Rap1 and Canoe/afadin are essential for establishment of apical–basal polarity in the *Drosophila* embryo

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ABSTRACT The establishment and maintenance of apical–basal cell polarity is critical for assembling epithelia and maintaining organ architecture. *Drosophila* embryos provide a superb model. In the current view, apically positioned Bazooka/Par3 is the initial polarity cue as cells form during cellularization. Bazooka then helps to position both adherens junctions and atypical protein kinase C (aPKC). Although a polarized cytoskeleton is critical for Bazooka positioning, proteins mediating this remained unknown. We found that the small GTPase Rap1 and the actin-junctional linker Canoe/afadin are essential for polarity establishment, as both adherens junctions and Bazooka are mispositioned in their absence. Rap1 and Canoe do not simply organize the cytoskeleton, as actin and microtubules become properly polarized in their absence. Canoe can recruit Bazooka when ectopically expressed, but they do not obligatorily colocalize. Rap1 and Canoe play continuing roles in Bazooka localization during gastrulation, but other polarity cues partially restore apical Bazooka in the absence of Rap1 or Canoe. We next tested the current linear model for polarity establishment. Both Bazooka and aPKC regulate Canoe localization despite being "downstream" of Canoe. Further, Rap1, Bazooka, and aPKC, but not Canoe, regulate columnar cell shape. These data reshape our view, suggesting that polarity establishment is regulated by a protein network rather than a linear pathway.

INTRODUCTION

Polarity is a fundamental property of all cells, from polarized cell divisions in bacteria or fungi to the elaborate polarity of neurons. Among the most intensely studied forms of polarity in animal cells is epithelial apical–basal polarity (Goldstein and Macara, 2007). Polarity of epithelial sheets is key to their function as barriers between body compartments and is also critical in collective cell

migration and cell shape change during morphogenesis, as cytoskeletal and apical–basal polarity often go hand in hand. Loss of apical–basal polarity is a hallmark of metastasis (Wodarz and Nathke, 2007). We have made significant advances in defining the machinery required for cell polarity in many settings, but fundamental questions remain unanswered.

Cadherin–catenin complexes, which assemble into adherens junctions (AJs) near the apical end of the lateral cell interface, are critical polarity landmarks that define the boundary between apical and basolateral domains (Gumbiner *et al.*, 1988). Studies in *Caenorhabditis elegans* and *Drosophila* identified other key regulators of apical–basal polarity (Goldstein and Macara, 2007; Lynch and Hardin, 2009; Harris and Tepass, 2010; Laprise and Tepass, 2011). In the textbook view, the apical domain is defined by the Par3/Par6/atypical protein kinase C (aPKC) and Crumbs/Stardust(Pals1)/PATJ complexes (Assemat *et al.*, 2008), whereas Scribble, Dlg, Lgl, and Par1 define the basolateral membrane. Complex cross-regulatory interactions between apical and basolateral proteins maintain these mutually exclusive membrane territories (e.g., Bilder *et al.*, 2003; Tanentzapf and Tepass, 2003; Laprise

Abbreviations used: AJ, adherens junction; aPKC, atypical protein kinase C; Arm, Armadillo; Baz, Bazooka; Cno, Canoe; DEcad, DE-cadherin; Dzy, Dizzy; Ed, Echinoid; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; IF, Immunofluorescence; M, maternal; MT, microtubule; MZ, maternal/zygotic; WT, wild type.

et al., 2009; Wang et al. 2012). These proteins also regulate other types of polarity during morphogenesis (St Johnston and Sanson, 2011). For example, fly Par3 (Bazooka [Baz]), aPKC, and AJ proteins are planar polarized during fly convergent extension, thus regulating polarized cell movements (Zallen, 2007).

Polarized cytoskeletal networks also play key roles in establishing and maintaining apical–basal and planar polarity. These networks are believed to be physically linked to apical junctional complexes. The earlier model suggesting that cadherin–catenin complexes link directly to actin via α-catenin is now viewed as oversimplified (Drees et al., 2005; Yamada et al., 2005). Instead, different proteins are believed to mediate this connection in different tissues and at different times (e.g., Abe and Takeichi, 2008; Cavey et al., 2008; Sawyer et al., 2009).

Among the linkers is Canoe (Cno)/afadin, an actin-binding protein that binds transmembrane nectins via its PDZ domain (Mandai et al., 1997). Although originally hypothesized to be essential for cell adhesion, subsequent work supports a model in which afadin modulates adhesive and cytoskeletal machinery during cell migration in vitro (Lorger and Moelling, 2006; Miyata et al., 2009; Fournier et al., 2011) and the complex events of mouse gastrulation (Ikeda et al., 1999; Zhadanov et al., 1999). Afadin has two N-terminal Ras association domains for which the small GTPase Rap1 is the major binding partner (Linnemann et al., 1999), and afadin and Rap1 are functionally linked in both flies and mice (Boettner et al., 2003; Hoshino et al., 2005). Rap1, Cno, and the Rap1 guanine nucleotide exchange factor (GEF) Dizzy/PDZ-GEF are all essential for maintaining effective linkage between AJs and the apical actomyosin cytoskeleton during apical constriction of Drosophila mesodermal cells during fly gastrulation (Sawyer et al., 2009; Spahn et al., 2012). Rap1 regulates Cno localization to the membrane (Sawyer et al., 2009). Cno plays a related role during convergent extension, although its role is planar polarized during this process (Sawyer et al., 2011). Cno also regulates collective cell migration, signaling, and oriented asymmetric divisions (e.g., Boettner et al., 2003; Carmena et al., 2006, 2011; Wee et al., 2011). The Rap1/Cno regulatory module is also important in disease, as afadin and Rap1 are implicated in congenital disorders of the cardiovascular system (Glading et al., 2007) and cancer metastasis (Fournier et al., 2011). It remains unclear whether these diverse roles all involve junction-cytoskeletal linkage or whether some are independent functions.

The small GTPase Rap1 plays diverse cellular roles. Mammalian Rap1 isoforms are perhaps best known for regulating integrin-based cell matrix adhesion (Bos, 2005; Kim et al., 2011), but Rap1 also regulates cell–cell AJs in both Drosophila and mice (Kooistra et al., 2007; Boettner and Van Aelst, 2009). In murine endothelial cells, for example, Rap1, its effector Krit1, and VE-cadherin form a complex that regulates endothelial cell junctions and stabilizes apical–basal polarity (Glading et al., 2007; Lampugnani et al., 2010; Liu et al., 2011). In Drosophila imaginal disc cells, Rap1 regulates the symmetric distribution of DE-cadherin (DEcad) around the apical circumference of each cell (Knox and Brown, 2002). Rap1 carries out these functions via a diverse set of effector proteins, including Krit1, TIAM, RIAM, and Cno/afadin (Kooistra et al., 2007; Boettner and Van Aelst, 2009). Thus Rap1 and its effectors are candidate proteins for regulating interactions between AJs, polarity proteins, and the cytoskeleton during polarity establishment and maintenance.

The early Drosophila embryo provides among the best models of establishing and maintaining apical–basal polarity (Harris, 2012). Flies start embryogenesis as a syncytium, with 13 rounds of nuclear division without cytokinesis. Membranes then simultaneously invaginate around each nucleus, forming ~6000 cells in a process known as cellularization (Figure 1A, right). Before cellularization, the egg membrane is already polarized (Mavrakis et al., 2009) and serves as a polarity cue for underlying nuclei. This ultimately becomes the apical end of the new cells. Epithelial apical–basal polarity is initiated during cellularization (Harris, 2012). In the absence of cadherin–catenin complexes, cells form normally but then lose adhesion and polarity as gastrulation begins (Cox et al., 1996). These data and earlier work from cell culture (Gumbiner et al., 1988) suggested that AJs are the initial apical cue. However, we found that Bazooka (Baz)/Par3 acts upstream of AJs in this process (Harris and Peifer, 2004). Strikingly, Baz and DEcad apically colocalize in spot AJs from cellularization onset (Figure 1A, right; cadherin–catenin complexes are also enriched in "basal junctions" just above the invaginating actomyosin front; Hunter and Wieschaus, 2000). In the absence of Baz, DEcad loses its apical enrichment and redistributes all along the lateral membrane, whereas in the absence of AJ proteins, Baz remains apically localized, and a subset of cells retains residual apical–basal polarity, although cell shapes are highly abnormal (Harris and Peifer, 2004). Cadherin–catenin and Baz complexes form independently before cellularization, and Baz then helps position DEcad in the apicolateral position, where spot AJs will form (McGill et al., 2009).

This places Baz atop of the polarization network (Figure 1A, left), raising the question of how it is positioned apically. Two cytoskeletal networks play important roles in initial Baz positioning (Harris and Peifer, 2005). Disrupting dynein led to spreading of Baz along the lateral membrane, suggesting that polarized transport along microtubules (MTs) plays a role. Depolymerizing actin also destabilized apical Baz, as did significantly overexpressing Baz, suggesting that an actin-based scaffold with a saturable number of binding sites anchors Baz apically. Whereas both actin and MTs are required for initial Baz polarization, they are not the only cues. Mislocalized Baz is rerecruited or restabilized apically at gastrulation onset if either initial cue is disrupted, suggesting a third cue (Harris and Peifer, 2005) perhaps involving aPKC/Par6 (Hutterer et al., 2004) or Par1 (McKinley and Harris, 2012). Thus the current model for initial establishment of apical–basal polarity involves a relatively simple pathway in which Baz is positioned apically and then positions other apical polarity players (Figure 1A, left). However, once initial polarity is established, events become more complex, with a network of mutually reinforcing and inhibitory interactions between apical and basolateral polarity complexes leading to polarity elaboration and maintenance.

These were significant advances, but the proteins directing apical accumulation of Baz remained unknown. Work on apical constriction in the fly mesoderm (Dawes-Hoang et al., 2005; Martin et al., 2010), convergent extension during gastrulation (Bertet et al., 2004; Zallen and Wieschaus, 2004), establishment of anterior–posterior polarity in one-cell C. elegans embryos (Munro et al., 2004), and apically constricting Drosophila amnioserosal cells (David et al., 2010) suggested that a complex network of interactions link AJs, the apical polarity proteins Baz and aPKC, and the actomyosin cytoskeleton. Our recent work on the roles of Canoe and Rap1 in mesoderm apical constriction (Sawyer et al., 2009) and convergent elongation (Sawyer et al., 2011) suggested that they also fit into this network. These data led us to explore whether Rap1 and Cno play roles in initial apical positioning of AJs and Baz and thus in the establishment and early maintenance of polarity.

RESULTS

Rap1 is required for initial apical positioning of AJs

In the current model for apical–basal polarity establishment, apical Baz directs apical positioning of AJs and aPKC, whereas a polarized
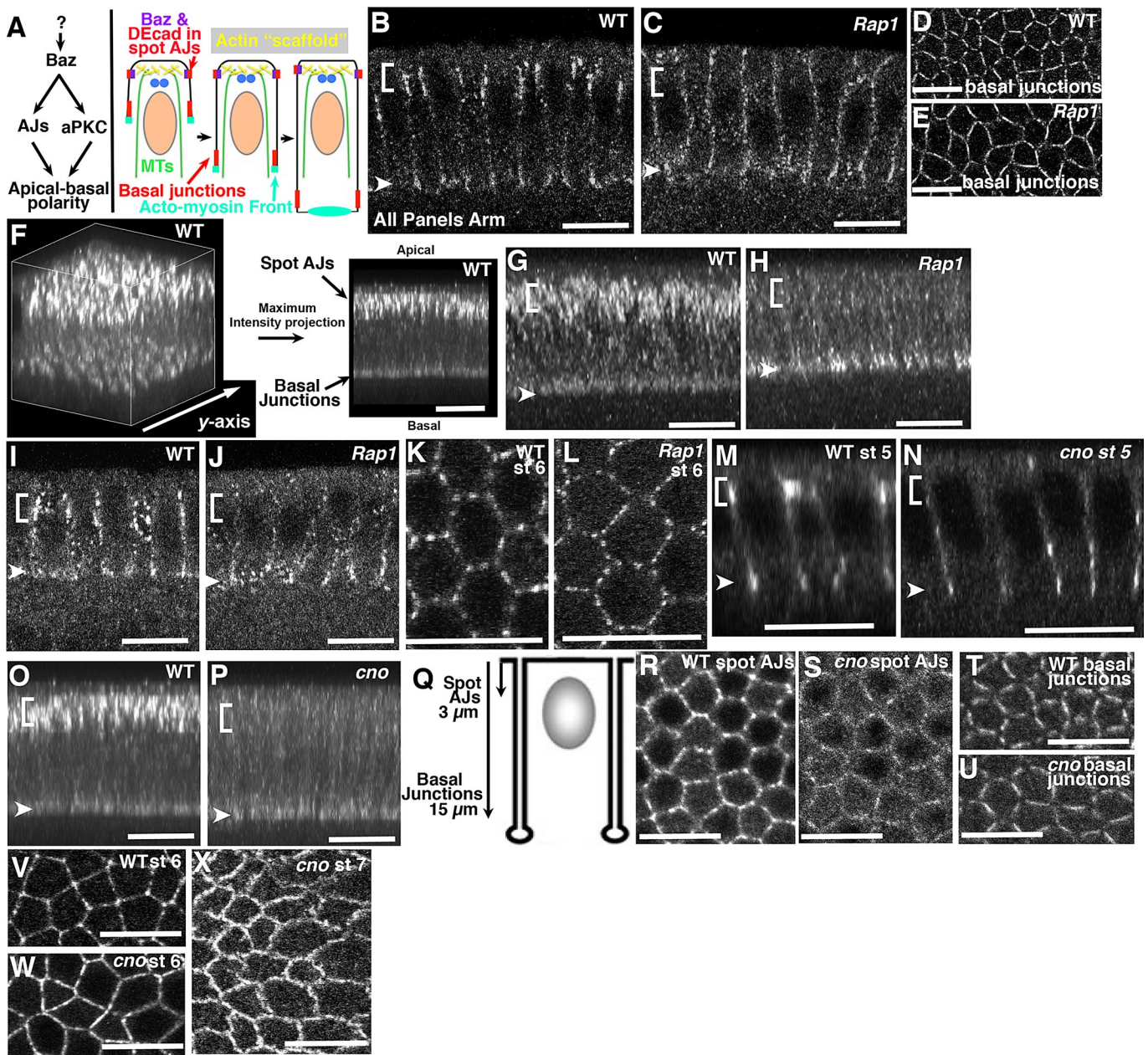
FIGURE 1: Rap1 and Cno are required for initial assembly of apical adherens junctions. (A) Diagram of current model of polarity establishment (left) and of initial apical–basal polarization during cellularization (right). (B–X) AJ protein Arm. (A–H) Late cellularization. (B, C) Apical-basal cross-sections. In WT (B), Arm is enriched at nascent apical spot AJs (bracket) and in basal junctions (arrowhead). In contrast, in *Rap1* mutants, apical spot AJ enrichment is reduced or lost (C, bracket). (D, E) Basal junctions remain essentially unchanged in *Rap1* mutants (D vs. E). (F) Approach for creating projections of cross-sections. Image stacks were collected and maximum-intensity projections created along the *y*-axis. This makes enrichment at forming apical junctions and basal junctions more readily apparent. (G, H) Projections highlight loss of apical Arm enrichment in *Rap1* (H, bracket) vs. WT (G, bracket), whereas basal junction enrichment remains (arrowheads). (I, J) Reduced apical enrichment in *Rap1* mutants (J, brackets) is already present at midcellularization. (K, L) Apical views, stage 6 (gastrulation onset). Apical spot AJs are present in both WT (K) and *Rap1* mutants (L). (M–T) Late cellularization. (M, N) Single apical–basal cross-sections. (O, P) Projections of apical–basal cross-sections (as in F). Arm is enriched in both spot AJs and basal junctions in WT (M, O, brackets), whereas Arm enrichment in spot AJs is lost in *cno* mutants (N, P, brackets), although basal junction enrichment remains (arrowheads). (Q) Planes of surface views in R–U. (R, S) The uniform enrichment of spot AJs in WT (R) is reduced in *cno* mutants (S), whereas basal junctions remain relatively unaltered (T vs. U). (V–X.) Apical spot AJs are visible in stage 6 WT (V) and *cno* mutants (W), and AJs are present in stage 7 *cno* mutants (X). Scale bars, 10 μm.

cytoskeleton is important for Baz apical localization and/or retention (Harris and Peifer, 2004, 2005). However, the proteins directing Baz apical positioning remained unknown. On the basis of Rap1's

roles in regulating cell–cell and cell matrix adhesion (Kooistra *et al.*, 2007; Kim *et al.*, 2011) and AJ:actin linkage during apical constriction (Sawyer *et al.*, 2009) and its presence at the plasma membrane
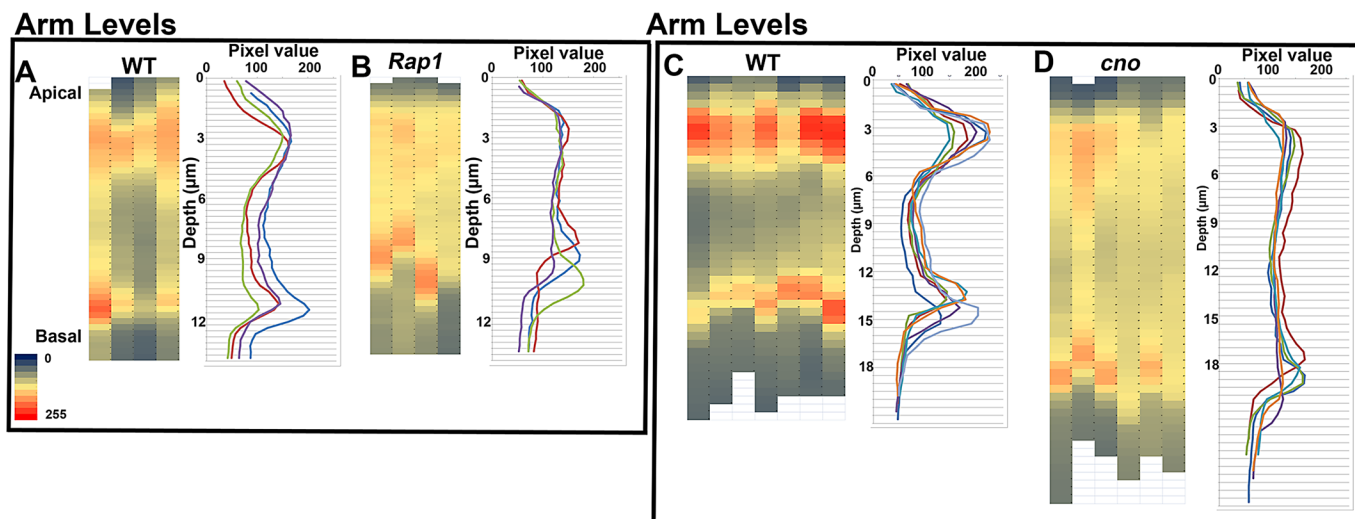
FIGURE 2: Rap1 and Cno regulate apical positioning of Arm in forming apical junctions. The Plot Profile option in ImageJ was used to measure average image intensity in projected cross-sections, and data were displayed either as heat maps illustrating intensity with different colors (left, apical is on top; each column is a different embryo) or graphically, displaying pixel intensity vs. depth from the apical surface (right, each line is a different embryo). Note that since we use embryos from more than one experiment, these quantitative measures are useful for comparing signal intensity along the apical–basal axis within an embryo, but absolute intensities between embryos vary due to variations in staining and imaging.

in syncytial and cellularizing embryos (Sawyer *et al.*, 2009; Supplemental Figure S1, A–C), we hypothesized that Rap1 might be part of the mechanism regulating initial apical positioning of AJs and Baz. To test this hypothesis, we used the FLP-recombinase target (FRT)/dominant female sterile approach (Chou *et al.*, 1993) to generate embryos completely lacking maternal (M) Rap1, using the null allele *Rap1rv(R)B1* (called *Rap1* hereafter), and crossed them to *Rap1/+* fathers. We cannot distinguish maternal/zygotic (MZ) mutants (*Rap1MZ*) from embryos receiving paternal wild-type *Rap1* (*Rap1M*) until mid–late gastrulation.

Cadherin–catenin complexes are already at the membrane in wild-type (WT) syncytial embryos (Grevengoed *et al.*, 2003). As cellularization begins, they form puncta in the egg membrane. These are recruited into apicolateral spot AJs as membranes invaginate (Figure 1A; Harris and Peifer, 2004; McGill *et al.*, 2009; Figure 1B, bracket indicates Armadillo [Arm] = fly β-catenin). AJ proteins also accumulate in basal junctions (Figure 1, A and B, arrowheads, and D; Hunter and Wieschaus, 2000) just behind the actomyosin rings at the front. Lower levels of DEcad and Arm are also found all along the newly formed lateral membrane. The twin apical and basolateral enrichment of cadherin–catenin complexes by the end of cellularization is readily apparent in maximum-intensity projections of many cross-sections (Figure 1F), which overlay the forming AJs of many nascent cells.

We tested the hypothesis that Rap1 helps to regulate initial apical positioning of AJs. *Rap1MZ* mutants end embryogenesis with a fragmented cuticle (Supplemental Figure S2, A vs. B; Sawyer *et al.*, 2009), suggesting that epithelial integrity is disrupted, but this could be due to early effects on polarity establishment or much later effects on polarity maintenance. When we examined initial apical positioning of spot AJs during cellularization, we found that it was significantly disrupted in *Rap1* mutants. Although AJ proteins still accumulated in basal junctions (Figure 1, C, arrowhead, and E), the apical enrichment of AJ complexes was substantially reduced during late cellularization (as seen in individual cross-sections; Figure 1, B vs. C, brackets). The difference was accentuated in projected cross-

sections overlaying AJs of many cells (Figure 1, G vs. H, brackets), where, in *Rap1* mutants, AJ puncta localized all along the lateral cell interface. Differences began as early as midcellularization (Figure 1, I vs. J, brackets). We verified that this alteration was due to loss of Rap1 by maternally knocking down Rap1 using RNA interference (RNAi; Supplemental Figure S1, C vs. D)—this also led to loss of apical enrichment of AJs during cellularization (Supplemental Figure S1, E–H). To quantitate alterations in AJ positioning and to compare multiple embryos, we used ImageJ Plot Profile to measure average image intensity in projected cross-sections from four embryos at late cellularization. These are displayed as heat maps (Figure 2A, left; intensity is color coded) or graphically (Figure 2A, right) from apical (top) to basal (bottom). Although there was some variability between embryos, the dual peaks of Arm at forming AJs and basal junctions are readily apparent in WT (Figure 2A). In contrast, in *Rap1* mutants, the apical peak of Arm at assembling spot AJs was essentially gone, although the basal junction peak remained in three of four embryos. In fact, many embryos appeared to have elevated Arm accumulation in basal junctions, including both *Rap1* mutants (Figure 1, G vs. H, arrowheads) and embryos with *Rap1* RNAi knockdown (Supplemental Figure S1, G vs. H). This could be a direct effect or the result of an elevated cytoplasmic pool due to failure to correctly assemble spot AJs. Some apical spot AJs were seen in *Rap1* embryos at gastrulation onset (Figure 1, K vs. L; stage 6), suggesting that the initial defect in their positioning may be partially rescued later; we explore this later. Taken together, these data suggest that Rap1 is required for initial apical positioning of AJs.

Cno is also required for initial apical positioning of AJs

Rap1 has many effectors mediating its diverse functions (Kooistra *et al.*, 2007), including Cno/afadin. Cno regulates AJ:actin interactions during gastrulation (Sawyer *et al.*, 2009, 2011). Furthermore, Cno is positioned to affect early polarization (Supplemental Figure S3, A–G), given that, like Baz (Supplemental Figure S3, L–K) and AJ proteins (Supplemental Figure S3, A and G; McCartney *et al.*, 2001), Cno already localizes to the egg plasma membrane in syncytial

embryos (Supplemental Figure S3A) and to developing apical junctions during cellularization (Supplemental Figure S3, B and C; Sawyer et al., 2009). Finally, Rap1 is required for effective localization of Cno during cellularization and early gastrulation (Sawyer et al., 2009). Given their parallel roles in many events, we tested the hypothesis that Cno is the Rap1 effector regulating initial apical AJ positioning.

To do so, we examined cno[R2] maternally null mutant embryos (hereafter cno; as with Rap1, we can definitively identify the 50% zygotically rescued embryos only late in gastrulation). Strikingly, whereas WT cellularizing embryos exhibited enrichment of cadherin–catenin complexes in both spot AJs and basal junctions (Figure 1M), cno mutants lost apical AJ protein enrichment (Figure 1, M vs. N, brackets; O vs. P, brackets; R vs. S). In contrast, basal junctions appeared normal (Figure 1, M vs. N, arrowheads; O vs. P, arrowheads; T vs. U). We quantitated these changes in multiple embryos, as we had with Rap1 mutants. In WT, peaks of Arm were readily apparent both in assembling apical AJs and in basal junctions (Figure 2C), whereas in cno mutants the apical peak was blunted or lost (Figure 2D). Apical spot AJs are seen later in gastrulating cno embryos (Sawyer et al., 2009), even those in which mesoderm invagination had stalled (see Figures 4–10 later in the paper), suggesting that this initial defect may be partially rescued later. Taken together, these data suggest that both Rap1 and its effector Cno regulate the initial positioning of AJs during establishment of apical–basal polarity.

Rap1 and baz exhibit strong dose-sensitive genetic interactions in epithelial integrity

These data are consistent with the possibility that Rap1 and Cno directly regulate AJ positioning or that they act on the upstream regulator of AJ positioning, Baz/Par3. One way to assess whether two proteins work together in a cell biological process is to look for dose-sensitive genetic interactions, such that reducing levels of one protein enhances the effect of lowering levels of the other. We adopted an approach used by Harris's lab to screen for proteins working with Baz in epithelial development (Shao et al., 2010), using zygotic mutants to reduce rather than eliminate gene function. Embryos retaining maternal Baz but lacking zygotic Baz slowly run out of Baz protein. Although they correctly establish apical–basal polarity (unlike baz[MZ] mutants), a subset of embryos lose full epithelial integrity later and end up with holes in the cuticle (explaining the name bazooka), ranging from minor (27%) to significant (71%), but almost all retain large portions of intact cuticle (Figure 3, A and B). This genotype is sensitized for alterations in apical–basal polarity regulators, and its phenotype is significantly enhanced by heterozygosity for known regulators, including Crumbs, aPKC, and DEcad (Shao et al., 2010).

We thus hypothesized that if Rap1 is an important player in apical–basal polarity, it would also enhance baz. Rap1 heterozygotes are viable and fertile, and even Rap1 zygotic mutants survive embryogenesis normally on maternally supplied Rap1, dying as late larvae/pupae. We thus assessed whether reducing Rap1 enhanced the effect of reduced Baz levels in baz zygotic mutants. Strikingly, even the small reduction in Rap1 levels in embryos maternally and zygotically heterozygous for Rap1 enhanced the defects of baz zygotic mutants, leading to stronger disruption of cuticle integrity (Figure 3B, top vs. middle genotype). Further reducing Rap1 levels by removing zygotic Rap1 caused an even stronger enhancement (Figure 3B, top vs. bottom genotype). To confirm that this was due to effects on AJ integrity, we visualized Arm at stage 10, focusing on the amnioserosa, as it is especially sensitive to reduced Baz levels (Shao et al., 2010). Although only

12% of baz zygotic mutants had defects in amnioserosal AJ integrity (Figure 3, C and D, illustrates the two classes and Figure 3E, top, is the quantitation), this increased to 64% when Rap1 was reduced maternally and zygotically (Figure 3E). Taken together, these data are consistent with the hypothesis that Rap1 and Baz cooperate in maintaining epithelial integrity; similar dose-sensitive genetic interactions exist between cno and baz (Sawyer et al., 2011).

Rap1 and Cno act upstream of Baz/Par3, regulating its apical positioning

These genetic data are consistent with the hypothesis that Rap1 and Cno act together with Baz to maintain epithelial integrity. During WT cellularization, Baz helps to recruit AJ proteins into apicolateral complexes, the spot AJs (McGill et al., 2009). Baz remains in this apical position throughout cellularization (Figure 1A); however, unlike AJ proteins Baz does not assemble into basal junctions (Harris and Peifer, 2004; McGill et al., 2009). Although it is clear that cytoskeletal interactions help to position Baz apically (Harris and Peifer, 2005), proteins mediating this remained unknown. We considered two mechanisms by which Rap1 and Cno might mediate the initial apical positioning of AJs. First, as Baz is required for apical AJ positioning (Harris and Peifer, 2005), Rap1 and Cno may be the missing players acting upstream of Baz. Alternatively, Rap1 and Cno might not affect Baz localization but instead might directly position AJ proteins. To distinguish between these mechanisms, we examined the initial apical positioning of Baz in Rap1 and cno mutants.

When we examined Baz localization in Rap1 mutants, we found the exclusively apical localization of Baz during cellularization was substantially disrupted. In WT, Baz is restricted to apical complexes (Figure 4, A' and C). In contrast, in Rap1 mutants, Baz still formed membrane-associated puncta, but its restricted apical localization was lost, and Baz was redistributed all along the lateral membrane, both at the end of cellularization (Figure 4, B' and D) and earlier during midcellularization (Figure 4, E vs. F; during earlier stages of cellularization, it was impossible to distinguish between the "normal" Baz localization and that which would occur if it was "all along the lateral membrane," as they are overlapping). Baz was basal to its normal position in spot AJs (Figure 4, I3 vs. J3, arrows) and also more apical (Figure 4, I1 vs. J1, arrows). Consistent with the partial zygotic phenotypic rescue seen in later embryos (see later discussion), whereas all embryos had defects in Baz localization, the degree of disruption of Baz fell into two overlapping classes, which we hypothesize represent Rap1 maternal mutants (or Rap1[M]) or Rap1[MZ] mutants; these are present at a roughly 1:1 ratio in the progeny (four of eight embryos scored in each class). In the most severe class, which we suspect are the Rap1[MZ] mutants, Baz apical localization was almost completely lost. This loss of apical Baz enrichment was also seen in embryos with maternal Rap1 RNAi knockdown (Supplemental Figure S1, I–N). Strikingly, we saw a very similar disruption in apical enrichment of Baz in cno mutants (Figure 4, G vs. H; I vs. K). We quantitated the effects of both mutants on Baz localization, once again measuring relative intensity of Baz from the apical to the basal ends of the cells in multiple embryos. WT embryos uniformly displayed a sharp apical peak of Baz at the position of the forming spot AJs (Figure 4L). In contrast, this sharp apical peak was lost in Rap1 mutants. Rap1 mutants fell into two phenotypic categories of equal frequency and different severity, likely representing Rap1[MZ] mutants (Figure 4M) and zygotically rescued embryos (Figure 4N). However, in both classes exclusive apical Baz enrichment was lost; this is particularly apparent when the levels in all the embryos in each
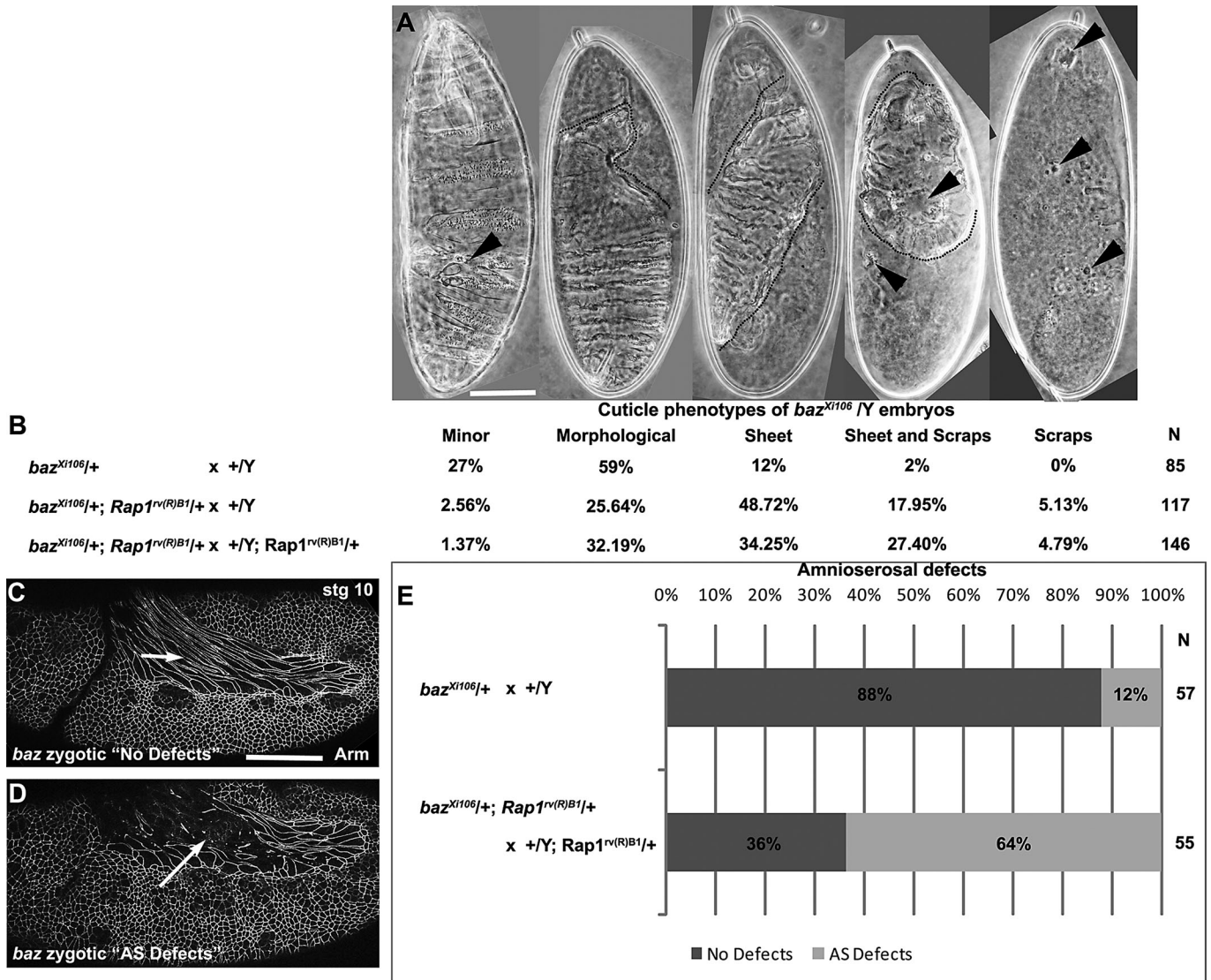
FIGURE 3: Reducing Rap1 levels enhances the effects of reducing Baz function on epithelial integrity. (A) Cuticle preparations illustrating the range of defects in epithelial integrity seen in embryos with reduced Baz levels (zygotic *baz* mutants; embryos were left inside the vitelline eggshell). These range from nearly WT, with only minor cuticle holes (Minor, arrow), to strong defects in the head (Morphological), to half the cuticle remaining (Sheet), to smaller sheets of intact cuticle (Sheet and Scraps), to only fragments of cuticle remaining (Scraps). (B) All embryos have reduced maternal Baz (mothers are heterozygous). We assessed the phenotype of the ~25% of embryos that die because they are *baz* zygotic mutant. Most *baz* zygotic mutants (top row) have only mild to moderate cuticle defects. Reducing maternal Rap1 levels by 50% (middle) significantly enhances the epithelial defects of *baz* zygotic mutants. Further reducing Rap1 levels (heterozygous mothers; 25% of progeny are zygotic *Rap1* mutant) further enhances the epithelial defects of *baz* zygotic mutants. (C–E) Analysis of amnioserosal AJ integrity (Arm) in progeny of crosses in B. In the cross generating zygotic *baz* mutants (E, top) 88% have an intact amnisoserosa at stage 10, whereas 12% of embryos display defects in Arm localization within the amnioserosa as Baz levels run down (C, arrow, vs. D, arrow; E, top). Reducing maternal and zygotic Rap1 substantially enhances the frequency of these defects, with 64% of embryos with amnioserosa defects (E, bottom). Scale bars, 75 µm.

phenotypic category were averaged (Figure 4Q). In *cno* mutants, Baz also became distributed all along the apical–basal axis (Figure 4, O vs. P). There was no obvious zygotic rescue of this phenotype, as the six embryos we quantitated did not fall into two clear classes, and averaging the distribution in the entire set of embryos emphasized the loss of apical enrichment (Figure 4R). Thus both Cno and Rap1 are essential for the initial apical positioning of Baz, putting them atop the current hierarchy of factors mediating the establishment of apical–basal polarity.

Rap1 and Cno are not essential for basic cytoskeletal organization during cellularization

These data suggest that Rap1 and Cno act upstream of Baz to regulate its apical positioning. One mechanism by which this could occur is by regulating cytoskeletal polarity, which is established before cellularization. As cellularization begins, the centrosomes in each cell are positioned above the nuclei and nucleate a basket of MTs with their plus ends deeper in the cytoplasm (Figure 1A). Meanwhile, actin rearranges from a cap above each nucleus into a network of
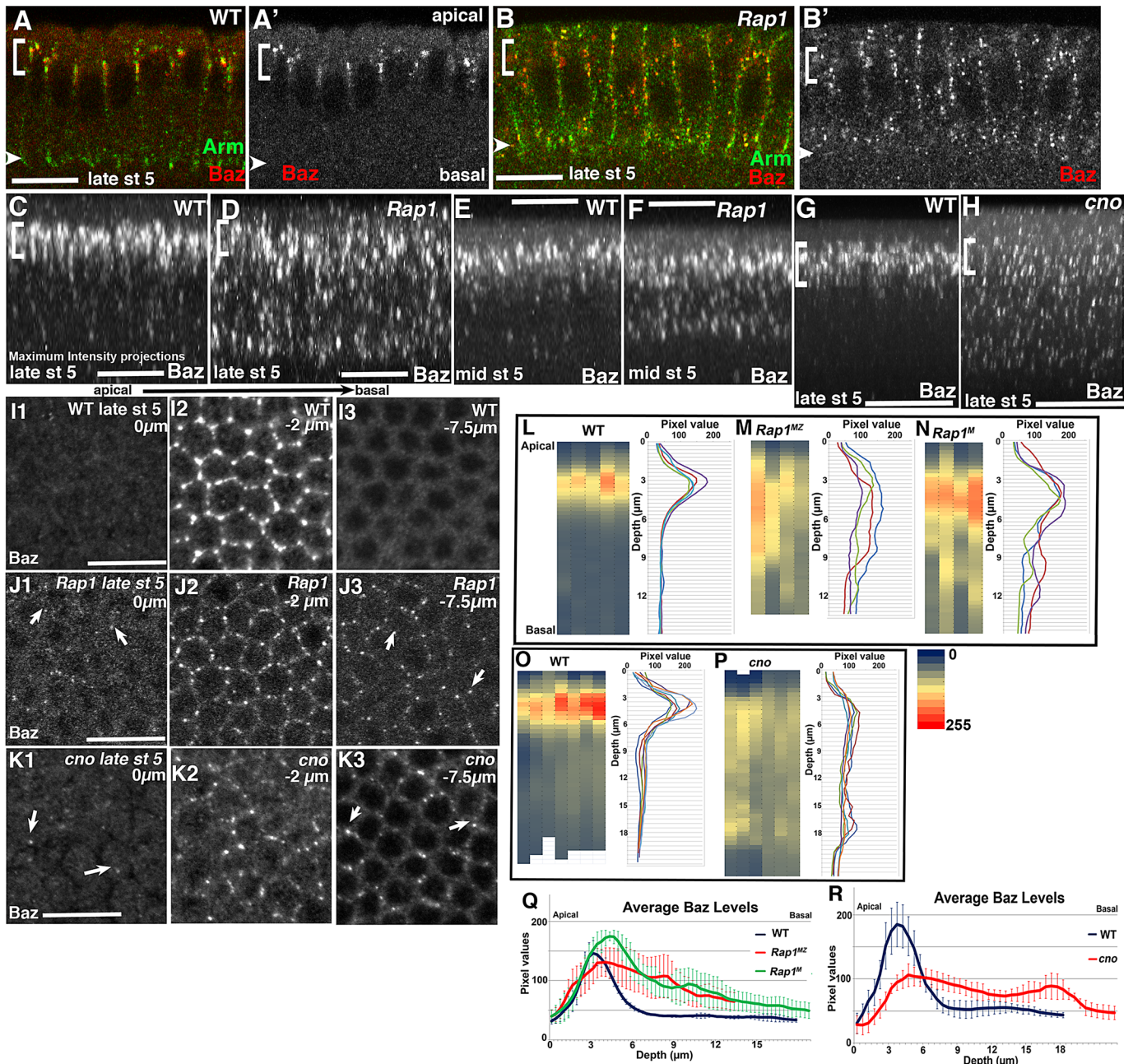
FIGURE 4: Rap1 and Cno are required for initial apical enrichment of Baz. (A, B) Late cellularization. In WT Baz is restricted to forming apical junctions (A, bracket), whereas in *Rap1* mutants apical enrichment is lost (B, bracket) and Baz puncta are all along the lateral border down to the basal junctions (B, arrowheads). (C–H) Maximum-intensity projections along the *y*-axis of cross-sections. In late cellularization (C, D, G, H), this highlights exclusively apical Baz enrichment in WT (C, G) and reduced apical restriction in *Rap1* (D) and *cno* mutants (H). (E, F) Reduced apical restriction of Baz in *Rap1* mutants begins to become apparent by midcellularization. (I–K). Surface sections at different apical–basal depths, as indicated. Note that whereas Baz puncta are relatively tightly localized to apical junctions in WT (I), they are found both apical and basal to this position in *Rap* (J) or *cno* mutants (K). Scale bars, 10 μm. (L–P) Quantitative analysis of changes in Baz localization along the apical–basal axis, as in Figure 2. We measured average image intensity in projected cross-sections of multiple embryos. Data are displayed as heat maps illustrating intensity with different colors (left, apical is on top; each column is a different embryo) or graphically, displaying pixel intensity vs. depth from the apical surface (right, each line is a different embryo). (Q, R) Plots displaying the average Baz image intensity in embryos of different genotypes (apical is to the left).

actin and myosin at the embryo cortex, which begins contracting, pulling in membrane around each cell as the cellularization front moves inward (Figure 1A, actomyosin front). A pool of actin also remains at nascent apical AJs (Figure 1A, actin scaffold). Baz positioning requires both the apical actin scaffold and dynein-directed MT transport toward what will become the apical domain (Harris and Peifer, 2005). We thus tested the hypothesis that Cno and Rap1 regulate Baz via roles in organizing actin or MTs.
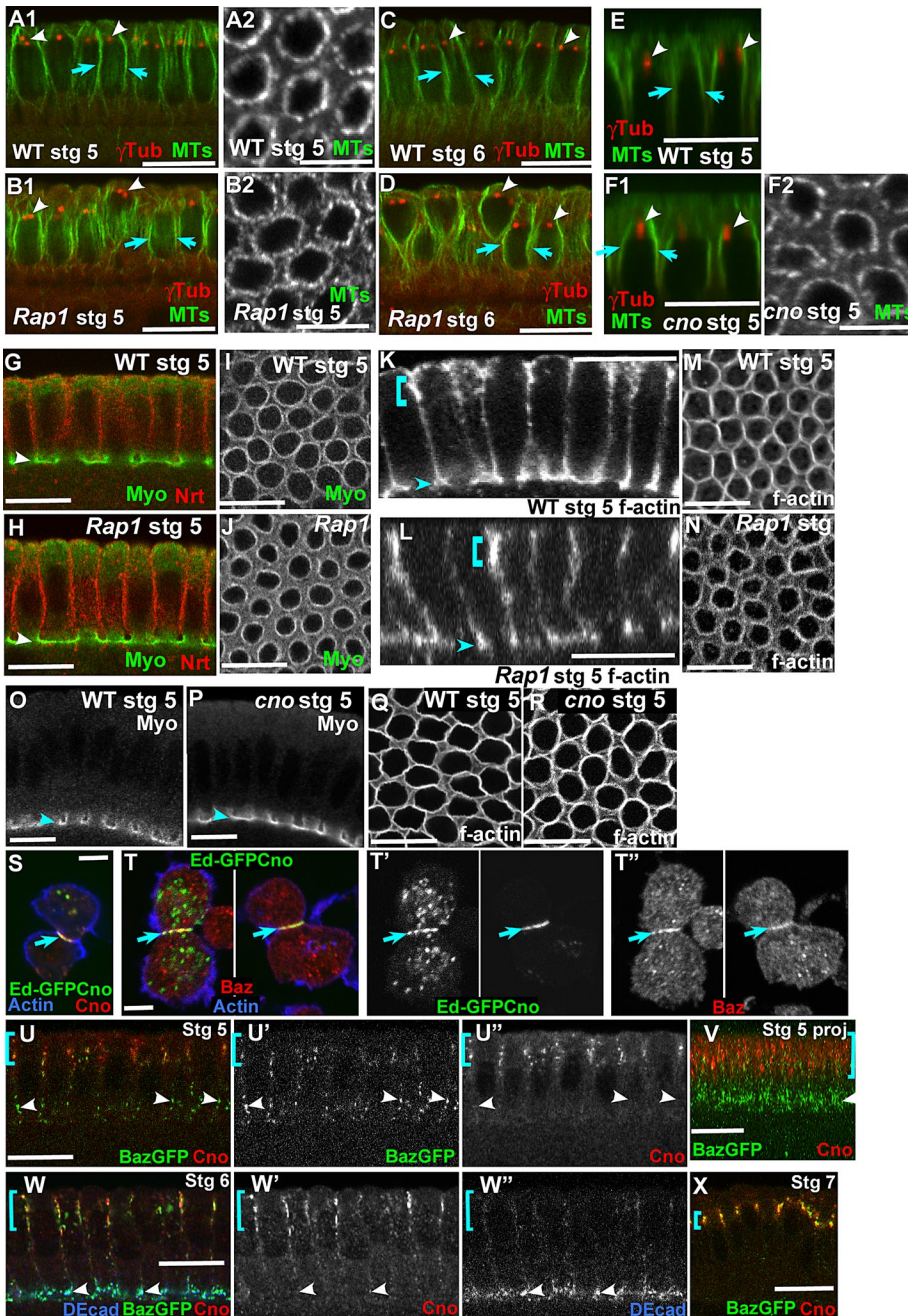
FIGURE 5: Neither *Rap1* nor *cno* mutants have apparent defects in organization of the microtubule or actin cytoskeletons during cellularization, but Cno can recruit Baz to new locations. Genotypes, antigens, and embryonic stages indicated: stage 5, cellularization; stage 6, gastrulation onset. F-actin was detected with phalloidin. (A1–F2) MTs and centrosomes visualized in apical–basal sections (apical up, A1, B1, C–F1) or in cross-sections of nascent cells at level of nuclei (A2, B2, F2). WT, *Rap1*, and *cno* mutants all generate similarly polarized MT cytoskeletons during cellularization, with apical centrosomes (A1, B1, E, F1, arrowheads) and bundled MTs forming baskets projecting basally along the lateral surface (A1, B1, E, F1, arrows; A2, B2, F2, in cross-section). Even after *Rap1* mutants begin to lose columnar cell shape and some cells have enlarged (B1, right arrowhead) or reduced apical ends, the MT cytoskeleton remains polarized. (C, D) At gastrulation onset (stage 6) *Rap1* mutants retain a MT cytoskeleton with apical centrosomes (arrowheads) and MT baskets (arrows) even as they further lose columnar cell shape. (G–J, O, P) WT, *Rap1*, and *cno* mutants all exhibited myosin enrichment at the cellularization front (G, H, O, P, arrowheads) and form myosin rings (I, J). (K–N, Q, R) Actin is similarly localized in WT, *Rap*, and *cno* mutants. Actin accumulates both in rings at the cellularization front (K, L, arrowheads; M, N, Q, R, in cross-section) and at nascent apical junctions (K, L, brackets). (S, T) *Drosophila* S2 cells transfected with Ed:GFP-Cno. (S) The extracellular domain of the fusion protein links cells, thus placing Cno at the cell–cell junction (arrow).

To test this mechanism, we examined the organization of the actin and MT cytoskeletons in *Rap1* and *cno* mutants during cellularization. We first examined MT and centrosomal polarity. In WT, centrosomes are apical to each nucleus (Figure 5A1, arrowheads) and baskets of MTs extend basally (Figure 5A1, arrows); these baskets are also visible as rings of bundled MTs in cross-sections of forming cells (Figure 5A2). In *Rap1* mutants we saw no obvious defects in either apical centrosomal positioning (Figure 5B1, arrowheads) or in MT baskets projecting basally (Figure 5, B1, arrows, B2). As we will explore in detail, defects in apical cell shape began to appear during cellularization in *Rap1* mutants, but centrosomes were positioned apically above nuclei even in misshapen cells (Figure 5, A1 vs. B1, arrowheads; C vs. D, arrowheads). Similarly, we saw no apparent defects in apical centrosome positioning (Figure 5, E vs. F1, arrowheads) or formation of MT baskets in *cno* mutants (Figure 5, A2 vs. F2). Thus the MT cytoskeleton becomes correctly organized in the absence of Rap1 or Cno, suggesting that their effects on Baz localization do not result from indirect effects on MTs.

We also examined actin and myosin during cellularization. The fact that mutant embryos cellularize correctly already suggested that there were not major defects. Myosin accumulated correctly at the cellularization front in both *Rap1* (Figure 5, G vs. H, arrowheads) and *cno* mutants (Figure 5, O vs. P, arrowheads), and to the closing myosin rings (Figure 5, I vs. J). Actin accumulated at the cellularization front in both *Rap1* (Figure 5, K vs. L, arrowheads; M vs. N) and *cno* (Figure 5, Q vs. R) mutants. Actin also accumulated normally at nascent apical AJs (Figure 5, K vs. L, brackets). Consistent with this, myosin is correctly recruited apically in the mesoderm of both *Rap1* and *cno* mutants at gastrulation onset and initiates constriction (Sawyer et al., 2009). Taken together, these data suggest that there are not substantial disruptions of the actomyosin or MT cytoskeletons in

(T) Endogenous Baz is then recruited to this site (arrows). (U–X) Embryos of the indicated embryonic stages overexpressing BazGFP using matGAL4. (U, W, X) Single apical–basal cross-sections. (V) Projected cross-sections. Overexpressed BazGFP localizes all along the apical basal axis and accumulates basally (U–W, arrowheads). There it can recruit DEcad (W″, arrowheads) but does not displace Cno from the apical region of the cell (U–W, brackets). (X) As gastrulation proceeds, BazGFP relocalizes apically, where it now overlaps with Cno. Scale bars, 10 μm.

either *Rap1* or *cno* mutants, making it less likely this indirect mechanism explains their effects on apical–basal polarization.

Cno can recruit Baz, but they do not obligatorily colocalize

Another mechanism by which Cno could regulate apical Baz localization is by recruiting it apically. The Doe lab recently developed an elegant protein recruitment assay (Wee *et al.*, 2011). They fused full-length Cno–green fluorescent protein (GFP) to the extracellular and transmembrane domains of the nectin-like adhesion protein Echinoid (Ed) and expressed this fusion in cultured *Drosophila* S2 cells. These hemocyte-like cells are nonadhesive, but homophilic interaction via Ed's extracellular domain can bring two such cells together at an adhesive contact. One can then assess whether another protein is recruited there; the Doe lab found that Cno recruited Pins. We transfected Ed-CnoGFP into S2 cells and confirmed that both the GFP tag and Cno localize to the resulting cell contacts (Figure 5S, arrow). Strikingly, this was sufficient to recruit endogenous Baz to the same site (Figure 5T, arrows). These data are consistent with the hypothesis that Baz is recruited apically in part by Cno, although it does not distinguish between direct or indirect recruitment.

Although these data support a possible role for Cno in Baz recruitment, things in vivo are likely to be significantly more complex. For example, whereas Cno and Baz both localize to spot AJs, their localizations, although overlapping, are not precisely coincident (Sawyer *et al.*, 2009). We further examined this by mislocalizing Baz in vivo. When BazGFP is expressed at low levels during cellularization, it localizes to spot AJs, but when it is overexpressed, it then localizes all along the apical–basal axis and accumulates near the basal junctions (Figure 5, U–W, arrowheads; Harris and Peifer, 2005). Although this mislocalized Baz can recruit DEcad and other AJ proteins (Figure 5, W and W″, arrowheads; Harris and Peifer, 2005), it does not recruit Cno from its normal apical location (Figure 5, U–W, brackets). Strikingly, BazGFP is restored to a normal junctional localization during gastrulation (Figure 5X; Harris and Peifer, 2005) and then overlaps with Cno in apical junctions. Thus there is no obligatory colocalization of Baz and Cno, suggesting that each may have multiple binding partners of different affinities, as we previously speculated for Baz and DEcad.

Rap1 and Cno are important for Baz localization during gastrulation, but other cues partially restore apical Baz

The foregoing data suggest that Rap1 and Cno regulate the initial establishment of apical–basal polarity by helping position Baz and AJs. However, loss of Cno (Sawyer *et al.*, 2009) does not cause the same early, dramatic disruption of the ectodermal epithelium seen when either Baz or AJ proteins are lost (Cox *et al.*, 1996; Tepass *et al.*, 1996). Two mechanisms could be at play: 1) Although the presence of Baz is clearly required for epithelial polarity, perhaps its apical restriction is not essential, or 2) alternatively, other cues might restore more normal Baz localization when additional polarity cues like the aPKC/Par6 module come into play at gastrulation onset to reinforce and elaborate initial polarity (Hutterer *et al.*, 2004; Harris and Peifer, 2005).

To test these alternate hypotheses, we examined Baz localization in *Rap1* or *cno* mutants at gastrulation onset (stage 6) and as germband extension began (stage 7). In both mutants, 50% of embryos are potentially zygotically rescued. As WT gastrulation begins, AJs and Baz continue to colocalize (Harris and Peifer, 2004, 2005). These apical junctional complexes tighten along the apical–basal axis (Figure 6A), and during stage 7 they move to the apical end of the lateral cell interface (Figure 6B). We found previously that *Rap1* mutants lost apical Baz enrichment during cellularization. As gastrulation

commenced (stage 6; Figure 6, C and E) and germband extension began (stage 7; Figure 6, D and F), Baz continued to be mislocalized in *Rap1* mutants, with many Baz puncta remaining basal to the apical junctions. However, there was clearly some restoration of apical Baz, both in presumptive *Rap1^{MZ}* (Figure 6, C and D) and presumptive zygotically rescued embryos (Figure 6, E and F; we divided embryos into classes on the basis of phenotypic severity; 7 of 14 stage 6 and 6 of 12 stage 7 embryos had the less severe phenotype). There was similar partial rescue of apical Baz enrichment in *cno^{MZ}* mutants, with subtle restoration of apical Baz enrichment at stage 6 (Figure 6, G vs. I), and significant restoration of apical Baz, albeit with remaining mislocalized Baz, at stage 7 (Figure 6, H vs. J). Presumptive *cno^{M}* mutants had slightly less severe phenotypes, with more complete restoration of apical Baz (Figure 6, K and L; 9 of 17 stage 6 and 8 of 15 stage 7 embryos had the less severe phenotype). We once again quantitated Baz localization in multiple embryos, confirming our qualitative observations. The WT profiles show the sharpening of the apical Baz peak during gastrulation from stage 6 (Figure 6, M and S) to stage 7 (Figure 6, P and V). In *Rap1* mutants, an apical Baz peak began to reappear at gastrulation onset (stage 6; Figure 6, N and O), even in presumptive *Rap1^{MZ}* mutants (Figure 6N), but some Baz remained mislocalized, broadening and lowering the peak. By stage 7, most Baz was apical (Figure 6, Q and R), but even in the least severe mutants (Figure 6R) the apical peak did not sharpen as it does in WT (Figure 6P). In *cno* mutants the situation was similar: an apical Baz peak began to reappear by stage 6 (Figure 6, T and U) and strengthened at stage 7 (Figure 6, W and X). Averaging the individual embryos revealed that the overall degree of rescue was somewhat more complete in *cno* than in *Rap1* mutants (Supplemental Figure S4, A vs. C, and B vs. D). It is possible that the slight remaining apical Baz enrichment we saw in many *Rap1* mutants at cellularization plays a part in the postgastrulation rescue, although the residual apical Baz enrichment was even more subtle in cellularizing *cno* mutants. Thus Rap1 and Cno play continuing roles in apical Baz localization, but additional cues that come into play at gastrulation onset partially restore apical Baz enrichment.

Rap1 and Cno are required for proper organization of Baz into planar-polarized junctional belts

In the *xy*-plane, junctional protein localization is more complex. As the germband extends (Figure 7A), spot AJs and associated Baz smooth into less punctate, more continuous belt AJs (Tepass and Hartenstein, 1994), and both Baz and AJ proteins become planar polarized, with enrichment along dorsal-ventral (Figure 7, B and B′, arrowheads; B′ close-up, arrowheads) versus anterior–posterior cell borders (Figure 7, B and B′, arrows; B′ close-up, arrows; Bertet *et al.*, 2004; Zallen and Wieschaus, 2004). In *cno^{MZ}* mutants, Baz planar polarization is significantly accentuated, with near loss of Baz on anterior–posterior borders (Sawyer *et al.*, 2011). We found that in *Rap1* mutants, Baz localization was similarly altered, with the most severe embryos (likely *Rap1^{MZ}*, based on strength of mesoderm invagination defects) having cortical Baz significantly reduced overall, and virtually lost on anterior–posterior borders (Figure 7, C and D, D′ close-up, arrows). This coincided with separation of myosin from the anterior and posterior cortex (Figure 7G arrows), as occurs in *cno^{MZ}* mutants (Sawyer *et al.*, 2011). In less affected *Rap1* mutants (presumptive *Rap1^{M}* mutants; Figure 7, E and F), Baz cortical localization was less reduced, but Baz remained more punctate along the cortex than in WT (Figure 7, B′ vs. F′; B′ vs. F′ close-ups). Thus both Cno and Rap1 are required for proper maintenance of Baz localization during gastrulation, regulating both its apical–basal and its planar polarity.
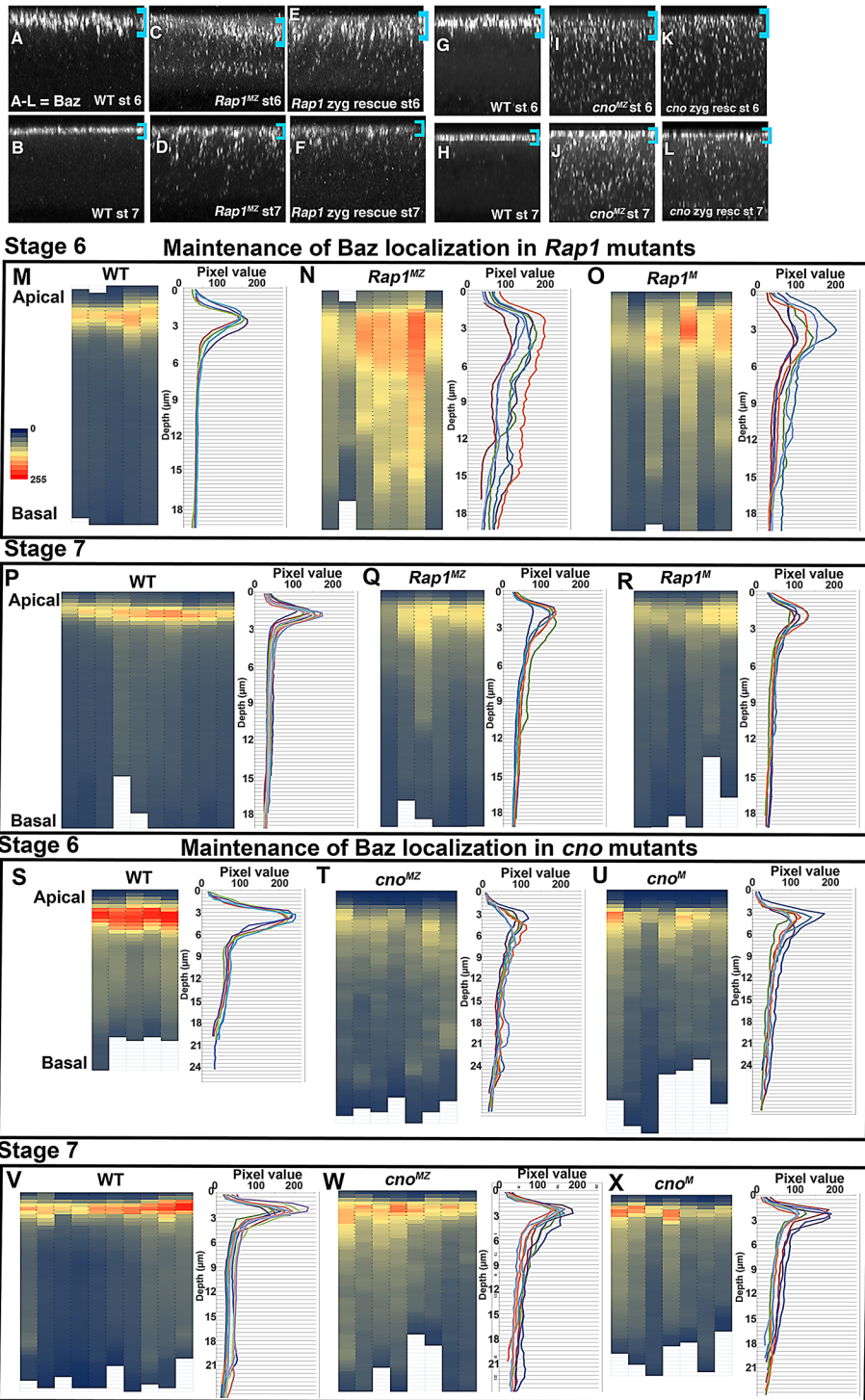
FIGURE 6: Rap1 and Cno are required for normal Baz localization during gastrulation, but other cues partially restore apical Baz. Genotypes and antigens indicated. (A–L) Baz localization in apical–basal sections through embryos of indicated stages. We cannot distinguish maternal/zygotic and zygotically rescued mutants at this stage; we thus divided embryos into two classes on the basis of phenotypic severity and show representative examples of each class. (A, B, G, H) In WT, Baz is apically localized at gastrulation onset (stage 6; A, G) and tightens up and moves to the extreme apical end of the cell during germband extension (stage 7; B, H). (C, D, I, J) In presumptive *Rap1^MZ* (C, D) and *cno^MZ* mutants (I, J), Baz slowly becomes enriched apically but significant mislocalized Baz remains. (E, F, K, L) In maternally mutant but zygotically rescued *Rap1^M* (E, F) and *cno^M* (K, L) embryos, restoration of apical Baz proceeds more completely than in maternal/zygotic mutants, but rescue remains incomplete. Scale bars, 10 μm. (M–X) Average image intensity along the apical–basal axis in projected cross-sections was assessed as in Figure 2, and data were displayed either as heat maps illustrating intensity with different colors (left,

Loss of Rap1 or Cno leads to disruption of epithelial integrity by the end of gastrulation

We next tested whether this junctional disruption affected epithelial integrity or whether the partial rescue of Baz localization coincided with restored epithelial architecture. As germband extension continues in stage 8, ectodermal cells undergo patterned mitosis, rounding up, reducing cortical AJ proteins (Supplemental Figure S2C) and Baz (Supplemental Figure S2, E and F) during mitosis, and then rebuilding apical junctions and resuming a columnar shape. Our previous work revealed that whereas cells can assemble and maintain AJs in some tissues in Cno's absence (e.g., the dorsal epidermis), in other tissues, such as the ventral epidermis, there are problems reestablishing apical AJs after cell division (Supplemental Figure S2, D and D close-up, brackets), and the tissue ultimately loses epithelial integrity, disrupting the ventral cuticle (Sawyer *et al.*, 2009). *Rap1^MZ* mutants had similar or more severe cuticle defects; in many, even the dorsal epidermis was fragmented (Supplemental Figure S2B). To determine when loss of Rap1 affected epithelial architecture, we examined postgastrulation *Rap1^MZ* mutants. In WT stage 8 embryos (Supplemental Figure S2E), nondividing cells have strong apical Baz localization, and even dividing cells have continuous but lower-level junctional Baz (Supplemental Figure S2F', arrow). In contrast, junctional Baz was substantially less continuous in *Rap1* mutants. In *Rap1^MZ* (Supplemental Figure S2I; 10 of 21 stage 8–9 mutants had this strong phenotype), junctional Baz was weak in the dorsal ectoderm (Supplemental Figure S2, F' vs. J', arrows), and in the ventral ectoderm Baz localized only to junctional fragments (Supplemental Figure S2, F' vs. J', arrowheads). Zygotically rescued embryos (Supplemental Figure S2G; 11 of 21 stage 8–9 mutants had the weaker phenotype) had similar but less severe defects (Supplemental Figure S2H',

apical is on top; each column is a different embryo) or graphically, displaying pixel intensity vs. depth from the apical surface (right, each line is a different embryo). Genotypes and stages are indicated. Because this analysis did not allow us to definitively distinguish zygotically rescued embryos, we binned the embryos into the most severe and least severe (they should be present in a 1:1 ratio) and labeled these as presumptive maternal/zygotic or zygotically rescued embryos.
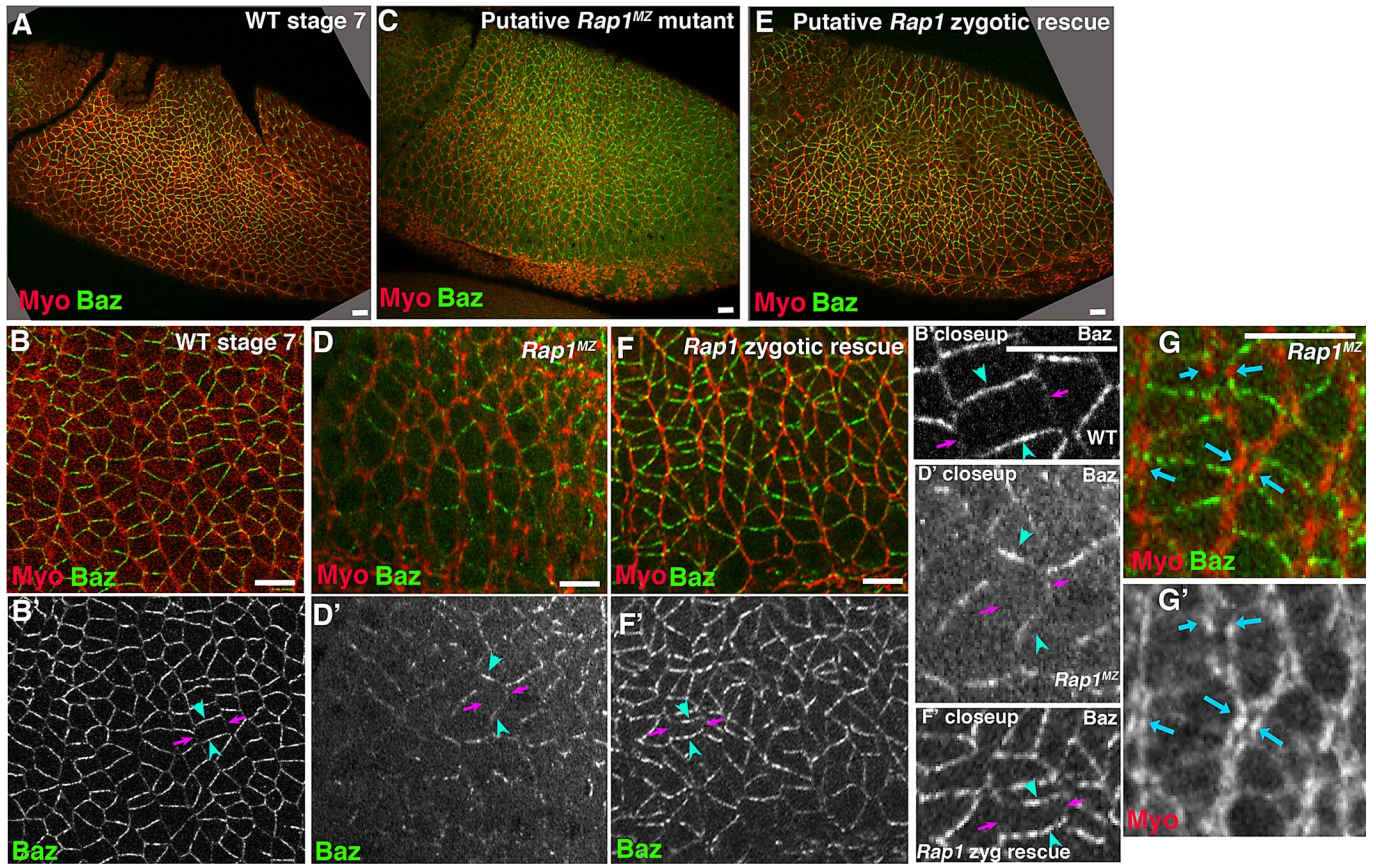
FIGURE 7: Rap1 is important for maintaining Baz localization in planar polarized apical junctions during gastrulation. (A–G) Surface views of stage 7 embryos. (A, C, E) Lateral views. (B, D, F) Close-ups of lateral epidermis. (D', E', F') Further close-ups. (A, B) Baz becomes planar polarized in WT, with stronger accumulation on dorsal ventral boundaries (arrowheads) and weaker on anterior–posterior borders (arrows). (C, D) Presumptive *Rap1^MZ* mutant (determined as in Figure 6 legend). Overall accumulation of Baz at cortex is reduced, Baz is lost from anterior–posterior borders (arrows), and the remaining staining is discontinuous. (E, F) Presumptive zygotically rescued *Rap1* mutant. Cortical Baz is more prominent but still less continuous than in WT. (G) Myosin cables detach from anterior–posterior boundaries in *Rap1* mutants (arrows), as we previously observed in *cno^MZ* mutants. Scale bars, 10 µm.

arrows, arrowheads). Thus the partial rescue of apical Baz localization in *Rap1^MZ* is not sufficient to allow ventral ectodermal cells to maintain AJ integrity during gastrulation, consistent with the fragmented cuticle. These data suggest that Cno and Rap1 are important to maintaining epithelial integrity in morphogenetically active tissues.

Baz and aPKC are not essential for apical Cno enrichment but play roles in Cno positioning

Our data support the hypothesis that Rap1 and Cno act upstream of Baz, ensuring its restriction to nascent spot AJs. In this view, Rap1 and Cno fill the missing place in a linear model of polarity establishment, with Baz then acting upstream of both AJs and aPKC to ensure their apical positioning (Figure 1A). In this linear model, neither Baz nor aPKC should be essential for positioning Cno, as both are "downstream" of it. In contrast, later junctional maintenance/elaboration does not involve a linear pathway, but instead relies on an interlocking network of positive and negative interactions (e.g., Bilder *et al.*, 2003; Tanentzapf and Tepass, 2003). To test the alternate hypotheses that polarity establishment behaves in a linear manner or that Baz and Cno fit into an interlocked network with feedback loops, we examined Cno positioning in embryos lacking Baz or aPKC.

We generated embryos with severely reduced levels of Baz using the new Valium RNAi lines (Ni *et al.*, 2009), expressing shRNAs targeting *baz* in the germline using maternal GAL4 drivers. We confirmed that this led to the expected lethality and disrupted cuticle integrity (unpublished data), consistent with very strong loss of function, and further confirmed that this treatment reduced Baz protein to background levels in situ (Supplemental Figure S5, A' vs. B'; C' vs. D'). To confirm that Baz reduction affected AJ assembly, we examined Arm localization. As we previously observed in *baz^MZ* mutants (Harris and Peifer, 2004), *baz* RNAi disrupted apical Arm enrichment in nascent spot AJs (Supplemental Figure S5, A'' vs. B'' brackets), leading to accumulation all along the basolateral axis, but without disrupting Arm enrichment in basal junctions (Supplemental Figure 5, A'' vs. B'', arrows).

We used *baz* RNAi to determine whether Baz helps to regulate Cno localization. In WT embryos, Cno is enriched in nascent spot AJs throughout cellularization (Figure 8, A and C2), with strong enrichment at tricellular junctions (Figure 8C2, arrows; Sawyer *et al.*, 2009). The Cno at tricellular junctions extends deeper into the cell, creating apical–basal "cables" of Cno at each tricellular junction, which are apparent in cross-sections (Figure 8A', maximum-intensity projection). At gastrulation onset, Cno moves further apically, but the cables remain prominent (Figure 8E', maximum-intensity
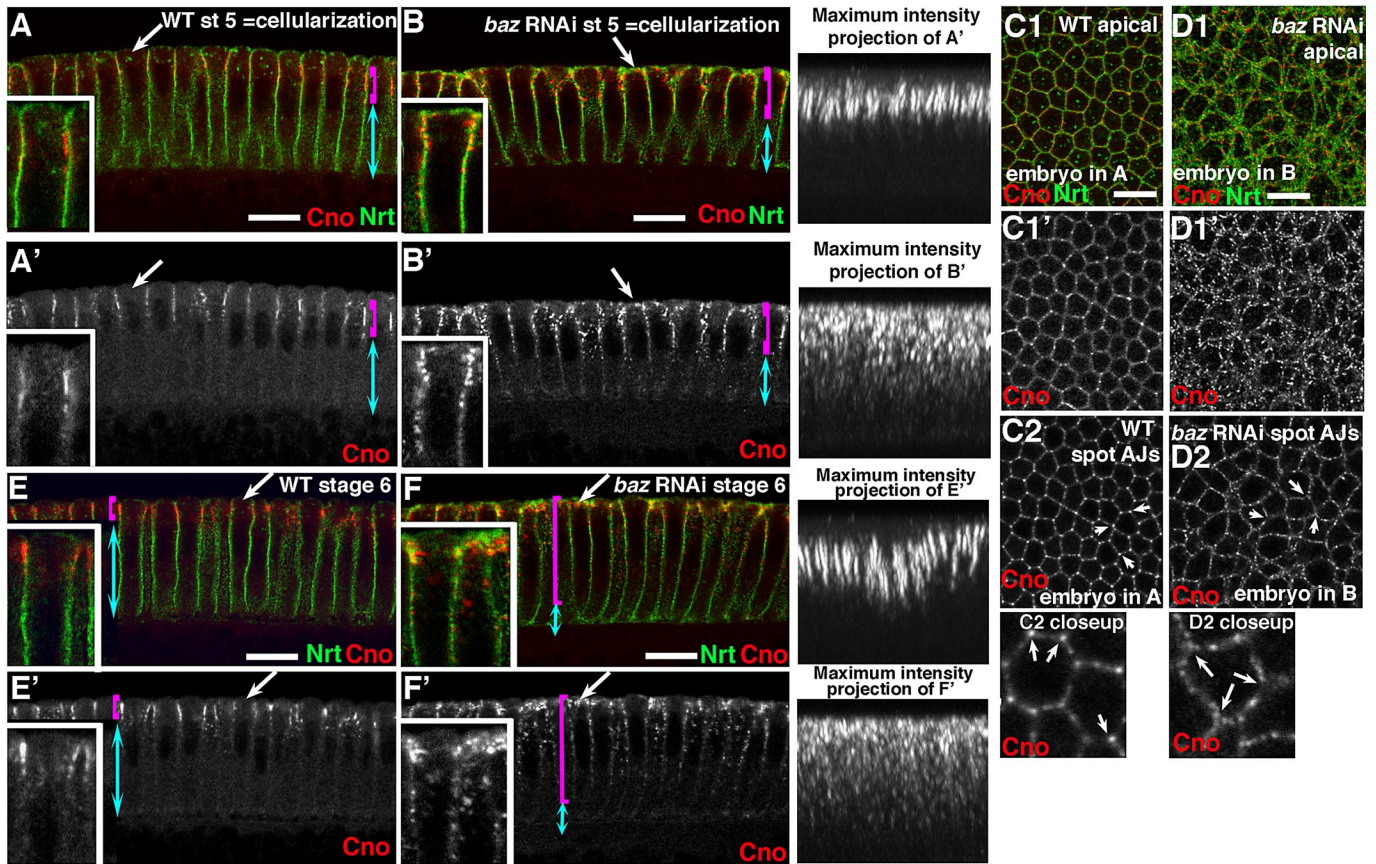
FIGURE 8: Baz is not required for Cno assembly into spot AJs but does regulate precise Cno localization during polarity establishment. (A–D) Late cellularization. (A, B) Apical-basal cross-sections. (C1, D1) Apical surface sections of embryo in A and B. (C2, D2) Surface sections at level of normal spot AJs. In WT (A, C), Cno localizes along the apical end of the lateral membrane (A', bracket) to spot AJs (C2). In maximum-intensity projections of multiple apical–basal sections (A'), cables of Cno that localize to tricellular junctions are apparent. Cno is also largely removed from the apical surface during cellularization (A', E', arrows). Reducing Baz by RNAi (B, F) leads to Cno spreading more basally (B', bracket, vs. A', bracket), to loss of organized Cno cables (B', maximum-intensity projections), and to failure to exclude Cno puncta from the apical membrane (B', arrow, C1' vs. D1'), but Cno still continues to assemble into spot AJs (D2). (E, F) Gastrulation onset (stage 6). In WT, Cno remains in spot AJs (E', bracket), and the apical–basal cables at tricellular junctions become even more prominent (E', projections). *baz* RNAi leads to spread of Cno basally (F'), perturbs assembly of Cno cables at tricellular junctions (F' maximum-intensity projection), and allows Cno puncta to accumulate at the apical surface. (F', arrow and inset). Scale bars, 10 µm.

projection). Baz knockdown affected both cell shape and Cno localization. As documented later, in Baz-depleted embryos apical cell shape was already altered during cellularization, with the apicalmost region of the cells expanded or reduced in area. In Baz-depleted embryos, Cno was still largely restricted to membrane-associated puncta in the apical one-third of the cell (Figure 8B', brackets) and still accumulated in spot AJs (Figure 8D2). However, Cno was not as tightly focused where spot AJs normally form, instead spreading along the basolateral membrane (Figure 8, A' vs. B', brackets). Further, enrichment at tricellular junctions was reduced (Figure 8, C2 vs. D2 and close-ups), and thus the cables of Cno were essentially eliminated (Figure 8, A' vs. B', maximum-intensity projections). Finally, Cno was not properly restricted from the apical domain (Figure 8, C1' vs. D1'). All of these changes in Cno localization in Baz-depleted embryos were further accentuated as gastrulation began (Figure 8, E vs. F). Thus, whereas Baz is not essential for apical Cno enrichment, Baz depletion altered the precise positioning of Cno at tricellular junctions during cellularization and prevented the apical exclusion of Cno as gastrulation began.

These data suggest that a strictly linear model with Cno "upstream" of Baz is oversimplified.

We next extended this analysis to aPKC, which regulates polarity maintenance during gastrulation (Hutterer *et al.*, 2004). In aPKC's absence, AJs and Baz assemble into spot AJs during cellularization (Figure 9, A vs. B, E–H, Arm; Harris and Peifer, 2005), but at gastrulation onset, AJs and Baz abnormally coalesce on dorsal and ventral cell borders (Harris and Peifer, 2007) in an exaggerated version of their normal planar polarity. Cells then lose polarity, with Baz and AJs forming nonpolarized aggregates (Hutterer *et al.*, 2004; Harris and Peifer, 2007). To determine whether initial Cno apical localization is independent of aPKC, as predicted by the linear model, we generated embryos maternally and zygotically mutant for the strong aPKC allele aPKC[K06403] (*aPKC* hereafter; 50% of embryos receive paternal WT *aPKC* and cannot be distinguished before gastrulation).

We were surprised to find that loss of aPKC affected Cno localization during cellularization. In WT cellularization, there is a modest pool of Cno in the apicalmost region of the cell early (Figure 9A', arrow), both diffuse and in puncta that may be similar to the apical
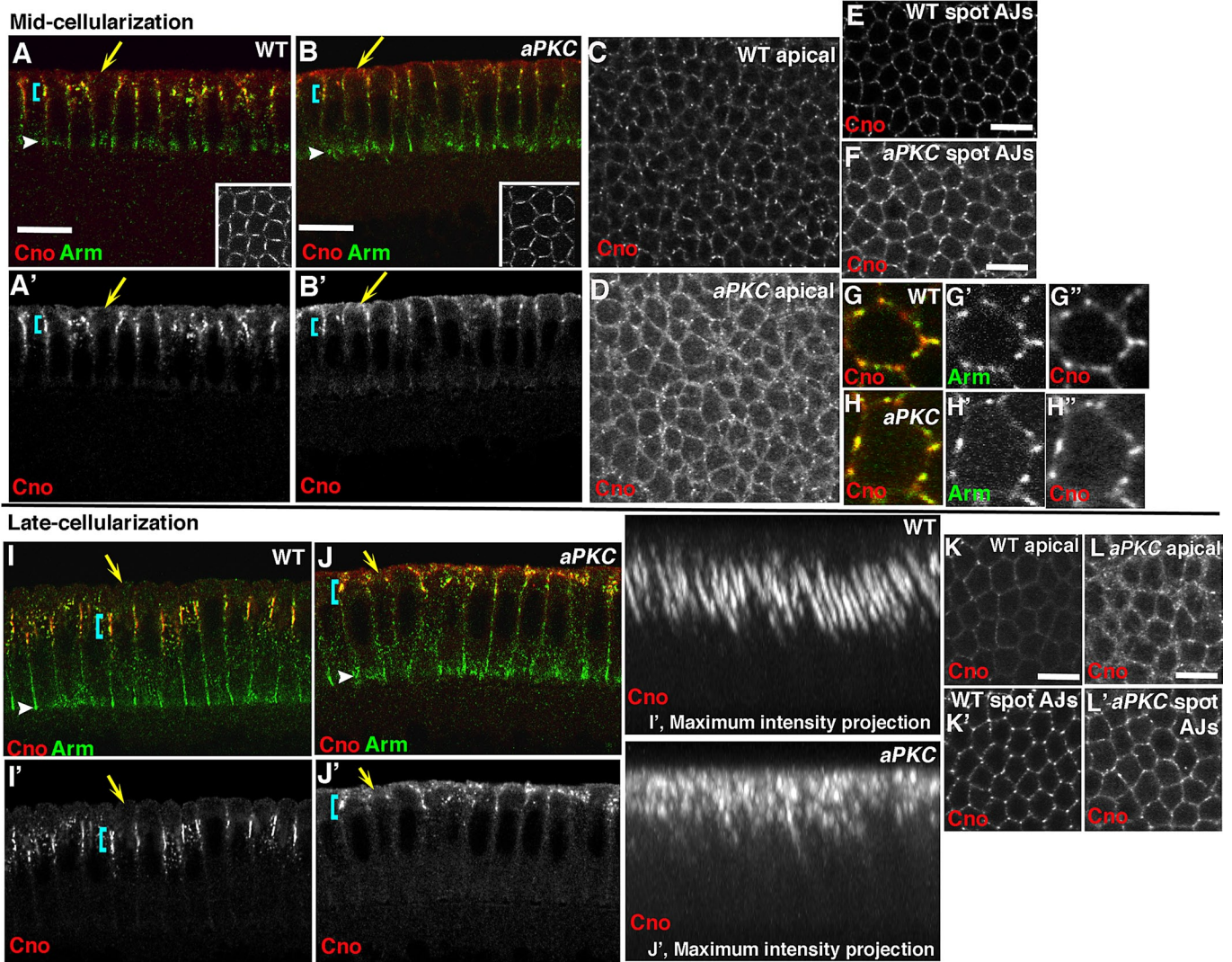
FIGURE 9: *aPKC* mutants fail to exclude Cno from the apical domain. (A, B, I, J) Apical-basal sections. (C–H, K, L) En face views. (A–G) Midcellularization. Cno accumulates along the apicolateral membrane (A, B, brackets) and in the apical region of the cell (A, B, arrows) in both WT (A') and *aPKC* mutants (B'). Elevated apical accumulation of Cno in *aPKC* mutants is already apparent in en face views (C vs. D), but Cno continues to accumulate in spot AJs (E, G vs. F, H), and Arm accumulation in basal junctions is not perturbed (A, B, arrowheads). (I–L) Late cellularization. Whereas in WT, Cno is removed from the apical region (I', arrow, K), Cno remains there in *aPKC* mutants (J', arrow, L). Scale bars, 10 μm.

puncta of DEcad and Baz seen at this time (McGill *et al.*, 2009). By late cellularization, most apical Cno disappeared (Figure 9, I', arrow, K), as Cno assembled into cables at tricellular junctions (Figure 9, I', bracket and maximum-intensity projection). Loss of aPKC did not prevent Cno from colocalizing with Arm in nascent spot AJs (Figure 9, E, G, and K' vs. F, H, and L') in roughly the right apical–basal position (Figure 9, B and J, brackets). However, loss of aPKC had two more subtle effects on Cno localization. First, the cables of Cno that normally assemble at tricellular junctions were disrupted (Figure 9, I' vs. J', maximum-intensity projections), mimicking the disruption in Baz-depleted embryos. Second, Cno was not lost from the apical region, as it normally is by late cellularization (Figure 9, I' vs. J', arrow; K vs. L). These data suggest that aPKC plays an unexpected early role in precisely positioning Cno during cellularization.

We were surprised that aPKC had such an early role because our earlier work (Harris and Peifer, 2005) suggested that it did not localize to the membrane until late cellularization and did not affect AJs until gastrulation. We thus reexamined when cortical aPKC

appears, exploring whether there was a membrane-associated pool of aPKC that we missed in our previous work. Cno (Supplemental Figure S3A), AJ proteins (Supplemental Figure S3A; McCartney *et al.*, 2001), and Baz (Supplemental Figure S3H) are all present at the membrane in metaphase furrows of syncytial embryos. We saw membrane-localized aPKC as early as syncytial stages, where it localized to metaphase furrows (Supplemental Figure S3L). Cortical aPKC remained during cellularization (Supplemental Figure S3, M–O; we confirmed the specificity of this membrane pool by determining that it is lost in *aPKC* mutants; unpublished data), when Cno, Arm, and Baz enter nascent spot AJs (Supplemental Figure S3, B–G and J). aPKC was not enriched in spot AJs during cellularization (Supplemental Figure S3N) but instead localized all along the lateral membrane (Supplemental Figure S3O); the apical aPKC visualized after heat fixation during late cellularization (Harris and Peifer, 2005) may be a more stable, perhaps cytoskeletally associated pool. Thus aPKC is positioned to regulate Cno localization during cellularization.

During gastrulation and after, aPKC helps to maintain the apical domain by mediating exclusion of basolateral and junctional proteins. Consistent with this, the elevated apical accumulation of Cno in *aPKC* mutants became even more accentuated at gastrulation onset (Supplemental Figure S6, A' vs. B', arrows). As gastrulation proceeded (stage 7), although Cno remained generally apically restricted, it did not condense into tight spot AJs in *aPKC* mutants (Supplemental Figure S6, C vs. D), and Cno levels became even more highly elevated in the apical membrane of *aPKC* mutants (Supplemental Figure S6, E vs. F; Cno also localized with Arm in the mislocalized spot AJs at the dorsal-ventral cell boundaries; Supplemental Figure S6H, arrowheads). As we observed with Baz depletion, this change in apical restriction of Cno in *aPKC* mutants coincided with alterations in apical cell shape, with the apicalmost regions of the cells expanded or reduced in area (see later discussion). Thus aPKC restricts Cno from localizing to the apical domain from cellularization onward, once again inconsistent with a simple linear hierarchy. Taken together, these data suggest that initial junctional assembly is not a simple linear pathway but instead that proteins are localized through a network of regulatory interactions, with, for example, Cno regulating Baz localization and Baz also regulating Cno positioning.

Rap1 plays a role in regulating cell shape that is Cno independent

Our data suggest that Rap1 and Cno regulate polarity establishment. However, Rap1 has other effectors besides Cno/afadin, suggesting that it might have Cno-independent mechanisms of regulating junctions and the cytoskeleton. In stage 5 *Rap1* mutants, we noted a defect in cell shape that was not apparent in *cno* mutants. During WT cellularization, as the actomyosin network constricts to draw membranes around each nucleus, the resulting cells are columnar in architecture, with relatively uniform cell areas from apical to basal, and with each cell similar in this regard to its neighbors (Figure 10, A–A''; cells are slightly more variable apically). In contrast, the cell areas of *Rap1* mutants were quite a bit more variable, with some cells enlarged or reduced apically and others enlarged or reduced basally (Figure 10, C–C'', arrows; cell areas in *Rap1* mutants were also on average overall larger, likely due to an elevated frequency of nuclear loss during syncytial stages; unpublished data). To quantitate these differences in columnar cell shape, we stained cellularizing embryos for the membrane protein neurotactin (Nrt) and took slices at three apical–basal positions: near the apical surface (0.9 µm deep; Figure 10, A–E), at spot AJs (3.0 µm deep; Figure 10, A'–E'), and at the level of the nuclei (6.9 µm deep; Figure 10, A''–E''). We then analyzed cell areas using ImageJ, measuring areas of hundreds of cells and comparing the largely uniform columnar cell area of WT (Figure 10A) with that of cells in *Rap1* mutants (Figure 10C). We then calculated the coefficient of variance (CV), which quantitates the degree of variability in cell area, and assessed the significance of CV differences using Tukey's HSD test to correct for multiple comparisons. This confirmed our visual impressions: *Rap1* mutant cell areas were more variable than those of WT (Figure 10F). The difference was statistically significant at the level of the nuclei (Figure 10H; CV = 0.20 vs. 0.09 in WT), and there was a trend toward more variability apically (CV = 0.29 vs. 0.23 and 0.20 vs. 0.15; Figure 10, F and G). This is also manifested in altered apical–basal positioning of nuclei (Figure 5, A vs. B; and unpublished data); whether these are a consequence or cause of altered cell shape remains to be determined. Thus Rap1 plays a role in initial establishment of columnar cell shape.

We next examined whether there is a continuing requirement for Rap1 in columnar cell shape or whether this effect was rescued at gastrulation onset. Whereas gastrulation results in significant changes in cell shape, most WT lateral ectodermal cells retain quite uniform apical areas during stages 6 and 7 (Supplemental Figure S7, A and F–H; Supplemental Figure S7, I and N–P). In contrast, cells in *Rap1* mutants continued to be significantly more variable in apical (Supplemental Figure S7, C, F, K, and N) and more basal cell areas (Supplemental Figure S7, C'', H, K', and O) at gastrulation onset (Supplemental Figure S7, C, F, and H) and during germband extension (Supplemental Figure S7, K, N, and O) Thus Rap1 plays an important role in both establishment and maintenance of columnar cell shape.

Because Rap1 has other effectors, we examined whether Cno shares Rap1's role in establishing or maintaining columnar cell shape. Visual inspection suggested that *cno* mutants had relatively uniform cell areas during cellularization, resembling WT (Figure 10, A–A'' vs. B–B''). To verify this, we quantitated cell shape. Apical cell areas in *cno* were less variable than in *Rap1* mutants and were not statistically distinguishable from WT (e.g., basal cell area CV = 0.12 for *cno* vs. 0.09 for WT and 0.20 for *Rap1*; Figure 10, A'', B'', C'', and H). These data suggest that Cno does not play a key role in establishing columnar cell shape. We also examined maintenance of columnar cell shape in *cno* mutants during gastrulation. As during cellularization, *cno* mutants retained more-uniform apical cell areas than did *Rap1* mutants at gastrulation onset (Supplemental Figure S7, A–A'' vs. B–B''); variation in cell area in *cno* mutants was not statistically different from WT (Supplemental Figure S7, F–H). During germband extension, although cell areas were more variable in *cno* mutants than in WT (Supplemental Figure S7, I–I'' vs. J–J''), these differences did not reach statistical significance (Supplemental Figure S7, N–P), unlike those in *Rap1* mutants. These data are consistent with the idea that Rap1 has a Cno-independent role in establishing and maintaining columnar cell shape.

Baz and aPKC also play early roles in cell shape regulation

Baz, aPKC, and AJ proteins are all required for *maintaining* columnar cell shape: cells lacking them ultimately round up as they lose adhesion. However, complete loss of cell adhesion and epithelial architecture occurs at different times in these different genotypes; most cells in embryos lacking Baz or AJs round up and fall apart at gastrulation onset (Cox *et al.*, 1996; Harris and Peifer, 2004), whereas *aPKC^MZ* mutants proceed farther, only fully losing epithelial architecture at the end of germband extension (Hutterer *et al.*, 2004; Harris and Peifer, 2007). Given our data suggesting that Rap1 may have a Cno-independent role in cell shape maintenance and the data suggesting that both Baz and aPKC are at the apical cortex before cellularization, we examined whether either Baz or aPKC also regulate the *establishment* of columnar cell shape.

Using the foregoing approach, we quantitated apical cell area during cellularization in *baz* RNAi and *aPKC* mutants. Strikingly, loss of Baz or aPKC affected initial establishment of columnar cell shape at stage 5, significantly increasing variability in apical cell area (Figure 10, A vs. D and E, F). However, unlike *Rap1* (Figure 10C''), there was less cell area variability in more basal sections of *baz* RNAi or *aPKC* mutants (Figure 10, D', D'', E', E'', G, and H). These data suggest that both Baz and aPKC are important for initially establishing columnar cell shape, in particular in the apicalmost region. Consistent with their known roles in maintaining epithelial architecture, both *baz* RNAi and *aPKC* mutants also had cell shape defects after gastrulation onset (Supplemental
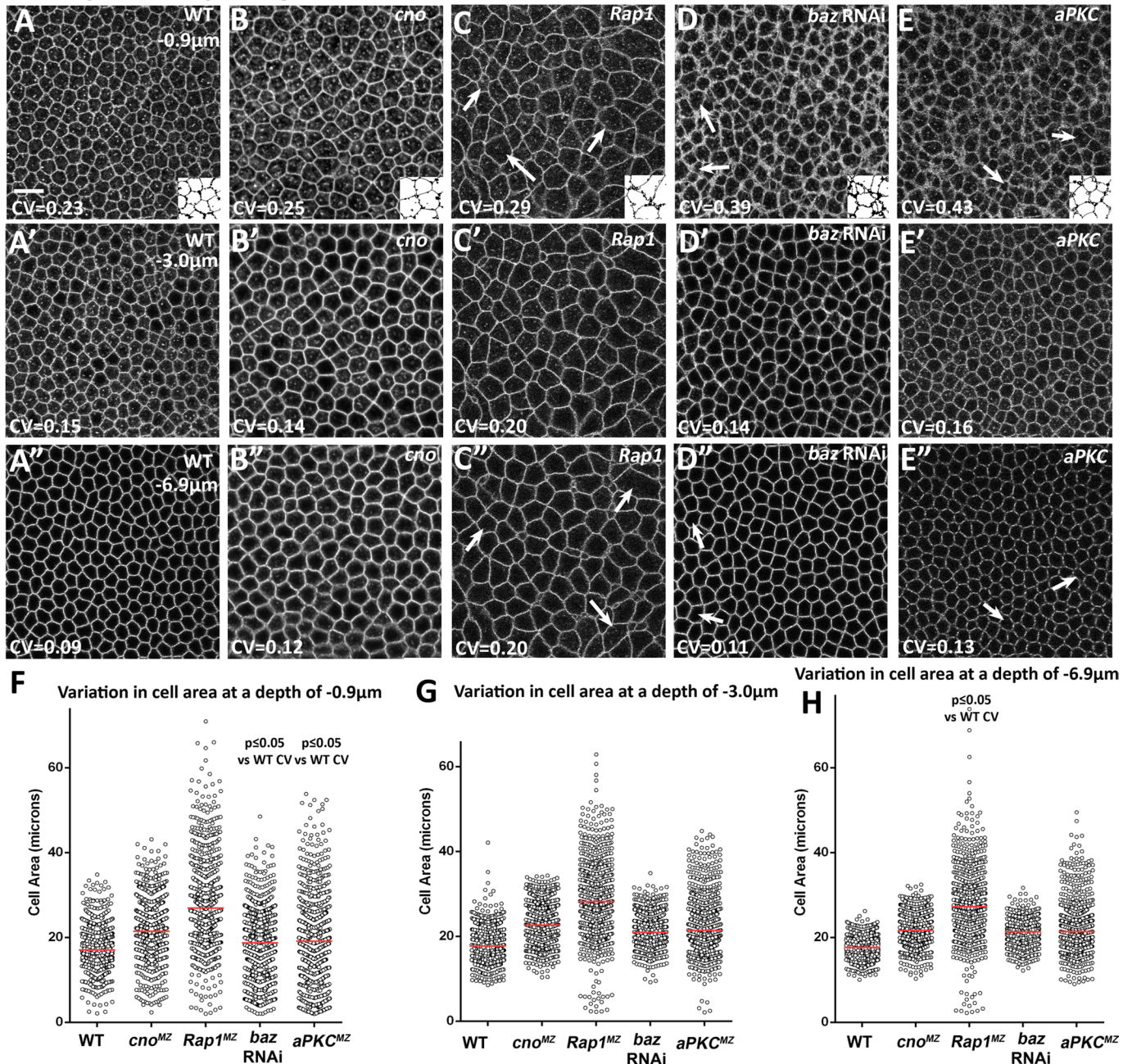
Cell shape variability at stage 5

FIGURE 10: Rap1, Baz, and aPKC play roles in establishing columnar cell shape during cellularization. (A–E) Cell shapes during late cellularization at three different apical–basal depths (0.9, 3.0, and 6.9 μm below the apical surface). Cells were stained with antibody to the membrane protein Nrt, background was removed and images were processed with a Watershed algorithm and thresholded (insets) to allow ImageJ to measure cell area. Representative embryos are shown. The degree of variation in cell shape is expressed by the coefficient of variation (CV). Arrows indicate examples of variable cell areas. (F–H) Beeswarm scatter plots of cell areas for all genotypes examined at the indicated depths. Significance of the degree of cell area variability (CV) was assessed by Tukey's HSD test to correct for multiple comparisons. Red lines indicate the median value. WT cells are essentially columnar (A–A″) and exhibit relatively little variation in cell area from cell to cell; what variation does exist is most prominent in the apicalmost slice. *cno* mutants (B–B″) also exhibit relatively uniform cell shapes. *Rap1* mutants (C–C″) are more variable in cell area than WT, and this difference reaches significance in the basal section. *baz* RNAi (D–D″) and *aPKC^MZ* mutants (E–E″) are significantly more variable in cell area than WT in the apicalmost region. Scale bar, 10 μm.

Figure S7, D, E, L, and M); once again their most striking and significant effects were on apical cell area (Supplemental Figure S7, F and N). Taken together, these data demonstrate that both

Baz and aPKC act early in cell shape establishment, further supporting the idea that a network of regulatory interactions is already in place during cellularization.

DISCUSSION

Rap1 and Cno are critical for positioning Baz/Par3 and AJs during polarity establishment

Drosophila embryogenesis provides a superb model for apical–basal polarity establishment and maintenance in vivo. The simultaneous formation of thousands of cells during cellularization allows one to view the process from start to finish with high resolution. Previous work suggested a hierarchy regulating polarity with Baz/Par3 protein at the top. In this model, Baz was positioned by cytoskeletal cues and then directed apical positioning of both AJs and the aPKC/Par6 complex (Harris and Peifer, 2004, 2005; Supplemental Figure S8A). However, proteins regulating Baz apical positioning remained unknown. Our data provide new mechanistic insights into this process. They demonstrate that the small GTPase Rap1 and the actin-binding protein Cno are essential for polarity establishment, regulating initial apical positioning of both Baz and AJs (Supplemental Figure S8B).

In regulating polarity establishment, Rap1 and Cno could act by several possible mechanisms. Their role in AJ positioning may be solely due to their effects on Baz localization, or alternatively Rap1 and Cno may independently affect the localization of both Baz and AJs. In the latter case, Cno may directly link AJs to the apical actin scaffold, as we suggested it acts in apical constriction (Sawyer *et al.*, 2009). Rap1 and Cno also clearly regulate Baz positioning. Because Baz apical positioning requires an apical actin scaffold and dynein-based MT transport (Harris and Peifer, 2005), we examined whether Rap1 and Cno act indirectly by regulating cytoskeletal organization. However, our data suggest that this is not the case: both the MT and actomyosin cytoskeletons appear normal in mutants. We thus think the most likely model is that Rap1 and Cno are required for anchoring Baz apically. Consistent with this, when we ectopically localized Cno to artificial cell–cell contacts in cultured fly cells, it was able to recruit Baz to that site. This could occur directly, for example, by Cno binding Baz, or indirectly, via unknown intermediaries. Strikingly, however, when we overexpressed Baz in cellularizing embryos, presumably saturating its apical binding sites, it accumulated basolaterally and recruited DEcad but not Cno to these ectopic sites. Thus Cno and Baz do not colocalize obligatorily. We think it likely that each has multiple binding partners and that when pools are limiting, as Cno may be in this latter experiment, ectopic Baz cannot recruit Cno away from a preferred binding site. Of course, it remains possible that Cno and Rap1 also regulate Baz positioning through effects on MT transport or, given Cno's apical localization, unloading at an apical docking site. It will be important to test these possibilities. As we discuss in more detail later, it will also be important to define the Cno- and Rap1-independent mechanisms that partially restore apical Baz localization after gastrulation onset.

Because Rap1 is uniformly distributed along the apical–basal axis during cellularization (Sawyer *et al.*, 2009; Supplemental Figure S1C), the most likely hypothesis is that it is locally activated apically by a GEF (Supplemental Figure S8B). A number of Rap1GEFs exist, many of which are conserved between mammals and flies. Recent work from the Reuter lab demonstrated that, like Cno and Rap1 (Sawyer *et al.*, 2009), the Rap1 GEF Dizzy (Dzy/PDZ-GEF) plays an important role in coordinated mesodermal apical constriction (Spahn *et al.*, 2012), suggesting that it is the GEF acting upstream of Cno and Rap1 in that process. They also suggest that Rap1 and Dzy help to regulate establishment of AJs (Spahn *et al.*, 2012). Although similar in outline, their analysis of AJs differs from ours in detail, as they see strong effects on DEcad localization without similar effects on Arm. This is surprising, since these two proteins of the cadherin–catenin complex generally localize very similarly at the cortex. However, these differences aside, their data are consistent with Dzy

acting with Cno and Rap1 in AJ establishment; it will be important to examine the effects of Dzy on Baz localization. It will also be important to determine how preexisting egg membrane polarity (Mavrakis *et al.*, 2009) is translated into localized Rap1 activity.

Establishing columnar cell shape—a Cno-independent role for Rap1?

In addition to the parallel roles of Rap1 and Cno in regulating initial apical–basal polarization, we identified a second role for Rap1 in establishing and maintaining columnar cell shape (Supplemental Figure S8B). Our data suggest that this is partially or completely Cno independent, and thus one of the many other Rap1 effectors may play a role in this process. It will be exciting to examine embryos mutant for other Rap1 effectors (Kooistra *et al.*, 2007), such as Krit1/Bili, TIAM/Stilllife, RIAM/Pico, or RhoL, to see whether they are required for establishing columnar cell shape. *baz* and *aPKC* mutants also had defects in establishing columnar cell architecture (Supplemental Figure S8B). It is possible that each protein provides an independent mechanistic input into this process. This is consistent with the observed differences in the details of how columnar cell shape is disrupted, with Baz and aPKC primarily regulating apical cell area, whereas Rap1 affects cell shape at multiple apical–basal positions. A more speculative but perhaps less likely possibility is that Rap1 uses Baz and aPKC as effectors in establishing columnar cell shape. Fly Rap1 can form a complex with aPKC and Par6 (Carmena *et al.*, 2011), and Rap1 acts upstream of cdc42/Par3/aPKC in regulating polarity of cultured neurons (Schwamborn and Puschel, 2004).

Having identified Rap1's direct effector(s) in regulating cell shape, we will need to move downstream. On the basis of analogies with other epithelial tissues in fly development, we hypothesize that establishing columnar cell shape involves regulating apical tension. Other small GTPases play key roles in this; for example, Rho and cdc42 have striking and opposing roles in apical tension regulation during fly eye development (Warner and Longmore, 2009a,b). In that context, Rho acts via separate effectors to maintain AJs and apical tension: it regulates tension via Rok, Diaphanous, and ultimately myosin contractility. It will be interesting to determine whether the defects in apical cell shape in the absence of Rap1, Baz, or aPKC also reflect unbalanced contractility in different nascent cells and which contractility regulators are involved. However, for now, this is speculative.

Cell polarity establishment—a network model

In our previous work, we suggested a linear hierarchy regulating polarity establishment, with Baz at the top, positioning AJs and aPKC (Supplemental Figure S8A; Harris and Peifer, 2004, 2005). Our present work extends this hierarchy, positioning Rap1 and Cno upstream of Baz in this process. However, our data further suggest that viewing polarity establishment as a linear process is significantly oversimplified (Supplemental Figure S8B). We now know that all of the relevant players—including the AJ proteins, Baz, Cno, and aPKC—are at the cortex in syncytial embryos before cellularization and the initiation of apical–basal polarity. This places them in position to cross-regulate one another. Consistent with this, our data suggest that viewing relationships with an "upstream–downstream" point of view misses important reciprocal interactions that occur as polarity is established. Two examples point this out most clearly. First, our earlier work suggested that localization of aPKC occurs "downstream" of Baz, as apical positioning of aPKC at gastrulation onset requires Baz function (Harris and Peifer, 2005). Our new data reveal that Rap1 and Cno are, in turn, "upstream" of Baz, and thus, if things work in a strictly linear manner, Rap1 and Cno should be "upstream" of aPKC. However, in contrast to this simple view, we

Fly stocks	Source
Rap1$^{rv(R)B1}$FRT3L^{2A}/TM3*twi*GFP	I. Hariharan (University of California, Berkeley, CA)
FRT82B *cno*^{R2}/TM3*twi*GFP	U. Gaul (Ludwig-Maximilians-Universität München, Munich, Germany)
aPKC^{K06403}FRT2R/CyO	C. Doe (University of Oregon, Eugene, OR)
UAS*baz*RNAi (stock # 35002)	Bloomington *Drosophila* Stock Center (Bloomington, IL)
UAS*Rap*RNAi (stock # 35047)	Bloomington *Drosophila* Stock Center

Antibodies/probes (species)	Dilution (IF)	Source
anti-ArmN27A1 (M-IgG2a)	1:50	Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA)
anti-Nrt (M-IgG2a)	1:100 (heat fixation)/ 1:25 (4% formaldehyde fixation)	Developmental Studies Hybridoma Bank
anti–DE-DCAD2	1:100	Developmental Studies Hybridoma Bank
anti-Rap1 (rabbit)	1:500	This work
anti-Baz (rabbit or guinea pig)	1:500	J. Zallen (Memorial Sloan-Kettering Cancer Center)
anti-aPKC (rabbit)	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA)
anti–α-Tubulin (clone YL1/2; rat)	1:250	Millipore (Billerica, MA)
anti–γ-Tubulin (clone GTU-88; M-IgG1)	1:500	Sigma-Aldrich (St. Louis, MO
anti-Zipper (myosin II heavy chain; rabbit)	1:1000	C. Field (Harvard University, Cambridge, MA) or D. Kiehart (Duke University, Durham, NC)
Alexa–phalloidin	1:500	Life Technologies (Carlsbad, CA)
Secondary antibodies: Alexa 488, 568, and 647	1:500	Life Technologies

TABLE 1: Fly stocks, antibodies, and probes.

found that precise positioning of Cno during cellularization requires aPKC—in its absence, Cno is not cleared from the apical region, and the apical–basal cables of Cno at tricellular junctions are not properly assembled. In a similar manner, Baz, which in a linear model is "downstream" of Cno, also regulates precise positioning of Cno during cellularization. aPKC and Baz also play important roles in Cno localization during the early polarity-maintenance phase beginning at gastrulation onset. Taken together, these data suggest that initial positioning of proteins along the apical–basal axis involves a network of protein interactions, similar to that previously suggested to regulate polarity elaboration during the extended germband phase and beyond, as cells develop the full suite of epithelial junctions (Bilder *et al.*, 2003; Tanentzapf and Tepass, 2003; Laprise *et al.*, 2009). It will now be important to define mechanisms by which aPKC and Baz act to precisely position Cno; two broad possibilities are that they act on Cno directly and that they modulate the fine-scale architecture of the actin cytoskeleton, with indirect effects on Cno. It will also be exciting to determine whether other polarity determinants, such as the basolateral proteins Discs Large, Scribble, or Lgl, or the basolateral kinase Par1 also play roles in polarity establishment, as they do in polarity maintenance. Consistent with this possibility, recent work from the Harris lab suggests that Par1 is important for the gastrulation-onset rescue of Baz localization in embryos in which early cues are disrupted (McKinley and Harris, 2012). Finally, it will be interesting to identify the cues that come into play at gastrulation onset, which partially restore apical Baz localization,

as part of the increasingly complex network of partially redundant regulatory cues that give polarity its robustness.

MATERIALS AND METHODS

Fly stocks

Fly stocks used in this study are listed in Table 1. Mutations are described at FlyBase (flybase.bio.indiana.edu). Wild type was *yellow white*. All experiments were done at 25°C unless otherwise noted. Stocks to make *Rap1*, *cno*, or *aPKC* germline clones were from the Bloomington *Drosophila* Stock Center (Indiana University Bloomington, IN). *Rap1* germline clones were made by heat shocking 48- to 72-h-old *hsFLP*^{1}/+; FRT3L^{2A}*Rap1*$^{rv(R)B1}$/FRT3L^{2A}*ovo*$^{D1-18}$ larvae for 3 h at 37°C. *aPKC*^{k06403} and *cno*^{R2} germline clones were generated similarly. Knockdown of *baz* or *Rap1* was carried out by crossing maternal GAL4-VP16 to UAS-*baz* short hairpin RNA interference (shRNAi) or UAS-*Rap1* shRNAi (VALIUM 20; Ni *et al.*, 2011), allowing overexpression of *baz*- or *Rap1*-targeted small interfering RNAs during oogenesis. Maternal overexpression of Baz was carried out by crossing female GAL4-VP16 flies to males carrying UAS-*baz*::GFP, allowing overexpression of GFP fusion proteins during oogenesis of the resulting female progeny.

Immunofluorescence

The following fixations were used: Baz, Arm, Cno, myosin, and Nrt, heat-methanol (Müller and Wieschaus, 1996); phalloidin, 12 min, 10% formaldehyde or 5 min, 37% formaldehyde; aPKC, α-tubulin,

γ-tubulin, Nrt, DEcad, or GFP were fixed in 4% formaldehyde for 20 min. Embryos were methanol devitillinized, or hand devitillinized for phalloidin. All embryos were blocked/stained in phosphate-buffered saline (PBS)/1% goat serum/0.1% Triton X-100 using the antibodies listed in Table 1 and mounted in Aqua-Polymount (Polysciences, Warrington, PA).

Ectopic expression of Ed:GFP-Cno in S2 cells

The full-length pMT-Ed:GFP-Cno construct was kindly provided by Chris Doe (University of Oregon, Eugene, OR) and is described in detail in Wee et al. (2011). Briefly, Drosophila Schneider (S2) cells were maintained in Schneider's medium with 10% fetal bovine serum at room temperature and transfected with 2 μg of cno constructs using Amaxa Nucleofection Kit V (Lonza, Walkersville, MD), and protein expression was induced by adding 0.5 mM $CuSO_4$ for 24 h. Cells were washed and resuspended in fresh media. Cell clustering was induced by shaking at 170 rpm for 2 h. Clustered cells, 200 μl, were transferred on 12-mm-diameter glass coverslips in a 12-well plate and allowed to adhere for 1 h. Cells were fixed for 20 min with 4% formaldehyde in PBS, followed by three washes with wash buffer (0.1% saponin in PBS) and two rinses with blocking buffer (0.1% saponin in 1% bovine serum albumin in PBS). Coverslips were incubated with primary antibodies (anti-Cno, 1:1000; anti-Pyd, 1:1000; anti-Baz, 1:500) in blocking buffer overnight and washed three times with wash buffer. Secondary antibodies (tetramethylrhodamine isothiocyanate–phalloidin, 1:1000; Alexa 647–conjugated anti-mouse or rabbit immunoglobulin G [IgG], 1:200) were incubated for 2 h at room temperature, and cells were washed three times, followed by mounting on a glass slide with Aqua-Polymount.

Image acquisition

Fixed samples were imaged with either the LSM 510 (40×/numerical aperture [NA] 1.3 Plan-Neofluar or 63×/NA 1.4 Plan-Apochromat oil immersion objectives) or LSM 710 confocal microscope (40×/NA 1.3 Plan-Neofluar oil immersion objective; Carl Zeiss, Jena Germany), together with LSM or ZEN2009 software. Photoshop CS4 (Adobe, San Jose, CA) was used to adjust input levels so that the main range of signals spanned the entire output grayscale and to adjust brightness and contrast.

Quantification of cell area variation

Embryos were heat fixed and stained with Nrt to mark cell membranes for measurement. Images were acquired as z-stacks with a 0.3- or 0.5-μm step using LSM 510 (63×/NA 1.4 Plan-Apochromat oil objective) or LSM 710 (40×/NA 1.3 Plan-Neofluar oil objective) microscope, respectively, with a digital zoom of 2. Slices at the three depths (0.9, 3.0, or 6.9 μm below the apical surface) were exported using LSM software. Background was subtracted from these slices in ImageJ (National Institutes of Health, Bethesda, MD) using a Gaussian filter before treatment with a Watershed algorithm to trace cell boundaries from Nrt staining. The Analyze Particles feature in ImageJ was used to measure cell areas of all outlined cells within the slice, but edges were excluded. CV values (SD of cell areas/mean of cell areas) were generated for each depth per embryo within Excel (Microsoft, Redmond, WA). CV values between genotypes were compared at each depth using statistical software from JMP (Cary, NC). Values for each genotype underwent a logarithmic transformation, making a more symmetrical distribution that could be analyzed using a generalized linear model that corrected for the comparison of multiple genotypes. Significance was then assessed using a Tukey's test to correct for multiple comparisons. Beeswarm plots of cell areas were generated in GraphPad Prism (GraphPad Software, La Jolla, CA).

Analysis of apical–basal positioning

Images from fixed embryos were acquired as z-stacks with a 0.3- or 0.5-μm step using LSM 510 (63×/NA 1.4 Plan-Apochromat oil objective) or LSM 710 (40×/NA 1.3 Plan-Neofluar oil objective) microscope, respectively, with a digital zoom of 2. ZEN2009 software was used to crop down stacks to a 708.8-μm area on the xy-axis, and the blocks of xyz images were used to create maximum-intensity projections through the y-axis (Figure 1F). With ImageJ software, projections were rotated 90° counterclockwise and analyzed using the Plot Profile function to generate values of average fluorescence intensity along the apical–basal axis. Values were exported to Excel to calculate averages and standard deviations. Graphs and heat map images were generated using Excel.

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