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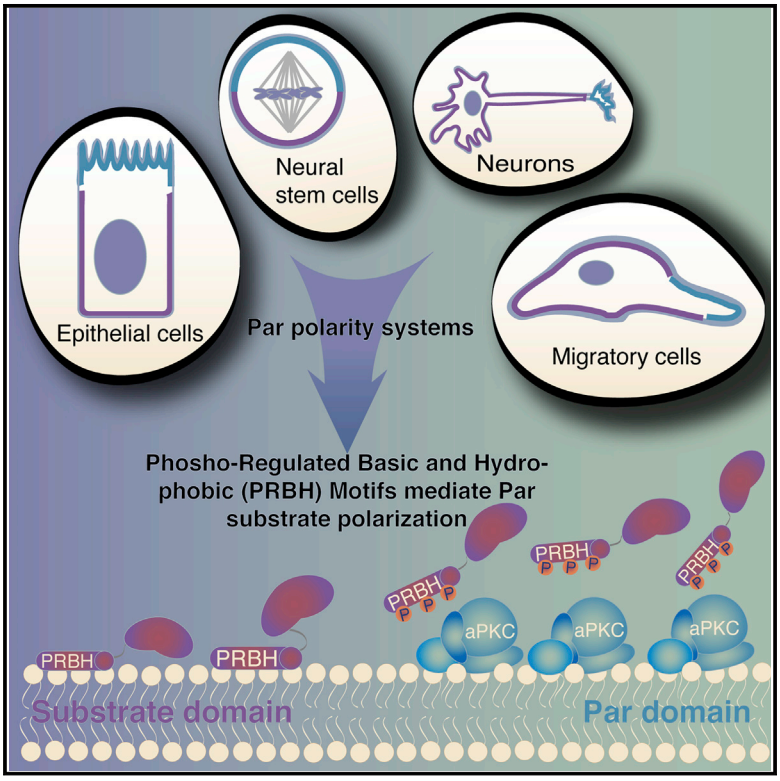
Developmental Cell

Establishment of Par-Polarized Cortical Domains via Phosphoregulated Membrane Motifs

Graphical Abstract



Highlights

- Diverse Par complex substrates directly bind phospholipids
- aPKC phosphorylation neutralizes substrate lipid binding
- Phosphorylation-induced displacement excludes substrates from Par cortical domain
- Many candidate phosphoregulatory motifs are present in animal proteomes

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In Brief

The mechanism of Par substrate localization and polarization has remained elusive. Bailey and Prehoda identify a phosphorylation-regulated motif that mediates cortical localization and also aPKC-mediated displacement of diverse Par substrates from the cortex. A bioinformatic screen demonstrates that candidate phosphoregulated membrane-association motifs are common in animal proteomes.

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Establishment of Par-Polarized Cortical Domains via Phosphoregulated Membrane Motifs

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SUMMARY

The Par polarity complex creates mutually exclusive cortical domains in diverse animal cells. Activity of the atypical protein kinase C (aPKC) is a key output of the Par complex as phosphorylation removes substrates from the Par domain. Here, we investigate how diverse, apparently unrelated Par substrates couple phosphorylation to cortical displacement. Each protein contains a basic and hydrophobic (BH) motif that interacts directly with phospholipids and also overlaps with aPKC phosphorylation sites. Phosphorylation alters the electrostatic character of the sequence, inhibiting interaction with phospholipids and the cell cortex. We searched for overlapping BH and aPKC phosphorylation site motifs (i.e., putative phosphoregulated BH motifs) in several animal proteomes. Candidate proteins with strong PRBH signals associated with the cell cortex but were displaced into the cytoplasm by aPKC. These findings demonstrate a potentially general mechanism for exclusion of proteins from the Par cortical domain in polarized cells.

INTRODUCTION

Animal cells are polarized in remarkably diverse ways, such as the specialized apical cortex of a simple epithelium and the leading edge of motile cells (Knoblich, 2010; Overeem et al., 2015; Tepass, 2012). Although these cells are dramatically different, they are polarized by the same molecular machinery known as the Par complex (Goehring, 2014; Goldstein and Macara, 2007). The capacity of the Par complex to direct diverse cell polarities derives in part from its ability to act on a multitude of downstream proteins. For example, in asymmetrically dividing neural stem cells, the Par complex polarizes fate determinants (Betschinger et al., 2003; Knoblich, 2010), whereas in epithelia it organizes junctional components (Suzuki et al., 2001; Tepass, 2012). The characteristic adaptability of the Par complex to regulate distinct classes of proteins suggests that there may be a common mechanism by which it acts on downstream factors, yet little is known about how Par activity is coupled to substrate polarity. Knowledge of this mechanism is important not only for our basic understanding of Par-mediated polarity, but it might also allow for the identification of novel Par-regulated proteins and provide insight into the evolutionary pathways underlying the wide range of polarities found among metazoa.

The Par complex polarizes cells by creating and maintaining mutually exclusive cortical domains. Upstream factors that can be cell-type-specific define the Par domain (Cuenca et al., 2003; Harris and Peifer, 2005; Martin-Belmonte et al., 2007; Rolls et al., 2003; Schober et al., 1999; Wodarz et al., 1999), and downstream proteins are excluded from this cortical area to create the substrate domain. While the Par complex can function through other mechanisms (Cline and Nelson, 2007; von Stein et al., 2005; Zhang and Macara, 2006), a key output is the activity of atypical protein kinase C (aPKC). Proteins that are directly downstream of the Par complex are often aPKC substrates, and phosphorylation is both necessary and sufficient for cortical displacement and concomitant removal from the Par domain (Atwood and Prehoda, 2009; Betschinger et al., 2003; Hao et al., 2006; Hurov et al., 2004; Smith et al., 2007; Suzuki et al., 2004; Wirtz-Peitz et al., 2008; Zhang et al., 2001). For proteins polarized by this mechanism, their phosphorylation must be coupled to release from the cortex. Although phosphorylation-coupled cortical release is critical to Par complex-mediated polarity, very little is known about the mechanisms by which Par substrates associate with the cortex and how phosphorylation is linked to this interaction. The lack of sequence homology or shared globular domain structure among Par substrates has made it difficult to identify a "polarity code."

The Par complex functions at the cell cortex, a complex organelle that includes the phospholipid bilayer and a meshwork of membrane-associated proteins and cytoskeletal elements beneath it (Engelman, 2005; Groves and Kuriyan, 2010; Morone et al., 2006). It has been unclear how these components might contribute to Par-mediated polarity, although actin polymerization is known to be required and protein-protein interactions have been implicated for certain substrates (Betschinger et al., 2005; Dho et al., 1999; Knoblich et al., 1997; Strand et al., 1994). Direct lipid binding has only been demonstrated for Numb PTB domain, however, this is not sufficient for cortical localization (Dho et al., 1999). Given the complexity of the cell cortex and the potential for diverse protein-phospholipid and protein-protein interactions, we set out to identify the interactions that retain Par-polarized proteins within their cortical domain and how aPKC phosphorylation regulates these cortical interactions to prevent entry into the Par domain.

RESULTS

Short, Charged, Hydrophobic Motifs Target Lgl, Mira, and Numb to the Cell Cortex

To determine if there might be a general mechanism of Par complex polarization, we selected three substrates with no

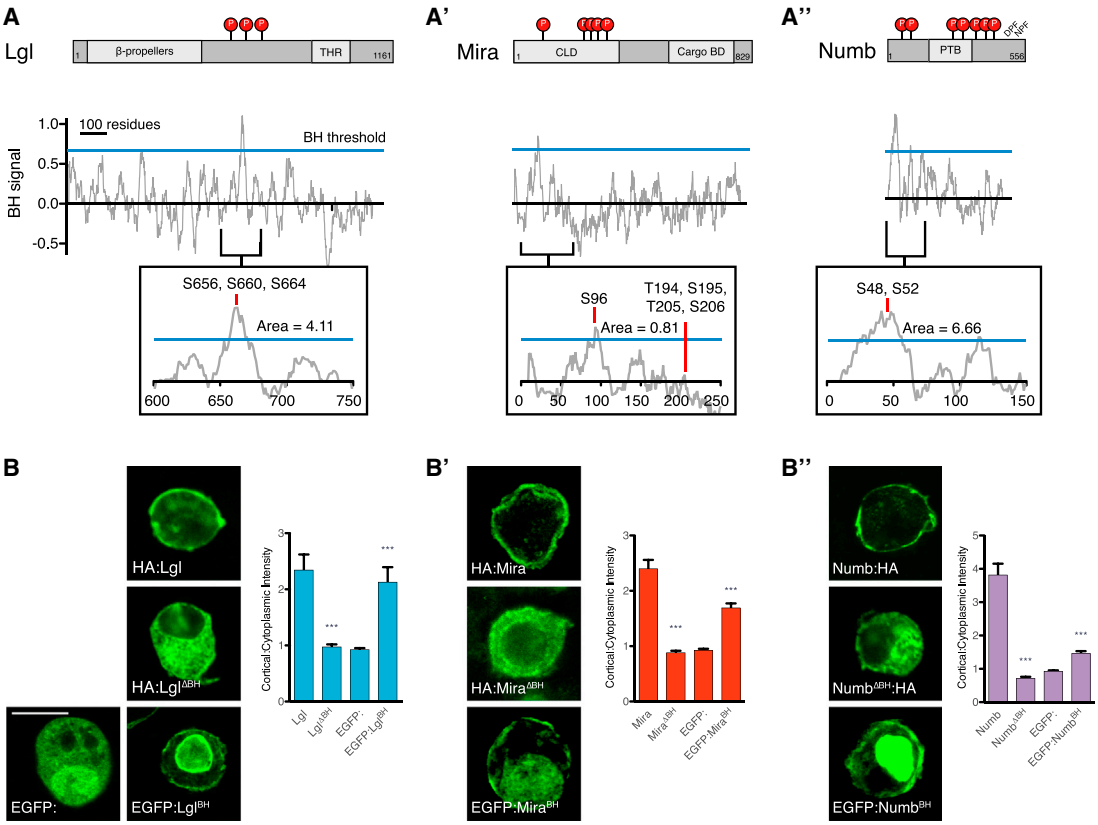

Figure 1. Lgl, Mira, and Numb Localize to the Cell Cortex through BH Motifs

(A–A″) Basic hydrophobic motif signal in Lgl (A), Mira (A′), and Numb (A″). Previous work identified domains with multiple potential cortical targeting domains in Lgl, Mira, and Numb. The BH-search algorithm was used to analyze the sequences of *Drosophila* Lgl, Mira, and Numb. The BH signal is shown across each protein's domain architecture. BH motifs are defined by peaks with a BH signal >0.6 (denoted by blue line) and the peak area indicates the strength of the peak's BH character. Each protein has a single BH motif that contains at least one aPKC phosphorylation site. THR, tomosyn homology region; CLD, cortical localization domain; Cargo BD, cargo binding domain; PTB, phosphotyrosine binding domain; DPF, α-adaptin-binding motif; NPF, Eps15-binding motif.

(B–B″) BH motifs are necessary and sufficient for cortical targeting of Lgl (B), Mira (B′), and Numb (B″). Deletion constructs were used to determine if the BH motif is necessary and sufficient for localization to the S2 cell cortex. Representative images of the localization are presented for each protein when transiently transfected in S2 cells and characterized by either immunofluorescence for the HA epitope tag or by EGFP fluorescence. Localization to the cell cortex was quantified as an intensity ratio for the cortex versus the cytoplasm. At least 17 cells were quantified and the mean and the SEM are shown. Asterisks indicate $p < 0.0001$ as assessed by a t test to compare each ΔBH construct to its respective full-length control or each EGFP-fusion to its EGFP control. Scale bar, 10 μm.

apparent sequence homology or shared domain structure: Lgl, Mira, and Numb (Figures 1A–1A″) (Betschinger et al., 2005; Dho et al., 2006; Fuerstenberg et al., 1998; Matsuzaki et al., 1998). Lgl and Numb each have protein interaction domains: β-propellers, a tomosyn homology region (THR), and a phosphotyrosine-binding (PTB) domain, but they are not sufficient for cortical association in *Drosophila* (Betschinger et al., 2005; Knoblich et al., 1997). Cortical localization of Mira is specified by its NH_2-terminal 1–290 amino acids (Fuerstenberg et al., 1998; Matsuzaki et al., 1998), which does not contain any recognizable globular domains. These three diverse Par complex substrates lack clear globular domains that would mediate interactions with the cortex or membrane (Lemmon, 2008). Short stretches of basic and hydrophobic (BH) amino acids are known to interact with the membrane, so we analyzed the basic and hydrophobic character of the sequences using the BH scoring algorithm (Brzeska et al., 2010). Sequence windows with BH scores exceeding 0.6 are candidate phospholipid binding motifs and we found that Lgl, Mira, and Numb each contain a single region above this threshold (Figures 1A–1A″). Interestingly, each of these sequences overlaps with a known or predicted aPKC phosphorylation site (Atwood and Prehoda, 2009; Betschinger et al., 2003; Smith et al., 2007). We identified several aPKC sites with BH scores below the threshold, indicating that not all aPKC sites are BH motifs (see below for a more extensive comparison of BH motifs and the aPKC recognition sequence).

Given their short sequences, the BH scoring algorithm could have a high false positive rate, so we investigated whether the Lgl, Mira, and Numb BH motifs mediate cortical localization. Each of these proteins localizes to the cortex of cultured *Drosophila* S2 cells consistent with their ability to interact with the cortex opposite Par domains. Deletion of each protein's candidate BH motif caused a loss of cortical enrichment and strong cytoplasmic signal (Figures 1B–1B″). In some cases, BH

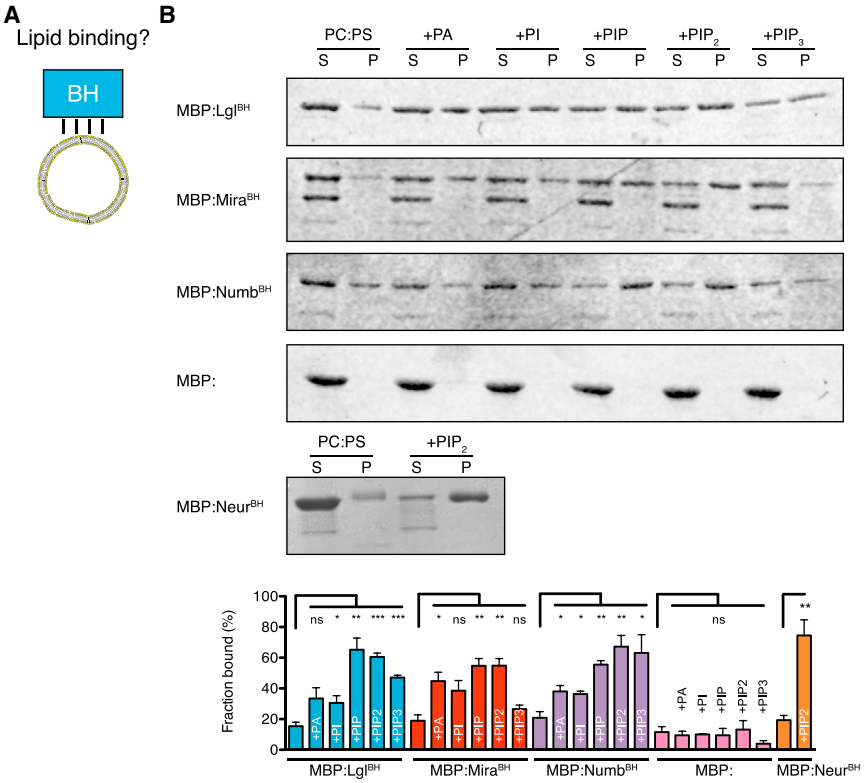

Figure 2. Phospholipid Binding Mediates Localization to the Cell Cortex

(A) BH motif phospholipid binding was tested using a cosedimentation phospholipid binding assay.

(B) The BH motifs of Lgl, Mira, and Numb bind directly to phospholipid vesicles. A representative Coomassie-stained SDS-PAGE gel from a lipid vesicle-binding cosedimentation assays is presented. Each protein was characterized with an NH_2-terminal maltose binding protein (MBP) fusion. The BH motif from Neuralized (Neur residues 68–88) was used as a positive control. The supernatant (S) and pellet (P) contain the unbound and bound fractions, respectively. All vesicles contained a 4-1 mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) to 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (PS). Lanes marked by +X contained a third lipid that was added to be 10% of the total lipid by mass. PA, L-α-phosphatidic acid; PI, L-α-phosphatidylinositol; PI4P, L-α-phosphatidylinositol-4-phosphate; PIP_2, phosphatidlyinositol-4,5-bisphosphate; PIP_3, 1,2-dioleoyl-*sn*-glycero-3-[phosphoinositol-3,4,5-trisphosphate]. The fraction bound expressed as a percentage is shown in the bottom panel. Error bars represent SEM from three independent measurements. Significance levels, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

See also Figure S1 for sequence analysis of Neur BH motif.

motifs often require additional multivalent interactions to associate with the membrane (Papayannopoulos et al., 2005; Swierczynski and Blackshear, 1996; Winters et al., 2005), so we tested whether each could function on their own. When EGFP is attached to stabilize these short, unstructured sequences (~30–70 amino acids), each BH motif was enriched at the S2 cortex (Figures 1B–1B″), although at reduced levels compared to the full-length proteins. These data indicate that cortical localization of Lgl, Mira, and Numb is mediated by their BH motifs. Although additional interactions are likely needed to reinforce cortical recruitment, this appears to be a common mechanism for BH-mediated membrane association (McLaughlin and Murray, 2005; Rohatgi et al., 1999; Winters et al., 2005).

Lgl, Mira, and Numb BH Motifs Bind Directly to Phospholipids

The key role of the Lgl, Mira, and Numb BH motifs in cortical localization suggests that protein-phospholipid interactions are, at least in part, responsible for the cortical association of Par substrates. To test for direct protein-phospholipid interactions, we purified the BH motifs as maltose-binding protein (MBP) fusions and tested whether they associate with giant unilamellar vesicles (GUVs) of various lipid compositions via a co-sedimentation assay (Figure 2A). Using the BH motif from Neuralized (Neur) as a positive control (Skwarek et al., 2007), we found that each of the BH motifs from Lgl, Mira, and Numb exhibited very little binding to vesicles containing phosphatidylserine and phosphatidylcholine alone, but interacted with these vesicles when they were doped with various negatively charged phospholipids (Figures 2B and S1). Although phosphoinositides with multiple phosphates were slightly preferred over other negatively charged lipids (e.g., phosphatidic acid [PA]), the level of specificity is small enough that we do not expect it to be physiologically relevant.

Par Substrate Cortical Recruitment Is Mediated by Electrostatic Interactions

The BH motifs of Lgl, Mira, and Numb have multiple positively charged residues that may confer favorable electrostatics for binding phospholipids. Polybasic regions can bind negatively charged phospholipids, including PS and phosphoinositides. To investigate the role of charge in cortical targeting, we mutated basic residues within the BH motif to the acidic residue aspartic acid; these mutations reduced the calculated BH signal to often eliminate BH motif identification (Figures 3A–3B″). We found that mutations that acidify the BH motif greatly reduce cortical localization of the full-length protein in S2 cells (Figures 3C–3D″). Further, this effect was not site-specific because mutations at multiple sites along the BH motif caused similar localization phenotypes (Figure S2A).

Consistent with their effect on cortical localization, charge swap mutations reduced BH affinity for liposomes (Figures 3E–3F″). To further test the role of electrostatics, we measured liposome interactions in the presence of 500 mM KCl, which reduces the entropic cost of displacing ions from the bilayer surface (McLaughlin, 1989). High ionic strength reduced the interaction between phospholipids and each BH motif (Figure S2B), supporting the conclusion that direct, electrostatic interactions with phospholipids mediate BH motif cortical enrichment.

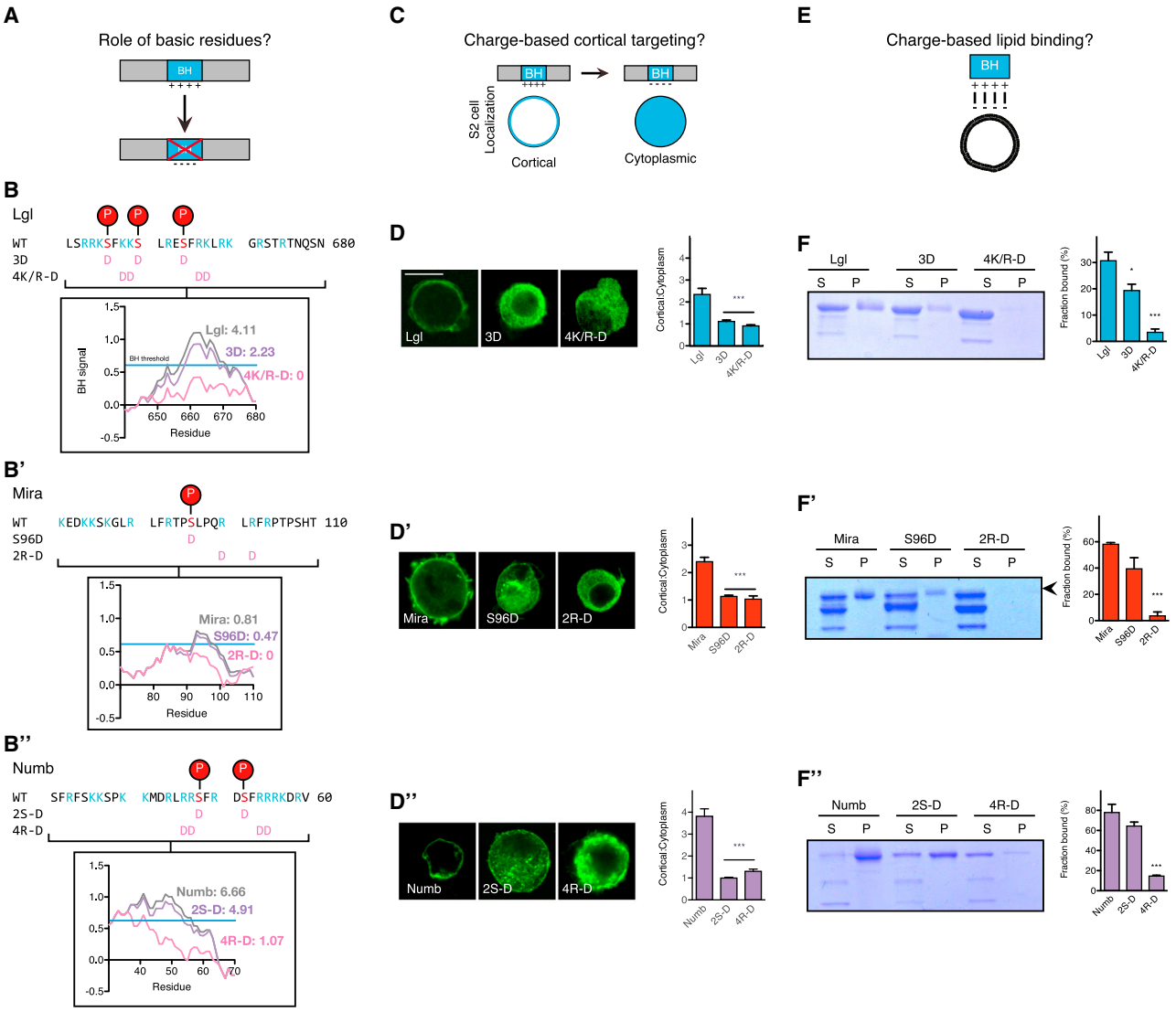

Figure 3. Electrostatic Interactions Mediate Lgl, Mira, and Numb Interactions with Phospholipids and the Cell Cortex

(A) BH motif point mutants test the role for charged residues in cortical localization.

(B–B″) Charged residues contribute to BH motif of Lgl (B), Mira (B′), and Numb (B″). The sequence of the BH motifs from Lgl, Mira, and Numb are displayed, highlighting basic residues (blue) and aPKC phosphosites (red). Terminology and location of the point mutants are displayed with pink aspartic acids. The inset shows the effect of each mutation on the BH signal and the area of the BH peak, as computed by BH-search. The BH threshold is displayed by a blue line with a BH signal of 0.6.

(C) S2 cell localization assay tested if BH motif charge mediates localization to the cell cortex.

(D–D″) Mutations to acidic residues reduce localization to the cell cortex in transiently transfected S2 cells. The localization of each full-length protein with point mutations was characterized by immunostaining for the HA epitope tag (located on each protein's NH_2 terminus), and the localization was quantified as a cortical to cytoplasmic signal intensity ratio for at least 16 cells (mean ± SEM). Scale bar, 10 μm. See also Figure S2A for additional point mutants.

(E) Lipid binding assays tested if BH motif charge disrupts phospholipid binding.

(F–F″) Acidic mutations reduce phospholipid binding. A Coomassie-stained gel from lipid vesicle binding sedimentation assays with vesicles of 4:1 PC:PS plus 10% PIP_2 is shown. S indicates supernatant and P the pellet fraction. Arrowheads mark the full MBP-BH protein while other bands are truncation products (e.g., MBP alone). The fraction of protein bound, quantified as the amount of protein in the pellet over total protein, was quantified in triplicate for each vesicle composition. The mean and SEM of the fraction bound are shown. $^{*}p < 0.05$, $^{***}p < 0.0001$. Significance was evaluated using a non-parametric t test relative to the unmutated BH motif.

See also Figure S2.

aPKC Phosphorylation Regulates BH Cortical Targeting and Phospholipid Binding

The Lgl, Mira, and Numb BH motifs contain verified and/or predicted aPKC phosphorylation sites. As charge swap mutations significantly reduce BH motif phospholipid binding and cortical association, we predicted that Par-induced polarization results when BH and aPKC phosphorylation site motifs are in close enough proximity that the phosphorylation(s) can sufficiently

influence BH electrostatics to reduce the affinity for the membrane. This would allow Par substrates to associate with the cortex via their BH motif (and any accessory interactions) in regions lacking the Par complex. However, upon entering the Par cortical domain, the BH motif would be phosphorylated by aPKC, altering electrostatic character and reducing membrane affinity. Consistent with this model, we observed that phosphomimetic Lgl BH had reduced PIP_2 binding, although no statistically significant difference was observed for Mira or Numb BH motifs (Figures 3F–3F″). However, expression of aPKC significantly reduced cortical enrichment of the motifs from all three proteins (Figures 4A–4B″), recapitulating the behavior of the full-length proteins. Non-phosphorylatable variants of each BH motif remain localized to the cell cortex when aPKC was expressed (Figures 4B–4B″ and S3A), indicating that phosphorylation is required for displacement. Furthermore, we found that aPKC phosphorylation inhibits BH interaction with PIP_2-containing vesicles, suggesting that disruption of this direct interaction is responsible for cortical displacement (Figures 4C–4D″). Addition of aPKC had no effect on non-phosphorylatable BH motif variants Lgl3A, MiraS96A, or NumbS48AS52A, in the absence of ATP, or with aPKC harboring a kinase dead mutation (K293W) (Figures S3B–S3D). The difference between the phosphomimetic and aPKC-phosphorylated proteins in binding PIP_2-containing vesicles likely arises from the higher negative charge density of the phosphorylated proteins. We conclude that aPKC displaces Lgl, Mira, and Numb from the cortex by phosphorylation inhibiting phospholipid binding.

A Bioinformatics Approach to Identifying Candidate PRBH Motifs

Our analysis of Lgl, Mira, and Numb suggests that phospho-regulated BH (PRBH) motifs mediate Par substrate polarization by coupling phosphorylation to membrane affinity. This coupling derives from the overlap of BH and aPKC phosphorylation motifs, and we used this criterion to search for putative PRBH motifs. We implemented an algorithm in the Python programming language (van Rossum, 2001) using the maximum BH score within a motif (Brzeska et al., 2010) along with a scoring system based on the aPKC phosphorylation site motif consensus sequence (Wang et al., 2012), modified to include Miranda (Figure 5A). We used the maximum BH score rather than the sum of BH scores within a motif to select for short, highly charged sequences like those found in Lgl, Miranda, and Numb. Additionally, to account for the observation that these sequences often contain multiple phosphorylations sites (Graybill and Prehoda, 2014), the PRBH score is increased by 0.2 times the number of phosphorylation sites. The weighting factor was set such that the BH and aPKC site score contributions to the overall PRBH score remained balanced. We used this algorithm to identify candidate PRBH motifs in several animal proteomes and numerically describe PRBH motifs by a maximum BH score (a metric of the basic character), aPKC site scores, and the number of aPKC sites identified (Figures 5B, 5C, S4A, and S4B; Table S1). The Lgl PRBH was identified as one of the top scoring motifs in the proteomes of human, fly, worm, and sponge, demonstrating that its charge and phosphosites are conserved in metazoan (Figures 5B, 5C, and S4A; Table S1). The algorithm also identified Numb and Mira, although with lower PRBH scores. It is important to note that for two reasons Par-polarized proteins likely represent a subset of PRBH-containing proteins. First, polarity is only one cellular process for which phosphorylation regulated membrane association is important. Second, as many kinases have overlapping specificity it is unlikely that all the identified PRBH motifs are solely aPKC substrates (especially those with low aPKC site scores). For example, two bona fide PRBH proteins regulated by the conventional PKC were identified: MARCKS and diacylglycerol kinase (DGK) (Luo et al., 2003; Topham et al., 1998; Überall et al., 1997). Finally, aPKC functions in other processes besides polarity (Farese et al., 2014; Standaert et al., 2001). While these effects increase the false positive rate for identifying polarity proteins, they also suggest that the algorithm will have utility outside of polarity.

To validate the algorithm, we selected several candidate PRBH motifs to determine if they are bona fide regulated membrane association elements. We selected candidates from the fly and human proteomes with a range of PRBH scores, aPKC site scores, and maximum BH scores (Figures 5B and 5C) to examine how well PRBH score correlates with activity. For each candidate, we tested if they can be phosphorylated by aPKC in vitro, whether they associate with the cortex in S2 cells, and whether aPKC can displace them into the cytoplasm. We found that each protein was indeed phosphorylated by aPKC in an in vitro kinase assay (Figure 5D). Membrane-associated guanylate kinase (MAGUK) p55 subfamily member 7 (MPP7) regulates tight junction formation (Stucke et al., 2007) and has a single, highly scored putative PRBH motif that includes two predicted aPKC phosphosites (Figures 5C and 5E). The candidate MPP7 PRBH was highly enriched at the cell cortex and aPKC expression inhibited cortical localization (Figure 5E). PIP82, a *Drosophila* protein involved in signal transduction downstream of photoreceptors (Suri et al., 1998), similarly contains two PRBH motifs, the stronger of which has high BH character and two predicted aPKC sites (Figures 5B and 5F). We observed that the PIP82 candidate PRBH sequence also localized to the S2 cell cortex and this localization was antagonized by aPKC (Figure 5F).

We also examined several lower scoring PRBH motifs to determine if they function as aPKC regulated cortical localization motifs. The putative PRBH motif on human casein kinase I γ (CKIγ-2) contains a strongly predicted aPKC site rated similar to MPP7, but has weak basic character as demonstrated by a low maximum BH height (Figures 5C and 5G). To our knowledge, its localization remain uncharacterized in polarized cells, but the Casein Kinase-associated protein Lrp6 localizes basolaterally in the *Xenopus* neuroectoderm (Davidson et al., 2005; Huang and Niehrs, 2014). CKIγ-2 PRBH localizes weakly to the cell cortex and has punctate localization suggesting it localizes to endomembrane organelles, as seen previously for Casein kinase (Tomishige et al., 2009). Expression of aPKC reduced the cortical localization of the CKIγ-2 candidate PRBH and did not alter the distribution of CKIγ-2 between the cortex and cytoplasm (Figure 5G). The human tumor suppressor Adenomatous polyposis coli membrane recruitment protein (Amer1, also known as WTX) represses WNT/β-catenin signaling (Grohmann et al., 2007; Rivera et al., 2007; Tanneberger et al., 2011); it contains a strong BH signal but a weak aPKC phosphosite score (Figures 5C and 5H). Correspondingly, we found that the Amer1 PRBH is targeted

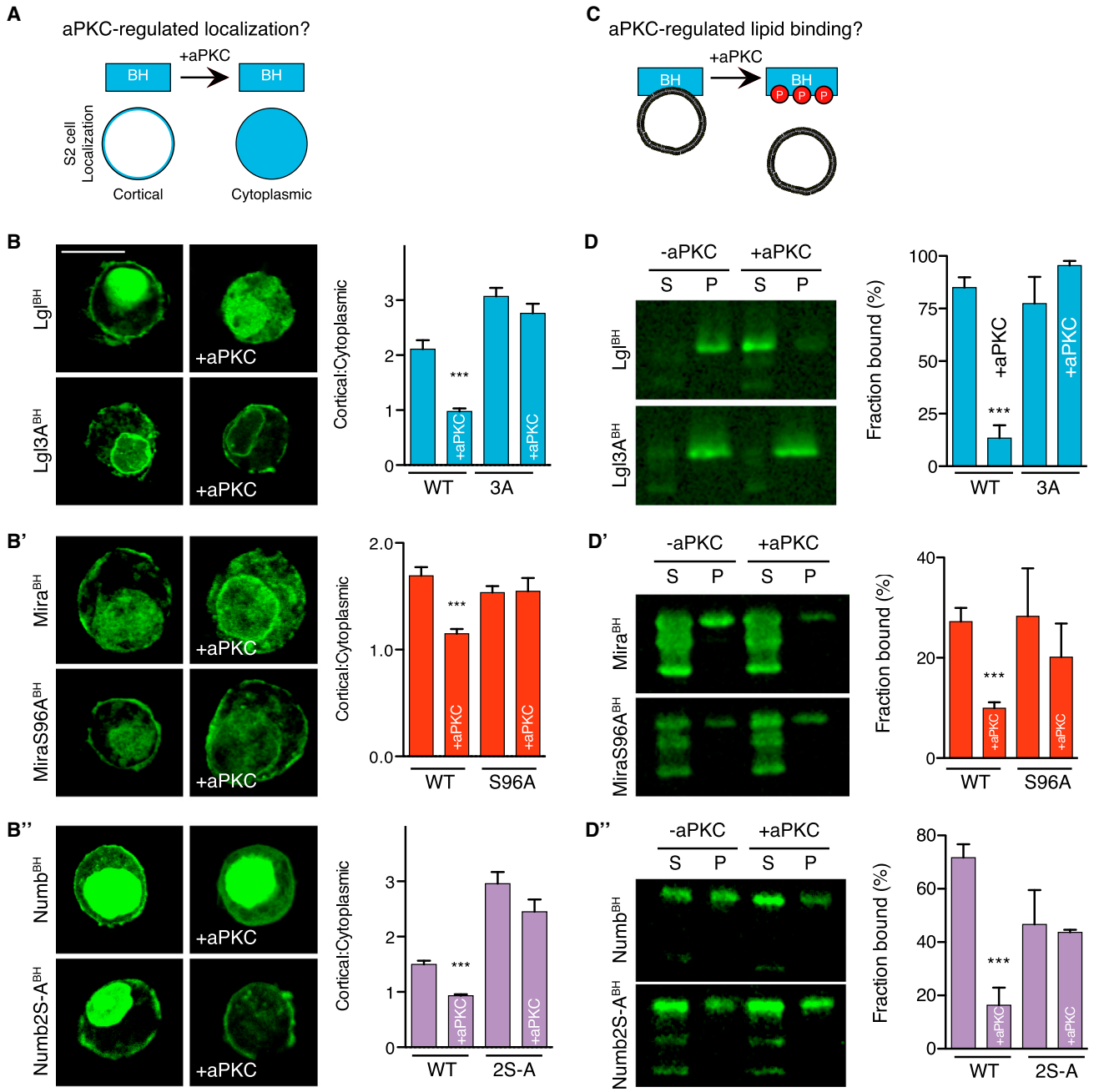

Figure 4. Phosphorylation Directly Inhibits Par Substrate BH Motif Interactions with Phospholipids and the Cell Cortex

(A) Schematic of aPKC regulation of BH motif cortical localization assay.

(B–B″) Phosphorylation by aPKC disrupts Lgl (B), Mira (B′), and Numb (B″) BH motif cortical enrichment. The localization of EGFP fused BH motifs both in the absence and presence (+aPKC) of aPKC is shown, along with that for BH motifs with phosphorylation sites mutated to alanine (3A, S96A, S48AS52A). Expression of aPKC was verified by immunostaining. Representative images from each transfection are shown. Expression of aPKC reduces cortical enrichment, and this reduction is dependent on the presence of the phosphorylation site. The mean and SEM are shown for measurements from at least 16 cells. Asterisks indicate $p < 0.0001$ as assessed by a non-parametric t test to compare each aPKC-expressing cell to its respective control without aPKC expression. Scale bar, 10 μm. See also Figure S3A.

(C) The effect of aPKC phosphorylation on phospholipid binding was tested with lipid cosedimentation assays.

(D–D″) Effect of aPKC on Par substrate BH motif binding to PC/PS/PIP_2 vesicles. Lipid-binding cosedimentation assays were performed in the presence of aPKC and ATP. Samples were analyzed by immunoblotting for the MBP tag. S marks the supernatant and P marks the pellet. The "S-to-A" mutations are: Lgl3A (D), MiraS96A (D′), and NumbS48AS52A (D″). All vesicles were composed of 4:1 PC:PS plus 10% PIP_2. The fraction of protein bound, quantified as the amount of protein in the pellet over total protein, was quantified in triplicate for each vesicle composition. The mean and SEM of the fraction bound are shown. *$p < 0.0001$. Significance was evaluated using a non-parametric t test relative to binding in the absence of aPKC.

See also Figures S3.

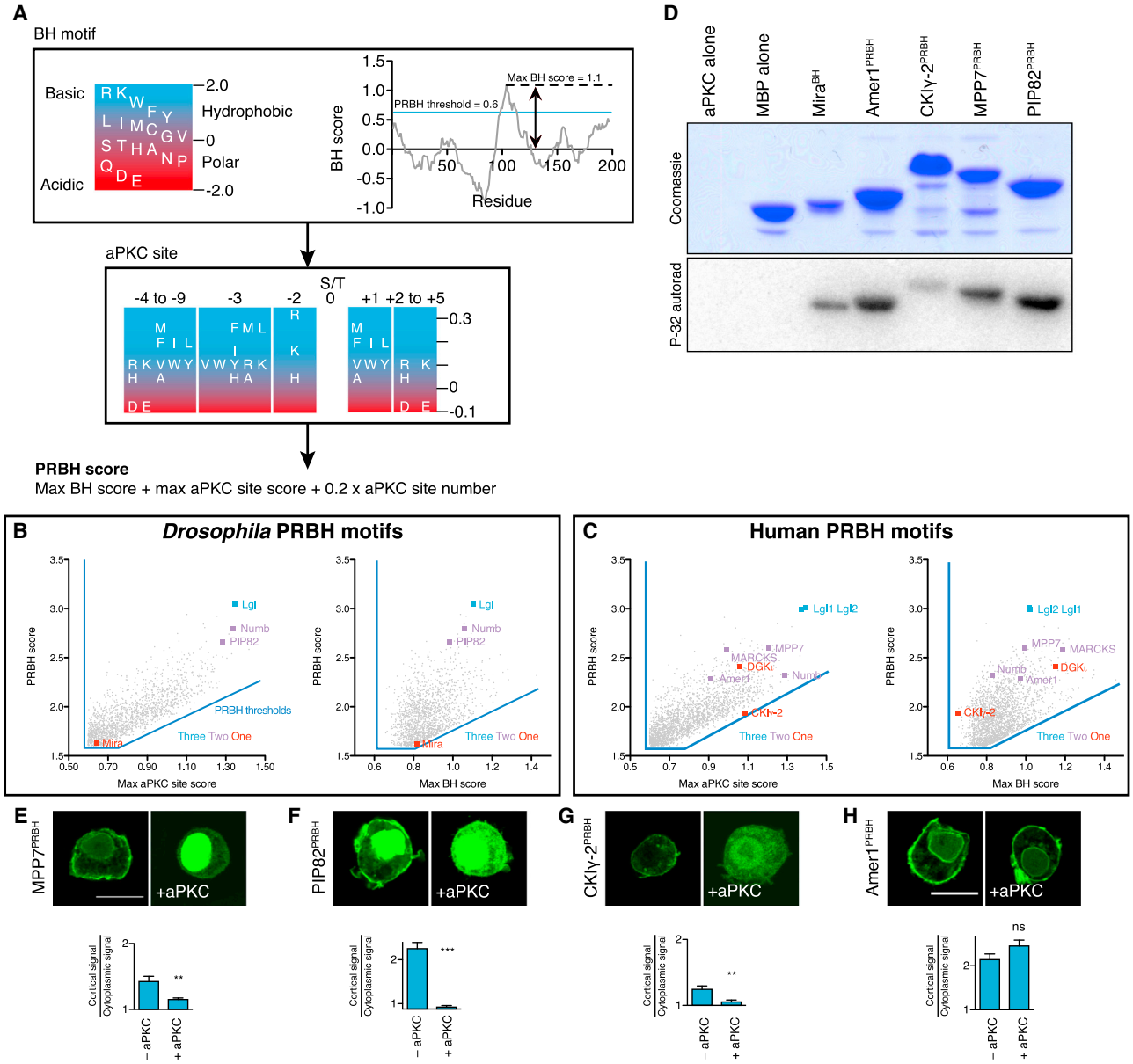

Figure 5. PRBHscreen Identifies Candidate aPKC-Regulated PRBH Motifs

(A) Schematic representation of bioinformatics identification of PRBH motifs from proteomic files. The BH motif scoring algorithm identifies sequences with strong BH character. Vertical position on a scale from −2 to 2 shows each residues' BH score. A representative BH plot has been included to depict the maximum BH score. aPKC consensus sites were identified and scored by scanning for S/T residues with preferred residues in the specific NH_2- and COOH-terminal sites. The scale shows the aPKC site score (ranging from −0.1 to 0.35) for each residue in the specified site. Unlisted residues have no effect on the overall phosphosite score. An overall PRBH score was assigned to sequences identified in both the BH and aPKC site identifying algorithm.

(B and C) PRBH score distribution from in the *Drosophila* and human proteome. Select PRBH motifs from the fly and human proteome are highlighted in (B) and (C), respectively. Gray points display each identified putative PRBH motif with its respective PRBH max BH score and max aPKC site score. Blue, purple and red denote the number of identified aPKC sites. Blue lines mark the PRBH threshold values, sequences with scores less than these values are not candidate PRBH motifs. See also Figure S4 and Table S1 for analysis of the *Caenorhabditis elegans* and sponge proteomes.

(D) aPKC phosphorylates candidate PRBH motifs. A ^{32}P autoradiograph demonstrates that Amer1^{PRBH}, CKIγ-2^{PRBH}, MPP7^{PRBH}, and PIP82^{PRBH} are phosphorylated by aPKC. A Coomassie-stained loading control gel is displayed.

(E–H) aPKC inhibits localization to the cell cortex for several candidate PRBH motifs. Representative images are shown with a quantification of the cortical to cytoplasmic signal intensity ratio as the mean ± SEM. Cartoons show the constructs characterized with candidate aPKC phosphosites listed. Each protein was characterized as an EGFP-fusion protein. Statistical significance testing was performed using the t test of aPKC cotransfected cells to singly transfected cells. $^{**}p < 0.001$, $^{***}p < 0.0001$. Significance was evaluated using a non-parametric t test relative of the singly transfected cells to aPKC cotransfected cells. ns, not significant.

See also Figure S4.

to the cell cortex, but aPKC expression does not reduce its cortical localization (Figure 5H). However, the number of potential phospho-accepting residues within Amer1's PRBH makes it a prime candidate for regulation by another kinase. We conclude that the PRBH algorithm is a good predictor of aPKC-regulated cortical association, with higher scoring motifs more likely to exhibit this behavior. Furthermore, a more precise assessment of PRBH character can be made be directly comparing BH motif area, the number of aPKC phosphosites, etc.

DISCUSSION

The organization of the animal cell cortex by the Par complex involves two key steps (Goehring, 2014; Goldstein and Macara, 2007; Knoblich, 2010; Prehoda, 2009). The first involves the specification of the Par domain by upstream components such as the Rho GTPase Cdc42. In the second, diverse proteins must "plug into" Par polarity by occupying cortical areas that lack the Par complex. We have examined how the key output of the Par complex, aPKC activity, leads to cortical exclusion of the diverse array of Par-polarized proteins. Our approach was to examine the cortical interactions of three Par substrates that had no described sequence or domain similarities and to determine how aPKC phosphorylation modulates their cortical binding. Although these Par substrates do not have clear sequence homology, they each contain a "phospho-regulated BH" motif that couples aPKC phosphorylation to membrane affinity. This coupling is a direct consequence of the overlap of BH and aPKC phosphorylation sites in the PRBH motif as this allows phosphorylation to have a significant effect on the electrostatic character of the sequence. Using this defining feature, we developed a computational approach for identifying candidate PRBH motifs, which we validated that aPKC regulates cortical association of several hits from the human and fly proteomes. We expect this technique will be useful for identifying candidate polarity proteins and proteins involved in other cellular processes where regulated membrane association is important.

Multivalent Interactions Mediate Par Substrate Cortical Localization

We have found that Par substrate BH motifs localize to the cortex, but it is important to note that robust targeting requires elements outside of the BH sequence. While our data suggest that PRBH motifs are key regulatory elements for polarity, the requirement of additional interactions for high-affinity cortical interactions means that they are unlikely to lead to substrate polarity on their own. Similar "multivalent" interaction mechanisms has been observed with other BH motif-containing proteins that are regulated by phosphorylation and utilize accessory interactions to enhance their cortical targeting, including the yeast pheromone signaling protein Ste5 (Pryciak and Huntress, 1998; Winters et al., 2005) and the actin filament crosslinker MARCKS (Hartwig et al., 1992; McLaughlin and Murray, 2005; Thelen et al., 1991). In Ste5, protein-protein interactions provide additional cortex affinity, whereas MARCKS contains a myristoyl modification that works with its BH motif (George and Blackshear, 1992). The multivalent nature of protein-phospholipid interactions mediated by BH motifs may explain why protein-protein interactions are important to polarize some Par substrates. For example, Lgl interacts with non-muscle myosin II (Betschinger et al., 2005), and this interaction may cooperate with BH-phospholipid binding to yield the increased cortical interaction of full-length Lgl compared to its BH alone. Phospholipid binding by the BH motif and the PTB domain may robustly target Numb to the cell cortex (Dho et al., 1999). However, in order for cortical localization to be regulated, the accessory interactions must not be stable enough to mediate cortical targeting on their own, or alternatively they must themselves be regulated by phosphorylation (McLaughlin and Murray, 2005; Strickfaden et al., 2007). Future studies of candidate PRBH-containing proteins will determine which regulated interactions are used for Par-mediate polarity and how factors such as cortical binding affinities, multiple PRBH motifs, and cortical mobility cooperate to mediate substrate polarization.

PRBH: A Mechanism for Convergent Evolution of Par-Mediated Polarity?

Phosphorylation can regulate protein activity by several means, including allostery and conformational changes (Cohen, 2000; Johnson and Barford, 1993; Serber and Ferrell, 2007). In this type of regulation, phosphate attachment to a specific side chain alters protein structural features and/or dynamics that are important for catalytic or binding activity (Barford et al., 1991). This coupling requires a connection between the phosphorylation site and the native state energy landscape, such that the phosphorylation site sequence is typically highly conserved (Holt et al., 2009). Allosteric effects or conformational changes may induce the polarization of substrates that lack PRBH motifs (e.g., Baz/Par-3 and Par-1) (Hurov et al., 2004; Morais-de-Sá et al., 2010; Sotillos et al., 2004; Suzuki et al., 2004). In contrast to complex mechanisms like these, phosphorylation of many Par substrates alters activity by changing the bulk electrostatics of the phospholipid binding sequence (Serber and Ferrell, 2007). Here, phosphorylation must occur close to the binding sequence but does not have to be coupled to structural or dynamic changes in the protein (Holt et al., 2009). Because many sequences satisfy the requirements of (1) an electrostatic character sufficient for binding negatively charged phospholipids, and (2) one or more aPKC phosphorylation sites, we propose that the sequence path for convergent evolution of PRBH motifs could be fairly simple. An existing motif, either BH or aPKC phosphosite is likely to require a small number of mutations to retain existing function while causing a gain of the missing function. For example, a protein that is advantageously targeted to the membrane via a BH motif could require only a small number of mutations to become polarized with the introduction of one or more aPKC phosphosites.

Functions of Candidate PRBH-Containing Proteins

We identified several proteins whose cortical localization is regulated by aPKC: MPP7 and PIP82 and CKIγ-2. In epithelial cells, MPP7 localizes to the lateral cortex and mediates tight junction formation (Stucke et al., 2007). While previous work has not implicated aPKC in its regulation, its localization and aPKC's inhibition of cortical localization make it a strong candidate Par-polarized substrate. The localization of several Casein kinase paralogs that lack predicted PRBH motifs (Table S1) have been described (Davidson et al., 2005; Gross et al.,

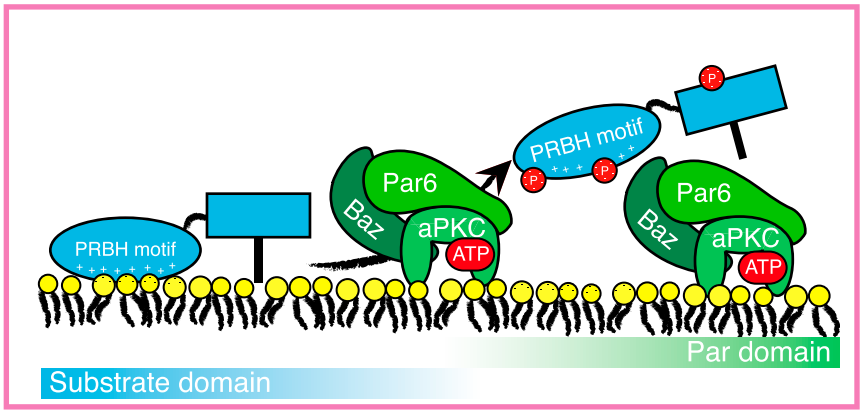

Figure 6. Model for Par Polarization by Phosphoregulated BH Motifs

PRBH motifs mediate localization to regions of the cell cortex opposite the Par complex. The box detail shows the molecular interactions occurring at the interface between the Par and substrate domains. Par substrates will localize to the cortex by electrostatic interactions with phospholipids until they encounter aPKC and become phosphorylated. Phosphorylation reduces their membrane affinity. Weak accessory interactions mediate localization to the cell cortex but they must allow the substrate to dissociate from the cell cortex, when the BH motif is phosphorylated.

1997), although the localization of the PRBH-containing CKIγ-2 in a polarized in vivo context is uncharacterized to our knowledge. However, recent work demonstrated that the CKIγ-associated protein Lrp6 localizes to the basolateral cortex in the neuroepithelium (Davidson et al., 2005; Huang and Niehrs, 2014), suggesting that CKIγ-2 may also exhibit this pattern of localization. Future work will need to address if aPKC indeed regulates CKIγ-2 localization in vivo. The localization and molecular function of PIP82 in *Drosophila* photoreceptor cells has not been described, but it is intriguing that light exposure causes it to be dephosphorylated (Suri et al., 1998). If the PIP82 PRBH is dephosphorylated during this process, it would be a mechanism for coupling cortical localization to light exposure. While we have identified many putative aPKC-regulated PRBH motifs, future work will need to address how this regulatory sequence is used cellular processes besides polarity. One particularly enticing PRBH protein is the retromer component Vps26 as it has been implicated in Par- and Scribble-mediated endosomal trafficking (de Vreede et al., 2014). Characterization of Vps26 and other PRBH proteins will likely emphasize the diverse functions of aPKC phosphorylation.

A Model for Protein Polarization Directed by the Par Complex

From these studies, we propose a model for Par complex function (Figure 6). In this model, Par-polarized substrates localize to the cortex via direct interactions between their BH motifs and membrane phospholipids. When a substrate enters the Par domain, either from the cytoplasm or by diffusion along the membrane, it becomes phosphorylated by aPKC. Addition of phosphates alters the electrostatics of the PRBH motif to reduce its affinity for phospholipids causing it to be displaced into the cytoplasm. Future work will be required to complete the "life cycle" of these substrates to understand the fate of Par substrates once displaced into the cytoplasm. Phosphorylation may be coupled to inactivation, degradation, or phosphatase-mediated re-association to the cortex.

EXPERIMENTAL PROCEDURES

Sequence Analysis and Computational Work

BH motifs were initially identified using BH-search: a computational algorithm described in Brzeska et al. (2010) and is available online (http://helixweb.nih.gov/bhsearch/). Domain analyses were performed using SMART (Letunic et al., 2015; Schultz et al., 1998). Figures were assembled using the Adobe Creative Suite. The PRBH algorithm was implemented using the Python programming language with standard Python libraries including numpy and matplotlib. The *Drosophila* and human non-redundant proteome were analyzed from the EMBL-EBI reference proteome files. Please see the Supplemental Information for the full script and a detailed description of the PRBH identification program. Briefly, BH motifs were identified using a previously described BH scoring metric (Brzeska et al., 2010). Residues surrounding S/T residues were scored based on the aPKC consensus sequence (Wang et al., 2012) with Miranda's aPKC recognition sequence included. Composite scores were assigned to sequences identified as BH motifs and aPKC consensus sequences. PRBH scores above a threshold value were analyzed.

Molecular Cloning and Cell Culture

All molecular cloning was performed as previously described (Graybill et al., 2012). Please see the Supplemental Experimental Procedures for details. Cell culture was performed as previously described (Lu and Prehoda, 2013). Briefly, cells were grown according to manufacturer's protocol, transiently transfected with Effectene (QIAGEN). Transfected cells were fixed and immunostained as previously described (Lu and Prehoda, 2013) and imaged with the following confocal microscopes: Olympus Fluoview FV1000 BX61 with a PlanApo N 60×/1.42 oil and a Leica SP2 confocal microscope with a 63×/1.40–0.60 oil CS objective. All proteins were tagged at their NH_2 terminus. Image analysis was performed with ImageJ.

Protein Expression and Purification

Protein expression was performed as previously described (Graybill et al., 2012). Briefly, MBP-tagged proteins were purified from BL21 (DE3) cells by amylose affinity purification. aPKC was purified from HEK293F cells transiently transfected with 293fectin transfection reagent (Life Technology). All His_6-tagged aPKC constructs were purified with Ni^{2+}-nitrilotriacetic acid resin purification from HEK293F cell lysates. Please see the Supplemental Experimental Procedures for a detailed description of this protein purification.

Biochemical Assays

All lipids were purchased from Avanti Polar Lipids. Giant unilamellar vesicles were prepared as previously described (Winters et al., 2005). Please see the Supplemental Experimental Procedures for details of on vesicle preparation and lipid cosedimentation assays. Pelleting assays were performed as previously described (Prehoda et al., 2000). Giant unilamellar vesicles and associated bound proteins were pelleted by ultracentrifugation and analyzed by SDS-PAGE. Kinase assays were performed as previously described (Atwood and Prehoda, 2009; Graybill et al., 2012). Briefly, aPKC and substrates were incubated in assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) at 30°C for 5 min before addition of 1 mM ATP doped with [γ-^{32}P] ATP (~1.0 × 10^5/nmol of ATP). The kinase reaction proceeded for 30 min before quenching with SDS loading dye. Samples were run on a 12.5%

acrylamide SDS-PAGE gel, then analyzed with the Storm 860 Molecular Imager and a phosphor screen (Molecular Dynamics).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.09.016.

AUTHOR CONTRIBUTIONS

All experiments were designed and conceived by M.J.B. and K.E.P. M.J.B. performed all experiments. Programming was performed by M.J.B. and K.E.P. The manuscript was written by M.J.B. and K.E.P.

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