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Role of phosphoinositides in cellular signaling, functions and diseases

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Abstract:

In this review we summarize the recent development in understanding the role of PIP2 in cellular function and signaling. We first discuss the effect of PIP2 on actin binding proteins addressing the mechanism of the actin cytoskeletal dynamics such as polymerization or depolymerization of the filamentous network or the coupling to membrane to generate forces. Next, we outline the role of PIP2 in membrane dynamics. We summarized how the membrane organization depends upon PIP2 in the presence of ions or transmembrane proteins that are sensitive to membrane curvature. We discuss how clathrin coated pits interact with adaptor proteins during the endocytosis process, which is facilitated by PIP2. Finally, we discuss the role of PIP2 in cell signaling and diseases.

Keywords: PIP2; membrane dynamics; disease; actin dynamics; phosphoinositides; signaling

Introduction

Phosphoinositides (PPIs), are inositol-containing glycerophospholipids bearing variable numbers of phosphate groups on their headgroups. PPIs are multifaceted molecules that have recently become an interesting player in regulating cell function due to their involvement in cellular functions such as actin dynamics, membrane trafficking, regulation of transmembrane proteins and signal transduction[1]. Although the total amount of PPI in eukaryotic cell membranes is low, they play critical roles in cellular dynamics by regulating multiprotein complexes [2,3]. Spatiotemporal regulation of PPI-mediated biological processes is achieved by interconversion (Figure1) of the phosphorylation states of PPIs by specific kinases and phosphatases, followed by recruitment of PPI-specific effectors. Inter-conversion of the phosphate groups is spatially controlled by phosphoinositide-metabolizing kinases and phosphatases as required for cellular function. PPIs generate seven possible isoforms by phosphorylating the inositol ring at position 3, 4 and 5. Three isoforms of PPIs with two phosphate groups connected to the inositol ring, phosphatidylinositol-(4,5)-bisphosphate (PI(4, 5)P_2), phosphatidylinositol-(3,5)-bisphosphate (PI(3, 5)P_2) and phosphatidylinositol-(3,4)-bisphosphate (PI(3, 4)P_2) are the focus of this review. PI(3,4)P2 and PI(3,5)P2 are produced by phosphorylation of PI3P and PI(4,5)P2 is produced by phosphorylation of PI4P or PI5P. The synthesis of PIP2 by phosphorylation of PI5P is regulated by PIP4K, which is one of the less studied pathways. (PI(4, 5)P_2) can be further phosphorylated to (PI(3,4,5)P_3) and (PI(3,4,5)P_3) is converted to (PI(4,5)P_2) by the enzyme phosphate and tensin homolog deleted from chromosome 10 (PTEN). PIP3 stimulates the activity of PDK1 and phosphorylates Akt. This PIP3/Akt pathway is intensively studied and regulates many crucial

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processes in cells, especially .PIP_3 and PTEN have been the subjects of excellent recent reviews, and the focus here is on PIP_2 [4].

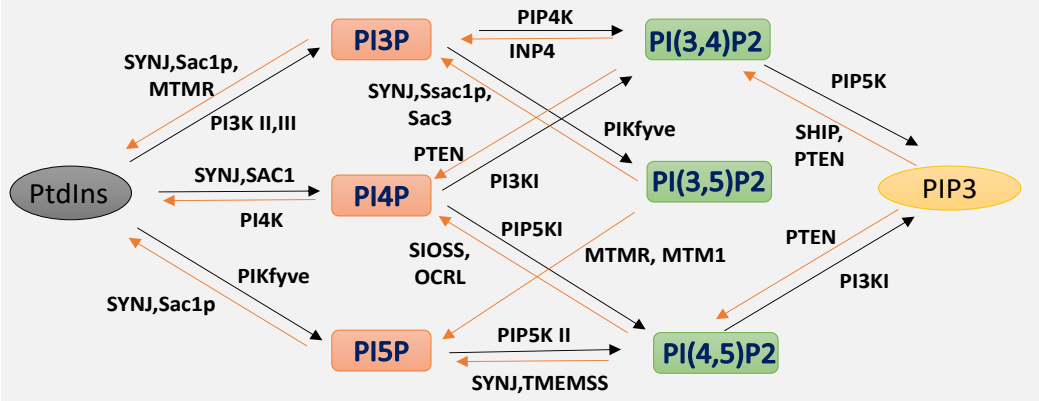

Fig1: Isoforms of phosphoinositides. By the action of PIK and phosphatase, phosphatidylinositol (PtdIns) and the three isoforms of PIP2 are formed, as indicated here. The specific action of PI3K I, II III and of the 3-phosphatases are also illustrated.

Phosphoinositides control intracellular trafficking, membrane dynamics and cytoskeletal organization by interacting with many different proteins. Studies showed that PIP_2 regulates other membrane phospholipids and their signaling function. The major role it plays in the cell membrane include cytoskeletal linkage, regulation of ion channels and intracellular trafficking. PI dynamics and mechanism are precisely controlled by kinase and phosphatase. Recent studies show the direct implication of these enzymes in diseases including liver cancer, glioblastoma or neurodegeneration[1,5]. Thus, many studies target phosphoinositide kinase inhibitors for pathological studies.

In this review we summarize the recent development in understanding the role of PIP_2 in cellular function and signaling. We first discuss the effect of PIP2 on actin binding proteins addressing the mechanism of the actin cytoskeletal dynamics such as polymerization or depolymerization of the filamentous network or the coupling to membrane to generate forces. Next, we outline the role of PIP_2 in membrane dynamics. We summarized how the membrane organization depends upon PIP_2 in the presence of ions or transmembrane proteins that are sensitive to membrane curvature. We discuss how clathrin coated pits interact with adaptor proteins during the endocytosis process, which is facilitated by PIP_2. Finally, we discuss the role of PIP_2 in cell signaling and diseases.

PIP_2 in actin dynamics

Cytoskeletal dynamics play an important role in many cellular functions such as force generation, intracellular transport or migration. Actin is the network inside the cell which is most responsible for cellular architecture providing the cell a mechanical scaffold [6–8]. Accumulated evidence suggests that membrane phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) regulates the function of many acting binding proteins including formin, gelsolin, cofilin, profilin, filamin, WASP,

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ezrin, α- actinin, and others, which control the dynamical organization of actin network[2,9–13]. PI(4,5)P_2 mostly inactivates the actin binding protein which inhibit actin polymerization and activates proteins which promote filamentous assembly[10,14]. Proteins bind to PIPs via numerus different structures, including the pleckstrin homology (PH) domain of phospholipase C-delta1, the Gag precursor protein Pr55 of HIV-1, phox homology (PX), C2, SH2, protein tyrosine binding, FYVE, PHD, GRAM, BAR, and espin N-terminal homology (ENTH)/ANTH domains , forming a large family of domains collectively [15,16].

Actin polymerization dynamics depend upon a variety of actin binding proteins. Actin dynamics depend upon the continuous attachment of G- actin at the barbed (+) end and dissociation at the pointed (-) end, and that defines the filament length. Cofilin is an actin binding protein that binds to both F-actin and G-actin and is a severing protein responsible for actin depolymerization (Figure 2). A study reported that cofilin binding to PI(4,5)P_2 via a specific pocket which is pH dependent. However, this result contradicts with recent finding showing that cofilin interaction with PIP2 is not pH dependent but the interaction of profilin with membrane, actin and multiple PIP2 headgroup (clustering) is affected a little when pH is increased [17]. Cofilin's activity depends on phosphorylation, which is regulated by Rho-GTPase and LIM kinase (LIMK) and by binding PPIs. The rho-family small GTPases, Rho, Rac and Cdc42, play a central role in regulating actin reorganization through their various downstream effectors[18]. LIMK1 and LIMK2 are activated by the GTPase-dependent protein kinases ROCK and PAK1 by phosphorylation of Thr508 and Thr-505, respectively, in the activation loop of the kinase domain[19]. LIMK1 and LIMK2 both regulate actin cytoskeletal reorganization by phosphorylating and inactivating Cofilin/ADF [19,20]. Hence, cofilin is regulated by the signals from both the Rho and Rac pathways. Epidermal growth factor (EGF) induces sudden loss of PIP2 in membrane that activates local cofilin pool in

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membrane in carcinoma [21]. These altogether lead to dramatic turnover of actin monomer (F-actin).

Gelsolin is another actin severing and capping protein which binds to the barbed end of actin filaments[22]. The barbed end of the filament capped by gelsolin becomes available again through the binding of phosphatidylinositol lipids, such as PIP_2, leading to filament elongation. Three PIP_2 binding sites for gelsolin have been characterized. Two of the binding sites compete with F-actin and G-actin sites[23,24]. Thus, the severing function of gelsolin can be inhibited by $PI(4,5)P_2$ [22,24]. Gelsolin can bind to the cell membrane by $PI(4,5)P_2$ which abrogates the gelsolin interaction with actin. Not only does the amount of phosphoinositide alter the free or actin-bound-gelsolin in cells but also the lateral distribution of $PI(4,5)P_2$ controls inactivation of gelsolin [25,26]. Recent studies showed that ATP competes with $PI(4,5)P_2$ to bind with gelsolin[27,28]. Interaction of gelsolin with PIP_2 can be abrogated chemically *in vitro* by including profilin which is a competing $PI(4,5)P_2$ binding protein [23]. $PI(4,5)P_2$ binding to the gelsolin family of capping protein is enhanced by

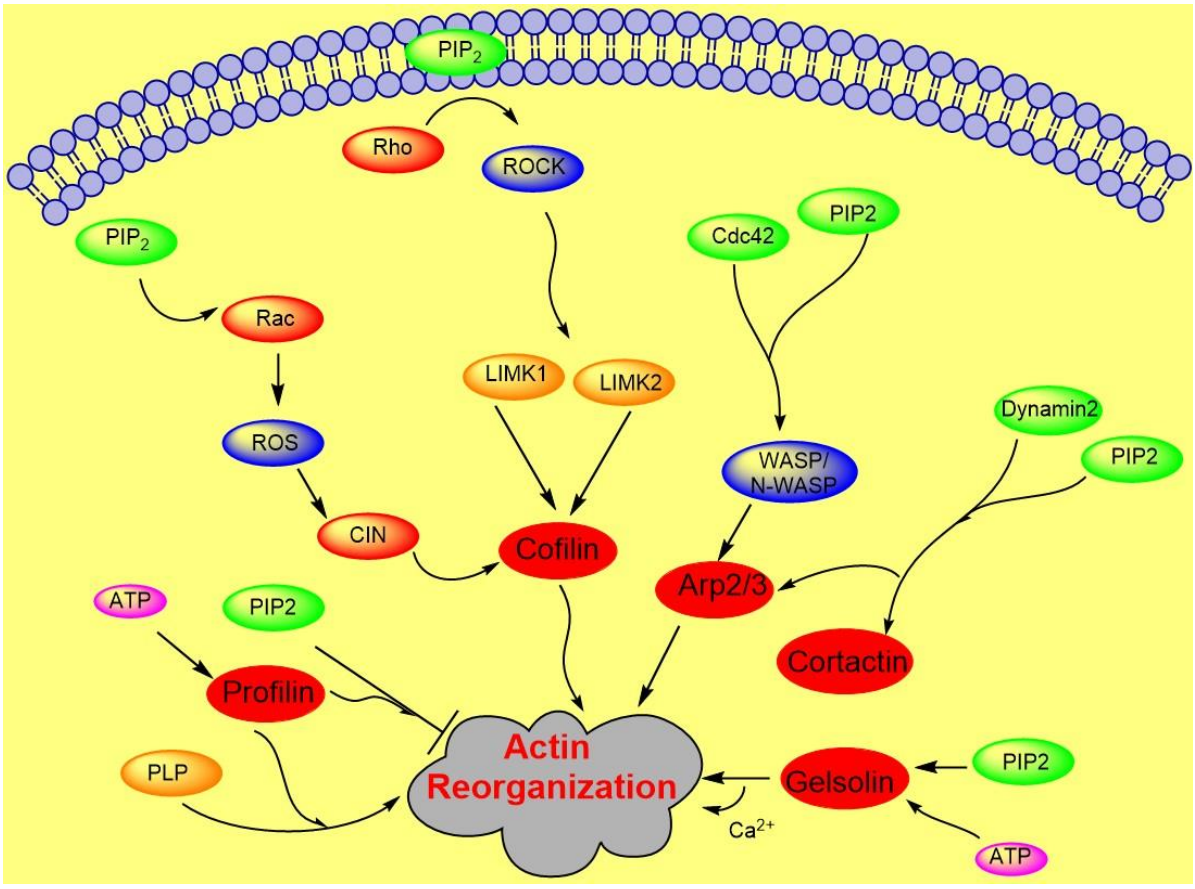

Fig2: Role of PIP2 in actin dynamics either by promoting polymerization or inhibiting severing. The figure summarizes gelsolin, profilin, cofilin, Arp2/3, and WASP dynamics in coordination with Rho-ROCK and Rac pathways.

calcium ions [22]. Ca^{2+} potentiates gelsolin's binding to the end of the filament and promotes the

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polymerization of monomeric actin into filaments [9,23]. Antibacterial activity of rhodamine B (RhB)-conjugated peptides based on the PI(4,5)P_2 binding site of gelsolin, which are cell membrane-permeant, has been shown to kill microorganisms such as Escherichia, Pseudomonas aeruginosa and Streptococcus pneumoniae [29,30].

Another important PPI-sensitive player for actin dynamics is the Arp2/3 complex, which regulates nucleation and branching of actin filaments. Lateral organization of PI(4,5)P_2 in lipid bilayer regulates nucleation [31]. Arp2/3 is primarily activated by Wiskott-Aldrich syndrome protein (WASP) family, multidomain proteins and PI(4,5)P_2 promotes this activation. WASP family proteins integrate PI(4,5)P_2 and other signals to regulate cytoskeletal response through the Arp2/3 complex. Moreover, PI(4,5)P_2 interaction with PH domain of WASP regulates the stabilization of WASP at the membrane. In Xenopus egg extracts N-WASP interacts with Cdc42, which is a small GTPase protein of the Rho family and is required for actin polymerization. Increase in N-WASP activity is coordinated by Cdc42 and PIP2 synergistically [14,32,33].

Profilin is another essential actin regulatory protein which interacts with many other proteins[34]. An *in vitro* study showed that profilin, isolated from platelets, binds to PI(4,5)P_2 along with other phospholipids in lipid bilayer [35]. Profilin regulates tyrosine kinase coupled PI(4,5)P_2 hydrolysis. PLC-γ1 hydrolyzes profilin bound PI(4,5)P_2 by competing inhibitory effect of profilin. Profilin binds to G-actin and increases the ATP binding to actin. This leads to ATP-actin binding at (+) end of filamentous actin. Profilin binding to actin competes with binding of PI(4,5)P_2. Profilin interacts with actin and poly-L-proline (PLP) stretches which is essential for profilin function in fission yeast. Profilin binds to both PLP and actin monomers simultaneously. In addition profilin binds to the cell membrane by PI(4,5)P_2 which prevents actin and PLP interaction[5,34].

A large body of literature shows that PIP_2 turnover regulates the activity of both gelsolin and profilin. It is clear by now that phosphoinositides and these actin binding proteins interact. However, the mechanism at the molecular level remains elusive. A recent study focuses on different actin binding proteins such as mDia2, N-WASP and gelsolin interaction with PIP_2 at the membrane by using molecular dynamics simulation and experimental approaches. The study showed that the cholesterol and PI(4,5)P_2 distribution alters the interaction between actin binding protein and PIP2. With large unilamellar vesicle containing PI(4,5)P_2 , a multivalent binding model it showed that PI(4,5)P_2 activates mDia2 and NWASP to nucleate straight and branched actin filaments, respectively, but inhibits gelsolin's ability to cap the fast-growing barbed end of F-actin or to sever the actin filament. Cortactin is also an actin associated protein that can bind and regulate Arp2/3 and N-WASP [36]. Cortactin mutant cells show reduced binding of Arp2/3 complex or dynamin2 to actin. By performing *in vitro* experiments it is shown that dynamin2 enhances nucleation of actin by Arp2/3 and cortactin in PI(4,5)P_2 containing vesicle [37].

PIP2 in adhesion dynamics:

PI(4,5)P2 binds to many focal adhesion proteins such as vinculin, talin and the focal adhesion kinase FAK. PI(4,5)P_2 serves as linkage to focal adhesion and actin binding proteins. There are

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actin binding proteins such as α-actinin, ezrin or filamin which also bind to focal adhesions. A synthetic peptide of α-actinin inhibits PLC-γ1 and PLC-δ1 activity and inhibition is induced by PIP2 competition[38]. PI(4,5)P2 binding to α-actinin is inhibited by the treatment of platelet derived growth factor resulting in actin depolymerization. A recent study showed that the architecture of the α-actinin-2 and 3 provides a suitable spatial orientation platform for PI(4,5)P2 bonding by performing molecular dynamics (MD) simulations[39]. In smooth muscle in which α-actinin was discovered, PI(4,5)P2 is found in large amounts which facilitates gelation of actin[40,41]. The length of smooth muscle depends upon the PI(4,5)P2 synthesis, which regulates inositol phospholipid turnover [42]. Filamin A is another crosslinker protein which forms contacts between focal adhesions and F-actin. Filamin is associated to the cell membrane by β integrins. PI(4,5)P2 bound to filamin A inhibits the gel formation of actin. Filamin has three isoforms called FLNa, FLNb and FLNc. It has been shown that FLNa is recruited by CD28 followed by lipid raft accumulation at the immunological synapsis in T lymphocyte activation. PI(4,5)P2 is essential for the clustering of lipid raft [43]. Ezrin is one of the ERM (ezrin,radixin,moesin) family proteins, which also forms linkages between the cellular membrane and cytoskeleton. Ezrin exists in both active and inactive states within cells. PI(4,5)P2 activates Ezrin by binding with it and becomes available for phosphorylation by Rho-kinase and many PKC isoforms [44]. Neutron scattering experiment showed for the first-time conformational changes of ezrin when simultaneously binds to PI(4,5)P2 and F-actin [45].

Focal adhesion kinase (FAK) is a protein tyrosine kinase implicated in many signaling pathways to regulate cellular functions including migration. When a cell binds to ECM, FAK is recruited to focal adhesion (FA) sites and undergoes conformational change, which is activated by phospholipids such as PI(4,5)P2 by unblocking the FERM domain and kinase domain. Simulation results show that FAK transiently binds to PI(4,5)P2 through electrostatic interactions[46]. Molecular dynamics simulation and fluorescence resonance energy transfer (FRET) experiments both showed that FAK binding to ATP decreases the FRET signal confirming that the PI(4,5)P2 binding acts in the reverse direction [47,48]. Phosphatidylinositol 4-phosphate 5-kinase type Iγ (PIP5KIγ) is required for efficient FAK activation and generates

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PI(4,5)P2 locally in FAs by PIP5KI*γ*. Thus, PIP2 is a strong mediator in integrin-FAK signaling pathways [47].

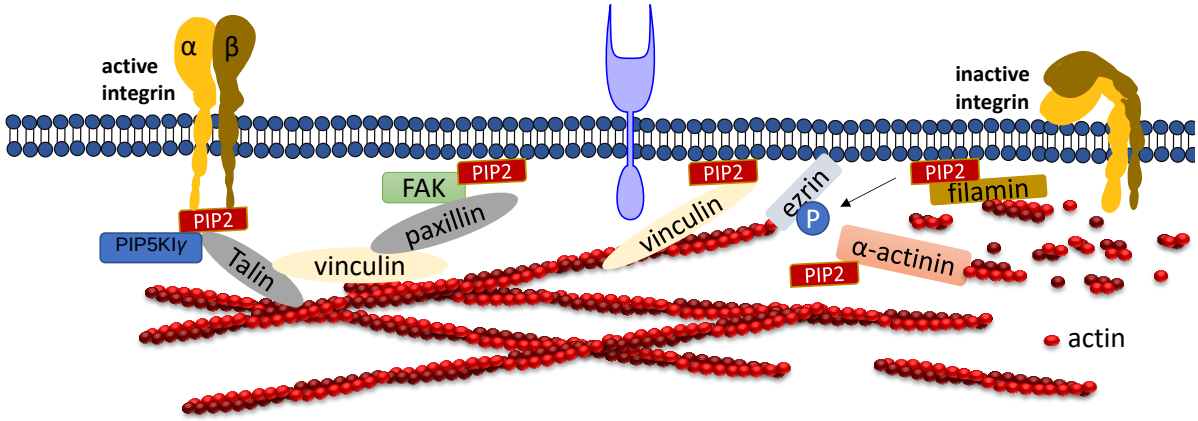

Figure 3: Role of PIP2 in regulating focal adhesion assembly. Depiction of adhesion molecules talin, vinculin, ezrin, filamin and a-actinin. PIP2 synergistically binds to both talin and integrin and activates both of them. Talin binds directly to actin or activates vinculin and facilitates its binding to actin. PIP2 also binds to FERM domain of FAK and binds to vinculin via paxillin. PIP2 negatively regulates cross-linking activity of filamin and the actin bundle formation mediated by α-actinin.

Talin plays a crucial role in activating integrins[49,50]. Within the cytosol talin is in an inactivated form, where C-terminal rod domain binds to N-terminal head domain. Many pathways lead to disruption of the interaction between talin's C-terminal and N-terminal including binding with PIP5KI*γ* which generates $PI(4,5)P_2$ from PI4P [51]. Ye *et al.* delineate a detailed account of $PI(4,5)P_2$ role in activating talin by using FRET. They showed interaction of talin with lipid bilayers is altered by $PI(4,5)P_2$ [52]. The FERM domain of talin-1 binds to the cytosolic domain of β_3-integrin weakly. However the interaction affinity increases three fold when it synergistically binds to acidic $PI(4,5)P_2$ [53–55]. Membrane bound talin recruits and activates vinculin. Vinculin localizes at the adhesion complex and interacts with PI(4,5)P2 to associate with the membrane [56]. Simulation data shows that PI(4,5)P2 is not required for vinculin localization at FAs but is needed for the activation of FA turnover during mechanotransduction processes[56]. Other studies mentioned that PI(4,5)P2 is required for FA formation, and vinculin phosphorylation and trafficking [57].

PIP2 in membrane dynamics and organization

Phosphoinositides are minor component of the lipid bilayer that forms the plasma membrane, constituting 1% of total cellular phospholipid. Eukaryotic cell plasma membranes maintain a balanced composition of sterols, phospho- and sphingo- lipids that is distinct from other cellular membranes, which is required for cellular integrity. All seven PIPs are spatially localized uniquely in the plasma membrane. However, PIP_2 is most abundant among all seven isoforms of PIPs. Many PIP2 binding proteins are characterized as high affinity ligands for these lipids to regulate signaling[58] and activated by agonists for numerous cell surface receptors [13]. Several studies reported that PIP_2 is highly enriched in the plasma membrane within segregated domains with

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an approximate size of 73 nm by showing PC12 cell staining with anti-PIP_2 antibody and high-resolution STED imaging [59,60]. The plasma membrane is fluid like with proteins and lipids co-existing within in a heterogeneous distribution. Also, the negative charge on PIP2 plays a crucial role in the interaction with membrane bound proteins.

Charge dependence and electrostatic interaction

Over 30 years the electrostatic properties of membranes have been highlighted in the literature. Many theoretical models have been proposed based on the smeared charge model of Gouy-Chapman theory, Finite-difference Poisson-Boltzmann (FDPB) method, based on dielectric properties of the solvent. Afterwards, it was proposed that flat lipid bilayers can be considered for the electrostatic calculations of the present PIP-based systems when proper choice of orientations are made, concluding that specific charge of PIP_2 with respect to the cell membrane is required for lipid signaling events to occur [61]. Effort has been made to understand the atomic level structural of $PI(4,5)P_2$ such as its protonation state and binding to cations, by using hybrid quantum mechanics and molecular mechanics simulation methods which determine the optimal geometry of $PI(4,5)P_2$ [62]. $PI(4,5)P_2$ has high negative charge density obtained by deprotonation of two phosphomonoester groups, which can range from -3e to -5e depending on pH and the counterions present, which brings the net lipid charge to - 3-99 ± 0.10 e. The charges on $PI(4,5)P_2$ regulate its interaction with proteins[31].

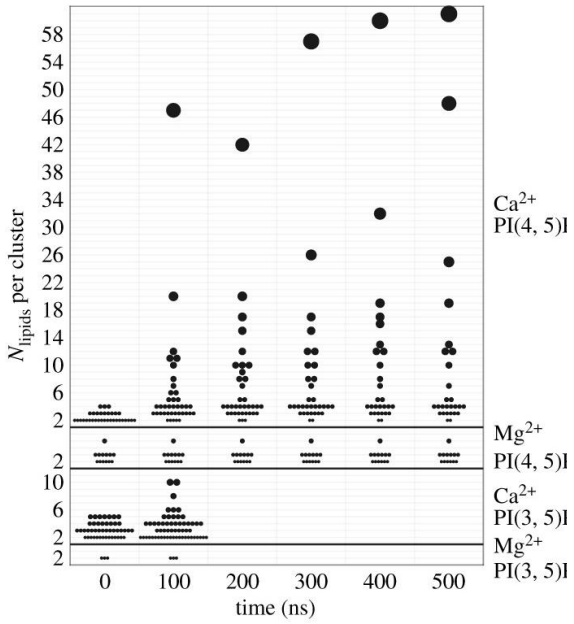

Figure4: Histograms of cluster of lipids which is also measured on the vertical axis. Only the unique combination of $PI(4,5)P_2$ and Ca^{2+} shows large and growing clusters. . The symbol area is proportional to the number of lipids in the cluster. (Bradley *et. al*)

Another important characteristic of $PI(4,5)P_2$ is that the different lateral organizations such as small clusters or large stable aggregates, which are interconvertible, within the region of the membrane are associated with diverse functionality. $PI(4,5)P_2$ turnover at the plasma membrane have been observed by immunofluorescence probes suggesting the evidence of spatially segregated of $PI(4,5)P_2$ pool. Non-homogeneous distribution of $PI(4,5)P_2$ in membrane is due to electrostatic interaction between neighboring lipids. Levental *et al.* showed that of $PI(4,5)P_2$ clustering depends upon the multivalency of the counterion and high charge density of the lipids by using monolayer lipid. Lateral organization on a large range of length scales can be remodeled when Ca^{2+} is introduced to $PI(4,5)P_2$ containing membrane monolayers at different concentrations. This leads to domain formation and reduces phase co-existence surface pressure in of $PI(4,5)P_2$ containing monolayer [63]. The formation of the domains or nano clusters has relevance in cellular function, and regulated by the

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Ca^{2+} ions in the absence of proteins [64,65]. Not only Ca^{2+} but other divalent ions such as Mg^{2+} and Zn^{2+} also affect lateral organization of $PI(4,5)P_2$ in the asymmetric membrane at physiological concentration which in turn regulate $PI(4,5)P_2$ protein interaction[65]. Bradley *et al*. have characterized multivalent lipid cation interaction by the number of lipids bound within a specific distance (called N-bridge), showing the largest cluster formations up to 60 lipids for the combination of $PI(4, 5)P_2$ and Ca^{2+} (Figure 4). The formation of clusters is also dependent on physiological trivalent ions such as Fe^{3+} and Al^{3+} [66]. These findings suggest that the electrostatic sequestration and condensation of $PI(4, 5)P_2$ by divalent and trivalent ions resulted in increasing the molecular packing and ordering the more disordered phase which has important biological relevance.

In the lipid bilayer, lateral distribution of PIP2 has been affected by both electrostatic interaction and cholesterol dependent phase mixing [26]. The cholesterol enriched region in the membrane forms heterogeneous nanoscale clusters having a size of 10 – 200 nm, known as lipid rafts which are compartmentalized in the plasma membrane and regulate different cellular functions [59]. Nano clusters of phosphoinositide, localized in the membrane can be visualized by fluorescently labeled pleckstrin homology (PH) domains, which allow $PI(4,5)P_2$ visualization by protein-domain–GFP chimeras in live cells and $PLC\delta_1$-PH at plasma membrane and OSBP-PH at Golgi membrane; and for $PI(3,4)P_2$ visualization with Akt-PH at plasma membrane [16,67].

PIP2 regulation in membrane curvature sensing and transport:

It is known that PIP2 interacts with many transmembrane proteins such as Bin-Amphiphysin-Rvs (BAR) domain proteins, curvature sensing proteins that are important in regulating membrane shape transitions during endocytosis and membrane trafficking [68,69]. These BAR domain protein interactions with $PI(4,5)P_2$ are charge dependent. By coarse grain modeling Li et al. showed that the electrostatic interaction between PIPs head group which contains large negative charges and many positive charged residues in the BAR is the origin of membrane binding [70]. $PI(4,5)P_2$ binds to both sides of BAR proteins to form membrane protrusion by synergistically binding to actin[71]. Experimentally and by simulation it has been shown that PIP2 has preference in binding to the negatively curved membrane over positively curved membranes (Figure 5) (cite).

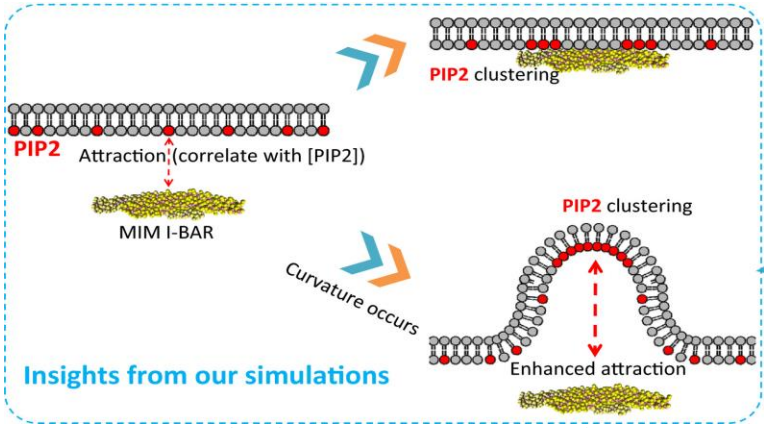

Figure5: PIP 2 molecules are necessary to recruit MIM I-BAR, which in turn can induce local PIP 2 clustering at its two ends after binding to the membrane (upper panel). Spontaneous bending of lipid membranes can re-distribute PIP 2 molecules to the negatively curved membrane areas (lower panel), which promotes the recruitment of MIM I-BAR and maintaining the curvature. [70]

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Thus, membrane curvature can promote the spatial regulation on PI(4, 5)P_2 binding and local enrichment of lipids. It has been shown *in vitro* and also in cells that the phosphoinositide binding domain of BIN1 targets the membrane by interacting with PI(4, 5)P_2 .The N-BAR domain of BIN1 clusters with PIP_2 to promote the recruitment of its downstream partner dynamin and responsible for membrane tubulation[72]. Amphiphysin1 (BIN1) in the PI(4,5)P_2 containing membrane induces curvature[73]. Membrane curvature sensing and generation of BIN1 is abrogated in membranes lacking PI(4, 5)P_2. However, BIN1 alone can initiate membrane tubulation. BIN1 membrane curvature sensing and generation show autoinhibition regulated by downstream ligands and PIP_2 [73]. A recent study demonstrates that mutation of BIN1 N-BAR impairs membrane T tubulation[74]. This affects regulation of muscle functioning or nuclear positioning leading to diseases like centronuclear myopathies [72,75].

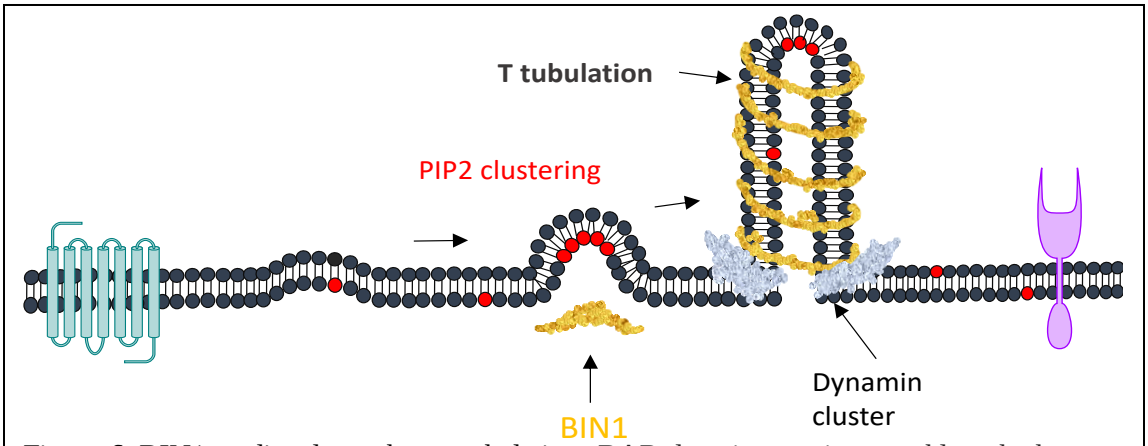

Figure 6: BIN1 mediated membrane tubulation. BAR domain proteins are able to both sense and induce membrane curvature. BIN1 clustering with PIP2 that promote dynamine recruitment and thus forms T- tubule. Binding of dynamin depends upon the amount of PI(4, 5)P_2 and enhanced by BIN1.

PI(4, 5)P_2 is a major regulator of voltage gated ion channels, in which PI(4, 5)P_2 binds to the transmembrane domain [76]. Kobayashi et al showed in skeletal muscle that PIP2 is a major activator of Ca2+ channels. Depletion of PI(4, 5)P_2 induces increases in voltage sensitivity and a decrease in voltage amplitude in K+ ion channels in *Xenopus* oocytes. PI(4, 5)P_2 controls both movement and stability of the channels by interacting through linkers[77]. ATP-sensitive K+ channel rundown, the process by which a channel steadily decreases in conductance until the channel inactivates, is induced by Ca2+, and this process is shown to be regulated by PI(4, 5)P_2. KCNQ is another family of channels that absolutely requires PI(4, 5)P_2. The importance of PIP2 in modulating KNCQ channels is well studied in neurons, showing that PI(4, 5)P_2 hydrolysis increases neuroexcitability and in cardiac arrythmias in patients by showing PI(4, 5)P_2 dependent channel activation [78,79].

A potentially important event that occurs at the cell surface, is the interaction between the lipid bilayer with Ras, a small GTPase and with its effectors. These interactions are shown by molecular dynamics simulation or FRET in live cells. RAS proteins such as H-RAS, N- Ras and

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K-Ras operate in the inner plasma membrane, and are mutated in many cancer types[80]. Recent studies have shown RAS enrichment in nano clusters within phosphatidylserine-rich regions. PI(4, 5)P_2 binds to RAS G-domain and KRAS4b HVR, which is one of the isoforms of RAS[81]. Experimental or computational studies showed the tight binding between PI(4, 5)P_2 and KRAS4b by measuring rotational dynamics by random amine labeling and by atomic force microscopy. Rotational dynamics of KRAS is important for signaling in cancer cells[81,82].

Intracellular trafficking

PPIs are spatially localized in different compartments in intracellular organelles [83]. For example, the Golgi is enriched with PI(3)P or PI(4)P, which are also enriched in early endosomes [84,85]. The Golgi plays a crucial role in membrane trafficking [86,87]. Phosphoinositide 3- kinase metabolizes PI(4, 5)P_2 to PI(3,4,5)P_2 which is important for vesicular trafficking[88]. New studies demonstrate that the cell surface membrane as major site of action for PIP_2 and the localization of it in different compartment is directly corelated to intracellular trafficking such endocytosis and exocytosis [89]. By specifically interacting with proteins, PIP_2 controls the formation and spatiotemporal organization of many protein complexes that are involved in intracellular trafficking.

Clathrin mediated endocytosis, in which cargo is packaged into vesicles with clathrin coating, plays a crucial role in cell signaling, migration and cell-cell interactions. PI(4,5)P_2 has been implicated in clathrin-mediated endocytosis[90]. However, clathrin does not directly bind to the membrane or cargos but to adaptor proteins such as adaptor protein 2 (AP2) or accessory protein AP180 and espin[91]. A recent study reported that during clathrin coated pit (CCP) assembly initiation AP2 is recruited to the plasma membrane and colocalizes with the nucleation complex which binds to both cargo and PI(4,5)P_2, when stained with anti-PI(4,5)P_2 antibody[88].

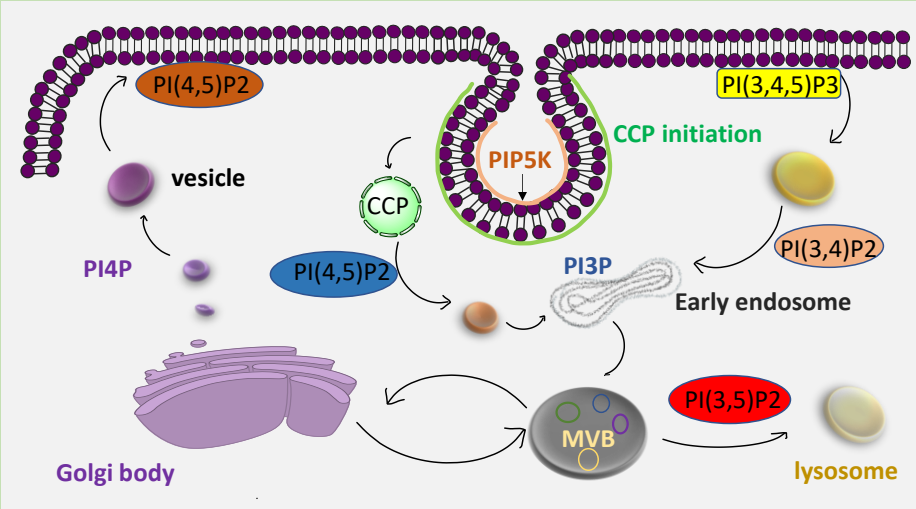

Figure 7: PIP_2 is involved in intracellular trafficking and vesicular transport. PIP_2 participates in both clathrin mediated (CCP) and non- clathrin mediated endocytosis. PI(3,5) P_2 is involved in exocytosis whereas PI(4,5) P_2 and PI(3,4) P_2 are involved in endocytic processes.

The formation of PI(3,4)P_2 by class II PI3-kinase C2α (PI(3)K C2α) spatiotemporally controls clathrin-mediated endocytosis. Depletion of PI(3,4)P_2 hinders the maturation of CCPs before fission. PIP5K is associated with the initiation of CCPs but its activity is not found to mature them.

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Another study shows that PI(4,5)P_2 is an established regulator of endocytosis. Endosomal PI(4,5)P_2 is required for the sorting of active epidermal growth factor receptor (EGFR) towards multivesicular bodies (MVB) and further termination of the signal (Figure 7). Sun *et al.* showed that type I gamma phosphatidylinositol phosphate kinase (PIPKIγ) is an enzyme that synthesizes PI(4,5)P_2 by phosphorylation of PtdIns4P and regulates EGFR sorting from endosomes to lysosomes. This was done by performing flow cytometry analysis and quantification of internalized Alexa Fluor 488-labelled EGF in control and PIPIγi5- knockdown cells [88,92]. PIPIγi5 interacts with sorting nexin 5 (ANX5) which is the effector of PI(4,5)P_2 ,in the early endosome, but cells lacking SNX5 still localize PIPIγi5 to endosomes[93]. SNX5 has been reported to inhibit EGFR degradation when overexpressed. However, knockdown of SNX5 does not affect EGFR trafficking to early endosomes, but blocks trafficking to the late endosome/lysosome [92,94]. On the other hand EGFR regulates Ras activity, which is implicated in PIP3 and MAP kinase pathways [95].

Another crucial role of PIP2 is in bidirectional homeoprotein trafficking. Homeoproteins are a class of transcription factor that predominantly resides in the nucleus. Chick engrailed 2 (EN2) is a homeoprotein that shuttles between the nucleus and cytosol. In the cytosol, EN2 associates with those membrane fractions enriched in cholesterol and glycosphingolipids. EN2 directly binds to PIP2. Dephosphorylation of PIP2 reduces EN2 secretion. Moreover, PIP2 is involved in EN2 internalization [96].

Phosphoinositides are interconvertible and the balance of production and usage is tightly maintained in a specific organelle. Contrary to the plasma membrane, the Golgi membrane has less PI(4,5)P_2 and high abundance of PI4P and PI4K enzymes [97–100]. There is a possibility that the plasma membrane PI4P pool is due to vesicular trafficking of PI4P from Golgi membrane. The recent discovery of lipid binding domains enables life time monitoring of lipid synthesis by fusing with green fluorescent protein GFP. GFP-tagged PKB/Akt PH and GFP-PH (PLCδ) are possible markers for live monitoring PIP_3 and PI(4,5)P_2. Utilizing recent advancements, a study demonstrated PI(4,5)P_2 level decreases in the plasma membrane when stimulated by angiotensin II (AngII) by showing the change in PLCδ$_1$PH-GFP expression level in HEK-293 cells and an increase of GFP in cytosol. Recovery experiment shows that Golgi PI4P eliminated cells recover slowly compare to control. These studies confirm that although PI4P takes part in the maintenance of the PI(4,5)P_2 level pool at plasma membrane, it is not requisite for the process[84].

Phosphatidylinositol 3,5-bisphosphate PI(3,5)P_2 is synthesized from PI3P by FYVE-domain-containing PI kinase (PIKfyve) in mammalian cells. FYVE domain appears to target the enzyme to PI3P -rich membranes[101]. However a similar process occurs in yeast called *Saccharomyces cerevisiae,* and the PI(3,5)P_2 synthesis is found to be processed by Fab1p. Since Fab1 is not responsible for the full synthesis, additional unknown effector proteins are expected to be involved. PI(3,5)P_2 is involved in vacuole to lysosome membrane trafficking and packaging of proteins in multivesicular bodies (MVBs) [99,102].

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PIP2 in signaling and diseases

Accumulating evidence suggests that $PI(4,5)P_2$ dysregulation contributes to cancer including melanoma, breast cancer, leukemia, prostate cancer. Literature suggests that $PI(4,5)P_2$ is implicated in many pathways and binds to signaling proteins such as lamellipodin/RAPH1, tandem PH domain-containing proteins TPP1 and TAPP2 and PIP3 binding proteins including protein kinase Akt/PKB. The PIP3-Akt signaling pathway is implicated in many diseases [103,104].

Phosphoinositides play a major role in intracellular signaling pathways which are implicated in carcinogenesis such as hepatocellular carcinoma (HCC) or melanoma. Thus, many signaling pathways are targeted for therapies including phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) pathways. Numerous proteins are regulated downstream of these pathways. Generally, pathways are activated by the alteration of the cell's microenvironment or genetic alteration [13]. Our recent work shows that when Huh7 cells, a hepatocellular carcinoma cell line, adhere to soft hyaluronic acid (HA) gels, they show similar behavior as cells adhered on stiff polyacrylamide gels by regulating phosphoinositide signaling. The result is confirmed by pAkt expression level by immunoblotting and by quantifying the total amount of PIP_3 on HA and PAA substrates by using mCherry Grp1, a fluorescent protein that specifically binds to PIP_3 [105]. PIP_3 which increases Akt activity via PDK1, is activated by Ras. PDK1 activates Akt by phosphorylation of threonine site. Overexpression of PAK, which is one of the downstream effectors of PIP3, is correlated with many cancer types such as ovarian cancer [80,106]. PI3K catalyzes PIP2 into PIP3[107] (figure5). One of the major downstream effectors of Akt is mTORc1 which is deregulated in many cancers when phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene dephosphorylates PIP3 to PIP2 [106].

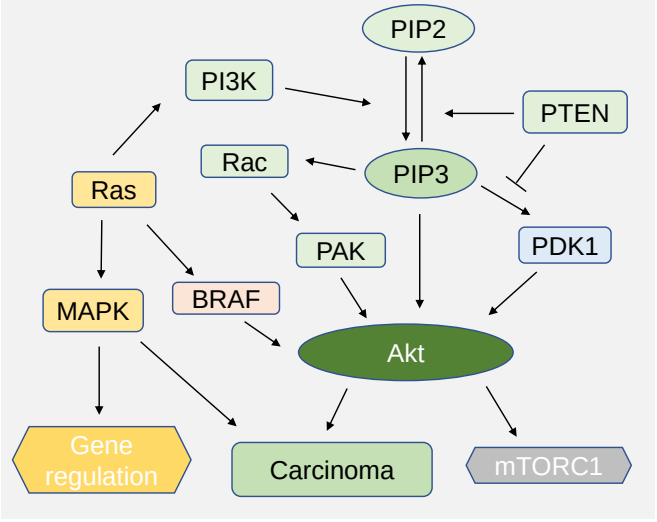

Figure 8: PIP_3 and MAP kinase pathways synergistically and independently regulates melanoma cancer or any other carcinoma. Ras regulates both PI3 and Akt kinase pathways. In addition, Ras independently regulates BRAF which is also implicated in Akt3 activity depicted in diagram.

The PIP3-Akt pathway also synergistically activates MAPK signaling pathway in melanoma cancer development. The MAPK pathway is one of the most investigated signaling pathways in melanoma cancer [107]. Thus, a series of inhibitors for these pathways are targeted for the therapeutics of melanoma. It has been observed that PI3K activity is increased in melanoma due to loss of PTEN (figure 8) or increased levels of Akt3 activity, and that plays a crucial role in

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early melanoma development. A recent study has shown that Akt3 phosphorylates B-Raf which is often mutated in melanoma cancer [95]. Lipid binding domains such as PH domains of Akt/PKB, are important in signaling, which depends upon PIP synthesis. Akt/PKB binds to $PI(3,4)P_2$ and PIP_3 to regulate cell survival and growth, which is independent of $PI(4,5)P_2$. In cases of melanoma, 50% of patients progress to metastatic stress due to upregulation of protein tyrosine phosphate (PTP) promoting cell migration and invasiveness [108]. PTPs bear phosphatase activity toward lipidic substrates, including phosphoinositides. PRL-3 is one of the dual specificity phosphatases which is associated with intracellular membranes and cellular migration[109]. PRL-3 dephosphorylates $PI(4.5)P_2$ and thus alters the phosphoinositide level in cells [110]. PRLs are overexpressed in many cancer types and has become the target of many cancer therapies including melanoma[108,109,111].

ORCL (Oculo-cerebro-renal syndrome of Lowe) enzymatic activity is found in many compartments in cells especially concentrated at the Golgi network. Mutation of ORCL causes oculo-cerebro-renal syndrome of Lowe which is an X-linked condition [112]. Lowe syndrome leads to many diseases including renal Fancomi syndrome, glaucoma, cataracts, blindness, mental retardation. ORCL is a key component of endocytic trafficking which is involved in clathrin coated pits and other binding motifs such as AP2, APPL1, Rab GTPase including Rab5, Rab6, Rab14. Therefore, inactivation of ORCL leading to deregulation of $PI(4,5)P_2$ level that influence trans- Golgi network and endosomal activity. Imbalance of PIP2 levels further affects actin dynamics and actin binding proteins. Moreover, ORCL controls reabsorption of proteins via PIP2 5 - phosphatase in renal proximal tubule cells (PTCs) [113]. Another regulator for Down's Syndrome is synaptojanin1 which acts on both $PI(4,5)\ P_2$ and $PI(3,5)\ P_2$, found in endocytic intermediate nerve terminals. Synaptojanin regulates the actin pool, de-coating of cathrin mediated endocytic vesicles and synaptic vesicles. In synaptojanin deficient mice the $PI(4,5)P_2$ level increases whereas a decreased level of cytosolic inositol 5 phosphatases in neurons is observed. Also an increased in clathrin coated vesicles in nerve terminals is observed [114]. Inositol polyphosphate-4-phosphatase ($INPP_4$) which binds to $PI(4,5)P_2$, shows a reduced level in an asthma mouse model due to restrictive stress [103]. Oxidative stress which is generated by reactive oxygen species (ROS) stimulates the accumulation of $PI(4,5)P_2$. ROS has been implicated in airway inflammation. $INPP_4$ deficiency also leads to cancer including breast cancer and neurodegeneration [104].

Table1: PIP_2 and enzymatic activity in different pathways in disease [100].

Phospho-inositides	Pathways/ functions	Enzymatic Activity	Disease implication	References
PIP3, $PI(4,5)\ P_2$, $PI(3,5)\ P_2$	PI3K-Akt	PI3K, PTEN 1,2	Melanoma cancer Cowden disease, pancreatic cancer, ovarian cancer.	(118)

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PI(4,5) P_2	Endocytic trafficking pathways	OCRL, 5 phosphatases	Oculo-cerebro-renal syndrome of Lowe: renal Fancomi syndrome, glaucoma, cataracts, blindness, mental retardation.	(112,113)
PI(3,5) P_2		MTM1, PI4P,	Myopathy.	(72,98,98)
PI(3,5) P_2		Fab1/PIKfyve kinase	Neuropathologies, Charcot-Marie tooth disease.	
PI(4,5) P_2 , PI(3,5) P_2	Endocytic pathways	Synaptojanin1,2	Bipolar disorder, Down syndrome, neuronal disorder.	(9,60,114)
PI(4,5) P_2		INPP$_4$	Asthma, nondegeneracy.	(5,103)
PI(4,5) P_2	Actin reorganization		Human immunodeficiency virus-1 (HIV-1).	(115,116)
PI(4,5) P_2	impairment of synaptic function	Amyloid-β peptide oligomers	Alzheimer's disease	117
PI(3,4) P_2. PIP3	Akt/PKB		Cell survival and growth, cancer.	(103,104, 119)
PI(4,5) P_2		PRL-3	Melanoma, colon cancer.	(108– 110)

Mutation of myotubularin (MTM) causes several disorders such as failure of skeletal muscle development. MTM related proteins, MTMR1-13 which is an inactive partner of MTMR2 causes the same mutation as active member. Each MTM protein regulates a specific pool of PI(3)P and PI(3,5)P_2 . Another disease where PI(4,5)P_2 regulation is important involves the human immunodeficiency virus-1 (HIV-1). Viral entry into the host cell requires actin cytoskeletal reorganization. Viral receptor clustering is regulated by actin adaptor proteins such as moesin, filamin A, gelsolin, tailn, vinculin, profilin, WASP, Arp2/3 that are controlled by PI(4,5)P_2. PI(4,5)P_2 production is regulated by HIV-1 attachment and promotes viral infection. Hence, the virus controls actin dynamics during cycle, by facilitating actin polymerization and depolymerization [115]. In HIV-1 infection, CD4 and coreceptors clustering at the cell surface is induced by glycoprotein g120 that facilitates virus envelope and cell membrane fusion. PI(4,5)P_2

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is required to recruit the gag protein at the cell membrane to facilitate invasion. A high density of $PI(4,5)P_2$ is not only required for HIV-1 recruitment but also to maintain glycoprotein at the membrane[116]. PIP2 plays a central role in many neuronal and synaptic functions by regulating endocytosis, exocytosis, cytoskeletal reorganization, and ion channels. In Alzheimer's disease, Amyloid- β peptide(Aβ) oligomers cause impairment of synaptic function. Elevation of Aβ in Alzheimer's diseased brain results in decreased levels of $PI(4,5)P_2$ [117].

Conclusion and outlook

This review summarizes the role of PIP_2 and other PPIs in cell membrane dynamics, focal adhesion, actin organization, intracellular signaling and disease. $PI(4,5)P_2$ regulates actin binding protein activity which either promotes polymerization and depolymerization of actin filament. Past evidence suggests that actin is connected to the membrane via actin binding proteins such as α-actinin or filamin which are regulated by phosphoinositides. These interactions also affect the binding of actin filaments with focal adhesion proteins such as paxillin, talin, FAK or vinculin. The distribution of PIP_2 in the membrane regulates cell signaling. PIP_2 activity depends upon the concentration of cholesterol and divalent ions such as Ca^{2+}, Mg^{2+} or Zn^{2+}. In addition, PIP_2 plays a crucial role in modulating many signaling pathways such as PIP3/Akt, mTORc1 or Rho dependent pathways that have implications in many diseases including cancer, neurodegenerative disease, or down syndrome.

Although PPIs are essential for many cellular functions, there are disparities in many processes which need further studies. PIP_2 plays an important role in actin reorganization and filament dynamics. However, the role of PIP_2 in any other cytoskeletal component has not yet been well studied. Among PIP_2 binding actin proteins, LIMK1 and LIMK2 play an overlapping role in actin reorganization in the Rho-ROCK pathway. Further studies are required to differentiate the functional role of LIMK1 and LIMK2. Moreover, it is inexplicit if members of ROCK and PAK family proteins function as LIMK- activating kinase. Cortactin shows dependencies on PIP_2 and Rac in dissociating from actin-myosin complex although, direct implication of PIP_2 in regulating cortactin still remains controversial [120] and other activators such as endocytic protein Abp1p remain unclear. It has been shown that the synthetic peptide of α-actinin inhibits PLC-γ1 and PLC-δ1. It is ambiguous whether PIP2 bound to α-actinin is hydrolyzed by activated PLC-γ1 or not. The interaction of vinculin and membrane is based upon either full length or tail domain of vinculin in lipid bilayers or in cells. However, a specific lipid binding site has yet to be discovered.

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