Phosphoinositides and Membrane Targeting in Cell Polarity

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Selective enrichment of the polyphosphoinositides (PPI_n), such as PtdIns(4,5) P_2 and PtdIns4 P , helps to determine the identity of the plasma membrane (PM) and regulates many aspects of cell biology through a vast number of protein effectors. Polarity proteins had long been assumed to be non-PPI_n-binding proteins that mainly associate with PM/cell cortex through their extensive protein–protein interaction network. However, recent studies began to reveal that several key polarity proteins electrostatically bind to PPI_n through their positively charged protein domains or structures and such PPI_n-binding property is essential for their direct and specific attachment to PM. Although the physical nature of the charge-based PPI_n binding appears to be simple and nonspecific, it serves as an elegant mechanism that can be efficiently and specifically regulated for achieving polarized PM targeting of polarity proteins. As an unexpected consequence, subcellular localization of PPI_n-binding polarity proteins are also subject to regulations by physiological conditions such as hypoxia and ischemia that acutely and reversibly depletes PPI_n from PM.

The fundamental role of phosphoinositides (PPI_n) in regulating the formation and dynamics of polarized cellular structures has been well recognized (Shewan et al. 2011; Balla 2013). During cell polarization, many studies have focused on how asymmetric distribution of PPI_n in the plasma membrane (PM) plays an instrumental role in specifying membrane domains such as apical versus basolateral, and how protein effectors that bind specific PPI_n such as PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 are involved in such processes (Gassama-Diagne et al. 2006; Martin-Belmonte et al. 2007; Fairn et al. 2011). However, it is also well known that PPI_n, because of their negatively charged nature, can at-

tract proteins carrying positive charges through nonspecific electrostatic interactions (Heo et al. 2006; Yeung et al. 2006; Hammond et al. 2012). Recent studies highlight that intracellular membranes are enriched with unique combinations of PPI_n that are key to define membrane identity and determine the degree to which the surface is negatively charged (Hammond et al. 2012). PPI_n such as PtdIns4 P and PtdIns(4,5) P_2 are of particular importance as their unique enrichment at the inner surface of PM makes it the most negatively charged membrane surface inside the cell (Hammond et al. 2012). Although such electrostatic binding to PPI_n are inherently non-structure-specific, it is nonetheless crucial

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to mediate the PM-specific targeting of a wide array of proteins carrying specialized domains enriched with positive charges, including MARCKS (Arbuzova et al. 2002; Morton et al. 2014), N-WASP (Papayannopoulos et al. 2005), small GTPases (Heo et al. 2006; Yeung et al. 2006), Src (McLaughlin and Aderem 1995), Ste5 (Strickfaden et al. 2007), and TRPV1 receptor regulator (Kim et al. 2008). Recent studies show that multiple polarity proteins contain positively charged domains such as polybasic domains that directly bind to PPIIn (Bailey and Prehoda 2015; Dong et al. 2015). The importance of such direct PPIIn binding in controlling the polarity protein subcellular localization and their functions in cell polarity has just begun to be appreciated. In this review, we will first focus on how PPIIn distributions are dynamically regulated in membranes. We will then discuss how electrostatic binding to membranes serves as a pivotal mechanism regulating the PM targeting of PPIIn-binding polarity proteins under both normal and stress conditions such as hypoxia. We will primarily focus on polarity proteins well characterized in *Drosophila* and mammalian cells for their roles in apicobasal polarization and asymmetric cell division.

BIOGENESIS OF PHOSPHOINOSITIDES

Although they are frequently relegated to the diminished status of simple, inert building blocks, lipids are in fact crucial to the biological function of membranes. Indeed, cells control lipid composition very tightly to define the functional properties of their membranes (Holthuis and Menon 2014). One family of lipids, the phosphoinositides (PPIIn), are especially important in the control of membrane function. These lipids are derived from one of the major phospholipids, phosphatidylinositol (PtdIns). This lipid contains a unique polyol headgroup, which presents three hydroxylated carbons (at positions 3, 4, and 5) to the cytosol that can undergo cycles of phosphorylation and dephosphorylation by various lipid kinases and phosphatases (Fig. 1). Collectively, these enzymes can add and remove phosphatases from 3-, 4-, and 5-positions on PtdIns in all possible combinations and configurations, leading to a family of seven PPIIn: three mono-, three bis-, and a single tris-phosphorylated species. Interconversion of these molecules can be extremely fast, enabling cells to rapidly modulate lipid signals, or else maintain steady-state accumulation of PPIIn at

Figure 1. Generation of plasma membrane (PM) phosphoinositides. The lipid phosphatidylinositol (PtdIns) may be phosphorylated at any of three hydroxyl groups on its cytosol-exposed six-membered polyol headgroup; the major plasma membrane phosphoinositides and their synthetic pathways are indicated.

specific compartments despite the rapid flux of lipids by vesicular and nonvesicular traffic. Once formed, the lipids can bind to hundreds of different protein partners with varying degrees of specificity to perform a dizzying array of cellular functions, from the gating of ion channels to the regulation of cell polarity and motility. This biology is vast, as are the numbers of protein effectors and enzymes that metabolize the lipids. In this review, we will restrict ourselves to the discussion of PM PIP₂ and their effectors that contribute to cell polarity. For a broader view of this large field, and an excellent historical perspective, we recommend the recent exhaustive review on the subject by Balla (2013).

The most abundant PIP₂ at the PM is PtdIns(4,5)P₂ and, in turn, the PM is where the majority of PtdIns(4,5)P₂ molecules are localized (Várnai and Balla 1998; Watt et al. 2002). Although this lipid is comparatively scarce in the cell compared with the more abundant structural phospholipids, this selective enrichment at the PM means it is relatively abundant, with a mole fraction on the cytosolic leaflet of between 1% and 5% (James et al. 2008). It is synthesized by two separate families of kinases, each with three paralogs; the PIP5K, which phosphorylate PtdIns4P at the 5-position; and PIP4K, which phosphorylate PtdIns5P at the 4-position (Fig. 1). Because PtdIns4P is around 100-fold more abundant in cells (Sarkes and Ramiah 2010), the former pathway has been assumed to be the predominant one. However, the capacity of PIP4K to function correctly has recently been elegantly shown to depend only on its capacity to synthesize PtdIns(4,5)P₂ and is unaffected by substrate preference for PtdIns5P or PtdIns4P (Bulley et al. 2015). This, coupled with the observation that most PtdIns4P is not required to synthesize PtdIns(4,5)P₂ (Hammond et al. 2012), means the question is open as to whether the PtdIns5P pathway is truly only a minor contributor to PM PtdIns(4,5)P₂ levels.

PtdIns4P is localized on several organelle membranes, including the PM, endosomes, and the Golgi (Hammond et al. 2014). Pharmacologic treatments that primarily target the PM pool indicate that ~80% of the cells' PtdIns4P

is in the PM (Bojjireddy et al. 2014). That being said, given PtdIns4P's enrichment on the *trans*-Golgi network (Weixel et al. 2005) and the fact that the Golgi as a whole contains less membrane surface area than the PM (Griffiths et al. 1989) means that, although there are more PtdIns4P molecules in the PM, they are probably most concentrated in the *trans*-Golgi network (i.e., this compartment may contain a higher mole fraction of PtdIns4P).

Synthesis of PtdIns5P is enigmatic. The best evidence to date places its synthesis at the hands of the lipid kinase PIKfyve, although it remains controversial whether synthesis occurs via direct 5-phosphorylation of PtdIns (Shisheva et al. 2015) or via a circuitous route via 5-phosphorylation of PtdIns3P followed by removal of the 3-phosphate (Fig. 1) (Zolov et al. 2012). Either way, it appears PtdIns5P synthesis occurs on endosomal membranes, yet may in fact accumulate in the plasma membrane (Roberts et al. 2005).

Once synthesized, PtdIns(4,5)P₂ has a number of metabolic fates. First, it can be dephosphorylated back into PtdIns4P by the large family of inositol polyphosphate 5-phosphatase (INPP5)-domain proteins, a reaction that frequently accompanies internalization of PtdIns(4,5)P₂ molecules by endocytosis (Hakim et al. 2012). PtdIns4P is itself dephosphorylated by Sac-family phosphatases, which either occurs on nascent or early endosomes through the synaptojanin or Sac2 enzymes (Chung et al. 1997; Hsu et al. 2015; Nakatsu et al. 2015) or else by the ER-associated Sac1 lipid phosphatase; although whether this occurs through activity of Sac1 on PM PtdIns4P at ER-PM contact sites (Dickson et al. 2016) or after nonvesicular transport of PtdIns4P from the PM to the ER (Chung et al. 2015) or both has not been definitively shown.

Second, PtdIns(4,5)P₂ can be hydrolyzed by phospholipase C (PLC), which cleaves it into the second messengers diacylglycerol and InsP₃. Depending on how robust PLC activation is, there can be no detectable change in the levels of PtdIns(4,5)P₂ after PLC activation (such as after EGF stimulation of 3T3 fibroblasts [Jackson et al. 1992]), transient reductions in the

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lipid levels that recover inside of a minute (e.g., after bombesin stimulation of the same cells [Jackson et al. 1992]), or rapid, sustained, and near complete depletion of the PM PtdIns(4,5) P_2 levels (as seen after activation of PLC-coupled muscarinic acetylcholine receptors [Willars et al. 1998]). In this latter case, PLC activity can be a means to control membrane PtdIns(4,5) P_2 levels, which can be important, for example, in gating inwardly rectifying potassium channels and thus controlling electrical excitability in neurons (Suh and Hille 2002). However, it is worth emphasizing that this is by no means the normal situation for every PLC-coupled pathway.

Finally, PtdIns(4,5) P_2 can be phosphorylated by the class I PI 3-kinases at its last available 3-hydroxyl to produce PtdIns(3,4,5) P_3 , which like diacylglycerol and Ins P_3 is an important second messenger molecule for cell signaling. It functions by recruiting and activating specific binding proteins such as Akt. PtdIns(3,4,5) P_3 generation tends to be transient (Jackson et al. 1992) or else polarized (Janetopoulos et al. 2004; Gassama-Diagne et al. 2006). This spatially or temporally restricted accumulation is controlled by phosphatases such as PTEN that remove the 3-phosphate to convert PtdIns(3,4,5) P_3 back to PtdIns(4,5) P_2 , or by members of the INPP5 family, namely, SHIP1 and SHIP2, that remove the 5-phosphate (Hakim et al. 2012). The resulting PtdIns(3,4) P_2 is converted to PtdIns3P on nascent endosomes by INPP4 enzymes (Shin et al. 2005a). It is important to note that, unlike PLC activity, the flux of PtdIns(4,5) P_2 molecules through the PI 3-kinase pathway is comparatively low, with at most 10% of the membrane PtdIns(4,5) P_2 converted (Jackson et al. 1992). Activity of PI 3-kinases and PTEN are therefore unlikely to directly contribute to the control of PtdIns(4,5) P_2 levels.

PHOSPHOINOSITIDE-DEPENDENT PM TARGETING

There are many classes of lipid-binding domains that recognize PPIIn with varying degrees of specificity and affinity. Lipid binding is often

necessary but very commonly not sufficient to dictate localization of the protein (Hammond and Balla 2015); in this regard, protein–protein interactions can still play a crucial role, as we shall see. Broadly speaking, there are two classes of PPIIn-binding protein (Hammond and Balla 2015): those with specific high affinity and usually 1:1 stoichiometric binding to a specific PPIIn such as the TRAF domain (Rousseau et al. 2013), and those showing more promiscuous binding. It is to this latter category that the polybasic domains found in several polarity proteins belong.

Polybasic domains specifically bind to the PM in cells (Heo et al. 2006; Yeung et al. 2006). These proteins have long been known to bind to membranes containing anionic phospholipids through nonspecific electrostatic interactions (Wang et al. 2002), which cooperate with some hydrophobic character of the peptide such as a lipid modification or the presence of bulky hydrophobic residues such as those found in Phe and Trp. In cells, the selective targeting of the PM by polybasic domain proteins depends on their highly concentrated positive charges, because graded reduction of the net charge on the K-Ras polybasic domain from +8 to +2 broadens the protein's localization to membranes with successively lower accumulations of anionic lipids (Yeung et al. 2006).

What is the determinant on the PM that so specifically recruits these highly positively charged peptides? The anionic phospholipid phosphatidylserine (PtdSer) is most concentrated on the cytosolic leaflet of the PM, and this is an essential part of this specific membrane targeting (Yeung et al. 2008). However, the relative enrichment of PtdSer over endosomal compartments is not sufficient to explain the highly selective PM binding of the polybasic domains. In addition, it appears there is a requirement for polyanionic lipids (i.e., the PPIIn) in maintaining selective binding (Heo et al. 2006; Yeung et al. 2008; Hammond et al. 2012). Because these interactions are based on nonspecific electrostatics, it is not surprising that experiments have suggested that this polyanionic lipid requirement can be met by PtdIns4P, PtdIns(4,5) P_2 , or even PtdIns(3,4,5) P_3 (Heo et al. 2006; Ham-

mond et al. 2012). However, given the higher charge density on PtdIns(4,5) P_2 versus PtdIns4P and the 10- to 100-fold excess of PtdIns(4,5) P_2 over PtdIns(3,4,5) P_3 , the former lipid is most likely the lipid that primarily interacts with polybasic domain proteins. In vitro, polybasic domains will specifically sequester PtdIns(4,5) P_2 even in a PtdSer-rich leaflet (Gambhir et al. 2004). In fact, reversible sequestration of the lipids by polybasic domain proteins such as MARCKS has even been suggested to be a mechanism to regulate lipid levels and thus signaling (McLaughlin and Murray 2005). Collectively, it therefore seems that PtdIns(4,5) P_2 is the primary lipid involved with targeting polybasic domain proteins to the PM.

PM TARGETING OF PPIIn-BINDING POLARITY PROTEINS

With few exceptions, polarity proteins typically need to specifically localize to apical or basolateral cortical/PM domains to play their regulatory functions in establishing and maintaining cell polarity (Tepass 2012; Rodriguez-Boulán and Macara 2014). Except for transmembrane polarity proteins such as E-cadherin and Crumbs, non-transmembrane polarity proteins were generally assumed to associate with cell cortex/PM by physically interacting with other polarity proteins and/or cortical actomyosin network (Rodriguez-Boulán and Macara 2014). For instance, protein interactions identified so far in *Drosophila* apical polarity complex aggregates to the transmembrane protein Crb, which appears to act as an anchor to localize Sdt (Bachmann et al. 2001; Hong et al. 2001), Patj (Shin et al. 2005b), Par-6, and aPKC (Hurd et al. 2003), whereas E-cadherin brings the cortical localizations of β -catenin and Baz(Par-3) to adherens junctions (Wei et al. 2005). Interaction with the actin cytoskeleton is also considered essential to localize polarity proteins such as Lethal giant larvae (Lgl) to the cell cortex. Because of the limited resolution of optical microscopy, terms “cortical” and “membrane” were often used interchangeably in the literature describing the subcellular localization of non-transmembrane polarity

proteins. Because no polarity proteins contain known PPIIn-specific-binding domains such as PH domains, little attention was paid to their potential PPIIn-binding property. Recent studies, however, began to reveal that several key polarity proteins in fact have domains that electrostatically bind to PPIIn, and such direct PPIIn binding can be a primary mechanism for their PM targeting.

Lgl (Lethal giant larvae)

Recent studies identified Lgl, Numb, and Miranda as the first polarity proteins that contain polybasic domains, which are essential for their PM targeting (Bailey and Prehoda 2015; Dong et al. 2015). Lgl belongs to the basolateral polarity protein group, which also contains Scrib and Dlg (Fig. 2A) (Bilder et al. 2000, 2003; Tantenzapf et al. 2000). Similar to Scrib and Dlg, Lgl plays dual roles as both a polarity protein and a tumor suppressor (Jacob et al. 1987). The precise molecular function of Lgl remains to be fully elucidated, but like many polarity proteins a key feature of Lgl is that its biological activity requires polarized localization to PM/cell cortex. A well-established mechanism limiting the localization of Lgl to basolateral PM is aPKC phosphorylation. In *Drosophila* epithelial cells, apically localized aPKC phosphorylates a highly conserved serine cluster in Lgl to prevent its localization at apical membranes (Betschinger et al. 2003; Plant et al. 2003; Hutterer et al. 2004). One early study suggests that phosphorylation of Lgl induces Lgl into a self-inhibitory conformation incapable of binding to cell cortex (Betschinger et al. 2005). However, this self-inhibiting model of Lgl was based on the primary sequence analysis that incorrectly predicted the carboxy-terminal half of Lgl as low complexity region. Later protein structure studies showed that Lgl and its yeast homolog Sro7 share a highly conserved structure composed of two β -propellers each containing seven WD40 blades, and the previously assumed low complexity region is actually the second β -propeller (Fig. 2B) (Hattendorf et al. 2007). It is notable though that somehow the incorrect structural model of Lgl persisted in Lgl literatures even

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Figure 2. Polyphosphoinositides (PPI)_n-binding polarity proteins. (A) Subcellular localization of key polarity proteins in epithelial cells and in dividing neuroblasts. Both cells were drawn apical side up. Only polarity proteins relevant to this review were drawn. In *Drosophila* epithelial cells, aPKC/Par-6 complex and Crb/Sdt (Pals1) complex localize to the apical membrane, and Bazooka (Baz) mostly localizes to apical adherens junction (AJ). Lgl and Par-1 localize to the basolateral plasma membrane (PM). In dividing neuroblasts, aPKC, Par-6, and Baz localize exclusively at the apical PM, with Numb and Miranda (Mir) at basal PM. (B) The conserved polybasic domain in Lgl. Shown at *bottom right* is the structure of Sro7, the yeast homolog of Lgl, which contains 14 WD40 blades (β -sheets are in green) organized in two β -propellers (Hattendorf et al. 2007). Blades 10 and 11 are enlarged at *bottom left*. The loop is in magenta. Shown at the *top* is the alignment of the loop regions between WD40 10D and 11A β -sheets in Lgl/Sro7. Positively charged Lys and Arg residues within the loop sequences are in red and polybasic domains are boxed, Trp/Phe/Leu residues are in green and aPKC-phosphorylatable serines are in blue. Ser residues were numbered based on *Drosophila* Lgl (dLgl) isoform A (gi24464586). Sequence alignments are modified from Hattendorf et al. (2007). (C) PPI_n-binding domains in Numb, Miranda, Baz, and Par-1. PPI_n-binding domains are in blue, except that in Baz the carboxyl-terminus regions that bind PPI_n are indicated by blue lines. Blue box in Baz indicates a conserved motif required for Baz PM localization in the absence of the amino terminus (Krahn et al. 2010b). Published phosphorylation sites are indicated by red vertical bars. Phosphorylation sites that regulate the polybasic domain-mediated PM targeting are in blue and those are conserved between *Drosophila* and mammalian Numb proteins are in bold. AJ, Adherens junction; PB, polybasic domain; PTB, phosphotyrosine-binding domain; UBA, ubiquitin-association domain; CLD, cortical localization domain; CargoBD, cargo-binding domain (Atwood and Prehoda 2009); OLG, oligomerization domain (Benton and St Johnston 2003a).

well after the Sro7/Lgl structure was published (Bailey and Prehoda 2015; Bell et al. 2015; Carvalho et al. 2015).

One breakthrough on the mechanism of Lgl PM targeting comes from two recent studies. They discovered that the short (~ 27 aa) motif containing all the phosphorylatable serines in Lgl is in fact a typical polybasic domain (Bailey and Prehoda 2015; Dong et al. 2015). More than half the residues of the motif are either Arg or Lys, in addition to several Phe, Trp, and Leu residues that are critical for enhancing the binding of polybasic domain to the bilayer of PM (Fig. 2B) (Heo et al. 2006). In vitro Lgl polybasic domain binds directly the liposomes containing PtdIns4P and PtdIns(4,5) P_2 . In vivo and in vitro data suggest that PtdIns4P and PtdIns(4,5) P_2 in PM are both required for Lgl's PM localization, whereas PtdIns(4,5) P_2 plays a more significant role (Dong et al. 2015). Loss of polybasic domain completely eliminates PM localization of Lgl in cultured mammalian cells, but a *Drosophila* Lgl mutant with all the Arg/Lys residues mutated in the polybasic domain still showed a significantly reduced PM localization in epithelial cells in vivo (Dong et al. 2015). The residual PM localization remains sensitive to the loss of PPIIn under hypoxia, suggesting a yet-to-be-identified hidden PPIIn-binding domain in Lgl may mediate partial PM targeting in the absence of primary polybasic domain. Alternatively, the mutant Lgl may weakly associate with unidentified protein(s) whose PM localization also depends on PPIIn. Overall, direct PPIIn-binding appears to be the primary mechanism localizing Lgl to PM, in spite of the fact that Lgl also interacts with multiple target proteins such as MyoII (Dahan et al. 2012), syntaxin (Musch et al. 2002; Gangar et al. 2005), aPKC, Dlg (Zhu et al. 2014), and possibly actin cytoskeleton (Betschinger et al. 2005). Intriguingly, the polybasic domain is nearly 100% conserved among *Drosophila* and mammalian Lgl proteins but is absent in yeast Sro7 (Fig. 2B), suggesting that this phosphorylatable polybasic domain (see below) is an evolutionarily acquired feature in metazoa to target Lgl to PM through direct and regulatable binding to PPIIn.

Numb and Miranda

Numb and Miranda are not required for apico-basal polarization; however, they are key components in establishing the asymmetric cell division of neuroblasts in *Drosophila* (Fig. 2A) (Knoblich et al. 1995; Shen et al. 1997). In dividing neuroblasts, both Numb and Miranda are localized exclusively to the basal PM/cortex and segregate into the smaller daughter cell GMC after division. An early structure–function analysis of *Drosophila* Numb showed that the first 76aa of the amino terminus is both required and sufficient for Numb PM/cortical association (Knoblich et al. 1997). It is now revealed that the amino terminus of *Drosophila* Numb in fact contains a phosphorylatable polybasic domain that can bind PPIIn (Bailey and Prehoda 2015). Furthermore, we showed that PM localization of Numb::GFP in *Drosophila* is sensitive to the depletion of PPIIn under hypoxia (Dong et al. 2015), suggesting that binding to PPIIn is likely the primary mechanism for PM targeting of Numb in *Drosophila*. However, interestingly the polybasic domain identified in *Drosophila* Numb is not conserved in vertebrates. Mammals have at least four Numb isoforms and two of them, p72 and p66, contain an 11aa insertion within in the PTB domain (Fig. 2C). The insertion creates a new and highly polybasic motif not presented in isoforms p71 and p65 or *Drosophila* Numb (Fig. 2C). It remains to be determined whether this alternatively spliced domain functions specifically as a PPIIn-binding polybasic domain, but an early study showed that only the isoforms p72 and p66 that contain the potential polybasic motif are membrane localized and can bind PtdIns4P and PtdIns(4,5) P_2 in vitro (Dho et al. 1999). Thus, although *Drosophila* and mammalian Numb proteins do not share the same polybasic domain, polybasic domain-mediated PPIIn-binding property appears to be well conserved for their PM targeting.

The polybasic domain in Miranda is also near the amino terminus (Atwood and Prehoda 2009; Bailey and Prehoda 2015). Miranda's polybasic domain is a weaker one based on its basic hydrophobic index, but it is nonetheless

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required for Miranda localization to PM in S2 cells (Bailey and Prehoda 2015). An early study showed that the first 114aa amino-terminal sequence that contains the polybasic domain is required for the PM localization of Miranda in *Drosophila* (Shen et al. 1998). However, it remains to be directly tested whether this polybasic domain is required for PM targeting of Miranda in vivo.

Baz/Par-3

Bazooka (Baz) and its worm/mammalian homolog Par-3 are the first polarity protein reported to show a direct PPIIn-binding property. In mammalian Par-3, the second PDZ (PDZ2) domain binds to PPIIn and is required for PM localization of Par-3 in MDCK cells (Wu et al. 2007). The PDZ2 domain contains a dozen conserved Arg and Lys residues and its structure suggests that the majority of these Arg and Lys residues form a positively charged surface that may bind electrostatically to any negatively charged phospholipids (Wu et al. 2007). Next to this surface, however, lies a polar pocket that only fits well the head group of PPIIn, making the PDZ2 domain selective in binding only PPIIn lipids without strong structural preference for a specific PPIIn. Interestingly, despite the fact that key PPIIn-interacting residues are well conserved in PDZ2 domain of Baz, PDZ2 is not required for PM localization of Baz in *Drosophila* epithelial cells (Krahn et al. 2010b). Instead, a ~300aa region at the carboxyl terminus is both required and sufficient for localizing Baz to PM in vivo and in S2R⁺ cell. This full 300–500aa carboxy-terminal region of Baz binds PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ liposomes in vitro (Fig. 2C) (Krahn et al. 2010b). Whereas the Baz carboxyl terminus shows stronger binding to PtdIns(3,4,5)P₃, the binding affinity measured is one or two magnitudes lower than proven PtdIns(3,4,5)P₃-specific-binding domains such as GRP1-PH (Manna et al. 2007). Such moderate binding affinity is consistent with the interactions between PPIIn and Baz carboxyl terminus being charge-based with a preference for PtdIns(3,4,5)P₃ in vitro attributable to its higher negative charge. PtdIns(4,5)P₂ and

PtdIns4P are most likely responsible for Baz localization to PM in vivo, as PtdIns(3,4,5)P₃ levels is only ~1% of PtdIns(4,5)P₂ in PM (Balla 2013) and depleting PtdIns(3,4,5)P₃ in *Drosophila* follicular cells does not affect Baz localization (Claret et al. 2014).

Neither Par-3 carboxyl terminus nor Baz PDZ2 domains has been experimentally shown for their PPIIn-binding potential. However, one study showed that Par-3 carboxyl terminus is also required for its PM localization in MDCK cells (Mizuno et al. 2003). It is possible that both PDZ2 and carboxy-terminal domains can bind PPIIn, but in different cell types and under different assay contexts one of them plays a more dominant role in localizing Baz/Par-3 to PM.

Par-1

Polarity protein Par-1 (or MARK in mammals) is a key Ser/Thr kinase that regulates the subcellular localization of multiple polarity proteins including Baz/Par-3 (Benton and St Johnston 2003b; Doerflinger et al. 2003). In *Caenorhabditis elegans* one-cell embryo and in polarized epithelial cells, Par-1 localizes to posterior or basolateral PM/cortical domain opposite to the PM domains occupied by Par-3(Baz) and aPKC (Suzuki 2015). Göransson et al. (2006) discovered that the carboxyl terminus of mammalian Par-1/MARK contains a so-called kinase-associated-1 (KA-1) domain that is required for the PM localization of Par-1 (Fig. 2C). However, it was unclear by then how KA-1, a domain only known for its potential function in kinase autoinhibition (Elbert et al. 2005), mediates the PM targeting of Par-1/MARK. The clue came years later in a survey on the yeast genome for phospholipid-binding proteins that led to the identification of a novel PPIIn-binding domain in yeast septin-associated kinases (Moravcevic et al. 2010). Structure analyzes of this PPIIn-binding domain revealed that it is remarkably similar to the KA-1 domain in kinases such as Par-1/MARK and MELK (Beullens et al. 2005). Although the primary sequence of KA-1 domain does not resemble a typical polybasic domain, its tertiary structure revealed strong Arg/Lys-enriched surface patches. Similar to

polybasic domain, KA-1 binds a broad array of negatively charged phospholipids including PtdSer and PPIIn, with little structure specificity to a particular phospholipid (Moravcevic et al. 2010; Hammond et al. 2012). The role of KA-1 in targeting Par-1/MARK to PM in mammalian cells is now well established (Moravcevic et al. 2010), but remains to be characterized in *Drosophila* cells. In particular, *Drosophila* Par-1 has several isoforms and one of them, N1S, is missing the KA-1 domain (Shulman et al. 2000) but still shows PM localization in at least follicular epithelial cells (Doerflinger et al. 2003). Studies suggest the carboxyl terminus of N1S (“spacer” region) is both required and sufficient for Par-1-N1S PM localization (Vaccari et al. 2005), but whether this region binds PPIIn remains to be determined.

REGULATION OF PPIIn-MEDIATED TARGETING OF POLARITY PROTEINS

At the first glance, direct binding to PM via electrostatic interactions between protein and PPIIn raises the question as how such a seemingly simple and nonspecific physical interaction can be reconciled with the fact that many polarity proteins not only localize to the PM, but are also further confined to specific PM domains. Nonetheless, several mechanisms may control the PPIIn binding to achieve the polarized localization of polybasic polarity proteins such as Lgl, Numb, and Miranda (Fig. 3A).

Phosphorylation-Dependent Regulation on Electrostatic PPIIn Binding

It has long been proposed that phosphorylation can function as an “electrostatic switch” to inhibit polybasic domains from binding to membrane (Mosior and McLaughlin 1991; McLaughlin and Aderem 1995; McLaughlin and Murray 2005; Mulgrew-Nesbitt et al. 2006). Phosphorylations of polybasic domain add negatively charged phosphate groups and therefore neutralize the positive charges required for polybasic domain to bind PPIIn. One classic example is the scaffolding protein MARCKS whose polybasic ED domain can be

phosphorylated by PKC (Hartwig et al. 1992; Arbuzova et al. 2002; Wang et al. 2002). As a polybasic protein, Lgl is remarkably similar to MARCKS as all the conserved phosphoserines identified in Lgl so far reside within the polybasic domain (Betschinger et al. 2003; Plant et al. 2003). Polybasic domains in Numb and Miranda also carry aPKC-phosphorylation sites (Bailey and Prehoda 2015; Smith et al. 2007). In either polarizing epithelial cells or dividing neuroblasts, phosphorylation by apically localized aPKC functions as an elegant mechanism to exclude Lgl, Numb, and Miranda to basolateral or basal PM (Wodarz et al. 2000; Betschinger et al. 2003; Wirtz-Peitz et al. 2008; Atwood and Prehoda 2009). This role of aPKC-phosphorylation in polarizing the PM localization of Lgl, Numb, and Miranda so far is well established, as loss of aPKC or more specifically its kinase activity (Kim et al. 2009) lead to all three proteins becoming localized around all PM domains (Wodarz et al. 2000; Betschinger et al. 2003; Wirtz-Peitz et al. 2008; Atwood and Prehoda 2009; Bell et al. 2015; Carvalho et al. 2015; Dong et al. 2015).

It is worth noting that, whereas in Lgl all the five to six aPKC-phosphorylation sites are clustered within the polybasic domain, in vitro phosphorylation assays have identified at least nine aPKC phosphorylation sites in Numb (Nishimura and Kaibuchi 2007; Smith et al. 2007) and ten in the first 300aa of Miranda (Atwood and Prehoda 2009) with the majority of them located outside the polybasic domain. The precise in vivo phosphorylation events required for aPKC to exclude Numb and Miranda remains to be fully determined. For instance, in Numb one particular phosphorylation site (S52) within the polybasic domain appears to contribute the most to apical exclusion of Numb by aPKC, but phosphorylations on four conserved sites outside the polybasic domain are required for full exclusion too (Smith et al. 2007). Similarly, eliminating the single phosphorylation site within the Miranda polybasic domain prevents aPKC from inhibiting its PM targeting, but eliminating four other phosphorylation sites ~100aa downstream of the polybasic domain show similar effects (Atwood and

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Figure 3. Regulation of polyphosphoinositides (PPI) binding in polarity proteins. (A) Three potential mechanisms that may regulate the plasma membrane (PM)-targeting of PPI-binding polarity proteins. Events depicted in allosteric regulation and structural masking of PPI-binding domain are hypothetical. (B) Hypoxia induces ATP reduction, loss of PPI in PM and loss of PM binding of Lgl and Numb. Lgl and Numb are the only two that are experimentally confirmed with loss of PM targeting under hypoxia. Baz remains on PM under hypoxia, likely through its interaction with protein complexes such as adherence junctions (AJs). PB, Polybasic domain.

Prehoda 2009). One way to interpret such data is that phosphorylation events outside the polybasic domain may be prerequisite for phosphorylation within the polybasic domain. However, it is also possible that in addition to neutralizing positive charges phosphorylation events may induce conformational changes that make polybasic domain less accessible to the PM, albeit experimental evidences of such mechanism remain to be shown.

Finally, excluding polybasic proteins to specific PM may involve more than passive and localized inhibition of PM binding. In *Drosophila* neuroblasts undergoing cell division, Miranda is at apical PM during interphase, localizes predominantly in cytosol during prophase, and only becomes concentrated to basal PM at metaphase (Erben et al. 2008). This dynamic relocation of Miranda appears to be

caused by a combination of both free diffusion and Myosin-dependent transportation of Miranda (Erben et al. 2008), and likely involves spatial-temporal regulation of aPKC activity (Wirtz-Peitz et al. 2008).

Structural Masking and Allosteric Regulation of PPI-Binding Domain

Although neutralizing positive charges via phosphorylation on polybasic domain efficiently and specifically blocks its binding to PPI, masking or exposing the PPI-binding domain in a target protein by protein modifications and protein-protein interactions can also be an effective mechanism. Direct experimental evidence of such regulation of PPI-binding domain controlling the polarized PM targeting of polybasic polarity proteins are still

lacking, but indirect evidences do suggest that polarity proteins such as Miranda, Par-1, and Baz could be the potential candidates subject to such regulations.

As mentioned earlier, aPKC-phosphorylation outside the Miranda polybasic domain could also inhibit Miranda PM localization (Atwood and Prehoda 2009), suggesting the possibility that phosphorylation-dependent protein conformation changes or protein interactions alter the accessibility of polybasic domains to bind PPIIn. Par-1 has more than a dozen identified phosphorylation sites but none are in the KA-1 domain (Göransson et al. 2006). Many of these phosphorylation events induce the binding of scaffolding protein 14-3-3 (Par-5) that sequesters Par-1 to the cytoplasm (Benton et al. 2002; Benton and St Johnston 2003b; Göransson et al. 2006). Although wild-type Par-1 is often localized both on the PM and in the cytosol in cells, a Par-1 mutant with all of the phosphorylation sites eliminated appears to be exclusively on the PM in cultured cells (Göransson et al. 2006). One model consistent with such data is that binding to 14-3-3 protein masks the KA-1 in Par-1 and prevents it from binding the PM (Fig. 3A). 14-3-3 can bury $>2000 \text{ \AA}^2$ surface on a target protein (Obsil et al. 2001) and masking events by 14-3-3 have been characterized in several 14-3-3-binding targets (Bridges and Moorhead 2005; Obsil and Obsilova 2011); for instance, binding of 14-3-3 inhibits Cdc25 nuclear import by specifically occluding the nearby NLS sequence (Yang et al. 1999). Nonetheless, further experiments are required to determine whether KA-1 is similarly masked in Par-1/14-3-3 interaction. The PPIIn-binding carboxy-terminal region in Baz contains the aPKC and Par-1 phosphorylation sites (Krahn et al. 2010b). Phosphorylation by Par-1 creates binding sites for 14-3-3 protein and sequesters Baz to the cytosol (Benton and St. Johnston 2003b). aPKC phosphorylation of Baz prevents its interaction with Stardust (Sdt, Pals1 in mammals), whereas eliminating the aPKC-phosphorylation site results in mislocalization of Baz into the cytosol together with Sdt (Krahn et al. 2010a; Morais-de-Sa et al. 2010).

Again, these data are consistent with a model that binding of 14-3-3 and/or Stardust controls the PM localization of Baz by masking its PPIIn-binding carboxy-terminal region (Fig. 3A).

Coincident Protein Interactions

Direct PPIIn binding often acts together with additional mechanisms to achieve robust and/or polarized PM targeting (McLaughlin and Murray 2005; Hammond and Balla 2015). For instance, lipid modifications such as myristoylation enhances the PM localization of MARCKS (Arbuzova et al. 2002), and for KA-1-domain-containing kinase Kcc4p, its physical interactions with septin restricts PM-bound Kcc4p to bud necks in dividing yeast (Moravcevic et al. 2010). As to the PPIIn-binding polarity proteins, we are still at the beginning of discovering and characterizing their PPIIn-binding property and there is a lot to be elucidated about how myriad protein interactions previously characterized may act coincidentally with direct PM binding to achieve polarized PM localization. Baz has many confirmed interactions with apical polarity proteins such as Par-6 (Lin et al. 2000; Petronczki and Knoblich 2001; Yamanaka et al. 2001), aPKC (Izumi et al. 1998; Wodarz et al. 2000; Soriano et al. 2016), Sdt (Krahn et al. 2010a; Morais-de-Sa et al. 2010), and adherens junction complex (through β -catenin; Wei et al. 2005). Such coincident binding to apical polarity proteins may not only act to bias Baz to apical PM, but apparently also function as a redundant mechanism to retain Baz on the PM in PtdIns(4,5) P_2 -deficient mutant cells (Claret et al. 2014) and in cells undergoing hypoxia-induced loss of PPIIn (Dong et al. 2015). Similarly, basolateral polarity protein Dlg may specifically bind partially phosphorylated Lgl (Zhu et al. 2014; Bell et al. 2015; Carvalho et al. 2015). Such phosphorylation-dependent interaction with Dlg is not required for Lgl PM localization in vivo (Dong et al. 2015), but it may act in concert with aPKC to reinforce the basolateral PM localization of Lgl. Finally, Numb has a potential myristoylation site (Knoblich et al. 1997). Although its elimination does not appear to affect Numb PM targeting in vivo,

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it may still function to enhance the electrostatic binding of Numb to PM.

Hypoxia and ATP Depletion Regulate PPIIn-Dependent PM Targeting

Whereas many studies focused on mechanisms regulating PPIIn-binding domains, it has been much less appreciated that PM localization of PPIIn-binding proteins can be sensitive to physiological conditions that dramatically alter PPIIn levels at the PM. For instance, ATP depletion acutely and globally diminishes PPIIn in PM in cultured cells (Poggioli et al. 1983). It is well established that hypoxia acutely and reversibly reduces intracellular ATP levels (DiGregorio et al. 2001), and our recent studies showed that hypoxia also induces acute and reversible depletion of membrane PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 (Dong et al. 2015). One significant consequence of such loss of PPIIn is that proteins that rely on PPIIn binding as their primary PM-targeting mechanism will dislocalize from the PM (Fig. 3B). Indeed, hypoxia acutely and reversibly inhibits the PM localization of Lgl and Numb in vivo. In *Drosophila* epithelial cells, both protein become mislocalized from PM to cytosol within an hour of acute hypoxia and relocalizes to PM within ~10 min of reoxygenation (Dong et al. 2015). The time courses of Lgl and Numb PM relocalization match well with the reduction and recovery of intracellular ATP levels (DiGregorio et al. 2001) and PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 levels at PM (Dong et al. 2015) under acute hypoxia and reoxygenation. Consistent with the fact that aPKC PM localization depends on PtdIns(4,5) P_2 (Claret et al. 2014), hypoxia also dislocalizes aPKC from PM (Dong et al. 2015). However, genetic reduction of PtdIns(4,5) P_2 does not acutely disrupt Baz PM localization (Claret et al. 2014) and Baz remains on PM under hypoxia (Dong et al. 2015), suggesting that in the acute loss of PPIIn interactions with apical polarity complex and junctional complex likely act as a redundant mechanism to retain Baz on PM. How PM localization of Par-1 and Miranda may be affected by hypoxia and loss of PPIIn have yet to be investigated.

Although many non-PPIIn-binding polarity proteins and complexes such as adherens junction complex and Crb complex retain their PM localizations under hypoxia (Dong et al. 2015), the dramatic loss of PM targeting of both apical and basolateral polarity proteins such as aPKC and Lgl is a clear indication that apicobasal polarity in epithelial cells can be severely compromised under hypoxia. How such polarity compromise affects epithelial cells undergoing hypoxia and ischemia/reperfusion process has not been investigated. In addition, mechanisms retargeting PPIIn-binding polarity proteins back to specific PM domains during reoxygenation (Dong et al. 2015) remain unknown but could be crucial to maintain/restore the cell polarity during posthypoxia recovery.

It is noteworthy that hundreds to thousands of proteins in *Drosophila* and mammalian genome carry potential polybasic domains (Bailey and Prehoda 2015; GR Hammond and Y Hong, unpubl.). Thus, global loss of PM PPIIn attributable to hypoxia or ATP depletion does not only disrupt the PM localization of a few polarity proteins, it could have profound consequences by dislocalizing a large number of PPIIn-binding proteins and their partners whose biological functions require meticulously controlled PM localization. It should be of great interest to determine how such transient but severe mislocalization of PPIIn-binding proteins may contribute to the potential disruption and recovery of polarity and functions of epithelial cells during hypoxia–ischemia/reperfusion in vivo.

CONCLUDING REMARKS

PPIIn such as PtdIns(4,5) P_2 and PtdIns4P are major constituents of PM phospholipids. Although in many cells they are not necessarily asymmetrically distributed among many PM domains, as we discussed in this review, PtdIns4P and PtdIns(4,5) P_2 can play a fundamental role in cell polarity by attracting PPIIn-binding polarity proteins specifically to PM. The physical nature of the electrostatic interactions between charged PPIIn-binding polarity proteins and PtdIns4P and PtdIns(4,5) P_2 in

PM are neither polarized nor specific to PPIIn species. However, as we discussed here, several elegant mechanisms can regulate such charge-based interaction to achieve polarized and controlled PM targeting of select polarity proteins. It must be emphasized that we are still at the beginning of appreciating the fundamental role of electrostatic binding to PPIIn as a means of targeting polarity proteins to the PM, and much still needs to be learned to integrate this mechanism with myriad protein–protein interactions that have so far been characterized in polarity pathways. Finally, such PPIIn-binding-mediated PM targeting also brings an unexpected consequence that, for several key polarity proteins, their PM targeting becomes sensitive to the loss of PPIIn induced by conditions such as hypoxia and ATP depletion, raising the question of how cell polarity may be compromised under hypoxia and ATP depletion as seen in ischemia in vivo.

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