Developmental Cell
Article

Rho-Kinase Directs Bazooka/Par-3 Planar Polarity during *Drosophila* Axis Elongation

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DOI 10.1016/j.devcel.2010.08.011

SUMMARY

Cell rearrangements shape the *Drosophila* embryo via spatially regulated changes in cell shape and adhesion. We show that Bazooka/Par-3 (Baz) is required for the planar polarized distribution of myosin II and adherens junction proteins and polarized intercalary behavior is disrupted in *baz* mutants. The myosin II activator Rho-kinase is asymmetrically enriched at the anterior and posterior borders of intercalating cells in a pattern complementary to Baz. Loss of Rho-kinase results in expansion of the Baz domain, and activated Rho-kinase is sufficient to exclude Baz from the cortex. The planar polarized distribution of Baz requires its C-terminal domain. Rho-kinase can phosphorylate this domain and inhibit its interaction with phosphoinositide membrane lipids, suggesting a mechanism by which Rho-kinase could regulate Baz association with the cell cortex. These results demonstrate that Rho-kinase plays an instructive role in planar polarity by targeting Baz/Par-3 and myosin II to complementary cortical domains.

INTRODUCTION

During axis elongation in *Drosophila*, the embryonic epithelium more than doubles in length along the anterior-posterior (AP) axis and simultaneously narrows in width along the dorsal-ventral (DV) axis. This structural transformation is characterized by a striking directionality in which cell movements are oriented perpendicular to the direction of tissue elongation, a process known as cell intercalation. Cell intercalation is essential for axis elongation in frogs, fish, chicks, flies, and ascidians (Keller et al., 2000; Solnica-Krezel, 2005). Intercalation in *Xenopus* and zebrafish requires Wnt-dependent activation of the Frizzled planar cell polarity pathway (Zallen, 2007; Roszko et al., 2009), whereas these cell rearrangements in *Drosophila* involve proteins that mediate contractility and adhesion (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006). Adherens junction proteins and the contractile machinery are enriched in complementary domains at the cell cortex, where they may participate directly in polarized cell movement. An asymmetrically localized contractile actomyosin network provides the driving force for cell rearrangement (Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009), and differential adhesion could influence local interactions between cells (Blankenship et al., 2006). Patterned gene expression along the AP axis provides the spatial information that directs planar cell polarity and cell rearrangement in *Drosophila* and *Xenopus* (Irvine and Wieschaus, 1994; Ninomiya and Winklbauer, 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006). However, the mechanisms that translate patterns of gene expression into asymmetric protein localization are not known.

Interactions between spatially localized protein kinases and their substrates are a common strategy for generating polarity in many cell types. In epithelial cells, the basolateral kinase PAR-1 restricts its substrate Bazooka/Par-3 (Baz) to the apical domain (Benton and St. Johnston, 2003) and the apical kinase atypical protein kinase C (aPKC) restricts its substrates Lgl and PAR-1 to the basolateral domain (Hutterer et al., 2004; Suzuki et al., 2004). During asymmetric cell division in *Drosophila* neuroblasts, aPKC phosphorylates Numb and Miranda, restricting their localization to the basal cortex (Wirtz-Peitz et al., 2008; Atwood and Prehoda, 2009). In each case, the localized activity of protein kinases inhibits the association of their substrates with the cell cortex, providing a dynamic mechanism for establishing and maintaining cellular asymmetry.

Rho-kinase is an activator of actomyosin contractility and a conserved regulator of cell shape and behavior (Winter et al., 2001; Marlow et al., 2002; Dawes-Hoang et al., 2005; Verdier et al., 2006; Wang and Riechmann, 2007). Rho-kinase activates myosin directly by phosphorylating the myosin regulatory light chain, as well as indirectly by inhibiting the myosin light chain phosphatase (Amano et al., 1996; Kimura et al., 1996). Rho-kinase has several substrates involved in cytoskeletal regulation, including LIM kinase and ERM proteins (Amano et al., 2000; Riento and Ridley, 2003). Mammalian Rho-kinase phosphorylates the polarity protein Par-3, disrupting its association with its binding partners Par-6 and aPKC in cultured cells (Nakayama et al., 2008). However, it is not known if the regulation of Par-3 by Rho-kinase is important for cell behavior in vivo. Moreover, the residue on mammalian Par-3 that is phosphorylated by Rho-kinase is not present in all Par-3 homologs, raising the question of whether this is a conserved mechanism of Par-3 regulation.

Here, we show that Rho-kinase is asymmetrically localized during axis elongation in the *Drosophila* embryo and that Rho-kinase activity is required for the planar polarized distribution of Baz. Rho-kinase localizes to boundaries between anterior

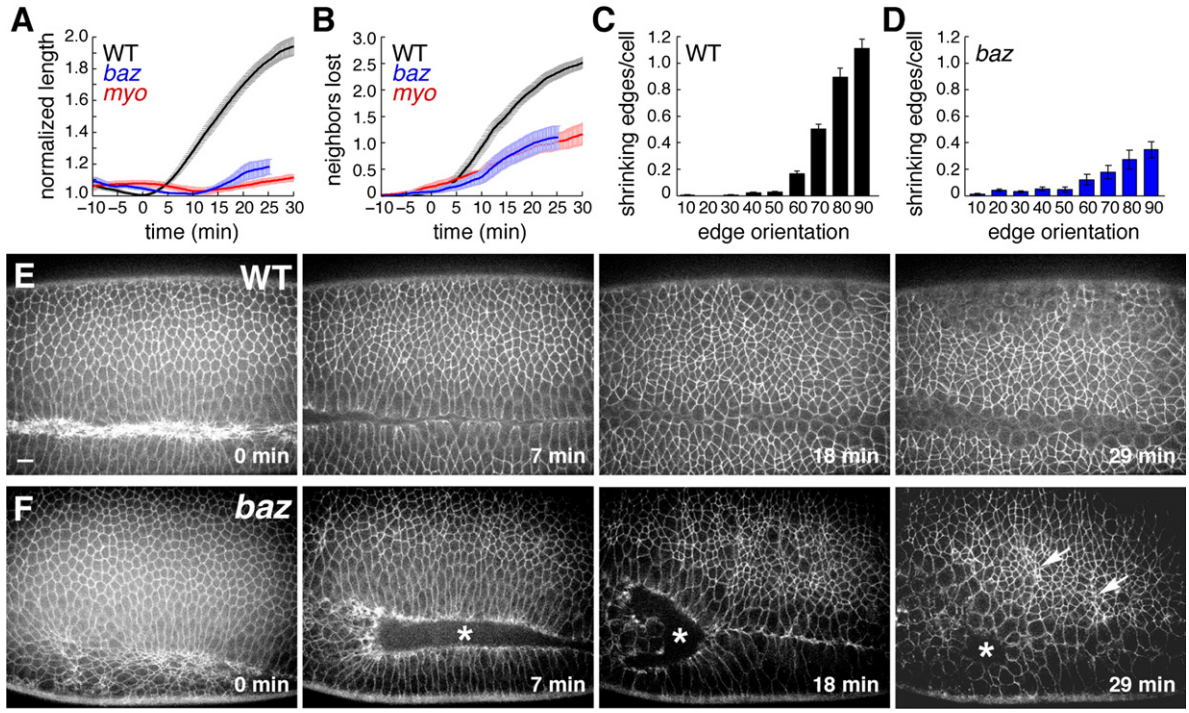

Figure 1. *baz* Mutants Have Defects in Cell Intercalation

(A–D) Cell behavior in time-lapse movies of wild-type and mutant embryos. (A) Germband elongation is strongly reduced in baz^{GD21} and sqh^{1} mutants. (B) Fewer neighbors are lost per cell in baz^{GD21} and sqh^{1} mutants. An average value was obtained for each embryo, error bars indicate the standard error of the mean across embryos (n = 10 wild-type, 5 baz^{GD21}, and 2 sqh^{1} embryos). Wild-type embryos were imaged with GFP:Resille (4), GFP:Spider (3), and E-cadherin:GFP (3), baz^{GD21} embryos were imaged with GFP:Spider, sqh^{1} embryos were imaged with E-cadherin:GFP. t = 0 is the onset of elongation in stage 7. (C) Contractile behavior in wild-type is biased toward edges perpendicular to the AP axis (angles in degrees, 0 for edges parallel to the AP axis). Bars indicate 10° bins starting at 0°–10°. (D) Contractile behavior in *baz* mutants is reduced and partially spatially deregulated: 66% of shrinking edges were oriented at ≥60° in *baz* mutants compared with 88% in wild-type; 16% were oriented at ≤30° in *baz* mutants compared with <1% in wild-type (n = 10 wild-type and 5 baz^{GD21} embryos, 33–161 cells/embryo). (E and F) Stills from movies of wild-type (E) and baz^{GD21} (F) embryos expressing Spider:GFP. Asterisks indicate defects in ventral furrow closure in *baz* mutants; arrows indicate clusters of apically constricting cells. Scale bar = 10 μm.

See Figure S1 and Movies S1 and S2.

and posterior cells complementary to Baz. Loss of Rho-kinase leads to ectopic Baz localization, and activated Rho-kinase is sufficient to exclude Baz from the cortex in a mechanism that requires the Baz C-terminal domain. The Baz C terminus has been shown to bind to phophoinositide membrane lipids (Krahn et al., 2010). We show that this domain can be phosphorylated by Rho-kinase and is required for Baz planar polarity. Baz, in turn, regulates the planar polarized distribution of myosin II and adherens junction proteins, and cell intercalation is disrupted in *baz* mutants. These results demonstrate that Rho-kinase plays an instructive role in planar cell polarity by targeting Baz/Par-3 and myosin II to complementary cortical domains.

RESULTS

Baz/Par-3 Is Required for Intercalary Behavior during Axis Elongation

Axis elongation in the *Drosophila* embryo is characterized by the planar polarized enrichment of myosin II and F-actin at interfaces between anterior and posterior cells and E-cadherin, β-catenin, and Baz/Par-3 at interfaces between dorsal and ventral cells (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006). Whereas polarized actomyosin contractility has been shown to play an essential role in orienting cell behavior (Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009), the role of Baz and adherens junctions is less clear. Elongation is reduced in embryos zygotically mutant for *baz*, but these embryos contain maternal *baz* mRNA and protein (Zallen and Wieschaus, 2004). To completely eliminate Baz activity, we generated embryos maternally and zygotically mutant for two null alleles, baz^{GD21} and baz^{FA50} (Experimental Procedures) (Denef et al., 2008). The zygotic lethality of baz^{GD21} and baz^{FA50} homozygotes was rescued by the *Dp(1;Y)W73* duplication that contains the *baz* open reading frame, indicating that each contains a single lethal mutation at the *baz* locus. Both alleles display no detectable Baz staining in embryos maternally and zygotically mutant for *baz*, referred to here as *baz* mutant embryos.

Axis elongation was severely defective in time-lapse movies of *baz* mutants (Figure 1A). Mutant cells engaged in fewer rearrangements (Figure 1B) and displayed a reduction in both neighbor exchange and rosette formation, two behaviors that require polarized actomyosin contractility (Bertet et al., 2004; Blankenship et al., 2006). Cell movements that occurred in *baz* mutants showed a moderate loss of directionality (Figures 1C

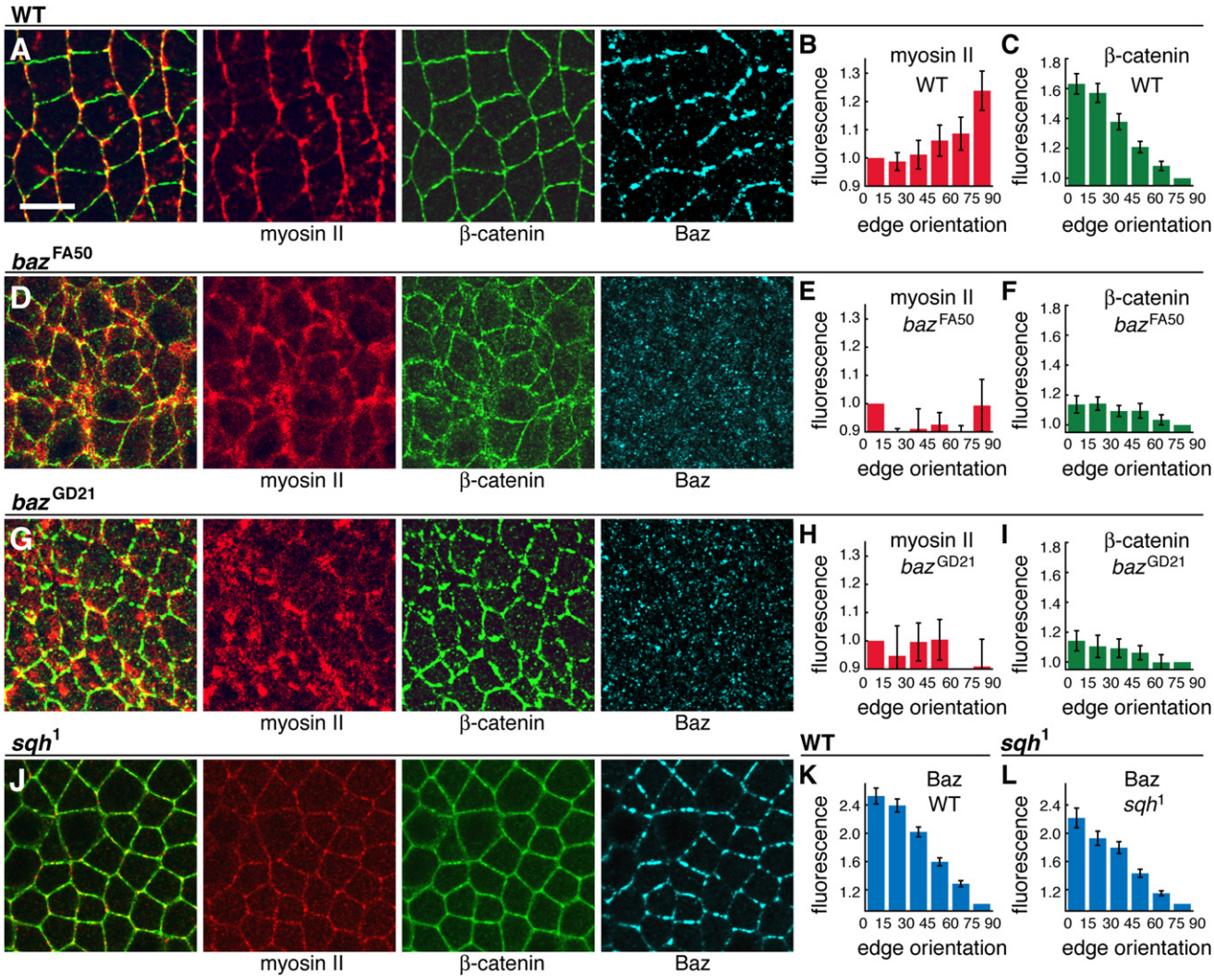

Figure 2. Baz/Par-3 Is Required for the Planar Polarized Localization of Myosin II and β-Catenin

(A, D, G, and J) Localization of myosin II (Zip heavy chain, red), β-catenin (green), and Baz (blue) in stage 7 wild-type (A), *baz*^{FA50} (D), *baz*^{GD21} (G), and *sqh*^{1} (J) embryos. Left panels show overlays of myosin (red) and β-catenin (green). Anterior left, ventral down. Scale bar = 10 μm. (B, E, and H) Myosin planar polarity was disrupted in *baz*^{FA50} ($p < 0.001$) (E) and *baz*^{GD21} ($p < 0.001$) (H) compared with wild-type (B) (n = 5 wild-type, 3 *baz*^{FA50}, 4 *baz*^{GD21} embryos). (C, F, and I) β-catenin planar polarity was disrupted in *baz*^{FA50} ($p < 0.001$) (F) and *baz*^{GD21} ($p < 0.001$) (I) compared with wild-type (C) (n = 11 wild-type, 3 *baz*^{FA50}, 4 *baz*^{GD21} embryos). (K and L) Baz planar polarity was retained in *sqh*^{1} mutants (L) compared with wild-type (K) (n = 11 wild-type, 14 *sqh*^{1} embryos). See Figure S2.

and 1D). Some cells began to apically constrict in late stage 7, leading to the formation of ectopic grooves in stage 8 (Figures 1E and 1F; see Movies S1 and S2 available online) (Muller and Wieschaus, 1996; Wodarz et al., 1999). These defects were not due to apoptosis, which was not present in wild-type or *baz* mutant embryos at these stages (Figure S1 available online). These results demonstrate that Baz is required for cell rearrangement and spatially regulated contractile behavior during axis elongation.

Baz/Par-3 Is Required for Myosin II and Adherens Junction Planar Polarity

To determine the basis of the defects in *baz* mutants, we analyzed protein localization in intercalating cells. In wild-type, myosin II is apically localized and concentrated at boundaries between anterior and posterior cells (AP edges) (Figures 2A and 2B) (Zallen and Wieschaus, 2004). Myosin apical localization occurred normally in *baz* mutant embryos (Figure S2) (5/5 *baz*^{GD21} and 6/6 *baz*^{FA50} embryos displayed apical myosin localization). By contrast, myosin planar polarity was strongly disrupted and myosin accumulated in an irregular fashion at edges of all orientations and at the medial apical cortex (Figures 2D–2I) (0/10 *baz*^{GD21} and 0/3 *baz*^{FA50} embryos displayed myosin planar polarity). Baz was also required for the planar polarized localization of adherens junction proteins (0/3 *baz*^{GD21} and 0/7 *baz*^{FA50} embryos displayed Arm/β-catenin planar polarity). Myosin planar polarity occurred normally in embryos where Arm/β-catenin was disrupted by RNAi and in embryos maternally and zygotically mutant for *arm*^{043A06}, even in cells that were visibly detached from their neighbors (Figure S2), suggesting

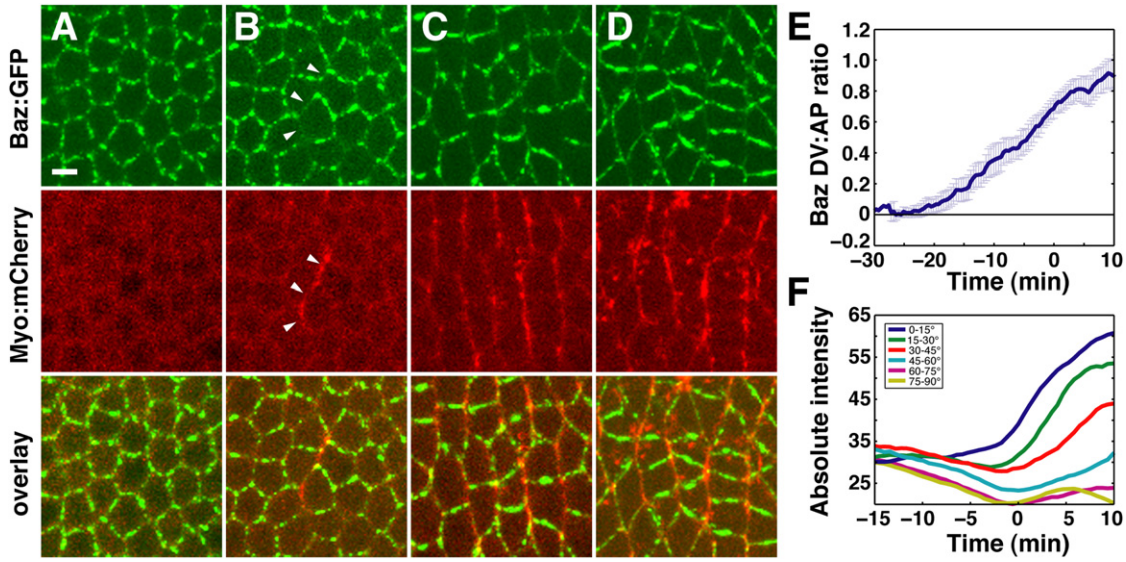

Figure 3. Baz/Par-3 Is Downregulated at AP Edges and Upregulated at DV Edges, Establishing Planar Polarity

(A–D) Movie stills of intercalating cells in an embryo expressing Myo:mCherry (red) and ectopic Baz:GFP (green). t = 0 is the onset of elongation in stage 7. (A) −11 min. (B) −4 min. (C) 3 min. (D) 9 min. (A and B) During stage 6, Baz:GFP is displaced from and Myo:mCherry accumulates at AP edges (arrowheads). (C and D) In stage 7, Myo:mCherry and Baz:GFP occupy AP and DV edges, respectively. Scale bar = 5 μm.

(E and F) Quantitation of ectopic Baz:GFP fluorescence intensity. (E) The $\log_2$ ratio of Baz:GFP intensity at DV edges (oriented at $\leq 30^\circ$ relative to the AP axis) relative to AP edges (oriented at $\geq 60^\circ$ relative to the AP axis) was averaged across all cells (n = 5 embryos, 764–2142 edges/embryo). Error bars indicate the standard error of the mean across embryos. t = 0 is the onset of elongation in stage 7. (F) Absolute edge intensities grouped by edge orientation in a single embryo. Baz is first downregulated at AP edges (yellow, purple) and then upregulated at DV edges (dark blue, green). See Figure S3 and Movie S3.

that the role of Baz in myosin regulation is separate from its role in adhesion.

Baz/Par-3 Is Downregulated at AP Edges and Upregulated at DV Edges, Establishing Planar Polarity

These results demonstrate that the asymmetrically localized Baz protein regulates the localization of myosin II and β-catenin in intercalating cells. We were therefore interested in identifying the upstream mechanisms responsible for Baz localization. The accumulation of Baz at DV edges could occur through a recruitment of Baz to DV edges, an inhibition of Baz association with AP edges, or a combination of both. To distinguish between these possibilities, we analyzed Baz localization in time-lapse movies of embryos expressing Baz:GFP and a Myo:mCherry fusion to the myosin regulatory light chain. Both fusion proteins are functional and correctly localized (Benton and St. Johnston, 2003; Martin et al., 2009; McGill et al., 2009).

Baz was apically localized in stage 5 whereas myosin apical localization was first detected in stage 6. Baz was initially present in a punctate unpolarized distribution at the apical cortex and became restricted to DV edges during stage 6, prior to the onset of intercalation (Figure 3A and 3B; Movie S3). By contrast, myosin was enriched at AP edges as soon as it was detected apically (Figure 3B). Myosin recruitment to AP edges corresponded to sites where Baz was depleted. Planar polarity was more prominent at the onset of intercalation in stage 7, when Baz was strongly localized to DV edges and myosin was predominantly AP (Figures 3C–3E). Quantitation of Baz levels in time-lapse movies revealed that Baz levels initially decrease at AP edges and later increase at DV edges (Figures 3F and S3). These results indicate that Baz planar polarity is generated from a uniform distribution through a dual mechanism in which Baz is first downregulated at AP edges, followed by an increase in Baz apical localization that is restricted to DV edges.

Rho-Kinase Is Required for Baz/Par-3 Planar Polarity

Because the loss of Baz from AP edges correlates with the recruitment of myosin, we asked if Baz planar polarity requires myosin activity. Although it is not possible to generate embryos lacking myosin, embryos maternally mutant for a hypomorphic mutation in the myosin regulatory light chain (sqh^1) (Karess et al., 1991) are severely defective for axis elongation and cell intercalation (Figures 1A and 1B). The myosin heavy chain was present at reduced cortical levels in these mutants and was no longer planar polarized (Figure 2J) (1/11 sqh^1 embryos displayed myosin planar polarity). Despite this mislocalization of the myosin heavy chain, Baz was concentrated in DV edges as in wild-type (Figures 2J–2L) (9/11 sqh^1 mutant embryos displayed Baz planar polarity). Thus, whereas Baz is necessary for myosin localization, myosin planar polarity is dispensable for Baz localization.

To identify alternative Baz regulators, we turned to Rho-kinase, which has been shown to phosphorylate Par-3 in cultured mammalian cells (Nakayama et al., 2008). Rho-kinase is required for myosin cortical localization in *Drosophila* (Figure S4), consistent with previous results in the embryo (Bertet et al., 2004; Dawes-Hoang et al., 2005) and other cell types (Winter et al., 2001; Dean and Spudich, 2006; Wang and Riechmann, 2007). To ask if Rho-kinase is required for Baz localization, we analyzed embryos maternally mutant for a null Rho-kinase mutation ($DRok^2$ mutants). Baz association with apical adherens junctions occurred normally in stage 6/7 $DRok^2$ mutants, but Baz planar polarity was strongly defective (Figures 4A–4H)

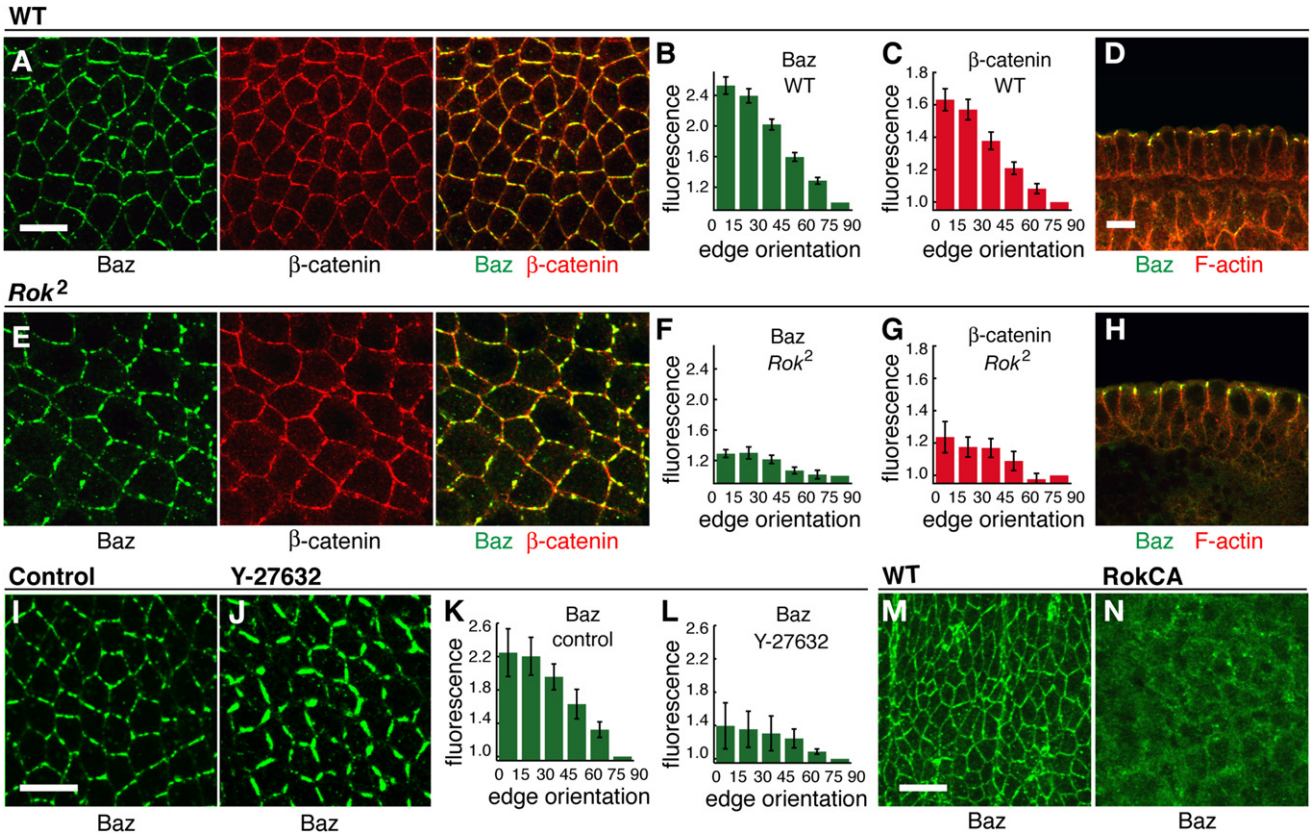

Figure 4. Rho-Kinase Is Required for Baz/Par-3 Planar Polarity

(A–H) Localization of Baz (green) and β-catenin (red) in stage 7 wild-type (A–D) and *DRok*^{2} mutant (E–H) embryos. Cross sections are shown in (D) and (H). Quantitation of Baz and Arm/β-catenin planar polarity in wild-type (B and C) and *DRok*^{2} (F and G). Baz and β-catenin planar polarity were strongly reduced in *DRok*^{2} mutant embryos compared with wild-type ($p < 0.001$) (n = 11 wild-type, 8 *DRok*^{2} embryos). Two classes of defects were observed, with the more defective class shown here. See Figure S4.

(I–L) Localization of Baz (green) in stage 7 embryos injected with water (I) or Y-27632 (J). Baz planar polarity was reduced in Y-27632-injected embryos (L) compared with wild-type (K) ($p = 0.002$) (n = 5 control-injected, 4 Y-27632-injected embryos).

(M and N) Localization of Baz (green) in the dorsolateral epidermis of a stage 14 control embryo (M) or an embryo expressing activated Rho-kinase (Rok CA, N). Scale bars = 10 μm.

(0/12 *DRok*^{2} mutant embryos displayed wild-type Baz planar polarity). Arm/β-catenin planar polarity was also defective in *DRok*^{2} mutants (0/12 *DRok*^{2} mutant embryos displayed wild-type β-catenin planar polarity). Similar defects were observed in embryos maternally mutant for the independent *DRok*^{1} allele (Figure S4). Two mutant classes with defects of varying severity were observed, consistent with a paternal contribution (Figure S4). These results demonstrate that Rho-kinase is required for the planar polarized localization of Baz, myosin II, and β-catenin in intercalating cells.

To ask if Rho-kinase activity is required for its role in Baz localization, we injected wild-type embryos with the Rho-kinase inhibitor Y-27632 at stages 7/8 after the onset of intercalation. Y-27632 led to a loss of cortical myosin (data not shown) and a rapid redistribution of Baz within 5–10 min of injection (Figures 4I–4L) (0/41 Y-27632 injected embryos displayed Baz planar polarity versus 10/10 control injected embryos), indicating an ongoing requirement for Rho-kinase during intercalation. Y-27632 has also been shown to inhibit aPKC (Atwood and Prehoda, 2009), but aPKC is unlikely to be responsible for these defects, because planar polarity is retained in *aPKC* and *Par6* mutants (Blankenship et al., 2006; Harris and Peifer, 2007).

Rho-Kinase Is Asymmetrically Enriched at AP Cell Borders Complementary to Baz/Par-3

To investigate the mechanism by which Rho-kinase regulates Baz localization, we first analyzed the distribution of Rho-kinase. Wild-type Rho-kinase tagged with HA was selectively enriched at AP boundaries of intercalating cells, colocalizing with F-actin (Figure 5A) (7/7 embryos). Because high levels of Rho-kinase often disrupted embryo morphology, we generated a Venus-tagged Rho-kinase variant mutated for a single conserved residue in the kinase domain that disrupts catalytic activity (Winter et al., 2001). Rho-kinase^{K116A} localized to the cellularization front in stage 5 and to the apical cortex in stage 6, in a region that overlaps with the adherens junctions (data not shown). At stage 6 and during cell intercalation in stages 7/8, Rho-kinase^{K116A} was present in a planar polarized distribution at AP edges (Figures 5C and 5D), similar to the wild-type protein (Figure 5A).

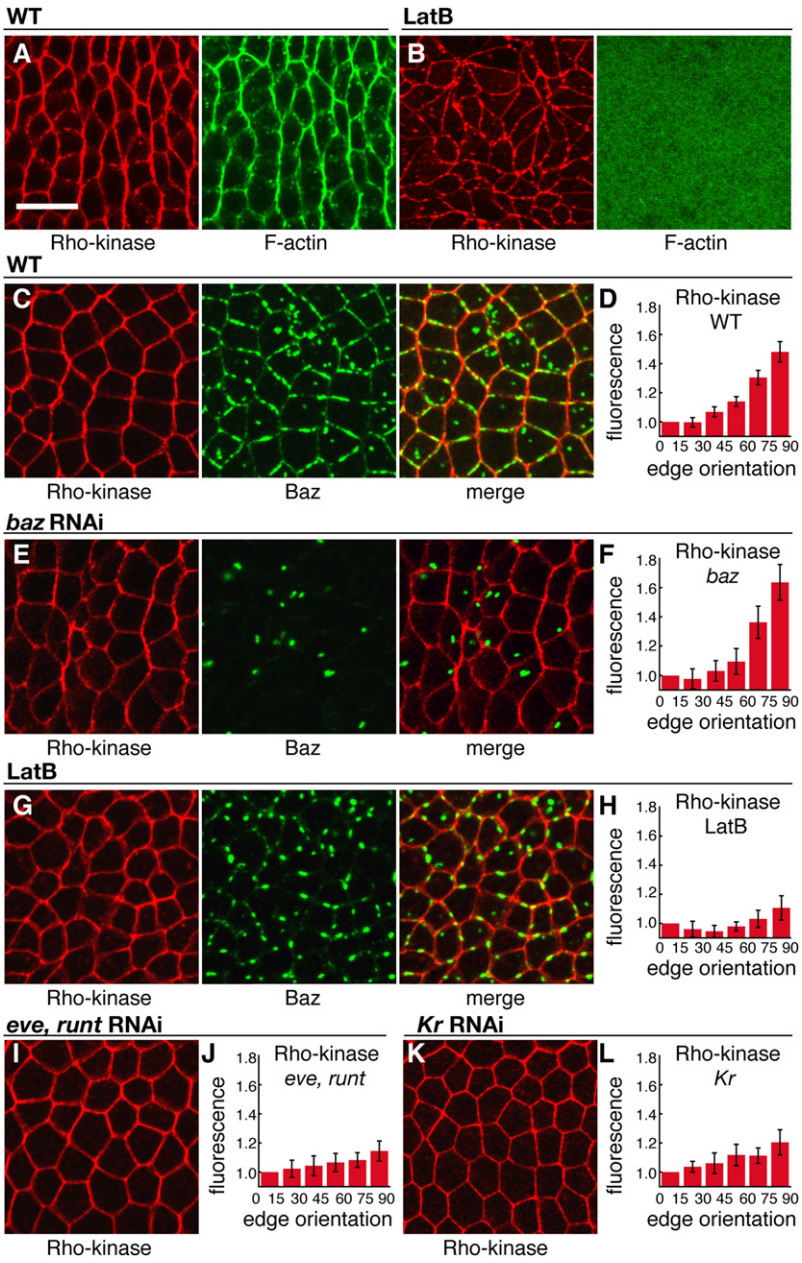

Figure 5. Rho-Kinase Is Planar Polarized in Intercalating Cells

(A and B) Localization of Rho-kinase (HA:Rok, red) and F-actin (phalloidin, green) in stage 7 wild-type (A) or LatB-injected (B) embryos.

(C–L) Localization of Rho-kinase (Venus:Rho-kinase^{K116A}, red) and Baz (green). Stage 7 wild-type embryo (C) or embryos injected with *baz* dsRNA (E), LatB (G), *eve* and *runt* dsRNA (I), or *Kr* dsRNA (K). Quantitation of planar polarity in wild-type (D) and injected embryos (F, H, J, and L) is shown. Rho-kinase planar polarity was not affected by *baz* RNAi (F) (n = 6 wild-type Venus:Rho-kinase^{K116A} expressing embryos, 7 *baz* dsRNA-injected embryos, $p = 0.29$). Rho-kinase planar polarity was reduced by injection of LatB (H) (5 embryos, $p < 0.001$), *eve* and *runt* dsRNA (J) (7 embryos, $p < 0.001$), or *Kr* dsRNA (L) (4 embryos, $p = 0.01$). Scale bar = 10 μm.

Rho-kinase planar polarity occurred normally in embryos depleted for Baz (Figures 5E and 5F) (16/16 *baz* dsRNA-injected embryos displayed Rho-kinase planar polarity versus 9/9 control injected embryos), which may explain why most shrinking edges in *baz* mutants are correctly oriented (Figure 1D). To ask if F-actin is required for Rho-kinase planar polarity, we injected embryos with latrunculin B (LatB), a toxin that inhibits actin polymerization. LatB injection during intercalation in stages 7/8 resulted in a rapid loss of Rho-kinase planar polarity and a reduction of Baz cortical localization within 5-10 min of injection (Figures 5B, 5G, and 5H) (0/13 LatB-injected embryos displayed Rho-kinase planar polarity versus 11/11 control injected embryos). These results indicate that an actin-dependent asymmetry is required for Rho-kinase planar polarity, but not for its cortical localization.

Spatial cues provided by the AP patterning system are necessary and sufficient for planar cell polarity (Zallen and Wieschaus, 2004). RNAi-mediated disruption of the pair rule genes *eve* and *runt* or the gap gene *Kruppel* resulted in a loss of Rho-kinase planar polarity (Figures 5I–5L) (0/10 *eve* and *runt* dsRNA-injected embryos and 0/4 *Kruppel* dsRNA-injected embryos displayed Rho-kinase planar polarity), indicating that asymmetric Rho-kinase localization requires the AP patterning system.

Rho-Kinase Is Sufficient to Displace Baz/Par-3 from the Cortex

Rho-kinase accumulates in cortical domains where Baz levels are reduced, suggesting that it may inhibit Baz association with the cortex. To test this model, we generated a variant of Rho-kinase (Rok CA) that terminates after 553 aa, resulting in constitutive kinase activity (Amano et al., 1996; Winter et al., 2001). Although we were unable to express high levels of Rok CA in the early embryo, presumably due to toxicity of the transgene, expression of Rok CA in late embryos resulted in loss of Baz from the cortex (Figures 4M and 4N) (1/6 embryos expressing Rok CA had cortical Baz localization versus 7/7 wild-type embryos). Rok CA also displaced Baz from the cortex in cultured cells (Figures 6A and 6B). Similar effects were observed when Baz was coexpressed with activated Rho1 GTPase (Rho^{V14}), an upstream activator of Rho-kinase (Figure 6C). Together, these results indicate that Rho-kinase is necessary and sufficient to exclude Baz from the cortex, consistent with an instructive role in regulating Baz localization.

The regulation of Baz by Rho-kinase could be direct or it could occur indirectly through alternative Rho-kinase effectors. To distinguish between these possibilities, we asked if Baz localization is affected by two known substrates of Rho-kinase, myosin II and Lim kinase. Expression of a phosphomimetic form of the

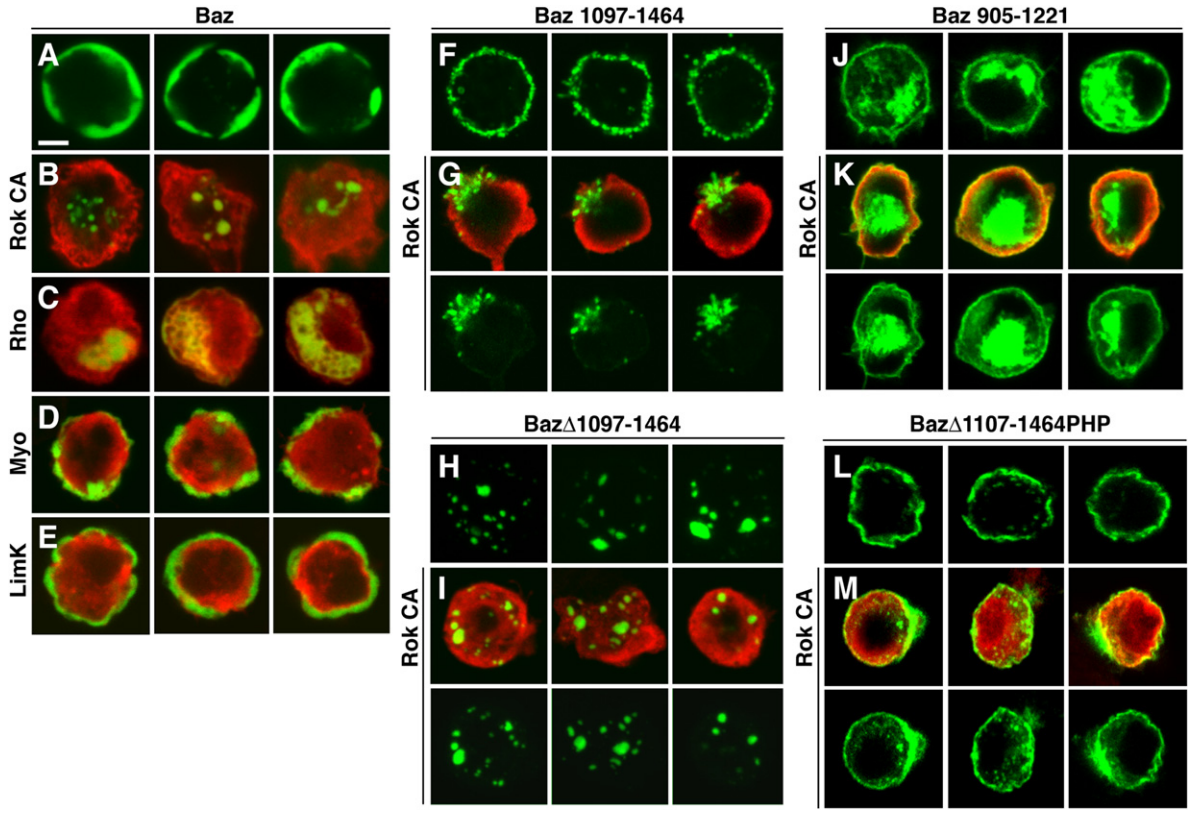

Figure 6. Rho-Kinase Regulates the Association of the Baz/Par-3 C-terminal Domain with the Cortex

(A–E) *Drosophila* S2R+ cells expressing Baz:Venus alone (green, A) or with activated versions of Rho-kinase (Rok CA red, B), Rho1 GTPase (Rho^{V14} red, C), myosin regulatory light chain ($sqh^{E20,E21}$ red, D), or Lim kinase ($limK^{E591}$ isoform C red, E). Baz:Venus was cortical in 78% of cells without Rok CA (n = 216) versus 5% with Rok CA (n = 284). Expression of Rho^{V14}, but not $sqh^{E20,E21}$ or $limK^{E591}$, led to a loss of Baz from the cortex.

(F–K) Cells expressing Baz:Venus variants alone (green, F, H and J) or with Rok CA (red, G, I, and K). (F and G) The Baz C terminus is sufficient for cortical localization (F) and is displaced from the cortex by Rok CA (G). Baz 1097-1464 was cortical in 99% of cells without Rok CA (n = 190) versus 2% with Rok CA (n = 178). (H and I) Deletion of the Baz C terminus (BazΔ1097-1464) abolished Baz cortical localization in the absence or presence of Rok CA. (J and K) An adjacent region of the Baz C terminus (Baz 905-1221) was not affected by Rok CA. Baz 905-1221 was cortical in 91% of cells without Rok CA (n = 137) versus 97% with Rok CA (n = 208).

(L and M) Cells expressing a GFP:Baz variant with the C terminus replaced with a heterologous PH domain. BazΔ1107-1464PHP localized to the cortex (L) but was not efficiently displaced by Rok CA (M). GFP:Baz cortical localization was reduced 5.5-fold from 94% to 17% by Rok CA. GFP:BazΔ1107-1464PHP cortical localization was reduced 1.4-fold from 74% to 54% by Rok CA (n = 183–257 cells/condition). Scale bar = 5 μm. See Figures S5 and S6.

myosin regulatory light chain can bypass the requirement for Rho-kinase in some cell types (Winter et al., 2001; Dean and Spudich, 2006; Monier et al., 2010). However, activated myosin regulatory light chain ($Sqh^{E20,E21}$) did not disrupt Baz cortical localization in cultured cells (Figure 6D) and did not rescue the effect of Y-27632 injection on Baz localization in vivo (data not shown). Similarly, activated Lim kinase ($LimK^{E591}$ isoform C) did not disrupt Baz cortical localization in cultured cells, despite effects on the actin cytoskeleton indicating that the protein was active (Figure 6E and data not shown). These results indicate that the regulation of Baz by Rho-kinase is unlikely to occur through myosin II or Lim kinase, although these or other Rho-kinase substrates could contribute to Baz localization in vivo.

Baz/Par-3 Planar Polarity Requires the C-terminal Domain, which Is Phosphorylated by Rho-Kinase In Vitro

Rho-kinase can phosphorylate mammalian Par-3 at Thr833 in the aPKC binding domain, disrupting its association with aPKC (Nakayama et al., 2008). This residue is not conserved in *Drosophila* or *C. elegans*, raising the question of whether this is a conserved mechanism of Par-3 regulation. To ask if Rho-kinase regulates the association of Baz with aPKC in *Drosophila*, we cotransfected S2R+ cells with Baz and aPKC with or without activated Rho-kinase. We found that Baz coimmunoprecipitated with aPKC in the presence or absence of activated Rho-kinase (Figure S5), suggesting that Rho-kinase is not sufficient to disrupt the Baz-aPKC interaction.

A conserved region in the Baz C-terminal domain has been shown to mediate Baz cortical association through a direct interaction with phosphoinositide membrane lipids (Krahn et al., 2010). Baz lacking its C-terminal domain fails to localize to the cortex, and the C-terminal domain alone is sufficient for cortical association (Figures 6F and 6H) (Krahn et al., 2010). We found that the C-terminal domain of Baz (aa 1097–1464) was efficiently excluded from the cortex by Rho-kinase, with residual accumulation in actin-rich membrane blebs (Figure 6G). This effect was

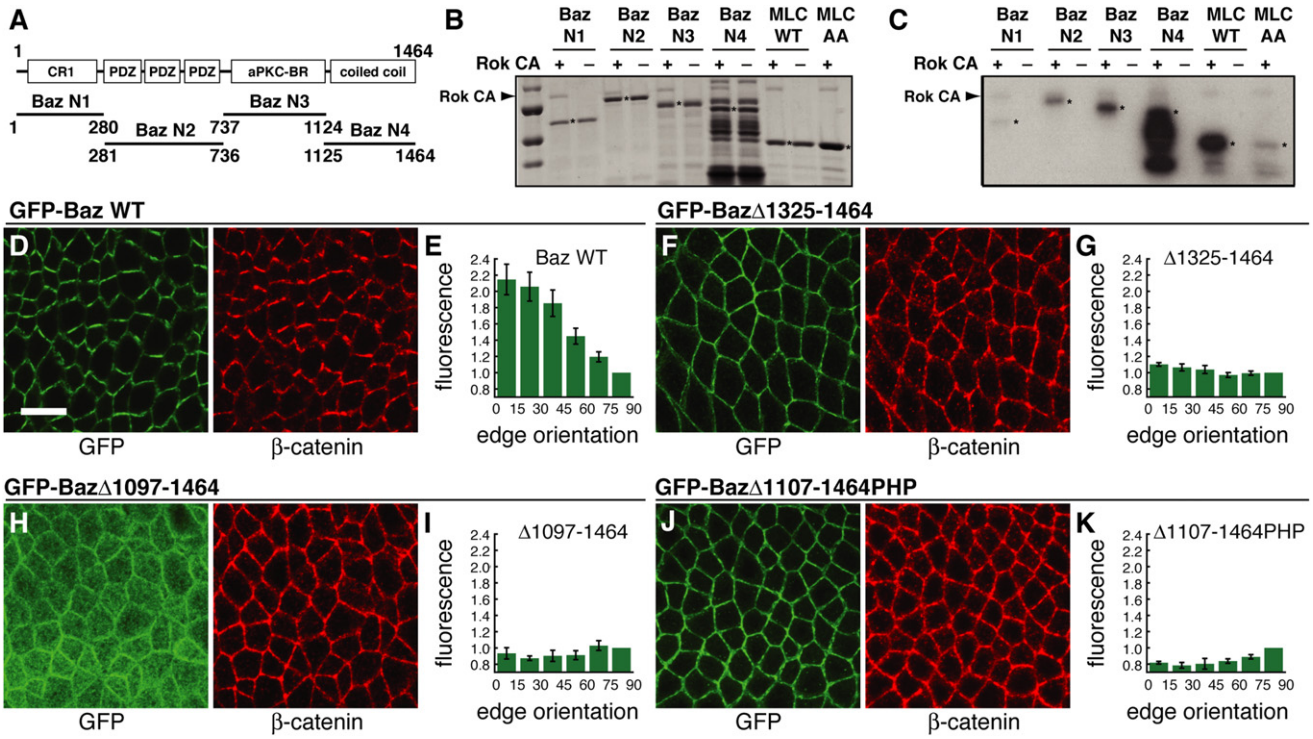

Figure 7. The Baz/Par-3 C-terminal Domain Is Necessary for Baz Planar Polarity

(A–C) GST-Baz fragments were incubated with activated Rho-kinase (Rok CA) and γ-^{32}P-ATP. (A) Domains in each fragment. (B) Total protein quantified by Coomassie staining. Molecular weight markers in kilodaltons (top to bottom): 100, 75, 50, and 37. (C) Phosphorylated proteins were detected by autoradiography. Arrowheads indicate Rok CA, which shows weak autophosphorylation. Asterisks indicate GST-Baz fragments, wild-type chick myosin regulatory light chain (MLC) (positive control), and unphosphorylatable chick MLC T19A,S20A (negative control).

(D–K) Localization of GFP-Baz transgenes in stage 7 embryos. Endogenous *baz* was knocked down via dsRNA injection and embryos were labeled with antibodies to GFP (green) and β-catenin (red). (D and E) Full-length GFP-Baz displays wild-type planar polarity (5 embryos). (F and G) GFP-BazΔ1325-1464 localized to the cortex but failed to localize asymmetrically (4 embryos, $p < 0.001$). (H and I) GFP-BazΔ1097-1464 lacking a larger region of the C terminus showed reduced cortical association and failed to localize asymmetrically (3 embryos, $p < 0.001$). (J and K) GFP-BazΔ1107-1464:PHP with the C-terminal 358 aa of Baz replaced with a heterologous PH domain restored cortical localization but failed to localize asymmetrically (6 embryos, $p < 0.001$). Scale bar = 10 μm. See Figure S6.

specific, because Rho-kinase did not interfere with the cortical localization of an overlapping Baz fragment that contains the aPKC-binding motif and part of the C-terminal domain (aa 905–1221) (Figures 6J and 6K). These results indicate that Rho-kinase acts on the Baz C-terminal domain to inhibit its association with the cell cortex.

Because mammalian Par-3 is a substrate for Rho-kinase (Nakayama et al., 2008), we asked if this regulation is conserved in *Drosophila*. We tested the in vitro kinase activity of human Rho-kinase 2, which is 75% identical to *Drosophila* Rho-kinase in the kinase domain, on Baz fragments purified from *E. coli*. The C-terminal region of Baz (aa 1125–1464), which has homology to coiled-coil domains (Nishimura et al., 2005), was efficiently phosphorylated by Rho-kinase (Baz N4, Figures 7A–7C). Baz N2 (aa 281–736), which contains the PDZ domains, and Baz N3 (aa 737–1124), which contains the aPKC binding region, were also weakly phosphorylated in this assay (Figures 7A–7C).

To test the role of the Baz C-terminal domain in vivo, we expressed truncated Baz proteins in intercalating cells. Because Baz can oligomerize through its N-terminal domain (Benton and St. Johnston, 2003; Mizuno et al., 2003), endogenous Baz levels were reduced by injecting dsRNA homologous to the *baz* 5′ untranslated region, which was not present in the transgenes. Full-length Baz localized correctly to DV edges (Figures 7D and 7E) (11/11 embryos displayed GFP-Baz planar polarity). By contrast, a deletion construct lacking the C-terminal 140 aa abolished Baz planar polarity (Figures 7F and 7G) (0/11 embryos displayed GFP-BazΔ1325-1464 planar polarity) but retained cortical localization as previously reported (Krahn et al., 2010). Similar results were obtained for a construct that removes a larger region of the Baz C-terminal domain (1/7 embryos displayed GFP-BazΔ1222-1464 planar polarity). A deletion construct lacking the entire C-terminal domain showed reduced cortical localization (Krahn et al., 2010), and the residual protein that reached the cortex failed to localize asymmetrically (Figures 7H and 7I) (0/5 embryos displayed GFP-BazΔ1097-1464 planar polarity). These results demonstrate that the C-terminal domain is necessary for Baz planar polarity in vivo.

The Baz C terminus binds directly to phosphoinositide membrane lipids, and replacement of this domain with a heterologous phospholipid binding pleckstrin homology (PH) domain from human phospholipase Cδ is sufficient to restore Baz cortical localization (Krahn et al., 2010). We found that this Baz variant was not displaced from the cortex by Rho-kinase in cultured cells (Figures 6L and 6M) and did not achieve a planar

polarized distribution in vivo (Figures 7J and 7K) (0/7 embryos displayed GFP-BazΔ1107-1464PHP planar polarity). These results suggest a model in which Rho-kinase regulates Baz planar polarity by inhibiting the association of the Baz C-terminal domain with the cell cortex. Consistent with this, Rho-kinase inhibits the association of the Baz C-terminal domain with phosphoinositide membrane lipids in vitro (Figure S6). In the absence of Rho-kinase, the Baz C-terminal domain (aa 1125–1464) bound to PIP, PIP_2, PIP_3, and phosphatidic acid. Phosphorylation by Rho-kinase efficiently disrupted this binding, and this effect was reversed by the Y-27632 Rho-kinase inhibitor. Together, these results demonstrate that the Baz C-terminal domain is necessary for Baz planar polarity and suggest that Rho-kinase regulates Baz localization by reducing the affinity of the Baz C-terminal domain for phosphoinositide membrane lipids.

DISCUSSION

The spatially regulated activity of protein kinases with multiple substrates provides an efficient strategy for the control of cell polarity in different contexts. Here, we show that Rho-kinase is an asymmetrically localized protein that plays an instructive role in planar polarity in the *Drosophila* embryo by excluding its substrate Baz/Par-3 from the cell cortex. Rho-kinase prevents expansion of the Baz domain, and Baz in turn directs the localization of contractile and adherens junction proteins that are required for axis elongation. The effect of Rho-kinase on Baz planar polarity appears to be independent of its role in regulating myosin II, because Baz localization is not affected in myosin mutants and activated myosin does not reproduce the effects of Rho-kinase in culture. Instead, Rho-kinase can directly phosphorylate the Baz C-terminal coiled-coil domain that is required for Baz association with the cortex. Deletions in the Baz C-terminal domain or replacement of the Baz C terminus with a heterologous phospholipid binding motif abolish Baz planar polarity in vivo. These results are consistent with a model in which Rho-kinase regulates the Baz C-terminal domain to inhibit Baz association with specific regions of the cell cortex.

Rho-kinase has been shown to phosphorylate mammalian Par-3 in cultured cells, disrupting its interaction with the Par complex proteins Par-6 and aPKC (Nakayama et al., 2008). The Par complex is necessary for some aspects of epithelial organization (Nagai-Tamai et al., 2002; Horikoshi et al., 2009; McCaffrey and Macara, 2009) but dispensable for others (Chen and Macara, 2005; Morais de Sa et al., 2010; Walther and Pichaud, 2010). Par-6 and aPKC are not required for Baz planar polarity in *Drosophila* (Blankenship et al., 2006; Harris and Peifer, 2007), suggesting that the role of Rho-kinase in this process is unlikely to occur through a similar mechanism. Here we provide evidence for a different mechanism of regulation involving the Baz C-terminal domain, which is phosphorylated by Rho-kinase in vitro and is necessary for Baz planar polarity in vivo. The Baz C terminus has been shown to bind directly to phosphoinositide membrane lipids, including PIP_3, PIP_2, and PIP (Krahn et al., 2010). We show that Rho-kinase inhibits the association of Baz with phosphoinositide membrane lipids in vitro, consistent with a model in which Rho-kinase directly regulates Baz association with the cortex. Alternatively, Rho-kinase could regulate Baz localization indirectly through other proteins that interact with the Baz C-terminal domain (Chen and Macara, 2005, 2006; Mertens et al., 2005). Despite these differences in mechanism, the regulation of Par-3 by Rho-kinase is a conserved feature of cell polarity in *Drosophila* and mammals.

The results presented here demonstrate that Rho-kinase is an asymmetrically localized protein that initiates a cascade of events required for the planar polarized distribution of contractile and adherens junction proteins in intercalating cells. The upstream signals that generate localized Rho-kinase activity are not known. Differences between cells conferred by striped or graded patterns of gene expression orient cell movement during axis elongation (Irvine and Wieschaus, 1994; Ninomiya and Winklbauer, 2004; Zallen and Wieschaus, 2004), and AP patterning genes expressed in stripes are necessary for the asymmetric localization of Rho-kinase. These findings raise the possibility that planar cell polarity may involve the local activation of a Rho GTPase signaling pathway. The *Drosophila* genome contains 21 RhoGEFs and 19 RhoGAPs that are candidate upstream regulators in this process (Hu et al., 2005). Rho GTPase pathways are activated by a number of upstream signals including G protein-coupled receptors, receptor tyrosine kinases, cytokine receptors, and cell-cell and cell-substrate adhesion. Identification of the signals upstream of Rho-kinase will help to elucidate the spatial cues that initiate planar polarity in the *Drosophila* embryo.

The role of Rho-kinase in planar cell polarity is reinforced by the effect of Baz on the localization of contractile and adherens junction proteins. The relationship between Baz and myosin II is complex. In the *C. elegans* zygote, a contractile myosin network carries PAR-3 to the anterior cell cortex (Munro et al., 2004). In other cell types, myosin appears to be dispensable for Baz localization (Simone and DiNardo, 2010). PAR-3 is required to sustain myosin contractility in *C. elegans* and *Drosophila* (Munro et al., 2004; David et al., 2010), and Baz promotes apical myosin localization in *C. elegans* gastrulation (Nance et al., 2003) and the *Drosophila* follicular epithelium (Wang and Riechmann, 2007). The ectopic cortical association of myosin in *baz* mutants and the complementary distributions of Baz and myosin in several tissues also raise the possibility of inhibitory effects of Baz on myosin (Zallen and Wieschaus, 2004; Major and Irvine, 2006; Simone and DiNardo, 2010). Baz could regulate myosin directly or indirectly through effects on apical-basal polarity (Goldstein and Macara, 2007) or the actin cytoskeleton (Chen and Macara, 2005, 2006; Mertens et al., 2005; Ramachandran et al., 2009).

Differential adhesion is sufficient to drive cell sorting in culture (Steinberg and Takeichi, 1994) and has been proposed to influence tissue morphogenesis in vivo (Irvine and Wieschaus, 1994; Zallen and Blankenship, 2008). We show that Rho-kinase and Baz regulate the planar polarized localization of the adherens junction protein β-catenin. Rho-kinase has been shown to downregulate adhesion in culture (Sahai and Marshall, 2002; Samarin et al., 2007), an activity that is thought to occur through myosin II, which can play positive and negative roles in junctional stabilization. The ability of Rho-kinase to exclude the Baz/Par-3 junctional regulator from the cortex suggests an alternative mechanism for the regulation of adherens junctions by Rho GTPases. These results suggest that Rho-kinase can both promote contractility and inhibit adhesion, providing a single

molecular mechanism linking cortical contraction with adherens junction disassembly during tissue morphogenesis.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

Embryos were generated at 20°C. Wild-type was *y,w* unless otherwise indicated. Alleles were *baz*^{FA50}, *baz*^{GD21} (gifts of N. Denef and T. Schüpbach), *arm*^{043A06} (Tolwinski and Wieschaus, 2001), *DRok*^{1}, *DRok*^{2} (Winter et al., 2001), and *sqh*^{1} (Karess et al., 1991). Embryos expressing UASp-Venus:-Rok^{K116A} (this work), UASp-HA:Rok (Wang and Riechmann, 2007), UASp-Baz:GFP (Benton and St. Johnston, 2003), or Baz transgenes (Krahn et al., 2010) were F2 progeny of UASp males × matαtub67;15 females (gift of D. St. Johnston). Germline clones were generated with the FLP-DFS system and *ovo*^{D2} FRT18E, *ovo*^{D1} FRT101 (Chou and Perrimon, 1996) or *ovo*^{D2} FRT19A (gift of N. Tolwinski). *baz* mutant embryos were identified by the absence of Baz staining.

baz alleles were sequenced by PCR amplification of all exons and splice junctions from GFP-negative larval progeny of FM7,Kr-Gal4,UAS-GFP heterozygous females and FM7,Kr-Gal4,UAS-GFP males and confirmed in independent PCR reactions from different larvae. The *baz*^{GD21} allele contains a 4-nt deletion of nt 793–796 in the open reading frame, causing a frameshift predicted to truncate the protein after 264 of 1464 aa, removing the PDZ domains, aPKC-binding region and C-terminal domain. The *baz*^{FA50} allele contains an 8-nt deletion of nt 2463–2470, causing a frameshift predicted to truncate the protein after 821 of 1461 aa, removing the aPKC-binding region and C-terminal domain.

Transgenic Lines

UASp-Venus:Rho-kinase^{K116A} was generated by cloning full-length *DRok* cDNA into the pENTR/D TOPO cloning vector and cloning Venus into the AscI site followed by site-directed mutagenesis (Stratagene Quik-Change system). Clones were recombined into pUASp-w-attB (gift of M. Buszczak) with the Gateway system (Invitrogen). pUASp-HA:Rok-CA was generated by amplifying the first 553 codons of the *DRok* cDNA and HA from pUASp-HA:Rok (Wang and Riechmann, 2007) and subcloned into pUASp. Transgenic lines for UASp-Venus:Rho-kinase^{K116A} were inserted in the attP40 site on chromosome II, and transgenic lines for UASp-HA:Rok CA were randomly inserted. (Genetic Services).

Immunohistochemistry

For antibodies to Arm/β-catenin, Baz, HA, and myosin, embryos were boiled for 10 s in 0.03% Triton X-100/0.4% NaCl, cooled on ice, and devitellinized in heptane/methanol. For antibodies to GFP, embryos were fixed for 20 min in 4% PFA (EMS) in PBS/heptane and devitellinized in heptane/methanol. For phalloidin, embryos were fixed for 1 hr in 4% PFA in PBS/heptane and manually devitellinized. Antibodies were used at the following concentrations: mouse anti-Arm, 1:50 (Developmental Studies Hybridoma Bank); guinea pig anti-Baz, 1:500 (made by J.A.Z. as described by Wodarz et al., 2000); rat anti-DE-cadherin, 1:100 (Developmental Studies Hybridoma Bank); rabbit anti-GFP, 1:100 (Torrey Pines); rat anti-HA, 1:500 (Roche); and rabbit anti-myosin II heavy chain (Zip), 1:1250 (gift of C. Field). Secondary antibodies conjugated to Alexa-488, Alexa-568, or Alexa-647 (Molecular Probes) were used at 1:500. Rhodamine-conjugated phalloidin (Molecular Probes) was used at 1:1000. Embryos were mounted in Prolong Gold (Molecular Probes) and imaged on a Zeiss LSM510 META confocal with a PlanNeo 40×/1.3NA objective. 1.0 μm Z slices were acquired at 0.5 μm steps. Maximum intensity projections of 2-3 μm in the apical junctional domain were analyzed.

Time-Lapse Imaging

Time-lapse imaging was performed with the following markers: GFP:Spider and GFP:Resille (gifts of Alain Debec), ubi-DE-cadherin:GFP (Oda and Tsukita, 2001), sqh-Sqh:GFP (Royou et al., 2004), UASp-Baz:GFP (Benton and St. Johnston, 2003), sqh-Sqh:mCherry (Martin et al., 2009), and a GFP insertion in the endogenous *baz* gene (Fly Trap). Embryos were dechorionated for 1 min in 50% bleach, washed in water, mounted on a YSI membrane with halocarbon oil 27 (Sigma), and imaged on a PerkinElmer RS5 spinning disk confocal with a Zeiss PlanNeo 40×/1.3NA objective. Z stacks were acquired at 1 μm steps and 15 s intervals. Maximum intensity projections of 2–3 μm in the apical junctional domain were analyzed.

Polarity Measurements

User-drawn, three-pixel-wide lines for all edges in a 50 μm × 50 μm region were used to calculate the mean pixel intensity and orientation for each edge. Intensities were averaged for all edges in a 15° angular bin after subtracting background (defined as the average value of cytoplasmic pixels ≥1 μm from the plasma membrane) with custom Matlab software. Mean intensity values in each bin were normalized to the mean intensity of edges parallel (0°–15°) or perpendicular (75°–90°) to the AP axis. A representative subset of images was quantified for each experiment. A mean value was obtained for each embryo, and error bars indicate the standard error of the mean in all figures. p values were calculated by comparing normalized mean intensities of edges in the 0°–30° or 60°–90° angular range in mutant and control embryos with the F test followed by the appropriate t test.

Drug Injection

Stage 7/8 embryos were dechorionated, glued to a coverslip, dehydrated for 5 min, covered in 1:1 halocarbon oil 27/700, and injected in the ventral perivitelline space with 100–200 pl of 10–100 mM Rho-kinase inhibitor Y-27632 (TOCRIS) in water or 10 mM LatB (Sigma) in DMSO. Control embryos were injected with water or DMSO. Drugs are diluted ~50-fold in the embryo. For immunostaining, embryos were washed off the coverslip with heptane 5–10 min after injection, fixed 1 hr in 4% PFA in PBS/heptane and manually devitellinized.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three movies and can be found with this article online at doi:10.1016/j.devcel.2010.08.011.

ACKNOWLEDGMENTS

We thank Andreas Wodarz for sharing information and reagents prior to publication, Natalie Denef and Trudi Schüpbach for the *baz* alleles, Nick Tolwinski for the *ovo*^{D2} *FRT19A* stock, and Mike Buszczak for the pUASp-w-attB plasmid. We thank Justina Sanny and Leah Greenspan for excellent technical assistance and Emily Marcinkevicius, Athea Vichas, Frederik Wirtz-Peitz, and Richard Zallen for helpful discussions and comments on the manuscript. This work was supported by a postdoctoral fellowship from Fundação para a Ciência e Tecnologia, Portugal (to S.S.), and a Burroughs Wellcome Fund Career Award in the Biomedical Sciences, March of Dimes Basil O'Connor Starter Scholar Award, Searle Scholar Award, W. M. Keck Foundation Distinguished Young Scholar in Medical Research Award, and NIH/NIGMS R01 Grant GM079340 (to J.A.Z.). J.A.Z. is an Early Career Scientist of the Howard Hughes Medical Institute.

Received: September 12, 2009
Revised: July 7, 2010
Accepted: August 17, 2010
Published: September 13, 2010

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