REVIEW ARTICLE

Out, in and back again: PtdIns(4,5)P_2 regulates cadherin trafficking in epithelial morphogenesis

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The morphogenesis of epithelial cells in the tissue microenvironment depends on the regulation of the forces and structures that keep cells in contact with their neighbours. The formation of cell–cell contacts is integral to the establishment and maintenance of epithelial morphogenesis. In epithelial tissues, the misregulation of the signalling pathways that control epithelial polarization induces migratory and invasive cellular phenotypes. Many cellular processes influence cadherin targeting and function, including exocytosis, endocytosis and recycling. However, the localized generation of the lipid messenger PtdIns(4,5)P_2 is emerging as a fundamental signal controlling all of these processes. The PtdIns(4,5)P_2-generating enzymes, PIPKs (phosphatidylinositol phosphate kinases) are therefore integral to these pathways. By the spatial and temporal targeting of PIPKs via the actions of its functional protein associates, PtdIns(4,5)P_2 is generated at discrete cellular locales to provide the cadherin-trafficking machinery with its required lipid messenger. In the present review, we discuss the involvement of PtdIns(4,5)P_2 and the PIPKs in the regulation of the E-cadherin (epithelial cadherin) exocytic and endocytic machinery, the modulation of actin structures at sites of adhesion, and the direction of cellular pathways which determine the fate of E-cadherin and cell–cell junctions. Recent work is also described that has defined phosphoinositide-mediated E-cadherin regulatory pathways by the use of organismal models.

Key words: cadherin, epithelial-to-mesenchymal transition (EMT), morphogenesis, phosphatidylinositol phosphate kinase (PIPK), PtdIns(4,5)P_2.

INTRODUCTION

The EMT (epithelial-to-mesenchymal transition) is the cellular mechanism by which a cell can alter its most fundamental characteristics in response to both intracellular and extracellular signalling events. Normal epithelial cells are polarized and form extensive contacts with their neighbours. However, there are key signals that entice an epithelial cell to dissolve these contacts and adopt mesenchymal-like characteristics. This process is essential for normal development, but, in many cancers, the cellular systems which regulate EMT can be hijacked. Consequently, misregulation of these systems leads to the dissociation of cells from the primary tumour, invasion into the vasculature and the establishment of satellite tumours in other organs. Impeding this morphogenic change is the transmembrane adhesion protein E-cadherin (epithelial cadherin), which, when displayed on the surface, will associate with the E-cadherin molecules of adjacent cells to form stable contacts. The strength of these cell–cell contacts, termed adherens junctions, is directly dependent upon the amount of E-cadherin at the plasma membrane. Recent work has underscored the importance of E-cadherin exocytosis, endocytosis and recycling as contributing factors to the overall stability of E-cadherin based cell–cell junctions. A growing body of research has granted credence to the notion that phosphoinositide signalling pathways tightly regulate these crucial activities. The present review will showcase the most current findings in the regulation of E-cadherin and adherens junction biology by PtdIns(4,5)P_2-mediated signalling pathways.

Phosphoinositide metabolism, diversity and signalling specificity

The cellular phosphoinositide signalling system, an intricate network of enzymes and phospholipid messengers, is a critical regulator of most, if not all, cellular processes. The cell generates and is able to utilize multiple phosphoinositides as unique messenger molecules in the transduction of extracellular or intracellular signalling. To further complicate matters, the existence of distinct cytoplasmic and nuclear phosphoinositide signalling networks have become apparent by observing the specific targeting of distinct isoforms of the enzymes that generate these lipids [1–7]. Figure 1 illustrates the cellular metabolic pathways resulting in the generation of specific phosphoinositides. The simplest inositol phospholipids are the phosphatidylinositol phosphates, PtdIns3*P*, PtdIns4*P* and PtdIns5*P*, which are singly phosphorylated on the inositol head group and have a unique set of effector proteins [8]. Each of these phospholipids can undergo a second or even third phosphorylation event, resulting in a multitude of distinct signalling molecules (Figure 1). The polyphosphoinositides PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 are generated by sequential phosphorylation of the inositol head group, or produced by the de-phosphorylation of PtdIns(3,4,5)P_3

Abbreviations used: AP, adaptor protein; Arf6, ADP-ribosylation factor 6; Arp2/3, actin-related protein 2/3 complex; Cdc42, cell division cycle 42; E, embryonic day; E-cadherin, epithelial cadherin; EEA1, early endosome antigen 1; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial-to-mesenchymal transition; ERM, ezrin/radixin/moesin; FERM, 4.1/ezrin/radixin/moesin; IL, interleukin; MDCK, Madin–Darby canine kidney; N-cadherin, neural cadherin; NF2, neurofibromatosis 2; N-WASP, neuronal Wiskott–Aldrich syndrome protein; PI3K, phosphoinositide 3-kinase; PIPK, phosphatidylinositol phosphate kinase; PIPKI, type I PIPK; PIPKII, type II PIPK; PIPKIII, type III PIPK; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SNX, sorting nexin; VE-cadherin, vascular endothelial cadherin.

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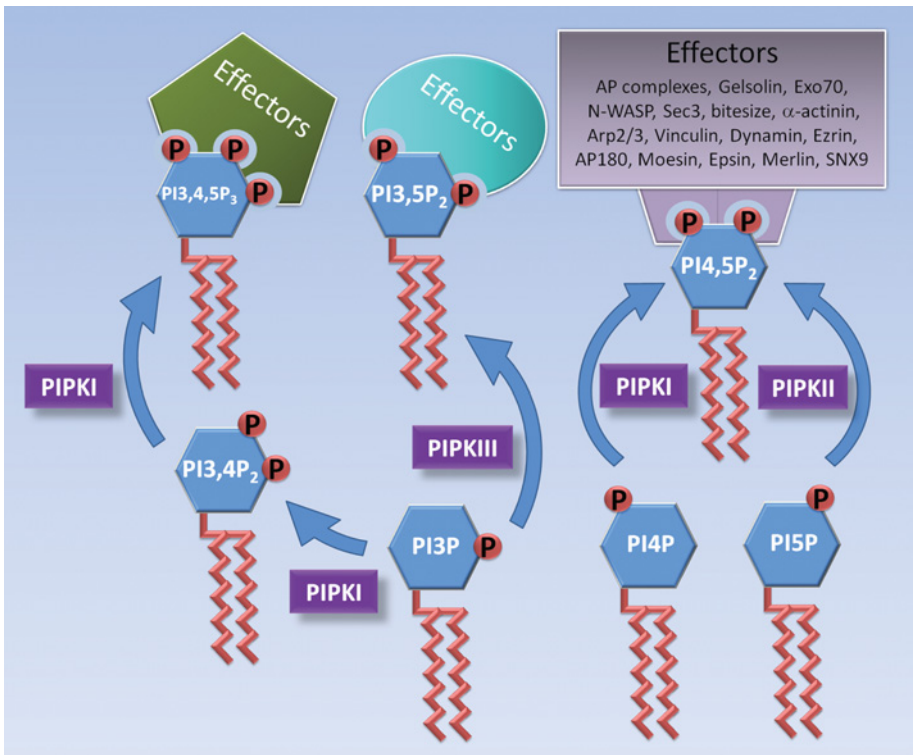

Figure 1 Specific phosphoinositide messengers regulate protein activities

Polyphosphoinositides can be generated from PtdIns3*P* (PI3P), PtdIns4*P* (PI4P) and PtdIns5*P* (PI5P) by enzymes of the PIPK family. Importantly, each of these polyphosphoinositides possesses a specific group of protein effectors within the cell, and the binding of these lipids to their protein partners acts to modulate protein activity. $PI3,4,5P_3$, PtdIns(3,4,5)P_3; $PI3,5P_2$, PtdIns(3,5)P_2; $PI3,4P_2$, PtdIns(3,4)P_2; $PI4,5P_2$, PtdIns(4,5)P_2.

[1,5–7,9–12]. The enzymes responsible for the generation of these lipids within the cell are as diverse as the signalling molecules they generate. There are three classes of PIPKs (phosphatidylinositol phosphate kinases): the PIPKIs (type I PIPKs) phosphorylate PtdIns4*P* to PtdIns(4,5)P_2, PIPKIIs (type II PIPKs) phosphorylate PtdIns5*P* to PtdIns(4,5)P_2 and PIPKIIIs (type III PIPKs) phosphorylate PtdIns3*P* to PtdIns(3,5)P_2 [1,6]. In addition, PIPKI has also been shown to phosphorylate PtdIns3*P* at the 4′- and 5′-hydroxy groups to generate PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vitro*. The PIPKIIs can also generate PtdIns(3,4)P_2 from PtdIns3*P* [1,6], although this activity is relatively low compared with that of the PIPKI isoform. The metabolism of PtdIns species by phosphoinositide phosphatases, such as PTEN (phosphatase and tensin homologue deleted on chromosome 10) and synaptojanin, also generate specific phospholipids in response to cellular signalling pathways [13]. The PI3K (phosphoinositide 3-kinase) family phosphorylates at the 3′-position of the inositol ring, and individual subclasses of these enzymes display substrate specificity to generate distinct 3′-phosphorylated species [14,15]. PI3K family members are involved in many different aspects of the regulation of E-cadherin and adherens junction biology, and this topic has been reviewed in depth elsewhere [15].

The PIPKs are a unique family of enzymes that shares little sequence homology with protein kinases or other lipid kinases [16,17]; however, the PIPK type I, II and III subfamilies display extensive sequence homology within their catalytic domains [1]. The greatest amount of sequence divergence between the subfamilies lies in their N- and C-termini, and this variability is key for the functional divergence of the kinases [6]. The multiple type I and type II PIPKs are classified as α, β and γ isoforms, and are still further divided by the existence of several splice variants within an individual isoform [6]. This ‘variety pack’ of PIPKs suggests that each isoform fills a functional niche within the cell.

PtdIns(4,5)P_2 diffuses rapidly in membranes, and its concentration does not fluctuate substantially in cells or at the plasma membrane [6]. This suggests that PtdIns(4,5)P_2 signals differently from second messengers, such as cAMP, whose cellular concentration changes considerably. There are hundreds of PtdIns(4,5)P_2 effectors within the cell, and these are present in most membrane compartments and in the nucleus [2,5–7]. These observations led to the hypothesis that the PtdIns(4,5)P_2 signal is generated in close proximity to its effectors [18], and is supported by the observation that the three type I PIPK isoforms localize to distinct regions of the cell [6]. The cellular targeting of the PIPKs in turn is dependent upon specific protein–protein interactions. A well-characterized example of this is the regulation of focal-adhesion dynamics via the site-specific generation of PtdIns(4,5)P_2 by PIPKIγ [19,20]. Focal adhesions, which are sites of integrin-mediated cellular contact with the extracellular matrix, are dependent upon the assembly of a multitude of structural and signalling molecules to effectively link the actin cytoskeleton to an integrin [21–24]. Assembly of focal adhesions is dependent on the scaffolding molecule talin [22,24]. Talin is a PtdIns(4,5)P_2 effector, and PtdIns(4,5)P_2 regulates the interaction of talin with integrins [25]. Through an association of the C-terminus of PIPKIγ with talin, PIPKIγ is targeted to focal adhesions [19,20], and the PtdIns(4,5)P_2 generated at these sites serves to activate and/or recruit other proteins to the adhesion complex [7]. The association of PIPKs with a targeting protein and their localization to a subcellular domain containing both lipid substrate and effector proteins is an emerging mechanism for regulating

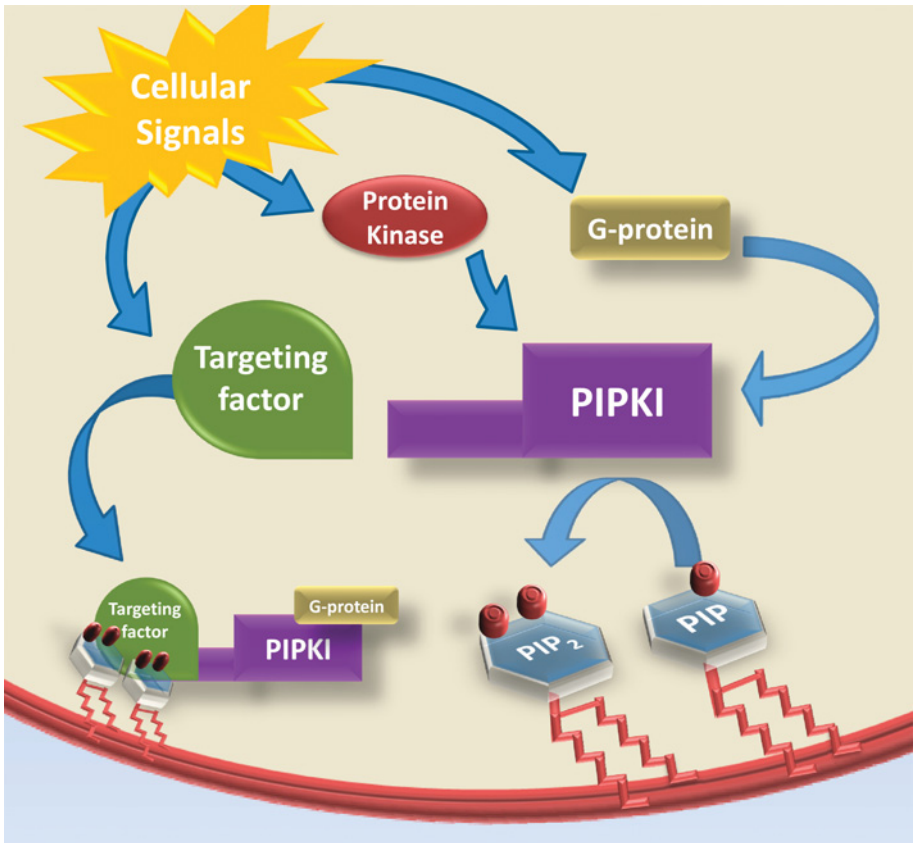

Figure 2 PtdIns(4,5)P_2 generation is spatially and temporally regulated

In order to act as a succinct signalling molecule, PtdIns(4,5)P_2 (PIP_2) is generated only where and when it is needed for function. PIPKI activity is modulated downstream of signalling cues such as G-proteins, tyrosine and serine phosphorylation, or other post-translational modifications. PIPKIs are targeted to discrete subcellular domains by the binding of proteins that can act as targeting factors. At these sites, PtdIns(4,5)P_2 is generated for use by its effectors. In addition, many proteins which physically associate with PIPKIs are also PtdIns(4,5)P_2 effectors. PIP, PtdIns*P*. An animated version of this Figure can be found at http://www.BiochemJ.org/bj/418/0247/bj4180247add.htm.

PtdIns(4,5)P_2 synthesis (for reviews, see [1,6,7,18]) (Figure 2). To further regulate the function of the PIPKs, serine and tyrosine kinases directly phosphorylate the PIPKs to influence their targeting, activity and/or protein–protein interactions [6,7,26,27]. Collectively, these strategies ensure that the appropriate lipid signalling molecule is generated exactly where it is needed for cellular function.

Classical cadherins and the organization of adherens junctions

The adherens junction and its associated proteins are an extensively studied subset of intracellular adhesion complexes [28–30]. The adherens junction is organized along the basolateral membrane of polarized epithelial cells, and is flanked by tight junctions at the apical–lateral border. Adherens junctions are crucial in establishing epithelial cell polarity, mediating cell–cell communications and providing a scaffold for intracellular signalling. The 'kingpin' of the adherens junction is E-cadherin, which is the most prominent member of the classical cadherin family. Classical cadherins are transmembrane glycoproteins that initiate homophilic *trans*-dimers with the same cadherin on opposing cells in a Ca^{2+}-dependent manner [30,31]. While the ectodomains of the classical cadherins are occupied by their association with cadherin molecules on adjacent cells, the cytoplasmic cadherin tail is engaged in courtship of the catenins and other cytoplasmic signalling molecules [32,33]. This functional association of the tail with cytoplasmic proteins is vital for E-cadherin to function as a cell–cell adhesion molecule [34]. In the case of adherens junction formation, the sequential binding of β-catenin and α-catenin to the E-cadherin cytoplasmic tail facilitates an uplink between E-cadherin and the actin cytoskeleton, while binding of p120-catenin to the juxtamembrane domain of E-cadherin stabilizes the assembled complex [32,33,35]. Interestingly, the mechanism by which E-cadherin is physically linked to actin remains a matter of debate [35,36]. Regardless, the molecular bridge which exists between E-cadherin and the cytoskeleton via regulation of the catenins provides the tensile strength required to hold cells together in the tissue microenvironment. Aside from roles as structural molecules, both β-catenin [37] and p120-catenin [38–40] act as potent signalling molecules by translocating to the nucleus and serving as transcriptional regulators, thereby forging a link between cell adhesion and gene expression.

Another classical cadherin, N-cadherin (neural cadherin), is normally expressed in mesenchymal cells and cardiac and neural tissues, and can also mediate adhesion between cells in a manner similar to that of E-cadherin. However, aberrant expression of N-cadherin can coincide with the down-regulation of E-cadherin in epithelial cells, and this process is known as the 'cadherin switch', a signature prelude to EMT [41,42]. The switch to N-cadherin expression is thought to influence an epithelial cell to exhibit aggressive migratory and invasive behaviour, although the signalling events which instigate these activities are not fully understood [41,42]. However, this phenomenon underlines the

importance of maintaining E-cadherin-based cell–cell contacts to the overall integrity of epithelial tissues.

The field of adherens junction biology has only begun to scratch the surface of the multitude of regulatory pathways that govern their formulation, maintenance and dissolution. Classical cadherins make up the cornerstone of adherens junctions, and the proper regulation of cadherin exocytosis, endocytosis, recycling and degradation is crucial for normal adherens junction biology. Each of these trafficking steps is dependent upon phosphoinositides and the enzymes that synthesize them.

TARGETING OF E-CADHERIN TO THE BASOLATERAL MEMBRANE

The regulation of exocytic targeting of newly synthesized E-cadherin to the plasma membrane is critical for the formation and maintenance of adherens junctions [43]. Multiple mechanisms exist for the efficient delivery of E-cadherin to the plasma membrane, and these pathways appear to be cell-line- or polarity-dependent. Although the factors which govern these exocytic mechanisms are not fully defined, these pathways have been shown to be regulated at key intersections by PtdIns(4,5)P_2 signalling.

Basolateral targeting of E-cadherin by a dileucine motif is regulated by PtdIns(4,5)P_2 signalling

The establishment of functionally distinct regions within the plasma membrane is a hallmark of epithelial cell polarity. The formation of specialized apical and basolateral zones are dependent upon the proper sorting of proteins, such as E-cadherin, and is required for the establishment of cell polarity. The clathrin AP (adaptor protein) family is thought to be the primary mediator of these protein-sorting events. Via recognition of dileucine- and tyrosine-based peptide signals, at least four different heterotetrameric AP complexes act as cargo adaptors to facilitate sorting of proteins within the *trans*-Golgi network and endosomal compartments (i.e. AP1, AP3 and AP4), and also mediate internalization of cell-surface receptors (i.e. AP2) [44–46]. In addition, AP complexes are regulated by phosphoinositides, in particular PtdIns(4,5)P_2 [47,48]. A conserved dileucine motif in the juxtamembrane domain of most classical cadherins was required for basolateral targeting of E-cadherin in LLC-PK1 cells, and this targeting was independent of association with β-catenin and p120-catenin [49,50]. However, conflicting reports raise questions as to whether the dileucine motif is required for proper sorting of E-cadherin. Miyashita and Ozawa [51,52] showed that E-cadherin lacking the complete cytoplasmic tail was trafficked successfully to the basolateral membrane, suggesting that this dileucine motif is not an absolute requirement for efficient sorting of E-cadherin. Also, mutation of the dileucine motif did not prevent the basolateral targeting of E-cadherin [51], but instead was required for the endocytosis of E-cadherin from the plasma membrane [52]. Moreover, an E-cadherin mutant lacking the β-catenin binding site was shown to accumulate in a cytoplasmic compartment and was subsequently targeted to the lysosome [51,52], indicating a requirement for β-catenin association for efficient basolateral targeting. This is consistent with the observation that the assembly of the E-cadherin–β-catenin complex occurs early in the exocytic pathway [53]. Further work is necessary to resolve these discrepancies and to more fully define the mechanisms utilized in the basolateral sorting of E-cadherin.

PIPKIγ is a signalling scaffold required for E-cadherin basolateral membrane targeting via AP1B

A requirement for PtdIns(4,5)P_2 has also been established in the basolateral targeting of E-cadherin via a tyrosine sorting motif recognized by the AP complexes. PIPKIγ directly associates with E-, N- and VE-cadherin (vascular endothelial cadherin), and co-localizes with E-cadherin at cell–cell contacts in MDCK (Madin–Darby canine kidney) epithelial cells [54,55]. The interaction site of PIPKIγ on E-cadherin was mapped by truncation and was shown to require amino acids 837–847 on its cytoplasmic tail. These residues are also within the β-catenin-binding site [54], suggesting that an association of β-catenin and/or PIPKIγ with E-cadherin in this region may be subject to regulation. Whether both PIPKIγ and β-catenin can form a ternary complex with E-cadherin remains to be defined (see below), but as β-catenin association regulates both trafficking and formation of stable junctions, it is likely to prove functionally significant.

PIPKIγ is required for adherens junction assembly, as the loss of PIPKIγ by siRNA (small interfering RNA) knockdown induced the accumulation of E-cadherin in a cytoplasmic compartment [54]. PIPKIγ-knockdown cells displayed a dramatic morphological transformation from that of polarized epithelia to a migratory mesenchymal phenotype [54]. Overexpression of PIPKIγ enhanced the targeting of E-cadherin to cell–cell contacts, whereas expression of a kinase-inactive analogue inhibited E-cadherin transport [54]. A particular splice variant of PIPKIγ, PIPKIγ661, contains a 26 residue C-terminal extension that includes a 'YSPL' sequence, which is a tyrosine sorting motif. Tyrosine sorting motifs have the general sequence Yxxϕ (where 'x' is any residue and 'ϕ' is a bulky hydrophobic residue) [56]. This sorting motif is recognized by the μ1B subunit of the AP1B complex [56,57] which is the epithelial-cell-specific version of AP1 that mediates the sorting of proteins targeted to the basolateral membrane [58–60]. PIPKIγ661 was observed to associate with AP1B *in vivo* and *in vitro* via its Yxxϕ sorting motif, and was able to recruit AP1B to sites of E-cadherin recycling after EGTA-induced E-cadherin internalization and calcium rescue [54]. Given the current data, it has been suggested that PIPKIγ661 acts as both a sorting adaptor and signalling molecule for the basolateral targeting of E-cadherin. By simultaneously associating with both E-cadherin and AP1B, PIPKIγ661 acts as a bridge to connect E-cadherin to the AP1B-coupled trafficking machinery (Figure 3) [54]. The generation of PtdIns(4,5)P_2 by PIPKIγ661 would then be able to locally regulate the activities of AP complexes and other trafficking proteins that are regulated by PtdIns(4,5)P_2. In this model, PIPKIγ is a dual-purpose molecule, and it will be interesting to determine whether similar PIPKIγ-regulated pathways exist which mediate the trafficking of other cell-surface receptors or adherens junction proteins. As the PIPKIγ/β-catenin-binding sites on E-cadherin partially overlap, it will also be important to determine the spatial regulation and function of β-catenin in this exocytic pathway.

The importance of the PIPKIγ661 association with E-cadherin is underscored by the discovery of a germline mutation in patients with hereditary diffuse gastric cancer [61]. A V832M mutation in the cytoplasmic tail of E-cadherin reduced the ability of cells expressing the mutant to form cell–cell contacts while increasing invasion of cells into a collagen matrix [62]. These phenotypes suggest a deficiency in this mutant to form effective cell–cell contacts. Although its association with β-catenin was normal, the V832M mutation inhibited the association of PIPKIγ661 with E-cadherin [54,62]. Also, a majority of the V832M mutant E-cadherin protein accumulated in cytoplasmic compartments, indicating a failure of the protein to be efficiently delivered to the plasma membrane [54]. These results demonstrate that the association of E-cadherin with AP1B via the PIPKIγ661 scaffold is required for its targeting to the basolateral membrane. In addition, the V832M mutation is within the β-catenin-binding site and, from the crystal structure of the β-catenin–E-cadherin complex,

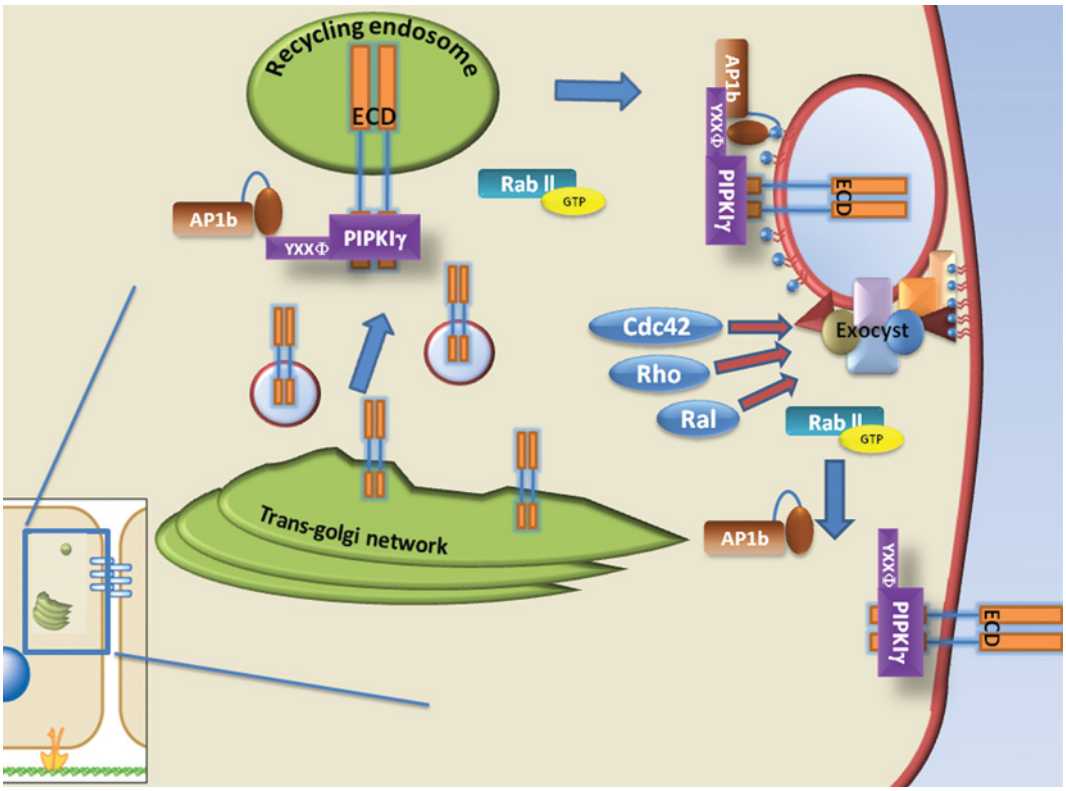

Figure 3 AP1B/PIPKIγ-dependent E-cadherin exocytosis

PIPKIγ661 acts as both a scaffolding and signalling molecule by linking E-cadherin (ECD) to AP1B, allowing for the trafficking of E-cadherin from the recycling endosome to the basolateral plasma membrane. At the plasma membrane, the exocyst complex assembles in a PtdIns(4,5)P_2-dependent manner to mediate fusion of the exocytic vesicle with the plasma membrane. Further regulation of PIPKIs and the exocyst at the site of exocytosis occur via the actions of the small G-proteins Ral, Cdc42 and Rho.

Val^{832} is facing away from β-catenin [63]. This structural arrangement could explain the loss of PIPKIγ binding upon mutation of Val^{832} and the relative indifference of β-catenin association to this change. However, the interaction of PIPKIγ with E-cadherin at this interface would position PIPKIγ to regulate both β-catenin and E-cadherin functions.

The PIPKIγ-knockout mouse created by Abrams's group [64] confirms the physiological importance of the PtdIns(4,5)P_2-generating enzyme in adherens junction biology. This mouse was embryonic lethal at E11.5 (where E is embryonic day), and displayed numerous developmental defects. Embryonic cardiomyocytes are rich in cadherin-based cell–cell junctions termed fascia adherens. Cardiomyocytes from E10.5 embryos displayed loss of actin cable organization in the sarcomere and failure of actin cables to associate with the fascia adherens, which coincided with the absence of N-cadherin from this region [64]. In addition, PIPKI$\gamma^{-/-}$ mouse embryos formed shorter and less organized adherens junctions in the central nervous system as compared with wild-type embryos, indicating that PIPKIγ is a core component for establishment of the embryonic nervous system [64]. Interestingly, although N-cadherin-knockout embryos exhibit a loss in adherens junction formation [65], N-cadherin-knockout myocytes still display organized actin bundle arrays that associate with the fascia adherens [64]. Conversely, a PIPKI$\gamma^{-/-}$ mouse created by the De Camilli group [66,67] displayed defects in neuronal vesicle trafficking and exhibited a decrease in PtdIns(4,5)P_2 production in neuronal cells, but was not embryonic lethal. The reason for the differences in the mouse phenotypes remain unclear, but both agree with the proposed role for PIPKIγ in the generation of a pool of PtdIns(4,5)P_2 required for cellular trafficking pathways.

The exocyst mediates targeting and fusion of E-cadherin-containing vesicles with the basolateral membrane

The exocyst complex, consisting of eight protein subunits, appears to direct membrane-protein-containing vesicles to their proper site of fusion on the plasma membrane [68]. The exocyst complex may dictate apical compared with basolateral delivery of exocytic vesicles [45,46], which could be an important determinant for defining the identity of membrane subdomains during epithelial cell polarization [69]. Interestingly, several exocyst components have been shown to be regulated by PtdIns(4,5)P_2. Exo70, which is thought to anchor the exocyst at sites of fusion in co-ordination with Sec3, directly binds PtdIns(4,5)P_2 to mediate its association with the plasma membrane [70,71]. PtdIns(4,5)P_2 association with Exo70 was also required for the recruitment of other exocyst components to the plasma membrane [71], suggesting that PtdIns(4,5)P_2 is a fundamental regulator of the exocyst complex. Accordingly, disruption of the PtdIns(4,5)P_2–Exo70 interaction disrupted the tethering and fusion of vesicles at the plasma membrane [70,71]. In addition, Sec3 required both PtdIns(4,5)P_2 and Cdc42 (cell division cycle 42) in order to perform its exocytic functions [72]. These results are in agreement with the observed roles for PtdIns(4,5)P_2 and PIPKIγ in fusion dynamics of neuronal exocytic vesicles [66,67,73,74]. In light of this, PIPKIγ or another PIPKI may associate with and regulate one or several exocyst components. Subsequently, the generation

of PtdIns(4,5)P_2 at the exocytic site could then drive multiple steps of exocyst-directed plasma membrane delivery. The exocyst is also subject to several levels of regulation by small G-proteins such as Rab, Rho and Ral [75,76], and there is evidence that small G-protein activity regulates PIPKI function [77]. Although specific regulation of PIPKI by these small G-proteins needs to be further defined, PtdIns(4,5)P_2 generation at multiple steps in exocytic pathways is required [54].

The precise mechanism by which the exocyst mediates the delivery of E-cadherin to the basolateral membrane of epithelial cells is not understood. However, one possibility is that the AP1B complex and its cargo proteins may utilize the exocyst for targeted fusion with the basolateral membrane [45,46]. PIPKIγ serves as a cargo adaptor to bridge E-cadherin and AP1B for the efficient basolateral targeting of E-cadherin [54]. The delivery and fusion of AP1B vesicles containing E-cadherin may also be mediated directly or indirectly by PIPKIγ and the exocyst complex (Figure 3).

In *Drosophila*, several components of the exocyst were shown to regulate the trafficking of E-cadherin from recycling endosomes to the plasma membrane [78]. When function-deficient exocyst components were introduced into *Drosophila* epithelial cells, E-cadherin accumulated in a compartment identified as a recycling endosome [78]. As the recycling endosome is an intermediate compartment in both the trafficking of biosynthetic proteins as well as those being recycled from the cell surface [79], it is possible that the exocyst is required for E-cadherin delivery to the plasma membrane in both cases [78,80]. The movements of biosynthetic E-cadherin were tracked in MDCK cells, and pre-exocytic E-cadherin accumulated in Rab11-positive recycling endosomes before it was targeted to the basolateral membrane [81]. Since the exocyst component Sec15 associates with Rab11 [82], it is possible that Rab11 may regulate the activities of the exocyst. In addition, Rab11 is probably a regulator of the transcytotic activities of the exocyst for targeting of recycled junctional components in polarized epithelial cells [83]. In light of the current evidence, the exocyst probably functions in multiple pathways to mediate the delivery of both biosynthetic and recycled E-cadherin to the basolateral plasma membrane, and PtdIns(4,5)P_2 signalling regulates these processes.

JUNCTION ESTABLISHMENT AND MAINTENANCE IS REGULATED BY ACTIN DYNAMICS AND PtdIns(4,5)P_2

The establishment, maturation and maintenance of adherens junctions is dependent upon the local organization of actin filaments and its regulatory proteins at sites of cell–cell contacts (see [84] for a review). The cytoplasmic domain of E-cadherin acts as a scaffold to organize these processes. E-cadherin associates with α-catenin, which in turn modulates links with actin filaments and actin-binding proteins, such as vinculin and α-actinin, to form a stable actin cytoskeletal ring around the apical/basolateral interface of polarized epithelial cells [85]. In addition, small G-proteins play an important role in the regulation of actin-binding and adherens-junction-targeted proteins, and are potent activators of PtdIns(4,5)P_2 generation via association with PIPKIs [77]. Recent work has also established the ERM (ezrin/radixin/moesin) family of PtdIns(4,5)P_2-regulated actin-binding proteins as important regulators of adherens junctions formation and stability (as described below). Many actin-associated proteins which drive actin polymerization and the assembly of actin filaments are regulated by PtdIns(4,5)P_2 [7,86], so PtdIns(4,5)P_2-generating enzymes are likely to be recruited to sites of newly forming or established cell–cell contacts in order to regulate the activities of these proteins and ensure the continued integrity of adherens junctions.

PIPKIγ co-ordinates with small GTPases to regulate N-cadherin junction stability and gelsolin activity

The PtdIns(4,5)P_2-binding domain of PLCγ1 (phospholipase Cγ1) was used to show that PtdIns(4,5)P_2 is generated at sites of N-cadherin junction formation as well as at mature contact sites, and that this generation required PIPKIγ [87]. Importantly, the appearance of locally concentrated PtdIns(4,5)P_2 pools coincided with physical contact between adjacent cells, indicating that N-cadherin *trans*-dimerization is likely to be the initiating factor for increased PtdIns(4,5)P_2 production [87]. Furthermore, the inhibition of PtdIns(4,5)P_2 synthesis by expression of kinase-inactive PIPKIγ greatly reduced the physical strength of N-cadherin-based contacts [87]. N-cadherin ligation is a potent activator of Rho GTPases [88], and RhoA and Rac1 are known to independently regulate the activity of type I PIPKs [89]. RhoA was found to recruit PIPKIγ to N-cadherin-based adhesions, while the activation of Rac1 stimulated localized PtdIns(4,5)P_2 production at these sites [87], indicating that the Rho GTPases are critical to the integrity of at least N-cadherin-based contacts. Gelsolin is a PtdIns(4,5)P_2-activated actin-severing and barbed-end capping protein which encourages dynamic rearrangements in the actin superstructure by increasing the number of actin filaments [90]. The enhancement of N-cadherin-based adhesions resulting from Rho-induced PIPKIγ targeting and increased PtdIns(4,5)P_2 synthesis is in part due to the enhanced dissociation of gelsolin from actin filaments. As a result, the barbed end of actin is uncapped, which positively regulates extension and strengthening of adhesive contacts [87,91]. Given that N-cadherin is mainly expressed in mesenchymal cells which are inherently less polarized and more motile than their epithelial counterparts [42], the dynamic regulation of actin growth and protrusion at N-cadherin contacts facilitated by gelsolin may represent a pathway that is less active in epithelial cells to preserve the stability of E-cadherin-based junctions.

As PIPKIγ is also present at E- and VE-cadherin-based adhesions, the Rho GTPases probably regulate the strength and stability of these contacts. Similar to the downstream signalling of N-cadherin ligation, Rac1 and Cdc42 are activated by the engagement of E-cadherin molecules on adjacent cells and this drives the actin nucleation activity of Arp2/3 (actin-related protein 2/3 complex) [92–94]. Likewise, the recruitment of PIPKIγ to contacts via activated Rac1 and/or Cdc42 would allow for the generation of the PtdIns(4,5)P_2 required to regulate actin assembly at nascent adhesions.

A pathway involving PIPKI, ezrin and Rac1 regulates adherens junction stability

The actin-binding, ERM-family protein ezrin is a potent regulator of cell migration and adhesion, and its activities are modified by PtdIns(4,5)P_2 binding in both Rho-dependent and -independent pathways [95]. Serine phosphorylation and PtdIns(4,5)P_2 binding to the N-terminal FERM (4.1/ezrin/radixin/moesin) domain disengages the head-to-tail intramolecular association of ERM proteins, which allows ERM proteins to bind specific surface receptors and the cytoskeleton [96]. The mechanism for ezrin activation independent of Rho is probably reliant on Rac1 and/or Arf6 (ADP-ribosylation factor 6) recruitment of PIPKI and the subsequent localized pooling of PtdIns(4,5)P_2 [97,98]. Rac1 was found to drive the localization of ezrin to N-cadherin cell–cell junctions, and the expression of PIPKIα could mimic this effect on ezrin localization [99]. Rac1 has also been suggested as a downstream effector of ezrin activation, as dominant-active ezrin induced the formation of lamellipodia which disrupted

cell–cell junctions in a Rac1-dependent manner [100]. In addition, dominant-active ezrin expression delayed the recycling of internalized E-cadherin to cell–cell contacts, and also inhibited the endocytosis of E-cadherin from the plasma membrane upon calcium withdrawal [100].

As Rac1 is activated upon the establishment of cadherin-based cell junctions [101,102], this suggests that Rac1 and ezrin may act to either promote or antagonize the formation of cadherin-based junctions. However, the signalling context in which ezrin and Rac1 are regulated to control junction stability still lacks definition. It is likely that normal activation of these pathways is influenced by the current state of junction establishment [101] and extracellular signalling by growth factors or other molecules. In support of this, ezrin and c-Src were shown to co-operate to induce dissolution of adherens junctions, promote cell scattering [103] and facilitate the metastasis of breast carcinoma cells [104]. The activation of c-Src downstream of growth-factor-receptor pathways has also been shown to regulate the activities of PIPKI [26,27,105]. One possibility is that Src stimulates enhanced PtdIns(4,5)P_2 generation by activating PIPKI, thereby making a local specific pool of PtdIns(4,5)P_2 available for ezrin activation. Additional work is needed to fully characterize this complex pathway and the overall effects it may exert on cadherin-mediated adhesion.

Merlin translates extracellular signals to stabilize cell–cell junctions

Another ERM-family member implicated in the regulation of junctional organization is the neurofibromatosis 2 locus gene product, NF2, also called Merlin. Mutations in the *NF2* gene correlate with specific human cancers, including Schwann cell tumours (Schwannomas), mesotheliomas, thyroid carcinomas and hepatocarcinomas [106]. Like ezrin, Merlin is subject to regulation of its activation state by PtdIns(4,5)P_2 association with its FERM domain and also phosphorylation of its C-terminus [96]. Merlin is targeted to and associates with adherens junction protein complexes [107], and associates with and stabilizes actin filaments *in vitro* [108]. NF2$^{-/-}$ primary cells display a striking loss of contact-inhibited growth and failure to form adherens junctions [107]. Therefore Merlin may serve as an important linker of junction components to the actin cytoskeleton at cell–cell contacts. Merlin, along with the ERM proteins, can also negatively regulate the ability of N-WASP (neuronal Wiskott–Aldrich syndrome protein) to signal for actin branching and assembly through Arp2/3 [109]. This suggests that when autoinhibition of N-WASP is relieved by PtdIns(4,5)P_2 binding, the Arp2/3 complex is able to nucleate actin assembly and branching at adherens junctions [110]. Nonetheless, the exact role of Merlin in junction formation has not yet been resolved. As Merlin is also able to inhibit EGFR [EGF (epidermal growth factor) receptor] signalling upon cell–cell contact [111], it is possible that Merlin-mediated EGFR inhibition could be a greater influence on the successful formation of contacts than its role in stabilizing actin at nascent adhesions [112]. In this model, Merlin could serve as a signal microprocessor by interacting with both growth factor and adhesive receptors, and by integrating, relaying and interpreting signals from both receptor types, the cell could be pushed into the appropriate polarity or migratory state [113]. Thus the function of Merlin parallels that of PIPKIγ661 in the regulation of both polarization and directional migration [7,54,114]. Further examination of the role of phosphoinositides in the regulation of Merlin is likely to yield functional links.

Interestingly, Merlin-mutated Schwannoma cells display erratic membrane ruffling at sites of cell–cell contact, while this type of ruffling only occurs transiently during the formation of mature adherens junctions in normal Schwann cells [32,115]. This abnormal ruffling was found to be driven by the non-directional activation of Cdc42 and Rac1, and the constitutive recruitment of these proteins with Arp2/3 to the plasma membrane [116]. Because of this ruffling, cells in contact with their neighbours release from other cells rapidly, and the N-cadherin, α-catenin and β-catenin molecules present at the plasma membrane are rarely assembled into mature adherens junctions [115,116]. The reintroduction of Merlin into these cells inhibited excessive membrane ruffling and adherens junctions appeared more mature [116], which suggests that Merlin is a stabilizer of adherens junctions. Although a putative role for PIPKI in the regulation of this pathway has not been determined, the excessive membrane ruffling observed in Schwannoma cells may at least in part be driven by abnormally high PtdIns(4,5)P_2 generation by activated PIPKIα downstream of Rac1 [117].

Drosophila bitesize spatially controls actin organization at junctions in co-ordination with moesin

A role for the synaptotagmin-like *Drosophila* protein bitesize in junction formation and stability in *Drosophila* embryos has recently been determined [118]. Loss of bitesize, a PtdIns(4,5)P_2-binding protein, resulted in normal E-cadherin recruitment to nascent junctions, but the overall stability of the junction was compromised due to the failure of actin filaments in the region to form a continuous network [118]. In *Drosophila* embryonic epithelia, adherens junctions do not automatically form at sites of cell–cell contact. Rather, the proper targeting and assembly of adherens junction components is likely to be directed by other, as-yet undefined, spatial cues [119]. Par-3 (a scaffolding protein thought to direct polarity), along with PtdIns(4,5)P_2, was identified as a critical spatial cue for bitesize recruitment to the adherens junction region, as the inhibition of PtdIns(4,5)P_2 in these embryos by neomycin injection recapitulated the bitesize-knockout phenotype [118].

Moesin, another PtdIns(4,5)P_2-activated protein and the only ERM-family member present in *Drosophila*, was recruited by bitesize to E-cadherin-containing adherens junctions [118]. Introduction of a dominant-negative mammalian ezrin into embryos resulted in a disorganization of E-cadherin at the junction and similar effects on epithelial integrity as the bitesize mutant [118]. This suggests that PtdIns(4,5)P_2 is a spatial cue and specific PtdIns(4,5)P_2-binding proteins are required for the proper organization and stability of adherens junctions. Although the role of skittles (the *Drosophila* orthologue of PtdIns4*P* 5-kinase) was not determined in this pathway, an alternative method of PtdIns(4,5)P_2 generation by PTEN (a Par-3-binding protein) [120] may function in this pathway. Regardless of the mechanism, it is clear that PtdIns(4,5)P_2 plays a critical role as a spatial cue for adherens junction formation and stability in the *Drosophila* embryo.

CADHERIN ENDOCYTOSIS: PtdIns(4,5)P_2 REGULATES REMOVAL OF ADHESIVE PROTEINS FROM THE CELL SURFACE

Cadherins integrated into a stable adhesive signalling complex at the plasma membrane are continuously fine-tuned by the regulated internalization of cadherins and their associated proteins. The endocytic turnover of cadherins does not affect the overall strength or stability of cell–cell adhesion, but rather serves to spatially reorganize individual contacts in response to a constantly changing microenvironment or to support the normal metabolic turnover of adhesive proteins [31,43]. Cadherins are also endocytosed in

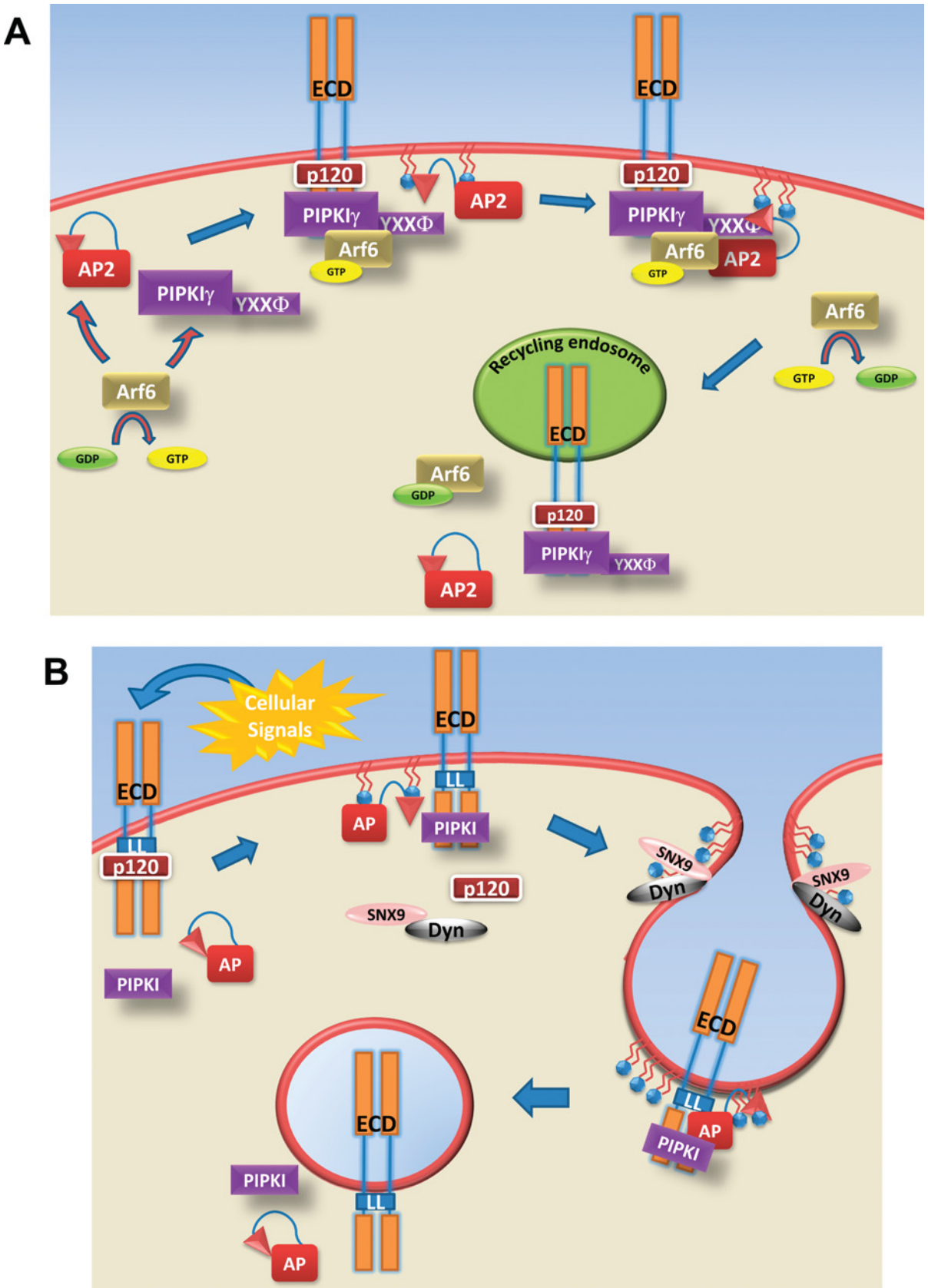

Figure 4 Tyrosine-compared with dileucine-facilited endocytosis of E-cadherin

The facilitation of E-cadherin internalization via two individual clathrin-dependent pathways suggests that the entry into one system compared with the other must be regulated via specific cellular signals. (**A**) AP2-mediated E-cadherin (ECD) endocytosis via the tyrosine sorting motif (Yxxϕ) and a PIPKIγ-scaffold may be the pathway in which E-cadherin could recycle back to the plasma

response to extracellular stimuli, such as growth factors, and prolonged stimulation of polarized epithelial cells can dissolve cell–cell contacts [31,85]. In this case, internalized E-cadherin is targeted for lysosomal degradation instead of being returned to the cell surface, resulting in a net loss of surface E-cadherin [31,121]. *In vivo*, the loss of E-cadherin-based contacts results in a migratory and invasive cellular phenotype, which is a hallmark of many metastatic tumours [41]. Given this fact, it is obvious that the amount of E-cadherin removed from the cell surface must be precisely tuned and subject to tightly regulated signalling pathways if a polarized cellular phenotype is to be maintained. The endocytosis of E-cadherin is accomplished by multiple mechanisms, including both clathrin-mediated and clathrin-independent endocytic pathways. As will be described below, the variety of signalling components which regulate E-cadherin endocytosis by these distinct pathways include PtdIns(4,5)P_2 and the enzymes which generate it.

Clathrin-dependent E-cadherin endocytosis is regulated by PtdIns(4,5)P_2

Receptors destined for endocytosis will associate with cytosolic adaptor proteins which catalyse the assembly of clathrin [122]. Assembly of the clathrin lattice is the preluding step to the formation of a clathrin-coated pit, which is the structural landmark of this endocytic pathway [123]. Several proteins present at these sites directly bind and are regulated by PtdIns(4,5)P_2, including AP complexes, AP180, epsin, Arp2/3 and dynamin, indicating that PtdIns(4,5)P_2 is a potent modulator of clathrin endocytosis [124]. In support of this, depletion of PtdIns(4,5)P_2 resulted in a loss of clathrin-coated pit formation and a dissociation of clathrin APs from the plasma membrane [125]. Accordingly, all three PIPKI isoforms have been implicated in the regulation of endocytic pathways via the site-specific generation of PtdIns(4,5)P_2 [6]. The AP2 complex is a multifunctional unit, as it serves as a docking site to link cargo to clathrin and also as a point of nucleation for clathrin lattice assembly [122]. PtdIns(4,5)P_2 facilitates the assembly of the AP2 complex on to cellular membranes [126], and is also a requirement for the binding of protein cargos containing a tyrosine sorting motif to the $\mu 2$ subunit of the complex [127] or cargos displaying a dileucine motif to the γ–$\sigma 1$ and α–$\sigma 2$ hemicomplexes [128]. The dependence of the AP2 complex on PtdIns(4,5)P_2 for function would suggest that AP2 itself closely associates with a PtdIns(4,5)P_2-generating enzyme. PIPKIγ661 was shown to directly associate with AP2 via the binding of the $\mu 2$ subunit to a tyrosine sorting motif in the C-terminus of the PIPKIγ661 splice variant [129]. There is also evidence that other sites of contact with PIPKIγ exist to facilitate the interaction with AP2, and that AP2 binding to PIPKIγ stimulates increased PtdIns(4,5)P_2 synthesis [130,131]. PIPKIγ661 expression was shown to greatly enhance the rate of E-cadherin internalization upon calcium withdrawal in polarized epithelial cells [54]. Expression of a kinase-dead PIPKIγ661 exhibited an opposite effect [54], reinforcing the concept that PIPKIγ and PtdIns(4,5)P_2 are critical regulators of endocytosis.

This endocytic pathway may also be regulated by Arf6, as Arf6 is a promoter of E-cadherin internalization [132] and PIPKIγ activity [133,134]. In addition, Arf6 directly associates with AP2 and promotes its recruitment to the plasma membrane, and this process is dependent on PtdIns(4,5)P_2 [135,136]. By a mechanism similar to that proposed for AP1B, PIPKIγ may act as a bridge to link E-cadherin to AP2 and the clathrin assemblage, thereby facilitating the endocytosis of E-cadherin from the cell surface (Figure 4A). In addition, positive regulation of PIPKIγ activity by Arf6 could stimulate complex assembly by providing the necessary PtdIns(4,5)P_2 for AP2-cargo binding and/or activation of other proteins required for efficient E-cadherin endocytosis. Although PIPKIγ–AP2 has been implicated in the regulation of other cell-surface receptors [129], direct evidence that links AP2 and PIPKIγ to the endocytosis of E-cadherin is lacking. However, it is likely that PIPKIγ–AP2 is a critical regulator of this process, and further work will elucidate these pathways.

The dileucine motif of E-cadherin facilitates endocytosis

PtdIns(4,5)P_2-regulated E-cadherin endocytosis via the AP complexes is probably facilitated by a second mechanism, as E-cadherin contains a dileucine sorting motif in the juxtamembrane region of its cytoplasmic tail (Figure 4B) [30]. As discussed earlier, p120-catenin associates with the juxtamembrane region of E-cadherin and is critical for the continued stability of cadherin-based junctions [30]. Loss of p120-catenin resulted in the rapid loss of E-cadherin and adhesive junctions, suggesting that p120-catenin can regulate the turnover of E-cadherin [137]. In addition, it is likely that p120-catenin is a critical regulator of the endocytosis of other classical cadherins. A fusion of the cytoplasmic tail of VE-cadherin to the IL (interleukin)-2 receptor was used to investigate the role of p120-catenin in VE-cadherin internalization [138]. The IL2–VE-cadherin fusion protein was rapidly internalized, and this required clathrin [138], suggesting that the dileucine motif on the cytoplasmic tail of VE-cadherin mediates associations with the endocytic machinery. In addition, association of p120-catenin with the tail of the chimaeric VE-cadherin blocked its internalization, indicating that p120-catenin is able to act as a membrane retention signal for VE-cadherin [138]. In a separate study, E-cadherin was uncoupled from p120-catenin via the mutation of several residues within the p120-catenin-binding site in the E-cadherin cytoplasmic tail [52]. In agreement with the previous study, p120-uncoupled E-cadherin is rapidly targeted to an early endosomal compartment, indicated by co-localization with EEA1 (early endosome antigen 1) and transferrin (markers of early endosomes) [52]. Importantly, mutation of the dileucine sorting motif on the p120-catenin-uncoupled E-cadherin resulted in the inhibition of endocytosis of the mutant [52], underscoring the importance of the dileucine motif in cadherin internalization. It is likely that p120-catenin can inhibit E-cadherin endocytosis possibly by masking the dileucine motif on E-cadherin and preventing its recognition by clathrin adaptors [52,138]. However, the endocytic AP(s) that associates with the dileucine motif of E-cadherin to link the E-cadherin complex to the clathrin lattice has yet to be determined. As PIPKIγ is a modulator of E-cadherin internalization, this clathrin-mediated endocytic pathway is likely to be dependent on and regulated by PtdIns(4,5)P_2 generation.

membrane after internalization. This pathway is subject to regulation by Arf6, as Arf6 can associate with AP2 and PIPKIγ and targets both proteins to the plasma membrane. As this pathway does not require dissociation of p120-catenin for endocytosis, E-cadherin would probably remain in a stabilized complex at the recycling endosome and be quickly returned to the cell surface, rather than being sent to the lysosome. In this model, the tyrosine-sorting-motif-mediated pathway could be a mechanism for constitutive E-cadherin endocytosis, as there is no net loss in E-cadherin. (**B**) In contrast, stimulated dissociation of p120-catenin from E-cadherin (ECD) would open up the dileucine (LL) motif for recognition by an as-yet undefined AP complex, and the destabilized E-cadherin complex would then be endocytosed in an SNX9- and dynamin (Dyn)-dependent manner. Generation of PtdIns(4,5)P_2 by a PIPKI would likely be required to activate the AP complex and to drive dynamin and SNX9 function at the site of vesicle fission. In this system, the fate of endocytosed E-cadherin is less clear. However, the identification of the specific clathrin adaptor(s) which mediate dileucine-based E-cadherin internalization will shed light on this pathway.

Endocytic accessory proteins are regulated by PtdIns(4,5)P_2: a connection to E-cadherin?

Various PtdIns(4,5)P_2-regulated accessory proteins such as SNX9 (sorting nexin 9), dynamin, AP180 and epsin facilitate efficient clathrin-mediated endocytosis [139]. Yet, it is unclear whether these proteins play a specific role in E-cadherin endocytosis, even though it is likely that the clathrin-mediated E-cadherin endocytic pathway would employ one or several of these proteins (Figure 3B). The GTPase dynamin is a prominent regulator of clathrin-mediated endocytosis, driving the fission of clathrin-coated pits from the plasma membrane in a PtdIns(4,5)P_2-dependent manner [139]. Dynamin is a known associate of cadherin-based junctional components [140], and is required for clathrin-dependent E-cadherin endocytosis [51,52]. Proper dynamin function is dependent upon its association with multiple accessory proteins, but SNX9 appears to be a fundamental regulator of dynamin activity. SNX9 associates with the core components of the AP2 complex [141,142], recruits dynamin to the plasma membrane [143], and is a promoter of dynamin activity [144]. SNX9 is also likely to co-ordinate actin dynamics and membrane remodelling at sites of endocytosis, as it is able to stimulate actin polymerization through N-WASP and Arp2/3 [145,146], and induce membrane tubulation [147]. SNX9 has also been shown to associate with and stimulate the kinase activity of PIPKIs [147], providing a mechanism for the generation of PtdIns(4,5)P_2 at these endocytic sites. Because of its intricate association with dynamin, it is likely that SNX9 is a crucial regulator of the trafficking of adherens junction components.

The endocytic accessory protein epsin is recruited to an assemblage of endocytic materials by PtdIns(4,5)P_2, and may function to induce invagination of the membrane to form a clathrin-coated pit in co-ordination with clathrin polymerization [148]. Interestingly, epsin can also simultaneously associate with PtdIns(4,5)P_2, AP2, clathrin and polyubiquitin chains, suggesting that epsin may act as a cargo adaptor for polyubiquitinated proteins destined for endocytosis [149]. The E3 ubiquitin ligase Hakai can polyubiquitinate E-cadherin to induce its endocytosis and disrupt cell–cell junctions, and this process is mediated by Src [150]. Given this, it is possible that epsin can bind ubiquitinated E-cadherin upon growth factor stimulation to facilitate its internalization [150–152]. It is important to note that this pathway would probably lead to the degradation of E-cadherin by the lysosome, and would therefore promote the overall dissolution of adherens junctions and the acquisition of a mesenchymal cellular phenotype (reviewed in [31,121]). However, a specific role for epsin and phosphoinositides in the growth-factor-induced endocytosis of E-cadherin has yet to be determined.

Non-clathrin-mediated E-cadherin internalization pathways also require PtdIns(4,5)P_2

Cell-surface receptors can also be internalized via cellular mechanisms that do not require the clathrin endocytic machinery [153,154]. E-cadherin seems to be no exception, as a Rac1-regulated internalization pathway that requires caveolae, but not clathrin, has been described in keratinocytes [155]. In addition, EGF signalling in tumour cells overexpressing EGFR resulted in the caveolin-dependent uptake of E-cadherin and disruption of cell–cell junctions [156]. There is also evidence that E-cadherin may be internalized via macropinocytosis. In non-polarized cells, surface E-cadherin not incorporated into cell–cell junctions does not co-localize with clathrin or transferrin (a marker of clathrin-dependent endocytosis) immediately after internalization [157]. However, shortly after internalization, E-cadherin enters the early endosomal system as observed by co-localization with EEA1 and transferrin [157]. This suggests that, although the mechanism for E-cadherin uptake from the cell surface may differ in clathrin-compared with non-clathrin-mediated pathways, it is likely that the trafficking routes post-internalization share common attributes. Macropinocytosis of an adhesion complex including E-cadherin, β-catenin and p120-catenin was also shown to occur downstream of EGF stimulation in semi-polarized MCF7 cells, and this was enhanced by expression of constitutively active Rac1 [158]. Activated Rac1 is a stimulator of PIPKI activity [77], and it is feasible to predict that a resulting increase in PtdIns(4,5)P_2 at these sites of clathrin-independent E-cadherin internalization could serve to regulate this process. However, a similar effect was not observed when cells were allowed to form polarized monolayers [158,159], demonstrating that internalization of E-cadherin via growth-factor-induced macropinocytosis is dependent on the status of adherens junction formation. Clathrin-independent E-cadherin pathways are still poorly defined, and even less is known about how these processes are regulated. However, it is quite likely that a specific PtdIns(4,5)P_2-generating enzyme is involved in regulating these alternative modes of E-cadherin internalization.

CONTROLLING THE FATE OF INTERNALIZED E-CADHERIN: A PtdIns(4,5)P_2-REGULATED PROCESS?

The mechanism of how internalized E-cadherin enters the endosomal system is a fundamental issue in cancer progression. The continued existence of adherens junctions is dependent on the timely return of cadherin to the plasma membrane. The balance of E-cadherin transcription and biosynthesis with degradation of endocytosed E-cadherin is a key mechanism that controls junction stability and strength. Stimulation of polarized cells by growth factors or other signals can cause an exodus of E-cadherin from the plasma membrane, resulting in cell depolarization. Loss of epithelial polarization is one of the first steps in EMT, and may lead to a once normal epithelial cell becoming migratory and invasive [160]. Therefore regulation of the amount of E-cadherin that is returned to the surface upon endocytosis is critical to maintaining cell–cell adhesion.

SNX1 regulates the post-endocytic trafficking of E-cadherin

Not all growth-factor-induced E-cadherin endocytosis results in the degradation of E-cadherin by the lysosome. SNX1, which contains a phosphoinositide-binding PX (Phox homology) domain, has been shown to regulate several intracellular trafficking pathways, and may function as either an instigator or an inhibitor of degradation of internalized receptors [161,162]. Treatment of non-polarized MCF-7 cells with EGF induces the macropinocytosis of E-cadherin, and the internalized E-cadherin is targeted to an endosomal compartment where SNX1 is present. [158]. Prolonged treatment of cells lacking SNX1 with EGF resulted in a significant increase in E-cadherin degradation, suggesting that SNX1 may function to re-route internalized E-cadherin from a degradation pathway [158]. In addition, SNX1-knockdown cells were unable to re-form cadherin-based junctions upon calcium depletion and restoration, indicating that the E-cadherin recycling pathway is dependent upon SNX1 [158]. Although the role of PtdIns(4,5)P_2 in this pathway has not been established, SNX1 is able to sense membrane curvature and induce the tubulation of membranes *in vivo* and liposomal substrates *in vitro* [163], and has been suggested to selectively bind 3-phosphoinositides *in vitro* [164]. As it is unlikely that 3-phosphoinositides are the only phosphoinositides in the membranes where SNX1 is

found, the local generation of other phosphoinositides, including PtdIns(4,5)P_2, may influence the activities of SNX1 *in vivo*.

The exocyst complex may regulate E-cadherin recycling

As described above, the exocyst complex is likely to be involved in the fusion of E-cadherin-containing vesicles at the plasma membrane. As recycling endosomes may harbour both biosynthetic as well as recycled E-cadherin, it is likely that the exocyst mediates the delivery of E-cadherin from this compartment to the plasma membrane regardless of its previous location. The quick recycling of E-cadherin mediated via the exocyst pathway may be important in the remodelling of cell–cell contacts during tissue morphogenesis. For example, in the *Drosophila* wing, the precise hexagonal packing of wing epithelial cells and hair organization is crucial for flight [80]. During polarization of these wing cells, cell–cell junctions are constantly remodelled via a dynamin-dependent process, which internalizes E-cadherin and associated junction proteins [80]. E-cadherin was shown to be recycled back to the remodelled junctions through Rab11-positive endosomes, probably via an exocyst-mediated pathway [80], suggesting that this pathway is a fundamental regulator of cell polarity in multiple systems. It is likely that such a recycling pathway in *Drosophila* mimics a similar pathway in mammalian cells to regulate epithelial cell polarity [69,83]. As PtdIns(4,5)P_2 is required for exocyst function, it is probable that a PtdIns(4,5)P_2-generating enzyme is associated with the exocyst complex. PIPKIγ has been shown to positively regulate the efficiency of E-cadherin recycling back to the plasma membrane [54], so it is possible that PIPKIγ may mediate this effect via regulation of exocyst components.

SUMMARY

The current literature positions PtdIns(4,5)P_2 as a fundamental regulator of adherens junction biology, as PtdIns(4,5)P_2 modulates cadherin basolateral targeting and exocytosis, maintenance of cadherin based cell–cell junctions, cadherin endocytosis and recycling. It is likely that the spatial and temporal targeting of the enzymes that generate PtdIns(4,5)P_2 is fundamental to adherens junction regulation, as the utility of these enzymes for both scaffolding and signalling purposes in cadherin trafficking has been demonstrated. Nonetheless, many questions surrounding the regulation of cadherin biology via PtdIns(4,5)P_2 remain to be answered. As PtdIns(4,5)P_2 is a signalling molecule which must be spatially generated, it will be important to further delineate the signalling pathways which target PtdIns(4,5)P_2 enzymes to their functional locations, and to determine the specificity and possible compensatory pathways that may occur between PIPKs in the performance of these duties. Also, since small G-proteins are critical regulators of PIPKs as well as multiple junctional, endocytic and exocytic components of these pathways, it will be critical to define how these positive and negative regulators conspire in the co-ordination of junction assembly. As these junctional complexes are such vital players in the establishment and maintenance of tissue integrity, revealing the nature of these signalling pathways will allow for the development of tools and utilities to fight the disease states caused by their misregulation.

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