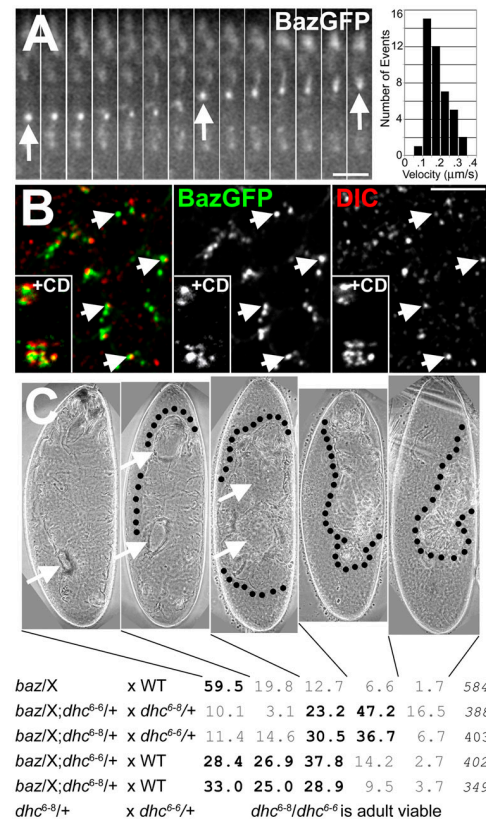


Figure 5. Baz positioning involves basal-to-apical transport and dynein. (A) BazGFP puncta moving apically at late cellularization (4 s intervals; Video S3). Velocity distribution in histogram (right). (B) Basal BazGFP puncta (green) overlap with DIC (red; arrows). Insets show overlap after CD treatment. (C) Cuticles. Most *baz* zygotic mutants have one cuticle hole (left, arrow). *dhc*⁶⁻⁶ mutations enhance the *baz* phenotype, producing larger holes (right, arrows), and overall loss of cuticle (right, outlined). Quantification below. Classes with >20% of dead embryos in bold. Bars, 5 μ m.

ectopic basal BazGFP puncta. Dynein intermediate chain (DIC) colocalizes with some ectopic basal BazGFP puncta (Fig. 5 B, arrows), in addition to localizing to other sites. DIC also localizes basally in WT embryos with a distribution indistinguishable from that in BazGFP-expressing embryos (unpublished data). Thus, the ectopic BazGFP has no gross effect on DIC localization, but appears to interact with DIC at sites where DIC normally accumulates. Surprisingly, CD treatment seems to enhance associations between DIC and basal BazGFP, perhaps by freeing the proteins from other sites (Fig. 5 B, insets). However, DIC has almost no overlap with apical BazGFP, localizing instead to more cytoplasmic regions (unpublished data). Thus, dynein is in position to function in the basal-to-apical transport of Baz, but this association may be lost in the apical domain.

If dynein plays an important role in Baz positioning, mutants affecting dynein motor function might enhance the severity of the *baz* zygotic mutant phenotype. *baz* zygotic mutants have maternal but no zygotic Baz, and should be sensitive to reduced function of their limited Baz pool. Thus, we tested the effects reducing the level of dynein heavy chain 64C (Dhc64C). *dhc*^{64C⁶⁻⁶/*dhc*^{64C⁶⁻⁸ trans-heterozygous mutants are adult viable (Robinson et al., 1999), whereas *baz*^{X106}/*Y* zygotic mutants (*baz* is X-linked) die as embryos, typically}}

with one hole in the embryonic cuticle, due to epithelial polarity defects (Wieschaus et al., 1984; Fig. 5 C, far left, arrow). When we reduced the maternal and zygotic dose of *dhc64C*, the *baz* cuticle phenotype was enhanced, exhibiting two large holes or one expansive hole deleting much of the cuticle (Fig. 5 C, moving right, arrows mark holes; cuticles outlined). The large fraction of embryos with an enhancement suggested that reducing maternal *dhc64C* might alone have effects. Indeed, crossing females heterozygous for *baz^{Xi106}* and either *dhc64C* allele to WT males also enhances the *baz* phenotype, although to a lesser extent (Fig. 5 C). These genetic interactions suggest that Baz and dynein may act in the same epithelial polarity pathway.

To directly test whether dynein functions in positioning Baz, we analyzed the Baz distribution in cellularizing *dhc64C^{m/z}* mutants. These mutants have defects in syncytial nuclear divisions (Robinson et al., 1999), but many undergo partial cellularization and initiate morphogenesis. In the mutants, centrosomes are positioned apically during cellularization (unpublished data), suggesting that basic MT polarity is retained. Baz and DE-Cad are recruited to early cellularization furrows in the *dhc64C^{m/z}* mutants (unpublished data), but during later cellularization, both are mislocalized basally along the full furrow length (Fig. 6 A, bracket; compare with WT, arrow). Baz and DE-Cad colocalize in many of the mislocalized puncta (Fig. 6 A, arrowhead). Thus, dynein functions to correctly position Baz and DE-Cad during cellularization; this could involve relatively direct dynein interactions or more indirect mechanisms. To our surprise, however, both Baz and DE-Cad become apically enriched as *dhc64C^{m/z}* mutants gastrulate (Fig. 6 D, arrows). Moreover, the *dhc64C^{m/z}* mutants produce large sheets of embryonic cuticle, indicative of relatively normal epithelial polarity (unpublished data; neighboring regions of missing cuticle are likely due to early syncytial defects). Thus, three mechanisms appear to position Baz during early development: an apical scaffold, dynein-mediated basal-to-apical transport and a third postcellularization activity.

aPKC and PAR-6 positioning is dynein independent

Although most models suggest that Baz acts with aPKC and PAR-6 to regulate polarity, our data show that they localize to different apical regions during much of embryogenesis. This prompted us to ask how closely Baz positioning is coupled to aPKC and PAR-6 positioning as polarity is established. We first asked whether dynein plays a role, examining aPKC and PAR-6 distribution in cellularizing *dhc64C^{m/z}* mutants. Remarkably, aPKC localizes in its normal apical position (Fig. 6 B, arrow), in contrast to Baz and DE-Cad, which mislocalize basally (Fig. 6 A and B). PAR-6 is cytoplasmic and cortical with minimal polarization (Fig. 6 C), as in WT (Fig. 1 E). Thus, aPKC positioning is largely dynein independent, and although it requires Baz, aPKC positioning is insensitive to the basal Baz mislocalization in *dhc64C^{m/z}* mutants.

We next assessed how dynein affects further polarization, examining gastrulating *dhc64C^{m/z}* mutants. At this stage, *dhc64C^{m/z}* mutants maintain apical aPKC (Fig. 6 E, arrow), and

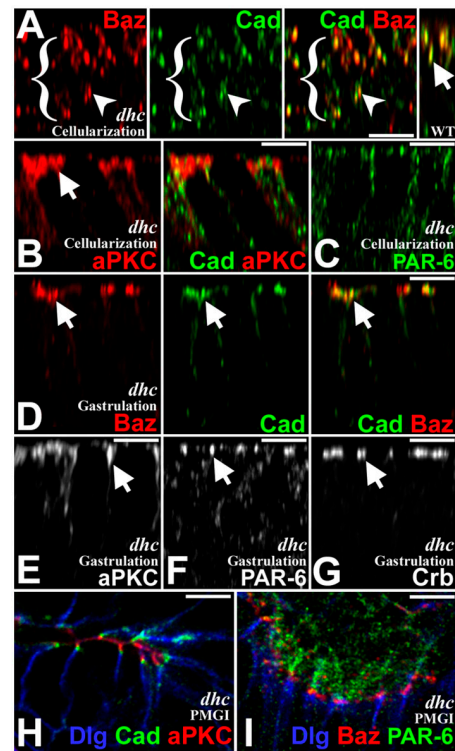


Figure 6. A specific role for dynein in positioning Baz and AJs. *dhc64C^{m/z}* mutants. (A–C) Cellularization. (A) Baz (red) and DE-Cad (green) mislocalize basally but often colocalize. WT, right. (B) aPKC (red) enriched apically. DE-Cad mislocalized basally (green). (C) PAR-6 (green) localizes as in WT. (D–G) Gastrulation. (D) Baz (red) and DE-Cad (green) are apical and colocalize. (E) aPKC apical. (F) PAR-6 apical. (G) Crb apical. (H and I) PMGIs. (H) aPKC (red) apical to DE-Cad (green) and Dlg (blue) segregates basally. (I) PAR-6 (green) above Baz (red) and Dlg (blue) basal. Bars, 5 μ m.

accumulate apical PAR-6 (Fig. 6 F, arrow) and Crb (Fig. 6 G, arrow). As described above, Baz and DE-Cad also accumulate apically at this stage. Apical aPKC and PAR-6 localize above DE-Cad and Baz in *dhc64C^{m/z}* mutants (shown in the PMGI; Fig. 6, H and I), as in WT (Fig. 1, I and J). Moreover, Dlg segregates basally, below DE-Cad and Baz (Fig. 6, H and I), as in WT (Harris and Peifer, 2004). Because *dhc64C^{m/z}* mutants develop many aspects of apical–basal polarity during gastrulation, dynein appears to play a specific role in positioning Baz and AJs during cellularization, whereas distinct mechanisms position aPKC and PAR-6.

DE-Cad, aPKC, and PAR-6 are recruited to sites of ectopic BazGFP

As Baz can directly bind aPKC and PAR-6 (Wodarz et al., 2000; Hutterer et al., 2004), we wondered what mechanisms keep the bulk of Baz segregated from aPKC and PAR-6 during cellularization and subsequent development. First, we tested whether Baz also forms complexes with DE-Cad by using Baz antibodies in immunoprecipitations (IPs) from 2 to 4 h BazGFP embryos. Both DE-Cad and aPKC coIP with Baz and BazGFP from these embryos (Fig. 7 A). GFP antibodies also coIP DE-Cad and aPKC from BazGFP embryos but not from control embryos, and reciprocally, DE-Cad and aPKC antibodies coIP

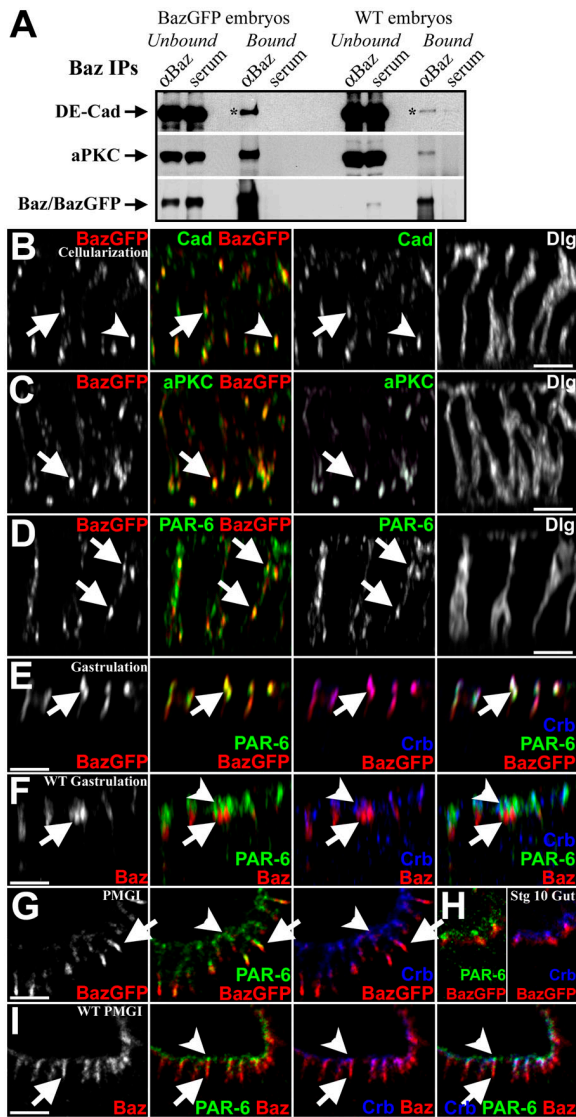


Figure 7. BazGFP overexpression recruits aPKC and PAR-6 early in development but they segregate later. (A) Baz IPs from 2 to 4 h BazGFP overexpressing and WT embryos probed for DE-Cad, aPKC, and Baz. 5% of unbound fractions and full bound fractions analyzed. Irrelevant rabbit serum used as control. (B–D) BazGFP (red) overexpressing embryos at cellularization. Dlg labels furrows. (B) DE-Cad (green), (C) aPKC (green), and (D) PAR-6 (green) at ectopic basal BazGFP puncta (red). (E) Epidermis at gastrulation. PAR-6 (green) and Crb (blue) at apical BazGFP (red). (F) WT epidermis at gastrulation. PAR-6 (green) and Crb (blue) above endogenous Baz (red). (G and H) BazGFP overexpressing embryos. (G) PMGI. PAR-6 (green) and Crb (blue) begin to segregate above BazGFP (red). (H) Stage 10 gut. PAR-6 (green) and Crb (blue) above BazGFP (red). (I) WT PMGI. PAR-6 (green) and Crb (blue) above endogenous Baz (red). Bars, 5 μ m.

BazGFP (unpublished data). Next, we used Baz antibodies in IPs from 2 to 4 h WT embryos (Fig. 7 A). DE-Cad and aPKC both coIP with endogenous Baz but at lower levels than with BazGFP overexpression. Thus, Baz can form a direct or indirect complex with DE-Cad, as it does with aPKC, and in each case Baz overexpression enhances the interactions.

We next tested whether Baz overexpression changes the localization of interacting proteins during cellularization. Strikingly, aPKC and PAR-6 are recruited to sites of ectopic basal

BazGFP accumulation (Fig. 7, C and D, arrows). As a result, aPKC is largely displaced from its normal apical position, whereas PAR-6 also shows cortical and cytoplasmic staining. Thus, aPKC and PAR-6 may normally interact with other partners during cellularization, but Baz overexpression appears to out-compete these interactions recruiting aPKC and PAR-6 to ectopic sites. DE-Cad is also recruited to ectopic BazGFP puncta, further supporting a close Baz-AJ relationship (Fig. 7 B, arrows). Some BazGFP puncta occur midway along the lateral membrane (Fig. 7 B, arrows), suggesting that BazGFP can recruit DE-Cad to ectopic sites, but BazGFP often colocalizes with DE-Cad near the base of cellularization furrows (Fig. 7 B, arrowheads), suggesting that Baz is recruited to basal junctions. Dlg is not recruited to BazGFP puncta (Fig. 7, B–D).

Although Baz overexpression can recruit aPKC and PAR-6 during cellularization, they do not normally colocalize during embryogenesis (Fig. 1). We hypothesized that other mechanisms might segregate these proteins as polarity is elaborated. Thus, we tested whether overexpressed BazGFP continues to recruit apical cues during gastrulation. In WT embryos, Crb accumulates at gastrulation, localizing with PAR-6 in the apical domain of the epidermis (Fig. 7 F, arrowheads) and PMGI (Fig. 7 I, arrowheads), above endogenous Baz (Fig. 7, F and I, arrows). In embryos overexpressing BazGFP, both PAR-6 and Crb are recruited to apical BazGFP (Fig. 7 E, arrows). However, at the same stage in the PMGI, Crb and PAR-6 begin to segregate (Fig. 7 G, arrowheads), localizing above BazGFP (Fig. 7 G, arrows). By stage 10, Crb and PAR-6 fully segregate from BazGFP (e.g., gut epithelium; Fig. 7 H). aPKC also segregates above BazGFP (unpublished data). Thus, Baz may be actively segregated from aPKC and PAR-6 after cellularization. Note, however, that we overexpressed BazGFP maternally and its levels decrease with development, so some segregation may occur due to lower BazGFP levels.

Crb is required to segregate Baz and AJs from aPKC and PAR-6

Crb plays a key role in maintaining the integrity of AJs and the apical domain after cellularization (Grawe et al., 1996; Tepass, 1996). We thus hypothesized that as Crb accumulates during gastrulation it may act to segregate Baz and AJs from aPKC and PAR-6. To test this, we analyzed the distribution of apical cues in *crb*² mutants. In stages 9 and 10 of *crb*² zygotic mutants, AJs fragment and become randomly positioned around the cell cortex and along the basolateral membrane as epidermal cells dissociate. We previously found that Baz colocalizes with AJ fragments (Harris and Peifer, 2004). Now we asked whether aPKC and PAR-6 localize at these fragments in the absence of Crb. Indeed, both aPKC and PAR-6 are recruited to fragmented AJs in the epidermis of *crb*² mutants (aPKC shows greater enrichment at these sites; Fig. 8 A, B, arrows). We next analyzed the gut epithelium of *crb*² mutants, which, in contrast to the epidermis, maintains basic epithelial structure at stage 10. Here, Arm, aPKC, and PAR-6 are extensively intermixed in the apical domain and colocalize in lateral puncta (Fig. 8 C, arrows), in contrast to their segregation in the WT gut (Fig. 8 C, inset). These data suggest that Baz, AJs, aPKC, and PAR-6 have direct

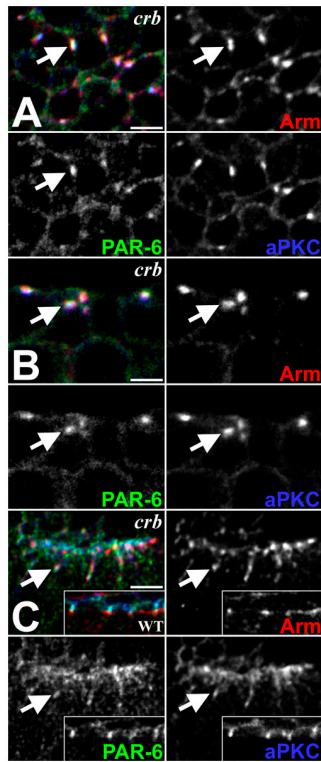


Figure 8. **Crb is required to segregate aPKC and PAR-6 from AJs after cellularization.** Stage 10 zygotic *crb* mutants. In embryo surface views (A) and cross section (B), PAR-6 (green), and aPKC (blue) colocalize with fragmented AJs (Arm, red). (C) Gut. PAR-6 (green) and aPKC (blue) intermix with AJs (Arm, red). In WT, they are above AJs (C, insets). Bars, 5 μm .

or indirect affinities for one another, and that Crb helps segregate Baz and AJs from aPKC and PAR-6 after cellularization.

Discussion

Our results frame a model of apical domain assembly during epithelial polarity establishment in *Drosophila* (Fig. 9). During cellularization, Baz acts as a primary polarity landmark that positions AJs and aPKC. Baz, itself, is positioned by two cues (an apical scaffold and dynein-mediated transport). Baz recruits and colocalizes with AJ proteins in a subapical region while helping direct aPKC to the extreme apical region (Fig. 9). During gastrulation, a third cue becomes important for Baz and AJ positioning. At this stage, aPKC becomes required for maintaining Baz and AJs. PAR-6 is also recruited to the extreme apical region and maintains Baz and AJs (Hutterer et al., 2004). Although Baz can interact with aPKC and PAR-6 at this stage, Crb blocks these interactions (Fig. 9). We propose that this interaction network establishes a robust, stratified apical domain from the earliest stages of epithelial development.

Cytoskeletal/cortical cues position Baz

AJs are often key polarity landmarks (Nelson, 2003). However, Baz positioning is AJ independent as epithelial polarity is first established in *Drosophila* (Harris and Peifer, 2004). Here, Baz appears to act as a primary polarity landmark, but what cues position Baz?

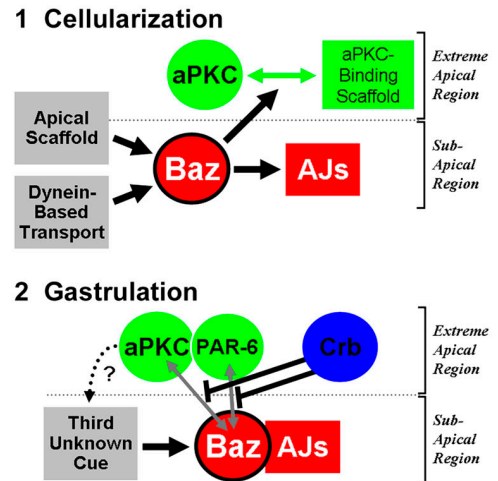


Figure 9. **A model of apical domain assembly during cellularization and gastrulation.** See Discussion for details.

Our data indicate that Baz is initially positioned by cytoskeletal cues that support an apical Baz-binding scaffold and mediate basal-to-apical Baz transport. The apical scaffold is saturable. Its function requires actin, as Baz becomes basally mislocalized after actin disruption. However, as Baz only overlaps the basal reaches of the apical actin network, it is unlikely that Baz simply binds actin. Interestingly, Baz remains largely membrane associated when actin is disrupted. One caveat is that there is some residual actin. However, the same treatment dissociates APC2 from the cortex (Townsend and Bienz, 2000; unpublished data). Actin is also required for PAR-3 cortical association in *C. elegans* one-cell embryos (Severson and Bowerman, 2003). During *Drosophila* cellularization, we speculate Baz may have other cortical anchors and that actin may control their distribution; of course actin is critical for many cellular processes and could play other roles in positioning Baz. It will be important to identify the apical scaffold for Baz.

Baz positioning also requires the minus-end-directed MT motor dynein. Our live imaging of BazGFP revealed basal-to-apical translocation of BazGFP puncta during cellularization. Baz-GFP that diffuses to ectopic basal positions appears to engage a preexisting, dynein-based, basal-to-apical transport system. Such a system was recently shown to transport Golgi vesicles apically during cellularization (Papoulas et al., 2005). Baz-dynein associations appear to cease once dynein brings Baz to the apical region, where Baz presumably docks with its apical scaffold. Although BazGFP puncta move slower than in vitro dynein velocity measurements (for review see King, 2000), dynein-mediated lipid droplet movements have similar speeds during *Drosophila* cellularization (Gross et al., 2000). In vivo, BazGFP puncta may be slowed because they form large cortical complexes. Indeed, DE-Cad, aPKC, and PAR-6 associate with these puncta and Baz oligomerization may promote complex assembly (Benton and St Johnston, 2003b). Further supporting a role for dynein, endogenous Baz is positioned near MT minus ends in WT embryos, but mislocalizes basally in *dhc64^{C^{m/z}}* mutants. *dhc64^C* mutations also enhance the *baz* mutant embryonic phenotype.

To our knowledge this is the first report of dynein positioning Baz or its homologues.

Our analysis of *dynein* mutants also revealed a third mechanism that can reposition Baz apically during gastrulation. Perhaps the apical Baz-binding scaffold is strengthened during this stage. Alternatively, a distinct polarizing mechanism may be activated, or, as we discuss below, aPKC and PAR-6 may be involved. Having three Baz positioning mechanisms may ensure proper Baz localization for regulating downstream polarity.

AJ positioning is coupled to Baz positioning

Baz acts upstream of AJs as epithelial polarity is first established in *Drosophila* (Harris and Peifer, 2004). We propose the following model in which AJ assembly may be coupled to Baz positioning. During cellularization, AJ proteins accumulate in both apical and basal junctions. Basal junctions form transiently near the base of each invaginating furrow. Baz is not required for basal junctions, but is required for recruiting AJ proteins into apical junctions (Harris and Peifer, 2004). Apical Baz may provide a landmark for apical AJ assembly.

Our data also suggest that Baz may be involved in ferrying DE-Cad to the apical domain via dynein-mediated transport. Dynein is required for correct apical positioning of both Baz and DE-Cad, and their colocalization in ectopic basal complexes in *dhc64C^{m/z}* mutants suggests they may normally be transported to the apical domain together. Indeed, Baz can form complexes with DE-Cad (Fig. 7 A) and Arm (Wei et al., 2005). Although most endogenous Baz is apical during WT cellularization, its basal mislocalization in *dhc64C^{m/z}* mutants suggests that some Baz may normally move basally. In fact, excess BazGFP displaced from the apical domain preferentially accumulates at basal junctions. We hypothesize that some Baz may normally interact transiently with basal junctions. From there, it may help ferry AJ proteins apically via dynein-mediated transport. MT motors have been previously implicated in AJ assembly. For example, dynein interacts with β -catenin and may tether MTs to AJs assembling between PtK2 cells (Ligon et al., 2001). Kinesin transports AJ proteins to nascent AJs in cell culture (Mary et al., 2002; Chen et al., 2003), and the mitotic kinesin-like protein 1 is required for apical targeting of AJs and other cues in *C. elegans* epithelia (Portereiko et al., 2004). It will be important to see if these targeting mechanisms have commonalities with AJ positioning in *Drosophila*, and if Baz homologues are involved.

Finally, we hypothesize that the third Baz–AJ positioning mechanism revealed in *dhc64C^{m/z}* mutants might be related to the normal maturation/stabilization of AJs at gastrulation. At this stage, precursory spot AJs fuse into continuous belt junctions around the top of each cell (Tepass et al., 2001). In mammalian cell culture, aPKC is required for such AJ maturation (Suzuki et al., 2002). Similarly, we find that aPKC is required for proper AJ and Baz positioning during *Drosophila* gastrulation, as previously shown for PAR-6 (Hutterer et al., 2004). Considering aPKC and PAR-6 are positioned apically as *dhc64C^{m/z}* mutants gastrulate, they might recruit Baz and AJs apically in this context as well.

Building a stratified apical domain

Based on their shared roles in polarity in *C. elegans*, characterized physical interactions, and colocalization in mammalian cells, Baz, aPKC, and PAR-6 are thought to function, at least in some cases, as an obligate tripartite complex (Ohno, 2001; Macara, 2004). Our data suggest that the bulk of cortical Baz and aPKC/PAR-6 do not form obligate complexes during epithelial development in *Drosophila*. Instead, aPKC and PAR-6 localize to an apical region above Baz and AJs, and are positioned there by distinct mechanisms. Baz/PAR-3 also segregates from aPKC and PAR-6 in other cell types. In *C. elegans* one-cell embryos, PAR-3, aPKC, and PAR-6 each localize in clusters on the anterior cortex, but these different clusters have limited colocalization (60–85% fail to colocalize; Tabuse et al., 1998; Hung and Kemphues, 1999). aPKC and PAR-6 colocalize without PAR-3 at the leading edge of migrating mammalian astrocytes (Etienne-Manneville and Hall, 2001). In *Drosophila* photoreceptors, Baz colocalizes with AJs below aPKC, PAR-6, and Crb (Nam and Choi, 2003). Even in polarized MDCK cells, aPKC and PAR-6 show some segregation above PAR-3, and although they mainly colocalize at tight junctions (Vogelmann and Nelson, 2005), mammalian PAR-3 can regulate tight junction assembly independently of aPKC and PAR-6 (Chen and Macara, 2005). Thus, in many contexts interactions between Baz/PAR-3, aPKC, and PAR-6 are dynamic and/or regulated.

Baz (PAR-3), aPKC, and PAR-6 often recruit each other to the cortex, but the assembly pathways vary. In *C. elegans* one-cell embryos, PAR-3, aPKC, and PAR-6 are mutually dependent for their cortical recruitment (Ohno, 2001). However, in *Drosophila* neuroblasts, Baz can be positioned without aPKC and PAR-6 (Rolls et al., 2003). Similarly, apical Baz is positioned without aPKC and PAR-6 during *Drosophila* cellularization. In contrast, apical aPKC recruitment requires Baz, whereas PAR-6 is largely nonpolarized at this stage. Given the lack of extensive colocalization of Baz and aPKC in WT embryos, Baz may control aPKC positioning indirectly, perhaps regulating binding to a separate apical scaffold. Alternately, cortical recruitment might involve cytoplasmic Baz–aPKC complexes. Apical PAR-6 accumulates at gastrulation, and this appears partially Baz independent. Indeed, *cdc42* recruits PAR-6 at this stage (Hutterer et al., 2004), and at the same time aPKC and PAR-6 become required for maintaining apical Baz (Fig. 2; Hutterer et al., 2004). Thus, although Baz is first positioned independently of aPKC and PAR-6, these cues soon develop complex interdependencies.

Although Baz can directly bind both aPKC and PAR-6 (Wodarz et al., 2000; Hutterer et al., 2004), at least two mechanisms keep them apart. During cellularization, Baz colocalizes with aPKC and PAR-6 when overexpressed, but normally it localizes with AJs below aPKC and PAR-6. This normal segregation may thus involve competition with other binding partners. After cellularization, Crb also becomes important for segregating Baz and AJs from aPKC and PAR-6. These segregation mechanisms help form a stratified apical domain from the earliest stages of epithelial development.

A stratified apical domain may strengthen the boundary between the apical and basolateral domains. This boundary

forms via reciprocal antagonism between polarity cues. For example, aPKC phosphorylates and excludes Lethal giant larvae (Lgl) from the apical domain in *Drosophila* epithelia and Lgl appears to repel PAR-6 from the basolateral domain (Hutterer et al., 2004). The Crb and Dlg complexes also have mutual antagonism (Bilder et al., 2003; Tanentzapf and Tepass, 2003). We propose that the subapical Baz-AJ region may insulate the apical and basolateral domains. For example, it may inhibit active aPKC from moving basally. Indeed, PAR-3 binding can block mammalian aPKC kinase activity (Lin et al., 2000). The Baz-AJ subapical region could also block basolateral cues, as AJs are required to segregate Dlg (Harris and Peifer, 2004). In this way, the Baz-AJ subapical region could help define a distinct apical-basolateral boundary.

To conclude, Baz appears to be a primary epithelial polarity landmark in *Drosophila*. It is positioned by multiple mechanisms, including an apical scaffold and dynein-mediated transport, and organizes a stratified apical domain, in which it colocalizes with AJs below its typical partners aPKC and PAR-6.

Materials and methods

Fly stocks and genetics

FlyBase describes mutations and constructs (<http://flybase.bio.indiana.edu>). *baz^{xi106}* m/z mutants were made by the FLP dominant female sterile method as in Harris and Peifer (2004). WT was *yellow white*. UAS-Baz-GFP flies (Benton and St Johnston, 2003a) were a gift of D. St Johnston (University of Cambridge, Cambridge, UK). They were crossed to a maternal GAL4-VPI6 driver for expression during oogenesis. Flies expressing the actin-binding domain of moesin fused to GFP were a gift of D. Kiehart (Duke University, Durham, NC). *baz^{xi106}*, *dhc64C⁶⁻⁶*, and *dhc64C⁶⁻⁸*, and *crb²* mutants were gifts of A. Wodarz (University of Dusseldorf, Dusseldorf, Germany), T. Hays (University of Minnesota, Minneapolis, MN), and the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN), respectively.

Embryo staining and treatments

For tubulin and γ -tubulin, embryos were fixed in 10:9:1 heptane/37% formaldehyde/0.5 M EGTA for 10 min. For other staining, embryos were fixed for 20 min in 1:1 3.7% formaldehyde in PBS/heptane. After methanol devitellinization, blocking, and staining was in PBS/1% goat serum/0.1% Triton X-100. Antibodies were: mouse mAbs against Arm (1:500), Crb (1:500; Developmental Studies Hybridoma Bank [DSHB]), Dlg (1:500; Covance), Dlg (1:100; DSHB), γ -tubulin (1:500; Sigma-Aldrich), tubulin (1:100; DSHB); rabbit pAbs against Baz (1:2,000; A. Wodarz) and ζ PKC (1:2,000; Santa Cruz Biotechnology, Inc.); and rat mAbs against DE-Cad (1:100; T. Uemura, Kyoto University, Kyoto, Japan) and PAR-6 (1:100; C. Doe, University of Oregon, Eugene, OR).

Drug treatments were performed as in Townsley and Bienz (2000). For CD treatments, dechorionated embryos were rinsed twice with 0.9% NaCl and incubated in 1:1 octane/10 μ g/ml CD (Sigma-Aldrich) in 0.9% NaCl for 30 min at RT with rocking. After removing both phases, embryos were rinsed twice with heptane and fixed immediately. The working solution of CD was prepared from a 1 mg/ml solution in DMSO. For controls, the embryos were treated with the DMSO carrier alone. For colchicine treatment, colchicine (Sigma-Aldrich) was used in the same way at 100 μ g/ml from a 10 mg/ml solution in ethanol. For controls, the embryos were treated with the ethanol carrier alone.

Image acquisition and manipulation

Fixed embryos were mounted in Aqua Polymount (Polysciences, Inc.) and imaged at RT with a 510 confocal microscope (Carl Zeiss MicroImaging, Inc.) with both 40 \times (Plan-Neofluor; NA 1.3) and 63 \times (Plan-Apochromat; NA 1.4) objectives and LSM 510 AIM software. Secondary Abs were Alexa 488, 546, and 647 (Molecular Probes). Unless otherwise noted, Adobe Photoshop 6.0 was used to adjust input levels so the main range of signals spanned the entire output grayscale and was used to adjust bright-

ness and contrast. We used bicubic interpolation for image resizing, but observed no changes to the data at normal viewing magnifications. Image deconvolution was performed on confocal stacks using a softWoRx Imaging Workstation (Applied Precision).

Time-lapse microscopy

Dechorionated, WT, homozygous Baz-GFP embryos were mounted in halocarbon oil (series 700; Halocarbon Products Corporation) on a gas-permeable membrane (petriPERM; Sartorius Corp.). Images were captured every 4 s with a Wallac Ultraview Confocal Imaging System (Perkin-Elmer) at RT with a 40 \times objective (Nikon Plan Fluor; N.A. 1.30), an ORCA-ER digital camera (Hamamatsu), and Metamorph software (Universal Imaging Corp.).

Immunoprecipitations

Dechorionated embryos were homogenized in extraction buffer (50 mM Tris, pH 7.5, 1% NP-40, 0.5 mM sodium vanadate, 3 mM hydrogen peroxide, Complete EDTA-free protease inhibitor cocktail [Roche]), and centrifuged to remove particulates. Samples were incubated with antibodies 1 h at 4°C. 10 μ l packed protein A Sepharose (Sigma-Aldrich) was added per 100 μ l sample for an additional 1.5 h at 4°C. After washing with extraction buffer, samples were separated by 8% SDS-PAGE, and immunoblotted.

Online supplemental material

Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200505127/DC1>.

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