aPKC Inhibition by Par3 CR3 Flanking Regions Controls Substrate Access and Underpins Apical-Junctional Polarization

Highlights

- Sequences flanking the Par3 CR3 consensus PKC site cooperate to inhibit aPKC
- A Par3 CR3 inhibitory arm disrupts aPKC P-loop/αB/αC contacts and αC-helix position
- Mutating either CR3 arm switches Par3 into an efficient aPKC substrate in vitro
- Equivalent Bazooka substitutions alter its apical localization to AJs in vivo

In Brief
Par3 is required for aPKC membrane recruitment, yet it polarizes to adherens junctions upon phosphorylation. Soriano et al. show that Par3 antagonizes active aPKC kinase by separating crucial N-lobe contacts. Disrupting high-affinity Par3 contacts switches it to an efficient aPKC substrate and polarizes Par3/Bazooka from apical domains to adherens junctions.

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aPKC Inhibition by Par3 CR3 Flanking Regions Controls Substrate Access and Underpins Apical-Junctional Polarization

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SUMMARY

Atypical protein kinase C (aPKC) is a key apical-basal polarity determinant and Par complex component. It is recruited by Par3/Baz (Bazooka in Drosophila) into epithelial apical domains through high-affinity interaction. Paradoxically, aPKC also phosphorylates Par3/Baz, provoking its relocalization to adherens junctions (AJs). We show that Par3 conserved region 3 (CR3) forms a tight inhibitory complex with a primed aPKC kinase domain, blocking substrate access. A CR3 motif flanking its PKC consensus site disrupts the aPKC kinase N lobe, separating P-loop/αB/αC contacts. A second CR3 motif provides a high-affinity anchor. Mutation of either motif switches CR3 to an efficient in vitro substrate by exposing its phospho-acceptor site. In vivo, mutation of either CR3 motif alters Par3/Baz localization from apical to AJs. Our results reveal how Par3/Baz CR3 can antagonize aPKC in stable apical Par complexes and suggests that modulation of CR3 inhibitory arms or opposing aPKC pockets would perturb the interaction, promoting Par3/Baz phosphorylation.

INTRODUCTION

Epithelial tissues are composed of sheets of polarized cells that are connected by adherens junctions (AJs) (Laprise and Tepass, 2011; St Johnston and Ahringer, 2010; Suzuki and Ohno, 2006). The plasma membrane of epithelial cells is segregated into apical and basolateral domains, with a prominent belt of AJs located at the interface of these two domains (Figures S1A and S1B). The atypical protein kinase C (aPKC in Drosophila or PKCζ isoforms in mammals), its binding partner Par6, and the small guanosine triphosphatase Cdc42 are three essential determinants of apical membrane identity in both Drosophila and mammals (Fletcher et al., 2012; Harris and Tepass, 2008; Hutterer et al., 2004; Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000). The aPKC-Par6-Cdc42 assembly can form a larger stable complex with Par3/Baz (Bazooka [Baz] in Drosophila) (known as the Par complex) at the apical membrane (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000). Association of Par3 with the basolateral membrane is prevented by phosphorylation of its lipid-binding domain by the basolateral kinase Par1 (Benton and St Johnston, 2003b).

Importantly, a distinct pool of Par3/Baz can also segregate away from apical aPKC-Par6-Cdc42 and localize to AJs (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). The role of Par3/Baz at AJs is thought to be essential as it involves defining the position of AJs during the establishment of epithelial polarity (Wang et al., 2012b) and possibly also remodeling of AJs as tissues undergo morphogenetic change (Walther and Pichaud, 2010). The regulation of this switch of Par3/Baz subcellular localization from the apical membrane to AJs has been shown in Drosophila to be dependent on aPKC phosphorylating Par3/Baz on serine 980 in vivo (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). This site is within a consensus PKC phosphorylation R-X-S-P motif and is equivalent to serine 827 of human Par3 (Figure S1D), both sites map to Par3/Baz conserved region 3 (CR3), a site of regulated protein interaction (Nagai-Tamai et al., 2002). However, how Par3/Baz switches from being a stable binding partner of aPKC in the Par complex to being a substrate of aPKC that segregates away from the Par complex remains unclear. In mammalian cells, a similar conundrum exists whereby Par3 is critical for the recruitment of PKCζ to the apical membrane and is known to be an in vivo substrate of PKCζ, but loss of Par3 in transformed epithelial cells can lead to PKCζ activation...
and can result in breast tumorigenesis and metastasis (McCaffrey and Macara, 2009; McCaffrey et al., 2012).

One complication in understanding the role of Par3/Baz in Drosophila epithelia is the presence of another key apical determinant, Crumbs (Crb) (Tepass, 1996). Like Par3/Baz, Crb can localize apically in a complex with Stardust (Sdt) (Bilder et al., 2003; Roh et al., 2003; Tanentzapf and Tepass, 2003; Tepass, 1996) and aPKC-Par6-Cdc42 (called the Crb complex) (Fletcher et al., 2012; Harris and Tepass, 2008; Morais-de-Sa et al., 2010). Par3/Baz and Crb-Sdt can therefore act in a semi-redundant fashion to specify the apical domain in Drosophila, such that either Par3/Baz or Crb-Sdt is usually sufficient to maintain polarity in Drosophila (Fletcher et al., 2012; Tanentzapf and Tepass, 2003). Similarly, Willin, a FERM-domain protein, has been implicated in another Par3-independent apical domain recruitment mechanism for Par6-aPKC (Ishiiuchi and Takeichi, 2011). The presence of Crb has been shown to promote Par3/Baz localization to AJs (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). However, in the absence of Crb, some Par3/Baz can still be phosphorylated by aPKC on S980 so that it localizes to AJs (Morais-de-Sa et al., 2010). These findings indicate that individual Par3/Baz molecules can localize either apically or junctionally without requiring any input from Crb. Thus, the paradoxical dual role of Par3/Baz molecules can localize either apically or junctionally (Morais-de-Sa et al., 2010). These findings indicate that individual Par3/Baz molecules can localize either apically or junctionally without requiring any input from Crb. Thus, the paradoxical dual role of Par3/Baz as either a Par complex component or an aPKC substrate appears to be an emergent property of these molecules themselves, although it is still uncertain how this property arises.

aPKC isoforms PKC\(\alpha\) and PKC\(\beta\) have regulatory regions distinct from those of other PKC isoforms, but share a conserved catalytic protein kinase domain (Parker and Murray-Rust, 2004). They are not responsive to diacylglycerol and have less well-defined activators (Limagola et al., 1994). Like many protein kinases, activation of aPKC requires activation-loop phosphorylation and an \(\alpha\)-helix conformation compatible with Lys-Glu salt-bridge formation to bind ATP and serve to align residues within the R spine (Kornev et al., 2008). Functionally validated aPKC substrates include Par3, LLGL2, ROCK1, and MARK2, and the Hippo pathway component Kibra (Betschinger et al., 2005; Buther et al., 2004; Hurov et al., 2004; Ishiiuchi and Takeichi, 2011). Sequences flanking the phospho-acceptor site in each aPKC substrate are rich in basic residues consistent with basophilic AGC kinase consensus sites derived from short peptide substrates (4–14 residues) (https://www.kinexus.ca). In these contexts aPKC phosphorylation inactivates substrates with basophilic membrane-binding motifs with embedded phosphorylation sites such that they are displaced from membranes (Bailey and Prehoda, 2015).

Here, we describe how Par3 CR3 recognizes and inhibits a nucleotide-occupied primed PKC\(z\). Two Par3 CR3 motifs flanking its PKC consensus site engage pockets within the PKC\(z\) kinase domain, one of which disrupts crucial N-lobe contacts required for catalytic activity. A second contact used by both aPKC inhibitors and substrates provides a high-affinity anchor point through a Phe-X-Arg motif. Together, both motifs cooperate to block aPKC substrate access and prevent phospho-transfer to Par3 CR3. Mutation of either motif switches Par3 from an inhibitor to an efficient substrate in vitro and redistributes equivalent Bazooka mutants to AJs in vivo. These data are consistent with high-affinity inhibitory interactions between Par3/Baz and aPKC preventing Par3/Baz phosphorylation and thereby promoting stable complex formation and apical localization. Modulation of the CR3 inhibitory arm by phosphorylation or engagement of the aPKC pocket by partner proteins would switch Par3/Baz to a more transient type of interaction, consequently enabling efficient phosphorylation of Par3/Baz by aPKC and subsequent relocalization to AJs.

RESULTS

The Par3 CR3 Region Inhibits Nucleotide-Bound Primed PKC\(i\): Kinase Domain through Two Flanking Arm Contacts

The human Par3 conserved region 3 (CR3, covering residues 816–834, defined hereafter as Par3\(\text{CR3}\)) is able to bind to PKC\(i\) (Nagai-Tamaia et al., 2002) and contains a phospho-acceptor site (P site) at residue serine 827 known to be phosphorylated by PKC\(i\) (Figures 1A and 1B). To characterize its interaction with PKC\(i\), we purified a "primed" active form of the human PKC-iota kinase domain (referred to as PKC\(i\)-KD\(\text{-2P}\)) and a partially primed low-activity form (referred to as PKC\(i\)-KD\(\text{-1P}\)), referring to the status of the two "priming" phosphorylation sites at t\(\text{PT}412\) and t\(\text{PT}564\) (Figures 1A and S2A–S2C). We then probed how efficiently they were able to phosphorylate Par3\(\text{CR3}\). Surprisingly, we found that Par3\(\text{CR3}\) strongly inhibited the catalytic activity of PKC\(i\)-KD\(\text{-2P}\) in vitro and could competitively block phosphorylation of a model substrate peptide, with an apparent 50% inhibitory concentration (IC\(50\)) of 0.45 ± 0.18 \(\mu\)M. In contrast, peptides from other known aPKC substrates such as Par1 were efficiently phosphorylated and were unable to inhibit (Figures 1B–1D). Using a fluorescence anisotropy assay, we found that the Par3\(\text{CR3}\) binds to PKC\(i\)-KD\(\text{-2P}\) with submicromolar affinity (K\(D\) of 0.47 ± 0.09 \(\mu\)M), as does an S827A mutant (K\(D\) of 0.97 ± 0.07 \(\mu\)M) (Figure 1E). PKC\(i\)-KD\(\text{-2P}\) is a good surrogate for an activated Par complex containing Par6-PKC\(i\)-Cdc42 complex that exhibits high activity in vitro and is also potently inhibited by Par3\(\text{CR3}\) (data not shown). In contrast, PKC\(i\)-KD\(\text{-1P}\) was not inhibited to the same extent and had a much lower affinity for Par3\(\text{CR3}\) (compare Figures 1D, S2D, and S2E). We conclude that a high-affinity Par3\(\text{CR3}\) targets PKC\(i\)-KD\(\text{-2P}\) and inhibits its catalytic activity.

To understand how Par3\(\text{CR3}\) could inhibit PKC\(i\)-KD\(\text{-2P}\), we determined the 2.0-Å crystal structure of a longer Par3 peptide (residues 816–841) bound to PKC\(i\)-KD\(\text{-2P}\) and Mg-AMPPNP (adenylyl imidodiphosphate) (Figures 2A and S3A; Table 1). The Par3\(\text{CR3}\) Peptide is well ordered in this structure and contains seven intramolecular hydrogen bonds (Figure S3B). It engages PKC\(i\)-KD\(\text{-2P}\) by adopting a "staple"-shaped conformation with two arms that flank the S827\(\text{Par3}\) phospho-acceptor site. Each arm binds in close proximity to opposite ends of the nucleotide, suggesting that recognition of aPKC is driven by nucleotide occupancy. The relative orientation of N and C lobes indicates a "closed" rather than "open" conformation. Par3\(\text{CR3}\) contacts extend from a pocket beneath the ribose-binding pocket of PKC\(i\)-KD\(\text{-2P}\) (site 1), across the G helix (site 2) through to the activation loop, \(\alpha\) and \(\alpha\)-helices of the PKC\(i\)-KD\(\text{-2P}\) (site 3) (Figures 2A and 2B). A total surface area of more than 1,305 \(\text{Å}^2\) is buried within the complex, consistent with a high-affinity inhibitory interaction. The nucleotide cleft is occupied by an Mg-AMPPNP.
nucleotide (Figure 2C). The conserved nucleotide-coordinating lysine (K283PKC) forms a salt bridge with the conserved xC-helix glutamate (E302PKC) side chain found in many active kinase conformers (Kornev et al., 2008). The terminal γ-phosphate of AMPNP is not observed in the structure, consistent with AMPNP being rapidly hydrolyzed under the crystallization conditions (see Experimental Procedures). A magnesium ion, equivalent to Mg2+ of PKA, is present, bridging both the xC and β phosphates of AMPNP (Adams and Taylor, 1993; Zheng et al., 1993). The Mg1 ion is not present, as frequently found in AGC kinase structures.

The amino-terminal part of Par3CR3 binds to site 1 (PKC<sub>KD-1P</sub>) through an F-X-R motif at positions −9 (F−9) and −7 (R−7) defined relative to the phospho-acceptor (P) site at serine 0 (equivalent to S827 of human Par3). F−9 lies deep within a hydrophobic cleft formed by M341PKC, M344PKC, and L381PKC beneath the nucleotide pocket (Figure 2C). In addition, the side chain of R−7 forms a salt bridge to D339PKC, just beneath the ribose ring of the AMPNP, while that of R−2 engages conserved residues Y419PKC and E445PKC (Figure 2C). As this motif does not appear to directly perturb αPKC catalytic residues, we refer to this element hereafter as the “affinity arm” of Par3CR3 (Figure 2C).

From site 1, the Par3CR3 backbone adopts two consecutive α-helices making side-chain and main-chain contacts to D378PKC, K380PKC, and T416PKC, preventing a catalytically competent orientation for nucleophilic attack on the ATP γ-phosphate. Glycine-rich loop residues S264PKC and Y265PKC side chains contact the CR3 main-chain atoms near the P site, as does the activation-loop main chain, to orient the Par3CR3 peptide and position the M+1 side chain into the known P+1 AGC kinase hydrophobic pocket (Figure 2C) (Pearce et al., 2010).

Site 3 contains carboxy-terminal flanking residues to the P site stretching from S+2 to T+6. We define this portion of Par3CR3 as the “inhibitory arm,” as it directly perturbs an active PKC<sub>KD-2P</sub> N-lobe conformation (discussed later). Residue K+4 directly contacts PT412PKC of the activation loop enhancing the recognition of mature, primed PKC<sub>KD-2P</sub>, but importantly not a partially primed PKC<sub>KD-1P</sub>. Crucially, the R+5 side chain is buried within a hydrophobic pocket beneath the regulatory xC helix. The pocket is lined by side chains from Y266PKC on the glycine loop and W298PKC of the xC helix, each making π-stacking interactions with the guanidine group of R+5 (Figures 2C and 3A). Both aromatic side chains are unique to αPKC isoforms from <i>Drosophila</i> to mammals. Finally, T+6 (equivalent to T833Par3, a known ROCK-driven phosphorylation site discussed later) lies adjacent to an acidic patch within the xB helix making side-chain and main-chain contacts to D295PKC, and a Mg ion (Figures 2C and 3A). Overall, the structure reveals that the Par3CR3 clamp involves an “inhibitory arm” and an “anchoring arm,” which together recognize and inhibit a nucleotide-bound PKC<sub>KD-2P</sub> conformer.

**Comparison of Par3CR3-Inhibited αPKC: Complex with an Active PKC: Conformer Reveals the Basis for Inhibition**

To fully understand how Par3CR3 inhibits PKC<sub>i</sub> and disrupts its activated state, we determined the structure of an “active”
conformer of PKCι for comparison (Figure 3B). Previous structures of PKCι kinase domain (PDB: 3A8W and 4DC2) (Takimura et al., 2010; Wang et al., 2012a) exhibited either a disordered or displaced αB-αC loop (Figures 3C and 3D). We captured an active mature PKCι conformation bound to the ATP analog 50-(β,γ-adenylyl methylene)diphosphonate (AMPPCP) at 1.8 Å (Figure S3C and Table 1). This analog was resistant to hydrolysis compared with AMPPNP. The structure has an ordered αB-αC loop and reveals side-chain contacts between Y265PKCι of the P loop and D295PKCι of the αB-αC loop. This interaction stabilizes Y265PKCι side-chain stacking with a rotamer of W298PKCι from the αC helix (Figure 3B). Other PKC isoform structures have a phenylalanine and cysteine, respectively, at these positions (Grodsky et al., 2006; Leonard et al., 2011; Xu et al., 2004). Structural comparisons suggest that Par3CR3 inhibitory arm not only separates P-loop contacts with αB-αC loop/αC helix but also

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RMSD, root-mean-square deviation; PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentanediol.

Developmental Cell 38, 384–398, August 22, 2016 387
hijacks Y265PKC and S264PKC side chains to directly form hydrogen bonds with CR3 main-chain atoms. Comparing the Par3CR3 inhibitory complex with 1ATP (PKA bound to Mg-ATP and PKI peptide) suggests that the R+5 side-chain guanidine group lies close to the Mg2 ion of an active kinase conformation (Adams and Taylor, 1993; Zheng et al., 1993), indicating another layer of Par3CR3 disruption of an active PKCi conformation. Furthermore, T+6 (equivalent to T833Par3), which makes direct contact with D295PKC, is a phospho-acceptor site targeted by the ROCK kinase, leading to a disruption of PKCi interaction with Par3 (Nakayama et al., 2008). This would predict, based on our structural comparison, that modulation of the “inhibitory arm” of Par3CR3 by ROCK kinase phosphorylation, or inaccessibility of the pocket to which it binds, could influence whether Par3 can inhibit PKCi or engages it as a substrate.

A Shared High-Affinity Anchor Motif Used by aPKC Substrates and Inhibitors

Our Par3CR3-PKCiKD-2P inhibitory complex differs significantly from a previous structure of an ATP-binding deficient and partially primed PKCi K283R mutant (PKCiKD-1P, PDB: 4DC2) bound to Par3CR3 (Wang et al., 2012a). In the absence of nucleotide, Par3CR3 residues +3 to +7 were disordered and, therefore, the CR3 region is lacking inhibitory site 3 contacts (Wang et al., 2012a). Consistent with this, Par3CR3 is unable to potently inhibit the partially primed PKCiKD-1P or bind with high affinity (Figures S2D, S2E, and S4B). We present evidence that our Par3CR3-PKCiKD-2P structure represents a Par3CR3-mediated inhibitory complex of mature PKCi. However, the structure reported by Wang et al. (2012a) most likely resembles a weaker and transient Par3-PKCi interaction relevant to a protein kinase-substrate interaction (Figure 4).

To explore and capture a substrate peptide bound to PKCiKD-2P, we used an artificial substrate (FKRQGSVRRR, referred to hereafter as F-X-Rshort peptide) (Figures S4A–S4E). This efficient PKCi substrate closely resembles the aPKC consensus motif identified from screening randomly oriented peptide libraries by Cantley and co-workers (Nishikawa et al., 1997). We therefore determined a crystal structure for F-X-Rshort bound to PKCiKD-2P in the presence of Mn-ADP and AlF3, a transition-state analog (Figure S4E). Manganese ions corresponding to Mg1 and Mg2 ions are present in the structure, and the AlF3 is positioned as expected to mimic the transition state for the γ-phosphate. Surprisingly, the structure revealed that the
F-X-R motif at F–5 and R–3 engages precisely the same site residue contacts (M341PKCi, M344PKCi, and L381PKCi, and D339PKCi) as the F–9 and R–7 contacts used by Par3CR3, despite their different position in the primary sequence (Figure S4F). Moreover, the R–3 side chain directly makes a hydrogen bond with the ribose hydroxyl, perhaps sensing nucleotide occupancy. We also observe an R+2 side-chain bridging contact between phospho-T412PKCi and G398PKCi main-chain carbonyl, making two key hydrogen bonds with these groups. We note that many aPKC substrates have an R+2 side chain, suggesting that direct contact with a phosphorylated activation loop may reflect a common interaction made by aPKC substrates.

From the Par3CR3 structure, it is evident that the F-X-R short peptide does not inhibit PKCi because it lacks a C-terminal inhibitory motif (Figures 4 and S5). Consistent with this, the Par1 peptide characterized as a good aPKC substrate also has an F-X-R anchor and a validated aPKC phosphorylation site, but lacks an obvious inhibitory motif (Figure 1C) (Hurov et al., 2004). In contrast, a Kibra-derived peptide (residues 919–978) containing a validated aPKC phosphorylation site has both an F-X-R motif anchor and a K-R inhibitory motif. As such, it is able to potently inhibit PKCi in vitro (Figures S5A–SSC), consistent with reports of Kibra inhibiting aPKC kinase activity in epithelial cells (Yoshihama et al., 2011). Indeed a related peptide from WWC2 protein, a poorly characterized Kibra homolog, also inhibits PKCi in vitro (Figures S5A–SSC). Taken together, these data indicate that an F-X-R motif anchor amino-terminal to an aPKC phosphorylation site can be found in both aPKC substrates and inhibitors at variable lengths in their primary sequence from the phospho-acceptor site. Furthermore, the C-terminal inhibitory arm bearing a K-R-T motif is unique to aPKC protein inhibitors such as Par3 and Kibra, and can be predictive of an inhibitory function (WWC2).

**Manipulating Par3 CR3 Flanking Arms In Vitro Switches Par3 from an Inhibitor to an Efficient PKCi Substrate**

Our results suggested that Par3 CR3 arms flanking the consensus PKC phosphorylation site cooperate to inhibit PKCi. To probe the individual contributions of each arm, we characterized Par3CR3 substitutions at critical contact residues in the affinity arm and the inhibitory arm for their impact on Par3CR3 affinity for PKCi and ability inhibit kinase activity. Two mutants were prepared: first, substitution of F-Q-R to A-Q-A in the site 1 affinity arm, referred to as A-X-A hereafter; and second, substitution of K-R-T to A-A-T of the site 3 inhibitory arm, referred to as A-A-T. Consistent with our crystal structure, either A-X-A or A-A-T mutation within Par3CR3 markedly reduce the CR3-binding affinity for PKCi and ability inhibit kinase activity. Two mutants were prepared: first, substitution of F-Q-R to A-Q-A in the site 1 affinity arm, referred to as A-X-A hereafter; and second, substitution of K-R-T to A-A-T of the site 3 inhibitory arm, referred to as A-A-T. Consistent with our crystal structure, either A-X-A or A-A-T mutation within Par3CR3 markedly reduce the CR3-binding affinity for PKCi and ability inhibit kinase activity. Two mutants were prepared: first, substitution of F-Q-R to A-Q-A in the site 1 affinity arm, referred to as A-X-A hereafter; and second, substitution of K-R-T to A-A-T of the site 3 inhibitory arm, referred to as A-A-T. Consistent with our crystal structure, either A-X-A or A-A-T mutation within Par3CR3 markedly reduce the CR3-binding affinity for PKCi and ability inhibit kinase activity.
Surprisingly, the in vitro kinase assay demonstrated that either an A-X-A or A-A-T mutation gave a substantial increase in Par3CR3 phosphorylation by PKC$^{i\text{KD-2P}}$, greatly enhancing the apparent $k_{\text{cat}}$ values (Figures 5A and 5C). The large effects observed for each mutant (57-fold for A-X-A Par3CR3 versus 18-fold A-A-T Par3CR3) suggest that these substitutions uncouple the ability of Par3CR3 to inhibit PKC$^{i\text{KD-2P}}$, resulting in access to the PKC consensus site at S827Par3 and efficient phosphorylation by PKC$^{i\text{KD-2P}}$. The magnitude of the increased $k_{\text{cat}}$ values allowed the measurement of a $K_M$ for the A-X-A Par3CR3 substrate ($K_M$ of 39 $\mu$M), which was not possible for wild-type Par3CR3. Combining both site 1 and site 3 mutations (A-X-A + A-A-T) within Par3CR3 generated a very poor substrate that was not detectably phosphorylated and had no measurable interaction with PKC$^{i\text{KD-2P}}$ (data not shown). These data indicate that while mutating either the anchoring arm or inhibitory arm switches Par3CR3 to an efficient aPKC substrate, the remaining arm must contribute sufficient binding affinity (both are basophilic) as mutating both arms generates a Par3CR3 that is neither a substrate nor an inhibitor. These striking results are also consistent with the notion that tight inhibitory binding of Par3CR3 to PKC$^{i\text{KD-2P}}$ must prevent its phosphorylation while weaker binding without the inhibitory interactions exposes its PKC site, switching it to a highly efficient in vitro PKC$^{i\text{KD-2P}}$ substrate.

Figure 4. Structural Comparison of aPKC-Substrate and aPKC-Inhibitor Interactions

(A) Top panel: cartoon depiction of the Par3CR3 (green) bound to PKC$^{i\text{KD-2P}}$ (omitted for clarity) highlighting the position of inhibitory –7 and +5 arginine residues flanking the nucleotide (gray sticks). Middle panel: schematic representation of the interaction sites of Par3CR3 with PKC$^{i\text{KD-2P}}$. Bottom panel: close up of the R+5 pocket occupied by Par3CR3 and the Y265 and W298 clamp residues of PKC$^{i\text{KD-2P}}$ (gray). The known phospho-acceptor site at serine 0 is shown in red for all panels.

(B) Top panel: cartoon depiction of the Par3CR3 (blue) bound to PKC$^{i\text{KD-1P}}$ K283R mutant (omitted for clarity) from Wang et al. (2012a) lacking inhibitory site 3 contacts, possibly reflecting a lower-affinity substrate-type interaction. Middle panel: schematic representation of the interaction sites of Par3CR3 with PKC$^{i\text{KD-1P}}$ K283R mutant. Bottom panel: close up of the “closed” R+5 pocket in which the clamp residues Y265 and W298 make direct contact (PKC$^{i\text{KD-1P}}$ shown in pink).

(C) Top panel: cartoon depiction of the FXRshort (purple) bound to PKC$^{i\text{KD-2P}}$, Mn-ADP, and AlF$_3$. This artificial substrate lacks inhibitory site 3 contacts but shares site 1 FXR motif. Middle panel: schematic representation of the interaction sites of FXRshort with PKC$^{i\text{KD-2P}}$. Bottom panel: close up of the ordered portion of the R+5 pocket including the glycine loop and Y265 but not the disordered W298 from the $\alpha$C helix (PKC$^{i\text{KD-1P}}$ shown in orange).

See also Figures S4A–S4D for the design and characterization of FXRshort peptide. See Figures S4E and S4F for refined FXRshort peptide electron density and a superposition of the Par3CR3 and FXRshort peptides bound to PKC$^{i\text{KD-2P}}$. 
Figure 5. Reducing the Par3<sub>CR3</sub> Affinity for PKC<sub>iKD-2P</sub> Promotes Efficient CR3 Phosphorylation In Vitro

(A) Summary table of $k_{cat}$, $K_m$, and $K_D$ constants between various Par3<sub>CR3</sub> mutants and PKC<sub>iKD-2P</sub>. Data are presented as mean ± SEM. n.d., not determined.

(B) Binding curves for Par3<sub>CR3</sub> and various Par3<sub>CR3</sub> mutants determined by fluorescence polarization (color coded as in A).

(C) PKC<sub>iKD-2P</sub> catalytic activity kinetic rate constants for Par3<sub>CR3</sub> and various Par3<sub>CR3</sub> mutants (color coded as in A). For further details of other inhibitory peptides similar to Par3<sub>CR3</sub>, see Figure S5.

(D) Co-immunoprecipitation (IP) of full-length Myc-Par3 or mutants (Par3-A-X-A and Par3-A (S827A)) and GFP-PKC<sub>i</sub> from HCT-116 cells shows that the F-X-R to A-X-A mutation dramatically reduces the interaction.

(E) Co-immunoprecipitation of GFP-PKC<sub>i</sub> or GFP-PKC<sub>i</sub>-D/D with Myc-Par3 also severely impairs the interaction. GFP-PKC<sub>i</sub>-D/D is a mutant replacing residues D330/D373 that interact with the F-X-R motif by alanine.

(F) Immunoblot (IB) using a phospho-S827-specific antibody indicates that Par3 and Par3 A-X-A mutant (but not Par3-A) are phosphorylated in HCT-116 cells. For details showing evidence of phosphorylation of A-X-A Baz mutant, see Figure S6.
To validate some aspects of these results, using full-length PKC\(_{\alpha}\) and Par3 in cells we undertook co-immunoprecipitation experiments of differentially tagged full-length forms of PKC\(_{\alpha}\) and Par3 expressed in transiently transfected HCT-116 cells. Endogenous PKC\(_{\alpha}\) was efficiently immunoprecipitated through exogenous wild-type Par3, while a full-length human Par3 bearing the site 1 A-X-A mutation showed substantially reduced interaction with PKC\(_{\alpha}\) (Figure 5D), consistent with in vitro data for the isolated CR3 domain. Note that endogenous PKC\(_{\alpha}\) retains binding to the non-phosphorylatable Par3-S827A (Myc-PAR-3-A) similarly to the wild-type but is unable to be turned over and remains tightly associated with PKC\(_{\alpha}\). Reciprocal co-immunoprecipitation of overexpressed exogenous wild-type GFP-PKC\(_{\alpha}\) efficiently pulled down endogenous Par3, whereas mutating residues D339PKC\(_{\alpha}/D382PKC\(_{\alpha}\) (GFP-PKC\(_{\alpha}\)-D/D) that directly contact the R–7 side chain of Par3 also markedly reduced the Par3/PKC\(_{\alpha}\) interaction (Figure 5E). Arm contacts identified from the crystal structure are therefore necessary for Par3 interaction with PKC\(_{\alpha}\). We developed a specific phosphorylated Par3 antibody to probe whether mutation of the A-X-A or K-H-T motif to A-A-T abolished interaction with PKC\(_{\alpha}\) completely as well as PKC\(_{\alpha}\)-mediated phosphorylation. While the A-X-A is less phosphorylated compared with wild-type (Figure 5F), we noted a large increase in ubiquitinylated Par3 (under conditions of proteasome inhibition), a likely consequence of Par3 phosphorylation in non-polarized cells (data not shown). Taken together, immunocomplex recovery from HCT-116 cells confirmed that (1) the contacts observed structurally indeed influence interaction in cells as predicted, and (2) “weakening” the strength of the aPKC-Par3 interaction through site-specific mutation prevents Par3 inhibition, leading instead to Par3 phosphorylation.

**Apical-Junctional Polarization of Par3/Baz In Vivo Is a Consequence of Switching between Inhibitory and Substrate-Binding Modes**

If the affinity of the Par3/Baz-aPKC interaction essentially determines the localization of Par3/Baz in epithelial cells, then the Par3cr3 substitutions within each arm (A-X-A or A-A-T mutants) characterized in vitro should affect the localization of Par3/Baz in vivo. To test this prediction, we mutated the CR3 region of full-length GFP-tagged *Drosophila* Baz in the F-X-R motif to A-X-A or the K-H-T motif to A-A-T and examined their apical domain or AJ localization in vivo. In the follicular epithelium, GFP-tagged wild-type Baz (GFP-Baz) co-localizes with aPKC at the apical membrane and also localizes to AJs (Figure 6A). Phospho-Baz is known to localize to AJs (Morais-de-Sa et al., 2010), and a GFP-tagged phosphomimic version of Baz (GFP-Baz S980E) expectedly fails to co-localize with aPKC at the apical membrane but instead localizes to AJs (Figure 6B) (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). Both the GFP-Baz A-X-A and A-A-T mutant localize similarly to the phospho-mimetic (Figures 6C, 6D, and 6I) and/or removing inhibitory elements from CR3 induces phosphorylation of Baz (as observed in vitro; Figure 5) and therefore results in its localization to AJs rather than stable Par complex formation at the apical membrane.

It was important to distinguish between whether the relocation of the Baz A-X-A or A-A-T mutants was due to exposure of the S980 site and phosphorylation as shown in vitro for Par3cr3, or simply due to a lack of interaction with aPKC. A combined A-X-A + A-A-T site mutation in vitro showed a complete loss of interaction of CR3 with PKC\(_{\alpha}\) and no phosphorylation of serine 827 (Figure 5A). An equivalent GFP-Baz A-X-A + A-A-T mutant also localized to AJs (Figures 6H, 6I, and 6M). Interestingly, this combined mutant showed distinct intracellular puncta in which the Baz-GFP mutant no longer overlapped with aPKC, suggesting that both proteins are mutually exclusive on the same membrane (Figure 6N).

We then sought to verify whether the A-X-A Baz mutant was indeed phosphorylated in *Drosophila* cells. Available phospho-antibodies against Par3 S827 and Baz S980 were previously raised against an epitope that included the F-X-R motif and therefore could not detect the Baz A-X-A mutant or its phosphorylation status (data not shown). Efforts to raise a Baz phospho-antibody against S980 peptides excluding the F-X-R motif were not successful. Therefore, we verified that the A-X-A Baz mutant was phosphorylated in *Drosophila* cells, by preparing transfected S2 cell extracts containing wild-type or A-X-A mutant Baz and probed S980\(^{\text{Baz}}\) phosphorylation status using dimethyl labeling and mass spectrometry. Previous efforts to identify the Bazcr3 phospho-site in wild-type and A-X-A mutant contexts using trypsin digest were unsuccessful due to cleavage at R979 and K984, yielding very short peptides. Therefore, Bazcr3 samples were first treated by in-gel reductive dimethylation, to generate the Bazcr3 peptide spanning the sequence (phospho)SISE(me2K)HHAALDAR. The dimethylation reaction modifies lysine ε-amino groups, thereby greatly reducing the ability of trypsin to cleave after lysines. This allowed capture of the phospho-Bazcr3 peptide, facilitated quantification of chromatographic peak areas, and identified phospho-peptides. The forward sample reaction used heavy (CD\(_2\)O with wild-type Baz mutant) or light (CH\(_2\)O with A-X-A Baz) reagents, reagents, resulting in a mass difference of 12 Da and an \(m/z\) difference of 4 for the triply charged target peptide (Figures S6A and S6B). The reverse sample used heavy (CD\(_2\)O with A-X-A Baz mutant) or light (CH\(_2\)O with wild-type Baz) reagents, and two control peptides were also used to test any differences in peptide recovery from the SDS-PAGE gel (Figures S6A and S6C). Recovery was poorer for all heavy-labeled reverse samples including both controls, although the data clearly showed that both wild-type and A-X-A Baz proteins were phosphorylated at S980\(^{\text{Baz}}\) (Figures S6C and S6D). Taken together, our data suggest that the A-X-A mutant can be phosphorylated by aPKC in vitro in HCT-116 and S2 cells. Moreover, it can be distinguished from the A-X-A + A-A-T combined mutant that is no longer a substrate for aPKC and fails to interact with it both in vitro and in vivo.

We next tested the idea that phosphorylation of Par3/Baz controls its localization simply by feeding back to block its binding to aPKC (as observed for phospho-Par3cr3 in vitro; Figure 5). A GFP-tagged phospho-mutant form of Baz (GFP-Baz S980A) is known to fail to localize to junctions and instead co-localizes perfectly with aPKC (Figures 6E, 6J, and S7) (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). We find that expression of this construct also disrupts cellular morphology, consistent with previously reported data (Morais-de-Sa et al., 2010). If the localization and morphology phenotypes of GFP-Baz S980A are caused by tight inhibitory binding to aPKC, it should be
Figure 6. Switching Par3/Baz from Apical to Junctional In Vivo

(A) GFP-tagged Par3/Baz (green) localizes to the apical domain (marked by aPKC in red) and also to AJs.

(B) Phosphomimic GFP-tagged Par3/Baz S980E (green) is largely excluded from the apical domain (marked by aPKC in red) and localizes to AJs.

(C) Low-affinity GFP-tagged Par3/Baz F-X-R to A-X-A mutant (green) is largely excluded from the apical domain (marked by aPKC in red) and localizes to AJs.

(D) Low-affinity GFP-tagged Par3/Baz K-H to A-A mutant (green) is largely excluded from the apical domain (marked by aPKC in red) and localizes to AJs.

(E) Phospho-mutant GFP-tagged Par3/Baz S980A mutant (green) co-localizes apically with aPKC (red) and also partially disrupts cell polarity, consistent with its inhibitory function. See also Figure S7 for evidence that Baz co-localizes with aPKC in the absence of kinase activity.

(F) Phospho-mutant GFP-tagged Par3/Baz S980A that also carries the F-X-R to A-X-A mutation (green) fails to co-localize with aPKC (red) and instead localizes to AJs.

(G) Phospho-mutant GFP-tagged Par3/Baz S980A that also carries the K-H to A-A mutation (green) fails to co-localize with aPKC (red) and instead localizes to AJs.

(H) The double mutant K-H to A-A and F-X-R to A-X-A localizes primarily to AJs.

(I) Apical section of GFP-BazAXA expressing follicle cell epithelium, showing junctional localization.

(J–M) Apical section of GFP-BazS980A (J) expressing follicle cell epithelium, showing mis-localization to the apical surface. Apical sections of (K) GFP-BazAXA S980A, (L) GFP-BazAA S980A, and (M) GFP-BazAA AXA-expressing follicle cell epithelium, showing restoration of junctional localization.

(N) Non-overlapping punctate localization of GFP-BazAXA AA (green) with aPKC (red). DAPI staining is shown in blue in (A)–(H) and (N). GFP-tagged Par3/Baz is shown in (A)–(H).
possible to reverse these phenotypes by introducing either the F-X-R or K-H-T site mutation to lower the affinity of this interaction. Accordingly, we find that GFP-Baz A-X-A or A-A-T S980A double mutants fail to co-localize with aPKC and instead localize to AJs and do not show polarity defects (Figures 6F–6L). These results strongly support the view that phosphorylation of Par3/Baz controls its localization through lowering its binding affinity for aPKC, because the phenotypic consequence of loss of phosphorylation can be reversed by mutations that reduce affinity. Consistent with our in vitro data, we propose that access to the phosphorylation site within Par3/Baz is in turn controlled by modulation of the high-affinity and inhibitory arms of the CR3 region.

**DISCUSSION**

Our results reveal the molecular basis for Par3 antagonism of aPKC through high-affinity inhibitory CR3 arm interactions that span both N and C lobes of the PKCζ kinase domain. Our structural and biochemical data provide a model that supports a mechanism explaining apical-junctional polarization of Par3/Baz in epithelial cells (Figure 7). Previous work has shown that...
apical localization of Par3/Baz depends on it being part of the Par complex, where Par3/Baz is not phosphorylated by aPKC, while junctional localization of Par3/Baz occurs when it is phosphorylated by aPKC (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). Formation of the Par complex with Par3/Baz is known to be crucial for apical membrane recruitment of aPKC-Par6 (Gao et al., 2002).

Here we provide an explanation for why Par3/Baz is not phosphorylated while engaged within the Par complex even though it can be phosphorylated when it separates from the Par complex. Our crystal structure of the Par3 CR3–PKCi kinase domain interaction reveals the basis for high-affinity Par3 Parsons contacts through the coordinated action of two short motifs flanking the PKC consensus motif R-X-S827-Ψ. Together these motifs cooperate to inhibit aPKC, one bringing high affinity and the other enabling inhibitory contacts. The observation that the same N-lobe pocket is closed in structures of partially primed PKCiPar3–1P with a peptide resembling an aPKC-substrate interaction supports a second mode of engagement of Par3. Access to this N-lobe pocket may dictate whether aPKC-interacting proteins with an R+5 hook can inhibit aPKC or are phosphorylated as substrates. The location of the pocket adjacent to the aC helix and the aPKC activation loop suggests a potential mechanism to regulate the decision to engage and phosphorylate or to be sensitized to Par3 CR3 inhibition in the case of a fully primed active aPKC conformer.

The precise mechanism determining this switch requires further study and is beyond the scope of these investigations. Potential regulatory influences could include the availability of Par3 inhibitory arms, competition with other aPKC substrates, the presence of binding partners adjacent to the aPKC aC helix, post-translational modifications of the Par3CR3 region (such as T833Par3 phosphorylation by the ROCK kinase), or even regulation of the aPKC activation loop (by PKD1 or by dephosphorylation by an unknown A-loop phosphatase). One or more of these factors, depending on the physiopathological status of the epithelium, could affect the Par3-binding mode. We do note that Kibra (and its homolog WWC2) contains an F-X-R motif and a K-R-T hook flanking its known aPKC phosphorylation site (between residues 911 and 978), suggesting that it too could act as dual-action inhibitor/substrate, consistent with biochemical data (Yoshihama et al., 2011). Similarly, many known aPKC substrates have an adjacent F-X-R motif, for example, Par1, Par2, and ROCK kinase, suggesting that the F-X-R may provide specificity and an affinity boost to these validated aPKC substrates (Hurov et al., 2004; Ishiuchi and Takeichi, 2011; Motegi et al., 2011; Suzuki et al., 2004).

Our evidence indicates that engineered lower-affinity interactions between the Par3/Baz CR3 domain and the aPKC kinase domain result in Par3/Baz CR3 phosphorylation (Figures 5A–5C). Mutation of either the F-X-R or K-H-T site that our structure shows are important for a high-affinity inhibitory interaction leads to a relocalization of Par3/Baz away from the apical domain (where the Par complex resides) to AJs, similar to a phosphomimetic S980E mutant in Par3/Baz (Figures 6A–6D). Combining both mutations further lowers the affinity, leading to a form of Par3/Baz unable to engage aPKC that cannot be phosphorylated by it. Such a mutant Par3/Baz also relocalizes to AJs. Thus, Par3/Baz that fails to form a stable inhibitory Par complex will localize to AJs either through aPKC-mediated phosphorylation or through a loss of interaction.

Why does phosphorylation of Par3/Baz cause its localization to AJs? Our findings show that phospho-Par3/Baz dramatically reduces its affinity for aPKC and thus the phosphorylation event precludes it from joining the Par complex. Phosphomimic S980E Par3/Baz is known to localize to AJs, just like the affinity-lowering A-X-A or A-A-T mutants of Par3/Baz (Figure 6). Furthermore, the behavior of phospho-resistant mutant S980A Par3/Baz (which only localizes with aPKC) can be reversed in A-X-A S980A or A-A-T S980A double mutant Par3/Baz (which only localizes to AJs) (Figure 6). Previous studies have proposed that the AJ localization of Par3/Baz results from exclusion from the apical domain upon aPKC phosphorylation combined with exclusion from the basolateral domain upon Par1 phosphorylation (Tepass, 2012). Taken together, our data stimulate a model in which aPKC-driven phosphorylation of Par3/Baz can be recapitulated simply by weakening the Par3/Baz interaction affinity by manipulating the sequences flanking the consensus PKC phosphorylation site (Figure 7).

Our findings implicate both Par3/Baz and Kibra as aPKC inhibitors that are also known substrates. An analogous situation arises for LGL, a known inhibitor of aPKC that is also phosphorylated at multiple serine sites (Bailey and Prehoda, 2015). There are also precedents for protein kinase dual-action inhibitor/substrates. The protein kinase A (PKA) regulatory subunit RIIα has an RRXS motif that is phosphorylated by PKA, leading to its stable association with and inhibition of PKA (Zhang et al., 2015). In this context the modification functions as part of a single-turnover phosphoryl transfer reaction. For Par3 and other F-X-R-containing proteins, a different role is likely whereby phosphorylation by aPKC provokes Par3/Baz dissociation, as shown in vitro using CR3 peptides and in vivo using phospho-mimetics. Another example is the cyclin-dependent kinase inhibitors p21/p27/KIP, which are able to both inhibit cyclin-dependent kinases as well as being efficient substrates (Russo et al., 1996).

Our findings suggest that aPKC is inhibited by Par3/Baz within the Par complex, yet it is known that the Par complex contains active aPKC kinase and can phosphorylate many substrates (such as Lgl and Par1 in Drosophila epithelia and Caenorhabditis elegans zygotes, and Miranda in Drosophila neuroblasts). One possible explanation for this open issue is that discrete functional states of the Par complex (Par6–aPKC-Cdc42–Par3) may exist. Par3-dependent recruitment of aPKC to apical membranes may evoke a higher-order oligomer consistent with known Par3 CR1-dependent oligomers (Benton and St Johnston, 2003a). Conversely, phosphorylation of T833Par3 by ROCK kinase or a lack of T412PKC phosphorylation by PDK1 would generate functionally distinct forms of the Par complex, unable to be inhibited by Par3 CR3 as discussed earlier. Equally, association of partner proteins close to the aPKC aC helix could also block the formation of an R+5 pocket and prevent CR3-mediated inhibition. Therefore, further experiments are required to characterize precisely which polarity signal(s) provoke Par3 phosphorylation by overcoming CR3 antagonism.

In conclusion, our findings provide a molecular basis for Par3-mediated antagonism of aPKC that affects apical versus junctional polarization of Par3/Baz in epithelia.
EXPERIMENTAL PROCEDURES

Protein Construct Design, Expression, and Purification
Mammalian plasmids pEGFP-PKCΔ382W, pEGFP-PKCΔ382D (D339A/D3382A), p-His-PKCΔ382 (Addgene, plasmid 19388), p-myc-PARP-3 (AXA (F818A/R822A), and p-myc-PARP-3 (S827A) included human PKCζ and Par3 cDNAs. Mutagenesis of PKCζ and Par3 was performed using QuikChange (Stratagene). Recombinant human PKCζ- iota kinase domain (PKCζKD) was prepared using a baculovirus encoding residues 248–596 (GenBank: NM_002740.5), fused to a glutathione S-transferase (GST) tag as described previously (Kjaer et al., 2013). See Supplemental Experimental Procedures for a detailed description. In brief, the protein was expressed in Hi5 cells by co-infection with the above virus and a PDK-1 virus using standard protocols (Oxford Expression Technology). The GST tag was used for affinity purification and removed by 3C protease cleavage using standard protocols. Two distinct phospho-species PKCζKD-2P and PKCζKD-1P were separated by ion-exchange chromatography (Hi-Trap Q column, GE Healthcare).

Enzymatic Assay and Fluorescence Anisotropy Binding Assay
The ADP–Kiss (DiscoverRx) was used to determine the Kcat app and KM app values for ATP against the PKCζKD-1P and PKCζKD-2P using a series of synthetic peptide substrates as described by Kjaer et al. (2013). The reactions were measured every 2 min for 30 min in a 384-well plate using a Safire plate reader (Tecan). The kinetic constants were determined by fitting the data to the Michaelis-Menten equation. Data are represented as mean ± SEM. Fluorescence anisotropy assays were performed to determine the Kd for each peptide labeled with a fluorescence tag following standard protocols using a Safire plate reader (Tecan). The anisotropy values were normalized and the Kd was determined using non-linear regression. All experiments were performed in triplicate and for at least three independent protein preparations.

Structure Determination of Nucleotide-Bound PKCζKD-2P Complexes
PKCζKD-2P was incubated with a 3-molar excess of nucleotide or analog with either Mg2+ or Mn2+ (see Table 1) and a 3-molar excess of peptide. Crystallization was performed using the hanging-drops method with 1:1 ratio of protein to reservoir solution (Oxford Expression Technology). The GST tag was used for affinity purification and removed by 3C protease cleavage using standard protocols. Two distinct phospho-species PKCζKD-2P and PKCζKD-1P were separated by ion-exchange chromatography (Hi-Trap Q column, GE Healthcare).

Cell Culture and Transfection
HCT-116 cells grown in McCoy’s 5A medium containing 10% bovine fetal calf serum and penicillin/streptomycin (Invitrogen) were transfected (10–20 μg DNA/20 μg lipofectamine) with the above virus and a PDK-1 virus using standard protocols (Oxford Expression Technology). Ovaries were dissected in PBS, fixed for 20 min in 4% paraformaldehyde in PBS, washed for 30 min in PBS/0.1% Triton X-100 (PBST), and blocked for 15 min in 5% normal goat serum/PBST (PBST/NGS). Primary antibodies were diluted in PBST/NGS and samples were incubated overnight at 4°C. Either optical cross-sections through the middle of egg chambers or apical sections of the follicular epithelium are shown. Primary antibodies used are described in Supplemental Experimental Procedures.

Cell Culture and Transfection
HCT-116 cells grown in McCoy’s 5A medium containing 10% bovine fetal calf serum and penicillin/streptomycin (Invitrogen) were transfected (10–20 μg DNA/20 μg lipofectamine) using a FuGene HD (Roche) according to the manufacturer’s instructions. The cells were then grown in normal medium for 36 hr.

Antibodies, Immunoprecipitation, and Immunoblotting
The following antibodies were used for immunoblotting: mouse monoclonal anti-PKCζ (recognition for human PKCζ), mouse monoclonal anti-Myc (9E10, Millipore), and rabbit polyclonal anti-GFP antibody (Santa Cruz Biotechnology). Anti-phospho-S827 Par3 antibody was raised in-house using an antigen lacking the F-X-R site of Par3. The Anti-phospho-Par3 antibody (Santa Cruz Biotechnology) was purified in-house using an antigen lacking the F-X-R site of Par3CR3. Immunoprecipitation and immunoblotting was carried out as described in Supplemental Experimental Procedures.

Dimethyl Labeling and Quantification of Bazooka Wild-Type and Mutant S980 Phosphorylation in S2 Cell Extracts
After SDS-PAGE, in-gel stable isotope dimethyl labeling was performed according to published protocols. The heavy reaction was performed using 13CD3O formaldehyde creating a mass difference of 6 Da per primary amine group between heavy and light dimethylated peptides. After extensive washing of gel pieces, the in-gel dimethylated proteins were then subjected to overnight in-gel trypsin digestion at 37°C. The following day peptides were extracted and subjected to another round of reductive dimethylation reactions aimed at methylating peptide N termini. Peptide mixtures were acidified and prepared for liquid chromatography-mass spectrometry analysis using an UltiMate 3000 high-performance liquid chromatograph coupled to a Q-Exactive mass spectrometer (Thermo Fisher). A targeted scan was performed for the S980-containing peptides and this was alternated with a top-10 data-dependent acquisition scan. Mascot-generated DAT files were converted to Skyline-compatible biblio.spec libraries, and heavy and light peak areas were extracted by Skyline software version 2.5.0.6079 (MacLean et al., 2010).

Accession Numbers
Coordinates and structure factors for PKCζKD-2P-Par3CR3, PKCζKD-2P-FXR2P180, and nucleotide-bound PKCζKD-2P have been deposited in the PDB with the accession numbers PDB: 5LI1, 5LIH, and 5LI9, respectively.

Supplemental Information
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.07.018.

Author Contributions
E.V.S. performed all stages of the Mg-AMPNP-Par3CR3-PKCζKD-2P peptide structure determination and refinement. A.P. refined the Mn-ADP-PKCDKD-FXR2P180 peptide structure. M.I. purified, crystallized, and refined the AMPPPCP-complexed PKCζKD-2P structure. E.V.S. and M.I. carried out kinetic assays and fluorescence anisotropy measurements. P.K. and S.K. assisted with protein production and virus preparation. A.E. prepared S2 cell extracts containing wild-type and mutant Baz protein. K.B. and B.S. performed the dimethyl-labeling and mass spectrometry analyses. B.K. prepared and characterized recombinant PKCζKD and PKCζKD proteins. P.S. prepared constructs for full-length human Par3 and mutants. N.O’R. purified all peptides used in this study. P.R. conducted co-immunoprecipitations using full-length par3 constructs for full-length human Par3 and mutants. N.O’R. performed all peptides used in this study. P.R. conducted co-immunoprecipitations using full-length Par3 with full-length PKCζ and raised the anti-phospho-Par3 antibody. M.L. prepared the PKCζ D/D mutant. G.F. and B.J.T. carried out all the Drosophila experiments. M.I. prepared the figures. N.O.M. and B.J.T. planned the project, designed experiments, and wrote the paper.

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