The Cellular Roles of Phosphatidylinositol 4,5-Bisphosphate Generating Enzymes in Association with IQ Motif Containing GTPase Activating Proteins

by

Suyong Choi

A dissertation submitted in partial fulfillment of the requirements for the degree

of

Doctor of Philosophy

(Cellular and Molecular Biology Graduate Program)

at the

UNIVERSITY OF WISCONSIN – MADISON

2014

Date of final oral examination: September 18 2014

The dissertation is approved by the following members of the Final Oral Committee:

Richard A. Anderson, Professor, School of Medicine and Public Health

Arnold Ruoho, Professor, Neuroscience

Alan C. Rapraeger, Professor, Human Oncology

William Bement, Professor, Zoology

Patricia J. Keely, Cell and Regenerative Biology

The Cellular Roles of Phosphatidylinositol 4,5-Bisphosphate Generating Enzymes in Association with IQ Motif Containing GTPase Activating Proteins

by

Suyong Choi

Laboratory of Professor Richard A. Anderson
School of Medicine and Public Health
University of Wisconsin – Madison

Phosphoinositide lipid messenger regulates a wide variety of cellular processes, including cell migration and intracellular signaling. Phosphoinositide synthesis at specific cellular compartments is regulated by the specific targeting of phosphoinositide modifying enzymes. Phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) is primarily synthesized by the type I phosphatidylinositol 4-phosphate 5-kinase (PIPKI) family of enzymes. There are several PIPKI genes and there exist several splice variants for each. The functions of PIPKI isoforms are regulated by interaction with signaling molecules. IQ motif containing GTPase activating protein 1 (IQGAP1) was identified as a novel interactor of PIPKI isoforms in this study. Therefore, my research focused on how IQGAP1 regulates the function of PIPKI isoforms. IQGAP1 interaction with γ isoform of PIPKI (PIPKIγ) controls cell migration. Mechanistically, PIPKIγ recruits IQGAP1 to the leading edge membrane of migrating cells. At the leading edges, IQGAP1 binds to $PI4,5P_2$ produced by PIPKIγ and the $PI4,5P_2$-binding relieves an intramolecular autoinhibitory interaction, facilitating actin polymerization to promote cell migration. IQGAP1 interaction with α isoform of PIPKI (PIPKIα) controls class I phosphoinositide 3-kinase (PI3K) activation. IQGAP1 mediates a physical association of PIPKIα with class I PI3K. In the IQGAP1-tethered complex,

$PI4,5P_2$ produced by PIPKIα is utilized as a substrate for class I PI3K for $PI3,4,5P_3$ generation. Furthermore, abrogation of IQGAP1 interaction with PIPKIα and class I PI3K by cell permeable peptides derived from the PIPKIα and class I PI3K binding sites on IQGAP1 inhibits Akt activation and cell survival. These studies provide evidence for a novel role for $PI4,5P_2$-generating enzymes in cellular regulation. The signaling pathways revealed in the current studies may be relevant to normal cellular function and potentially, defects in the pathways may alter cell migration and the class I PI3K signaling, resulting in pathological consequences.

Acknowledgements

There are several individuals who have contributed to my academic accomplishments. First, I would like to thank Dr. Richard Anderson for accepting me into his laboratory. Through his guidance and direction I have grown as an independent scientist and individual. I would also like to thank my thesis committee, Dr. Arnold Ruoho, Dr. Alan Rapraeger, Dr. William Bement and Dr. Patricia Keely for their constructive advice. The entire Anderson lab has provided assistance during my graduate career, and I would especially like to express my appreciation for Dr. Andrew Hedman, Dr. Nicholas Schill, Dr. Narendra Thapa and Dr. Yue Sun for their role in my scientific technical training and scientific discussions. The support provided by American Heart Association Predoctoral Fellowship (13PRE14690057) was also critical for my scientific training. Finally, I would like to thank my parents in South Korea for being there for me during my entire educational career, and especially thank my wife Oisun and a 5 month-old son Logan for being the most precious ones of mine.

Table of Contents

Table of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 1

Introduction

Targeted Generation of $PI4,5P_2$ by PIP Kinases Determines $PI4,5P_2$ Effector Function:

Though it only comprises a small percentage of membrane phospholipids, phosphatidylinositol is an important regulator of multiple signaling pathways. Phosphatidylinositol consists of two acyl chains oriented inside the lipid bilayer, and a polar 6 carbon head group that has hydroxyls at the 2, 3, 4, 5 and 6 positions that can be phosphorylated at the 3, 4 or 5 positions to generate 7 possible phosphoinositide species: phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$), phosphatidylinositol 3,5-bisphosphate ($PI3,5P_2$), phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$).

$PI4,5P_2$ is the most abundant species among the 7 known phosphoinositides [1, 2]. Due to its abundance, early studies focused on $PI4,5P_2$ as a substrate for phospholipases and phosphatidylinositol 3-kinases (PI3Ks) to generate other lipid messengers. In 1985, Anderson and Marchesi discovered that in erythrocytes $PI4,5P_2$ regulates the interaction of a cytoskeletal protein, band 4.1, with an integral membrane protein, glycophorin [3]. This work suggests that $PI4,5P_2$ has its own role in cellular regulation rather than being passively utilized as a substrate. After this initial discovery, hundreds of $PI4,5P_2$ effectors, whose function is directly regulated by binding to $PI4,5P_2$, have been identified. These include ion channels [4], receptors [5], Ras family small GTPases [6], actin regulatory proteins [7, 8], regulators of vesicular trafficking [9], scaffolds [10] and nuclear proteins [11]. Furthermore, recent advances in proteomics have identified unexpected proteins that associate with $PI4,5P_2$ [12-14].

An important feature of signaling molecules is that their availability at a specific time and location is tightly regulated to signal only when necessary [15]. In this view, $PI4,5P_2$ is a poor signal as early studies indicate that $PI4,5P_2$ is present in high concentrations and its level remains largely unchanged by extracellular stimuli. For example, in resting neutrophils

and erythrocytes, total cellular $PI4,5P_2$ concentrations are approximately 50 μM, while concentrations on the inner leaflet of the plasma membrane are estimated to be 5 mM, where the majority of $PI4,5P_2$ is found in the cell. Stimulation with fMLP, which activates phospholipase C (PLC), causes only a small drop in $PI4,5P_2$ concentration [2, 16]. Further, studies with $PI4,5P_2$-specific pleckstrin homology (PH) domains fused to GFP to probe the localized changes in $PI4,5P_2$ concentration reveal that $PI4,5P_2$ is uniformly distributed around the plasma membrane, both before and after stimulation [17]. However, the early studies are challenged by the discovery of a family of natively unstructured proteins that sequester $PI4,5P_2$ at the plasma membrane. Proteins such as myristoylated alanine-rich C-kinase substrate (MARCKS), growth-associated protein 43 (GAP43) and cytoskeleton-associated protein 23 (CAP23) contain basic clusters that mediate an electrostatic interaction with $PI4,5P_2$ [5, 18]. These proteins are present in concentrations of 1-10 μM, comparable to those of $PI4,5P_2$, and bind tightly to $PI4,5P_2$ (the dissociation constant is approximately 10 nM for MARCKS). Thus, a substantial fraction of $PI4,5P_2$ is masked by a family of natively unstructured $PI4,5P_2$ sequestering proteins and unavailable for binding to $PI4,5P_2$ effectors.

The discovery of $PI4,5P_2$ sequestering proteins raises a question of how $PI4,5P_2$ availability is regulated at a specific time and location. One direct way of increasing local $PI4,5P_2$ concentration could be achieved by resolving the sequestration. In line with this possibility, membrane association of $PI4,5P_2$ sequestering proteins is controlled by extracellular stimuli. For example, MARCKS translocates from the plasma membrane to the cytoplasm when cells are treated with phorbol myristate acetate or insulin, and there is concomitant accumulation of GFP fused $PI4,5P_2$-specific PH domains in the plasma membrane [5, 19, 20]. This strongly suggests that translocation of MARCKS frees $PI4,5P_2$. Another way of producing free $PI4,5P_2$ is direct synthesis from other phosphoinositides. $PI4,5P_2$ is synthesized in three different ways: phosphorylation at the 5 hydroxyl of the myo-inositol ring of PI4P by type I phosphatidylinositol phosphate kinases (PIPKIs),

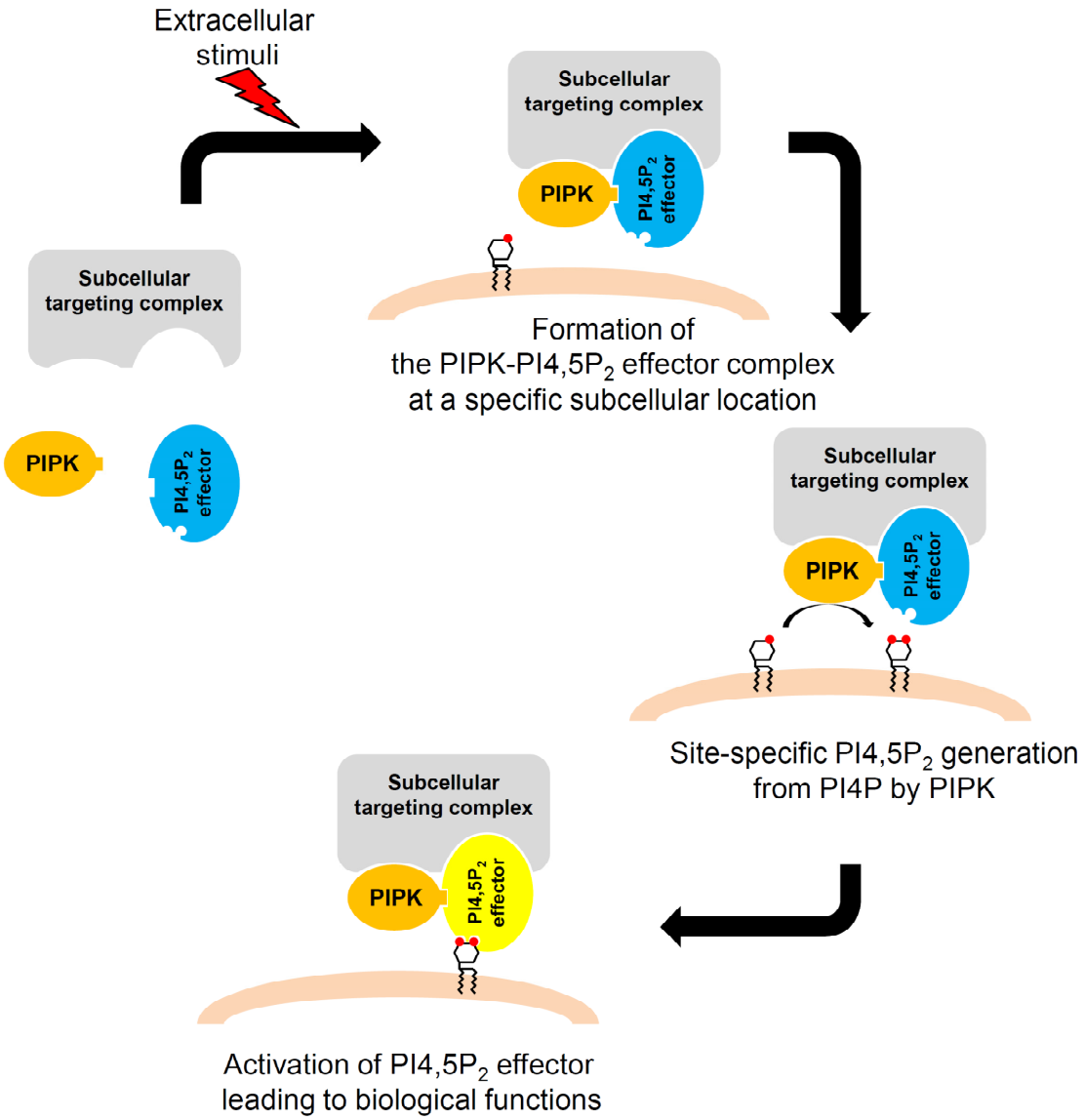
phosphorylation at the 4 hydroxyl of the myo-inositol ring of PI5P by type II PIP kinases (PIPKIIs), and dephosphorylation at the 3 hydroxyl of the myo-inositol ring of $PI3,4,5P_3$ by phosphatase and tensin homolog (PTEN). As cellular PI4P concentration is at least 20-fold higher than those of PI5P and $PI3,4,5P_3$ [1, 2], it is generally accepted that the majority of $PI4,5P_2$ is produced by PIPKIs [21].

In human, three distinct genes encode PIPKIs (*PIP5K1A* encodes PIPKIα, *PIP5K1B* encodes PIPKIβ, and *PIP5K1C* encodes PIPKIγ, nomenclatures for human and murine genes are not consistent, this manuscript only uses the human nomenclature). Moreover, each PIPKI gene undergoes alternative splicing, generating multiple splice variants [22]. All PIPKI isoforms contain a highly conserved kinase core domain with invariant catalytic residues in the middle suggesting that they catalyze the same reaction [21, 23, 24]. Despite their similarity in the kinase domain, each PIPKI isoform shows unique tissue and subcellular distribution. For example, by Northern analysis, PIPKIα, PIPKIβ and PIPKIγ splice variants have wide tissue distributions, but varying expression levels [25, 26]. The different isoforms also have distinct subcellular distribution. When overexpressed in cells, a large fraction of PIPKIα is found in membrane ruffles and the nucleus, whereas, PIPKIβ localizes at the perinuclear region likely at the intracellular organelles, such as the Golgi and endosomes [21]. PIPKIγ isoforms show diverse distributions, including the plasma membrane, focal adhesions, endosomes, cell-cell contacts, and the nucleus [7, 26, 27]. As the N- and C-terminal domains of PIPKIs are highly variable, it is suggested that distinct protein-protein interactions mediated by the N- and C-terminal domains target each PIPKI isoform to a specific subcellular location [23]. Interestingly, in many instances, proteins that target PIPKIs to specific locations are $PI4,5P_2$ effectors implying that $PI4,5P_2$ production is tightly linked to its usage. This concept fits well with basic principle of cell signaling, with a signal only being produced when and where it is needed.

As illustrated in Figure 1.01, compartmentalized $PI4,5P_2$ signal has great importance in cellular regulation. A wide variety of kinases and phosphatases exist to regulate the formation of $PI4,5P_2$ signal at specific compartments. Subsequently, these $PI4,5P_2$ signal can then regulate specific functions within the cell.

Figure 1.01. Central hypothesis: $PI4,5P_2$ synthesis and usage are linked by the association of PIPKs with $PI4,5P_2$ effectors. Extracellular stimuli promote the association of PIPKs with $PI4,5P_2$ effectors. By further association with the subcellular targeting complex, the PIPK/$PI4,5P_2$ effector complex is recruited to a specific location. $PI4,5P_2$ generated from PI4P by PIPKs then binds and activates $PI4,5P_2$ effectors. This assures that $PI4,5P_2$ signal is produced only where and when it is needed.

Figure 1.01


Extracellular stimuli
Subcellular targeting complex
PIPK
PI4,5P2 effector
Formation of the PIPK-PI4,5P2 effector complex at a specific subcellular location
Site-specific PI4,5P2 generation from PI4P by PIPK
Activation of PI4,5P2 effector leading to biological functions

A. Subcellular Distribution of PIP Kinases and PI4,5P_2 Generation:

Studies using PI4,5P_2-specific PH domains, such as the PH domain of phospholipase C δ1 (PLCδ1-PH), fused to GFP revealed that PI4,5P_2 is exclusively found in the plasma membrane, where it displays uniform distribution [28], and this distribution is unchanged by extracellular stimuli. For example, in migrating neutrophils and *Dictyostelium,* PI4,5P_2 distribution remains unchanged before and after chemotactic stimulation [17]. As these early studies were performed at low resolution without titrating expression of the probes, they hardly represent PI4,5P_2 distribution in living cells. We have observed that when GFP-PLCδ1-PH is expressed at low concentrations, it localizes to intracellular compartments with an accumulation at the leading edges in migrating carcinoma cells (data not shown). Examination at high resolution with electron microscopy followed by labeling cellular PI4,5P_2 with purified PLCδ1-PH fused to glutathione *S*-transferase demonstrated that PI4,5P_2 is present not only in the plasma membrane, but also in membranes of Golgi, endosomes, endoplasmic reticulum (ER) and the nucleus [29]. In line with this study, immunocytochemical analysis with a PI4,5P_2-specific antibody identifies multiple, distinct pools of PI4,5P_2 in the plasma membrane, Golgi and intracellular vesicles [30]. Additionally, this immunostaining approach reveals that in response to extracellular stimuli PI4,5P_2 in the plasma membrane actively redistributes. For example, PI4,5P_2 accumulates at the leading edges of fMLP-stimulated chemotacting neutrophils, that is likely mediated by a redistribution of PI4,5P_2-generating enzymes [31].

Detection of PI4,5P_2 at multiple subcellular locations is further supported by concomitant detection of PI4,5P_2-generating enzymes [21, 26, 32-34] and their substrate PI4P [30, 35] at the same locations. The majority of cellular PI4,5P_2 is generated by α, β and γ isoforms of PIPKIs and splice variants of each isoform are reported. In human, three α, four β and six γ splice variants have been described [22, 26, 32, 36, 37]. Amongst, PIPKIγ splice variants, PIPKIγi1 to PIPKIγi6, show strikingly diverse subcellular distribution. PIPKIγi1,

i3 and i6 are largely found in the plasma membrane [10, 37, 38], whereas PIPKIγi2 targets to the focal adhesions when overexpressed [32, 39]. PIPKIγi2 is also found at cell-cell contacts and endosomes [40, 41]. PIPKIγi4 is present in the nucleus and colocalizes with a nuclear speckle marker SC-35 [26]. PIPKIγi5 is found largely at the cell-cell contacts in confluent epithelial cells and colocalizes with E-cadherin [26, 42]. In E-cadherin negative mesenchymal-like cells, PIPKIγi5 localizes in intracellular compartments including early/late endosomes and lysosomes [26, 34].

Additionally, subcellular distribution of PIPKIs is altered by extracellular stimuli. When analyzed by fractionation, a substantial portion of PIPKIα and PIPKIγ is cytosolic [10, 43, 44]. In response to platelet-derived growth factor (PDGF) stimulation, PIPKIα translocates to membrane ruffles of the plasma membrane [43, 44] and in the nucleus [43]. In non-stimulated breast cancer cells, only ~30% of PIPKIγi1 is bound to membranes. Upon epidermal growth factor (EGF) or integrin receptor activation, membrane bound PIPKIγi1 increases ~2.5-fold and PIPKIγi1 accumulates at the leading edges of migrating cells [10]. Consistently, in phagocytic bone marrow-derived macrophages, PIPKIγi1 is recruited to the phagocytic cup where actin is actively polymerized [45]. PIPKIγi2 also accumulates at the leading edges of migrating breast cancer cells [33]. However, in chemotacting neutrophils and T lymphocytes, PIPKIγi2 targets to uropods at the rear of cells [46, 47]. PIPKIβ also targets to uropods of chemotacting neutrophils [48]. Taken together, the stimuli-induced targeting of PIPKIs to specific subcellular locations assures generation of PI4,5P_2 signal in the right place and at the right time.

B. PIP Kinases Regulate PI4,5P_2 Effectors in Cell Migration:

Cell migration is an essential process controlling many aspects of human physiology including morphogenesis during development, maintenance of tissue integrity and immune response. Consequently, aberrant cell migration is linked to pathological conditions such as cancer, mental retardation, atherosclerosis, and arthritis [7, 49-55]. Cell migration is initiated by extracellular signals such as cytokines, growth factors and extracellular matrix (ECM). These signals modulate many intracellular signaling pathways to eventually induce changes in the actin cytoskeleton and microtubules [54-56]. Early studies demonstrated that sequestration of PI4,5P_2 inhibits actin polymerization, whereas artificially increasing PI4,5P_2 levels enhance actin polymerization [17, 57-59]. Also, PI4,5P_2 is required for microtubule capture at the leading edge membrane [54, 60], but the exact mechanism remains unknown. PI4,5P_2 is now well established as a critical component in cell migration by controlling the targeting and activity of cytoskeleton regulators [7, 8, 27, 61]. Below, PI4,5P_2-regulated cytoskeleton regulators, whose functions are controlled by an association with PIPKIs will be discussed in detail.

Talin: In multicellular organisms, focal adhesion complexes are critical structural and functional units that enable cells to integrate signaling inputs from extracellular environments to regulate cell migration [7]. Integrin transmembrane proteins and the talin cytoskeletal protein play critical roles in cell migration [7]. PI4,5P_2 and PI4,5P_2 synthesizing enzymes control integrin activation by talin in the process termed as “inside-out” integrin signaling [62, 63]. Adhesion, spreading and migration, are all directly influenced by the activation state of integrins and talin in focal adhesion complexes [62, 63]. PI4,5P_2 generation by a focal adhesion-targeting variant of PIPKIγ, PIPKIγi2, is believed to provide a discrete pool of PI4,5P_2 required for talin activation during assembly of focal adhesion complex [32, 64].

Talin consists of a head domain (approximately 50 kDa) and a large rod domain (220 kDa) [65, 66]. The head domain contains a region that is responsible for β1-integrin tail binding. Next to this region, the N-terminal part of the head domain contains an atypical FERM domain, which is composed of four sub-domains, F0, F1, F2 and F3, arranged in a cloverleaf pattern [65, 66]. The rod domain contains multiple binding sites for vinculin and actin [65]. When inactive, the head domain interacts with the rod domain (in the middle segment), limiting the accessibility of the head domain for β1-integrin tail binding [65-67]. This type of autoinhibition is widespread among other FERM domain-containing proteins, including focal adhesion kinase (FAK) and moesin [67]. The F2 and F3 sub-domains in talin contain $PI4,5P_2$ binding sites and the availability of $PI4,5P_2$ is crucial for opening up the intramolecular autoinhibition of talin, promoting the head binding to β1-integrin tail [63, 65-67]. A NMR study indicates that the talin head domain has extraordinary affinity for $PI4,5P_2$ [63].

A key step in integrin activation is the binding of the F3 sub-domain to the membrane distal portion of β1-integrin tail via an interaction between the atypical PTB domain (in the F3 sub-domain) and the NPXY motif (in β1-integrin tail) [65-68]. Similarly, talin interaction with membrane proximal NPXY motif in β1-integrin disrupts an interaction with α-integrin, inducing structural rearrangements in the extracellular domain of the integrin that increase ligand binding [65-68]. Besides the F3 sub-domain, other domains in the talin head also contribute to integrin activation via interaction of positively charged patch in the F1 and F2 sub-domains of talin with negatively charged phosphoinositides including $PI4,5P_2$ in the plasma membrane [65-67].

Among the various isoforms of PIPKIs, PIPKIγi2 directly interacts with talin [32, 33, 64]. PIPKIγi2 binding to talin provides the mechanism for local enrichment of $PI4,5P_2$, which in turn induces the conformational change in talin exposing its integrin binding site [66, 69].

The WVYSPLH motif in the C-terminal tail of PIPKIγi2 interacts with the PTB domain in the F3 sub-domain of talin. The PIPKIγi2-talin interaction possibly induces talin activation as discussed above. However, unlike PI4,5P_2, PIPKIγi2 binding to talin does not affect the autoinhibitory conformation of talin as indicated by NMR and biochemical studies, although kinase activity of PIPKIγi2 is increased upon talin binding [70]. In addition, the ability of talin to homodimerize and the presence of additional β1-integrin binding sites in the talin rod domain may provide the mechanism for integrating talin, PIPKIγi2 and integrin into the same complex. Consistently, the ternary complex of PI4,5P_2, talin and integrin mediating integrin activation and clustering has been demonstrated [71]. Similarly, a biochemical study has shown the assembly of ternary complex of in migrating cells [33]. Src phosphorylation of PIPKIγi2 and β1-integrin provides an important regulatory mechanism in controlling talin binding with PIPKIγi2 versus β1-integrin [72]. Src phosphorylation of PIPKIγi2 promotes talin binding whereas Src phosphorylation of β1-integrin diminishes talin binding with integrins [72], although the functional role of this process remains to be defined in cell migration.

Rho family small GTPases: Rho family small GTPases, such as Rac1, Cdc42 and RhoA, are master regulators of the actin cytoskeleton and microtubules [54-56]. They have polybasic clusters allowing them to target to the plasma membrane for function. Studies with phosphoinositide-specific phosphatases reveal that multiple phosphoinositide species including PI4P, PI4,5P_2 and PI3,4,5P_3 mediate Rho family small GTPase association with the plasma membrane [6, 73] via an electrostatic interaction [5]. As PI4,5P_2, like other phosphoinositide species, is present in a limiting concentration for PI4,5P_2 effectors (see above), their plasma membrane targeting might require *de novo* synthesis of PI4,5P_2. Consistently, their plasma membrane targeting is controlled by an association with PIPKIs. Rac1 and RhoA are physically associated with all three PIPKI isoforms *in vitro* [74]. *In vivo*, PIPKIα is required for Rac1 localization at the plasma membrane [44], whereas PIPKIβ is recruited to the plasma membrane by Rac1 [75]. In addition, RhoA signaling is required for

the translocation of PIPKIβ to the plasma membrane [24, 76]. An association of Rho family small GTPases with PIPKIs regulates their function in cell migration. PIPKIα controls PDGF- and integrin-induced cell migration by regulating Rac1 translocation and activation at the leading edge membrane [44]. PIPKIα-mediated actin polymerization and membrane ruffle formation are dependent on Rac1 activity, and a PIPKIα-binding defective Rac1 mutant fails to mediate these processes [43, 44, 58] suggesting that PIPKIα and Rac1 mutually regulate their activity in actin polymerization. In neuronal cells, PIPKIα and PIPKIβ induce neurite retraction that are dependent on Rac1 or RhoA activity [76-78]. A PIPKIβ mutant that is unable to interact with Rac1 fails to translocate to the plasma membrane resulting in defective retraction [75]. Rac1-mediated PIPKIβ translocation to the plasma membrane facilitates neurite retraction as $PI4,5P_2$ promotes vinculin-dependent adhesion turnover [79, 80]. Although PIPKIs do not directly interact with Cdc42, PIPKIs are implicated in Cdc42-induced *de novo* actin polymerization. Cdc42 and $PI4,5P_2$ synergistically activate N-WASP to promote Arp2/3 complex-mediated actin polymerization [81]. Consistently, overexpression of PIPKIα or PIPKIβ induces N-WASP and Arp2/3 complex-dependent actin polymerization [82]. Furthermore, a physical interaction of PIPKIs with Arp2/3 complex is reported [7]. Taken together, Rho family small GTPases and PIPKIs mutually regulate their plasma membrane targeting and function in cell migration. This functional synergism is further supported by the observations that Rho GTPases stimulate the activity of PIPKIs [7, 74]. Conversely, PIPKIs regulate Rho GTPase activation [44], but the exact mechanism is still unknown.

Other effectors: Gelsolin is a dual function actin binding protein. Gelsolin severs actin filaments in the middle or caps the fast growing barbed ends of actin filaments [83]. The capping function of gelsolin is regulated by $PI4,5P_2$-binding that induces gelsolin dissociation from actin and allows actin filament assembly at the barbed ends [84]. $PI4,5P_2$ induces actin polymerization at the N-cadherin-mediated cell-cell contacts by regulating gelsolin [85]. As gelsolin associates with PIPKIγ [86], $PI4,5P_2$ produced by PIPKIγ directly controls gelsolin

dissociation from actin filaments and subsequent incorporation of actin monomers at the cell-cell contact sites [85].

The non-receptor tyrosine kinase Src regulates a plethora of cell functions. Src targets to the focal adhesions by interacting with many focal adhesion components, such as integrin, talin and FAK [87]. Src interacts with PIPKIγi2 and this interaction also regulates Src focal adhesion targeting and activation [88]. Kinase activity of Src remains suppressed by an intramolecular interaction of the SH2 domain with phosphorylated Tyr527 residue at the C-terminus [89, 90]. Interestingly, the PIPKIγi2 binding site on Src is within the C-terminal region including Tyr527 residue [88], suggesting that the interaction with PIPKIγi2 might activate Src by relieving the autoinhibitory intramolecular interaction. Polybasic residues at the N-terminus of Src medicate membrane binding through interaction with phosphoinositide species [91]. Mutating these residues to neutral amino acids inhibits Src activation by PIPKIγi2 [88]. Collectively, these studies suggest that PIPKIγi2 and $PI4,5P_2$ generation regulate Src recruitment to and activation at the focal adhesions.

For efficient cell migration, adhesions at the cell rear must be dissolved, which is largely mediated by RhoA-dependent actomyosin contractility and calpain-dependent proteolysis of adhesion components [50, 55, 56]. PIPKIβ and PIPKIγi2 target to the cell rear and regulate adhesion disassembly. In chemotacting neutrophils, PIPKIβ specifically localizes at the retractile tails of cells by an association with 4.1-ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 (EBP50). The EBP50 binding region on PIPKIβ is within the 83 C-terminal amino acids, which are not homologous to other PIPKI isoforms [92]. This PIPKIβ-specific interaction with EBP50 mediates PIPKIβ association with ERM proteins whose activities are regulated by $PI4,5P_2$-binding [83] and the Rho GDP dissociation inhibitor (RhoGDI) [47]. As ERM proteins inhibit RhoGDI leading to RhoA activation [93], localized $PI4,5P_2$ production at the cell rear controls cell contractility [47]. Calpain is a

calcium-dependent protease that cleaves several adhesion components at the trailing edges, such as vinculin and talin [94]. PI4,5P_2-binding to calpain enhances calpain activity by decreasing its calcium requirement for activation [95]. Although the calpain interaction with PIPKIs has not been reported, it is likely that calpain activity is regulated by PIPKIγi2 at the trailing edges of migrating cells [7]. In support of this possibility, PI4,5P_2 accumulates at the uropod of migrating neutrophils and overexpression of a kinase inactive form of PIPKIγi2 compromises uropod formation in which calpain activity is essential [47].

C. PIP Kinases Regulate PI4,5P_2 Effectors in Membrane Trafficking:

PIP kinases, and other phosphoinositide kinases and phosphatases have unique subcellular targeting and regulate specific processes. This compartmentalization is critical for proper membrane trafficking, as localized synthesis of phosphoinositides allows for regulation of specific effector molecules in trafficking pathways [27, 96, 97]. Within the cell, the numerous phosphoinositide species vary in location and quantity. This is regulated by multiple kinases and phosphatases, which maintain a distinct distribution for each lipid [24]. The precursor to all phosphoinositide signals, phosphatidylinositol (PI), is synthesized and primarily localized to the ER. From there, PI is distributed throughout the cell where it is further modified [98, 99]. The majority of each phosphoinositide is maintained at distinct compartments. These include, PI4P at the Golgi, PI3P and PI3,5P_2 at early and late endosomes [96, 97]. Though PI4,5P_2 is typically thought to be confined to the plasma membrane, recent evidence indicates diverse functions at multiple intracellular locations. Below, PI4,5P_2-modulated trafficking regulators whose functions are controlled by an association with PIPKIs will be discussed.

AP complex: Adaptor protein (AP) complexes are key components of clathrin coats in the post-Golgi and endocytic membrane trafficking pathways [100]. The formation of a clathrin coat is not through a direct interaction of clathrin with the membrane, and instead adaptor proteins are involved in recruiting clathrin. Each AP complex contains subunits that are responsible for phosphoinositide or cargo binding. The phosphoinositide and cargo binding is highly selective allowing for the formation of clathrin coats at specific membrane compartments. At the Golgi, clathrin coats are important in the formation of vesicles and fission events needed for cargo exit. Clathrin adaptors, including the AP1 complex, EpsinR and Golgi-localized γ-ear containing, Arf binding proteins (GGAs), bind to and are activated by PI4P, to recruit clathrin to the Golgi [101-104]. Loss of function studies for the PI4Ks, which generate PI4P, demonstrate the role of PI4P in recruiting clathrin coats. Knockdown of

the trans-Golgi network (TGN) associated PI4KIIα affects the trafficking of proteins from the Golgi and also the recruitment of the AP1 complex, which can be overcome by administering PI4P or PI4,5P_2 to knockdown cells [101]. The ability of PI4,5P_2 to rescue these phenotypes suggests that PIPKIs may also participate in Golgi functions [105].

Outside of the Golgi, PI4,5P_2 regulates several steps in trafficking to and from the plasma membrane. For example, in epithelial cells, there is a specific clathrin AP complex associated with basolaterial sorting, called the AP1B complex. PIPKIγi2 regulates the trafficking of cargo to the basolateral surface [106]. This is mediated through a YxxØ sorting motif (where x is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain) found in the PIPKIγi2 C-terminus that allows for specific association with the AP1B complex. Additionally, the formation of PI4,5P_2 is enhanced by association with AP1B. The PI4,5P_2 produced by PIPKIγi2 activates the AP1B complex, recruiting clathrin coats at the plasma membrane [106].

PI4,5P_2 has been well characterized in the regulation of endocytosis. Clathrin adaptors, such as the AP2 complex and Epsin1, regulate recruitment of clathrin to the plasma membrane at sites of endocytosis, and PI4,5P_2 is able to regulate the recruitment and activation of these proteins [40, 107-109]. The PIPKIs that generate PI4,5P_2 are important in this process. Knockdown of PIPKIβ was found to inhibit transferrin receptor endocytosis [110]. Additionally, there is a direct interaction between PIPKIγi2 and the AP2 complex. Increased expression of PIPKIγi2 enhances the endocytosis of transferrin, while a PIPKIγi2 kinase inactive mutant or siRNA knockdown of PIPKIγ inhibits endocytosis [40]. Besides their roles in clathrin recruitment, Epsin, and similar proteins, also regulate membrane deformation, and are able to elongate the membrane into tubules to assist in formation of vesicles [109]. Membrane deformation is also mediated by the actin cytoskeleton to generate force. PI4,5P_2 is known to regulate many actin regulating proteins,

including N-WASP and Arp2/3 complex. PI4,5P_2 can recruit these proteins to sites of endocytosis and stimulate actin branching and polymerization important for endocytosis [111]. At the final stages of endocytosis, PI4,5P_2 also recruits and activates the GTPase, dynamin, to complete fission and release of the endocytic vesicle from the plasma membrane [112].

Exocyst complex: The exocyst complex is a multiprotein complex essential for polarized delivery and tethering of secretory vesicles to specific domains of the plasma membrane for cell surface expansion and protein secretion [113]. It is thought to function before SNARE-mediated vesicle fusion with the plasma membrane [113]. Exocyst is composed of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) that interact with each other and are thought to organize into distinct sub-complexes. The subunits that define the targeting patch on the plasma membrane form one sub-complex and other subunits on a cargo-laden vesicle form the other sub-complex, and both sub-complexes function together at the final step of vesicle delivery/tethering on the plasma membrane [114]. Furthermore, the exocyst complex interacts with key regulators of vesicle trafficking such as Rab11, Arf, RalB and Rho GTPases [114].

In epithelial cells, PIPKIγ and the exocyst complex play crucial role in maintaining adherens junctions and apical-basal polarity by regulating endocytic trafficking of E-cadherin [115]. PIPKIγ directly associates with Exo70 and Sec6 subunits of the exocyst complex [33, 115]. Sec3 and Exo70 have clusters of basic residues (at the N-terminus of Sec3 and the C-terminus of Exo70) that mediate the interaction of the exocyst complex with PI4,5P_2 in the plasma membrane [116, 117]. This provides a mechanism for polarized delivery of E-cadherin to adherens junctions in the plasma membrane. However, in many carcinoma cells, E-cadherin expression is lost. In these cells, the exocyst complex redistributes from the adherens junctions to the leading edges and may regulate trafficking of other cargos [118]. In migrating cells, PIPKIγ and the exocyst complex colocalize at the leading edges [33]. The

loss of PIPKIγ profoundly affects the recruitment and localization of the exocyst complex, indicating that the interaction with PIPKIγ facilitates recruitment of the exocyst complex to the leading edges. In addition, the association of PIPKIγ with the exocyst complex is highly induced at the onset of cell migration [33]. This would ensure the efficient polarized recruitment and delivery of transmembrane proteins and signaling molecules that are required for nascent adhesion formation in migrating cells (Figure 2.03). Consistently, knockdown of PIPKIγ and the exocyst complex profoundly affect leading edge formation, polarized recruitment of integrins and cell migration [33, 113, 118, 119]. Furthermore, it is possible that these complexes may also mediate the polarized recruitment of growth factor receptors in migrating cells.

Sorting nexins: The sorting nexins are an evolutionarily conserved family of proteins that are found in eukaryotes that regulate a wide range of trafficking pathways, including lysosomal sorting, recycling pathways and endocytosis [120-124]. All sorting nexin family members contain a Phox homology (PX) domain, which mediates binding to phosphoinositides, especially PI3P [121, 125-128]. Therefore, the interplay of phosphoinositides, sorting nexins and the enzymes that generate phosphoinositide signals is essential for proper regulation of trafficking.

Sorting nexin 5 (SNX5) was identified as an interactor of PIPKIγi5 in a yeast two-hybrid screen [34], and the closely related SNX6 was also found to interact with PIPKIγi5 [42]. PIPKIγi5, SNX5 and SNX6 are found at early and late endosomes where PI3P and $PI3,5P_2$ are the most abundant phosphoinositide species. While most PX domains have specificity for PI3P, solution of the SNX5 PX domain crystal structure revealed binding specificity for $PI4,5P_2$, but not PI3P [129]. Additionally, a subset of sorting nexins, including SNX5 and SNX6, contain a phosphoinositide binding Bin/Amphiphysin/Rvs (BAR) domain [125, 127, 130]. At endosomes, production of $PI4,5P_2$ by PIPKIγi5 promotes an association

of SNX5 with Hrs, a subunit of endosomal sorting complex required for transport (ESCRT) complex that binds and regulates EGF receptor sorting to the intraluminal vesicles of the multivesicular body [34, 131]. The mechanism of how $PI4,5P_2$ regulates the SNX5-Hrs interaction is unclear. One possibility is that $PI4,5P_2$ binding on the PX and BAR domain of SNX5 induces conformational change that enhance Hrs binding. Additionally, $PI4,5P_2$ generation on endosomes may recruit SNX5 to specific regions of endosomes where SNX5 and Hrs can function to sort specific cargos [132-134].

SNX5 and SNX6 function with PIPKIγi5 to control endosome-to-lysosome trafficking of E-cadherin [42]. Treatment of polarized epithelial cells with hepatocyte growth factor initiates the disassembly of adherens junctions and eventual degradation of E-cadherin at lysosomes. PIPKIγi5 directly binds E-cadherin and promotes its lysosomal degradation, and $PI4,5P_2$ generation by PIPKIγi5 is required for this process [42]. However, SNX5 and SNX6 inhibit E-cadherin lysosomal targeting in this pathway [42]. The role for SNX5, SNX6 and PIPKIγi5 interaction in regulating receptor degradation to identify which cargos are affected and the specific mechanism for each pathway.

Additionally, other sorting nexins may be regulated by PIPKIs. For example, SNX9 binds to $PI4,5P_2$ and regulates endocytosis. PIPKIα, PIPKIβ and PIPKIγ were all shown to bind to SNX9. Thus, SNX9 represents another regulator of endocytosis that binds and is regulated by PIPKIs and $PI4,5P_2$ [135].

D. PIP Kinases Regulate Other Phosphoinositide Kinases:

Since phosphatidylinositol 4-kinases (PI4Ks) generate PI4P, a substrate for PIPKIs, the physical interaction of PIPKIs with PI4Ks might bestow functional synergism for $PI4,5P_2$ production. An association of type II PI4Ks (PI4KIIs) with PIPKIs is reported [136]. Following this discovery, ADP-ribosylation factor (ARF) small GTPase was shown to mediate the targeting of PI4KIIβ and an unidentified PIPKI to the Golgi [105]. The PIPKI that is responsible for $PI4,5P_2$ generation at the Golgi is likely PIPKIβ as it directly interacts with ARF1 and this interaction targets PIPKIβ to the Golgi [137]. Additionally, ARF1 enhances kinase activity of PI4KIIβ at the Golgi membranes [137] and likely that of PIPKIβ [7]. Taken together, the organization of PI4Ks and PIPKIs into multiprotein complexes specifically targeted to the Golgi membrane by ARF is an efficient way to spatiotemporally and efficiently coordinate the synthesis of $PI4,5P_2$. Furthermore, this organization might not be confined to the Golgi membrane as PI4Ks and their targeting factors are also found in other membrane compartments including the plasma membrane [138, 139].

PI3Ks generate $PI3,4,5P_3$ using $PI4,5P_2$ as a substrate. Thus, PI3Ks are potential effectors of $PI4,5P_2$ and the compartmentalized organization of PIPKIs and PI3Ks might be an efficient means for generating $PI3,4,5P_3$. However, the role of PIPKIs in $PI3,4,5P_3$ generation is underestimated as it had been believed that cellular $PI4,5P_2$ is present beyond the limiting concentration for $PI3,4,5P_3$ synthesis by PI3Ks [17]. As discussed above, the enzymatically available concentration of $PI4,5P_2$ at a specific time and location is much lower than estimated, thus *de novo* synthesis of $PI4,5P_2$ by PIPKIs might be required for efficient $PI3,4,5P_3$ generation by PI3Ks. In line with this possibility, in *Dictyostelium*, depletion of a PIPKI, that is responsible for approximately 90% of cellular $PI4,5P_2$ generation, results in attenuation of Akt activity, a readout of PI3K activity in the cell [140]. In human keratinocytes, knockdown of PIPKIα also attenuates extracellular calcium-induced Akt activation [141]. In this particular study, knockdown of PIPKIα reduces approximately 40% of global $PI4,5P_2$

levels in keratinocytes, but the reduction of $PI3,4,5P_3$ levels and Akt activation is much greater (up to approximately 90% reduction) suggesting that $PI4,5P_2$ and $PI3,4,5P_3$ synthesis might be locally organized and that only a specific pool of $PI4,5P_2$ (but not all) is available and responsible for $PI3,4,5P_3$ generation. In support of this possibility, we have observed that knockdown of single PIPKI isoform has no significant impact on global $PI4,5P_2$ levels in carcinoma cells (see chapter 3) and this is consistent with previous studies tested in other cells [41, 45]. The local organization of $PI4,5P_2$ and $PI3,4,5P_3$ synthesis is further supported by immunostaining analyses in migrating leukocytes [31]. PI3Ks are shown to localize at the leading edge membranes, whereas PTEN is present at the sides and rears of migrating cells [142-144]. This leads to an assumption that $PI3,4,5P_3$ concentration is higher at the leading edges, while $PI4,5P_2$ concentration is higher at the sides and rears. In contrast to this assumption, $PI4,5P_2$ concentration is also higher at the leading edges of migrating leukocytes when analyzed with a $PI4,5P_2$-specific antibody [31]. This is not resulted from membrane folds at the leading edges. Importantly, PIPKIα and PIPKIγ are colocalized with $PI3,4,5P_3$ at the leading edges suggesting that $PI4,5P_2$ synthesis by PIPKIs is coupled to $PI3,4,5P_3$ localization at the leading edges of migrating cell. However, the physical association of PIPKIs with PI3Ks still remains untested.

PI4Ks, PIPKIs and PI3Ks mediate sequential phosphorylation at the 4, 5 and 3 hydroxyl of the myo-inositol ring of PI, respectively, to generate $PI3,4,5P_3$. Organization of all three enzymes in the same multiprotein complex would more efficiently mediate the generation of $PI3,4,5P_3$. Knockdown of PI4KIIα or PI4KIIIβ in fibroblast cells reduces global PI4P and $PI4,5P_2$ levels, but only PI4KIIα knockdown reduces EGF-stimulated Akt activation [145] indicating that PI4KIIα-mediated local PI4P and $PI4,5P_2$ generation is required for PI3K dependent Akt activation. Also, sequestration of PI4P attenuates downstream signaling of Akt, without affecting other PI4P-mediated signaling, further suggesting that PI4P is

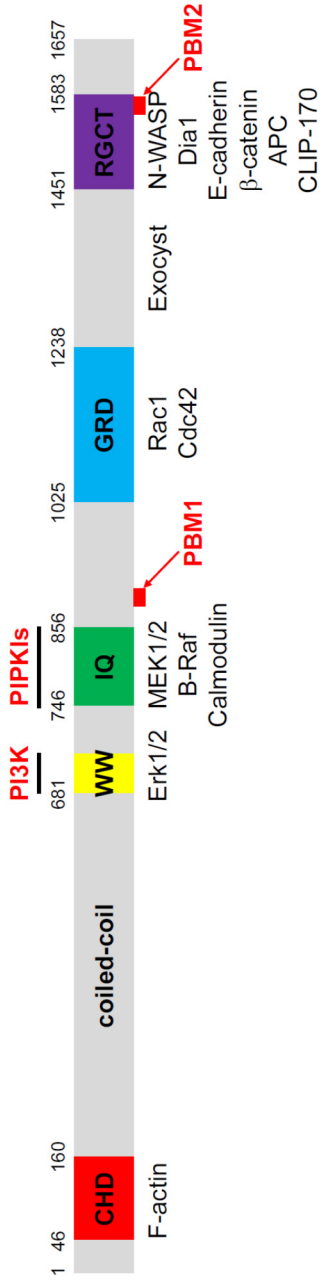
important for PI3,4,5P_3 generation by PI3Ks [146]. However, the physical association of the three sequential phosphoinositide kinases in a complex needs to be tested.

The Roles of IQGAP1 in Cellular Regulation:

IQGAP1 is one of three IQ motif containing GTPase activating protein family members expressed in mammals and it has wide tissue expression [147]. Since its discovery two decades ago [148], over 100 IQGAP1 interacting proteins have been identified. By interacting with various signaling molecules, IQGAP1 regulates many distinct signaling pathways (Figure 1.02). How IQGAP1 has such versatile cellular roles has remained an enigma. An unbiased quantitative study reveals that the messenger RNA and protein copy number of IQGAP1 is at least two orders of magnitude higher than those of its interacting proteins [149], which suggests that there may be multiple functional IQGAP1 containing complexes in a single cell. IQGAP1 contains five different domains that mediate protein-protein interactions: calponin homology (CHD), WW, IQ, GAP-related (GRD) and RasGAP C-terminal (RGCT) domains [147, 150-152], which all contribute toward complex formation. Below, the signaling pathways that are regulated by IQGAP1 will be summarized.

Figure 1.02. IQGAP1 domain structure and selected IQGAP1 interacting proteins. IQGAP1 interacts with many signaling proteins through its five domains. Proteins highlighted in red are newly defined in this study and discussed in detail in the following chapters. Also, two polybasic motifs (PBMs) are identified as regions mediating IQGAP1 interaction with phosphoinositides.

Figure 1.02


1
46
160
681
746
856
1025
1238
1451
1583
1657
CHD
coiled-coil
WW
IQ
GRD
RGCT
F-actin
Erk1/2
MEK1/2
B-Raf
Calmodulin
Rac1
Cdc42
Exocyst
N-WASP
Dia1
E-cadherin
β-catenin
APC
CLIP-170
PI3K
PIPKIs
PBM1
PBM2

Small GTPase signaling: The IQGAP1 GAP-related domain (GRD) has sequence similarity to GAP proteins, and preferentially binds to but it GTP bound forms of Rac1 and Cdc42 [153, 154]and stabilize them in the active state [155]. However, IQGAP1 lacks GAP activity [155]. As Rac1 and Cdc42 activity is indispensible for actin polymerization, IQGAP1 promotes actin polymerization at various cellular locations, including the leading edges of migrating cells [153, 154, 156], phagocytic cup [157] and cell-cell contacts [158].

Calcium signaling: IQGAP1 interacts with calmodulin in the both the absence and presence of calcium via the IQ domain. The binding of calmodulin regulates IQGAP1 interaction with other proteins such as small GTPases [159, 160]. As IQGAP1 is kept in an inactive conformation in unstimulated conditions [161, 162], extracellular signals might elevate calcium levels in cells that in turn induces calcium-calmodulin binding to IQGAP1 facilitating transition to an active conformation [163].

Mitogen activated protein kinase (MAPK) signaling: The role for IQGAP1 as a scaffold is best characterized in the MAPK pathway. IQGAP1 interacts with MAPK components Raf, MEK and Erk, and this interaction mediates sequential phosphorylation of downstream kinases by upstream kinases [164-166]. Activation of receptor tyrosine kinase stimulates the MAPK pathway through Ras. Consistently, IQGAP1 is shown to activate Erk downstream of K-Ras [167]. Furthermore, IQGAP1 interacts with an adaptor protein ShcA [168] linking receptor tyrosine kinase to the MAPK pathway via Ras. By organizing various components of the MAPK pathway, IQGAP1 mediates receptor tyrosine kinase signaling in various cell types [169-171].

Phosphoinositide signaling: In many cell types, IQGAP1 localizes to the leading edge membrane of migrating cells [10, 60, 152, 156, 161, 172], and this localization is controlled by an association of leading edge-targeted proteins such as Rac1, Cdc42, Dia1 and the exocyst complex [153, 154, 157, 173]. In addition to these factors, PIPKIγ binding also

regulates IQGAP1 leading edge targeting [10]. *In vitro*, the conserved region found in all six PIPKIγ interacts with IQGAP1 on the IQ domain. *In vivo*, PIPKIγ-binding is sufficient to recruit IQGAP1 to the leading edges as a PIPKIγ-binding defective IQGAP1 mutant is unable to target to the leading edge [10].

At the leading edge, IQGAP1 facilitates actin polymerization and recruits microtubules that are essential for membrane protrusion and polarity establishment of migrating cells, respectively [54, 55, 150, 161]. IQGAP1 interacts with N-WASP and the interaction relieves an autoinhibitory conformation exposing the VCA domain of N-WASP. The exposed VCA domain, then, activates Arp2/3 complex-mediated *de novo* actin polymerization [161]. IQGAP1 interacts with the microtubule plus end regulators, CLIP-170 and adenomatous polyposis coli (APC). By these interactions IQGAP1 brings microtubules to the leading edge [153, 154]. Interestingly, the N-WASP, CLIP-170 and APC interaction region on IQGAP1 is within the RGCT domain that is otherwise masked by an autoinhibitory interaction with the GRD domain [161, 162]. Rac1 and Cdc42 binding to the GRD domain *in vivo* or phosphorylation at Ser1441 and Ser1443, between the GRD and RGCT domains, *in vitro* relieves the autoinhibitory interaction [153, 154]. In addition to these factors, phosphoinositide binding also regulates IQGAP1 function by relieving the autoinhibitory interaction [10]. *In vitro*, IQGAP1 interacts with multiple phosphoinositide species with varying affinity [12, 13, 174] and the phosphoinositide binding site is a polybasic region located in the RGCT domain [10]. PI4,5P_2-binding on the RGCT domain relieves the autoinhibitory interaction and regulates N-WASP-Arp2/3 complex-dependent actin polymerization [10, 161]. Interestingly, when expressed in carcinoma cells, an IQGAP1 mutant that is defective in PI4,5P_2-binding induces multiple leading edges [10] suggesting that PI4,5P_2-binding is important for IQGAP1 to regulate cell polarity. Consistently, a Rac1/Cdc42 binding defective IQGAP1 mutant expressed in Vero epithelial cells also

induces multiple leading edges as its interaction with CLIP-170 is enhanced irrespective of the Rac1/Cdc42-binding status [154].

IQGAP1 activation requires Rac1/Cdc42-binding on the GRD domain, PIPKIγ-binding on the IQ domain, $PI4,5P_2$-binding on the RGCT domain, and phosphorylation at Ser1441 and Ser1443 in a stepwise manner. Phosphorylation at the serine residues, likely by PKCs, is required for Rac1/Cdc42-binding [162]. PIPKIγ-binding on the IQ domain is followed by phosphorylation at the serine residues [10], and finally both $PI4,5P_2$- and Rac1/Cdc42-binding are required for full activation of IQGAP1 [10, 163]. Based on these, we propose a mechanism of IQGAP1 activation in actin polymerization and microtubule recruitment at the leading edge.

Akt signaling: IQGAP1 interacts with Akt and is required for Akt activation [171, 175]. Also, IQGAP1 interacts with mTOR complex 1 and this interaction inhibits S6 kinase phosphorylation at Thr389 promoting Akt signaling [176]. However, the association of IQGAP1 with upstream regulators of Akt signaling pathways such as PI3K and PTEN remains to be defined.

Hypothesis and Thesis Plan:

Site-specific generation of PI4,5P_2 by PIPKIs provides an efficient means to regulate PI4,5P_2 signal at a subcellular compartment. Most studies on PI4,5P_2 have focused on its vast roles in the regulation of cellular processes such as cell migration, trafficking and signaling, while the implication of PI4,5P_2-metabolizing enzymes in these processes is not fully understood. Therefore, my research has focused on how PI4,5P_2-generating enzymes PIPKIs may regulate cellular functions via their association with other proteins. A proteomic analysis was used to identify potential interactors for PIPKIs, and one protein of interest was the signaling scaffold, IQGAP1. IQGAP1 interaction with PIPKIs was found to have affect icell migration and the class I PI3K signaling. Therefore, studies were performed to assess the mechanism for the phenotypes observed for PIPKIs and IQGAP1.

Chapter 2 describes the characterization of the interaction of PIPKIγ with IQGAP1. These proteins were found to mutually regulate cell migration. The mechanism of the functional mutuality of the two proteins in cell migration was intensively investigated. Surprisingly, IQGAP1 is a PI4,5P_2 effector and its function in cell migration is tightly regulated by the association with PIPKIγ.

Chapter 3 describes the characterization of the interaction of PIPKIα with IQGAP1. IQGAP1 was found to associate with PIPKIα and class I PI3K and the three proteins were found to regulate Akt signaling. The molecular mechanism of the IQGAP1-PIPKIα-class I PI3K in regulation of Akt signaling was investigated. Interestingly, our data suggest that IQGAP1 may function as a scaffold for the phosphoinositide kinase signaling pathway.

Finally, chapter 4 summarizes findings in the previous chapters to provide an overview for how PIPKIs and IQGAP1 regulate cellular processes. Also included is a description of future studies that will better characterize this pathway.

Chapter 2

The Roles of Gamma Isoform of Type I PIP Kinase and IQGAP1 in Cell Migration

Published in part as:

Suyong Choi, N. Thapa, A.C. Hedman, Z. Li, D.B. Sacks, R.A. Anderson, IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration, EMBO J 32 (2013) 2617-2630.

Chapter Summary:

Phosphatidylinositol 4,5 bisphosphate ($PI4,5P_2$) is a key lipid messenger for regulation of cell migration. $PI4,5P_2$ modulates many effectors, but the specificity of $PI4,5P_2$ signaling can be defined by interactions of $PI4,5P_2$-generating enzymes with $PI4,5P_2$ effectors. Here, we show that type Iγ phosphatidylinositol 4-phosphate 5-kinase (PIPKIγ) interacts with the cytoskeleton regulator, IQGAP1, and modulates IQGAP1 function in migration. Through intensive biochemical and cell biological studies, we reveal that PIPKIγ is required for IQGAP1 recruitment to the leading edge membrane in response to integrin or growth factor receptor activation. Moreover, IQGAP1 is a $PI4,5P_2$ effector that directly binds $PI4,5P_2$ through a polybasic motif and $PI4,5P_2$ binding activates IQGAP1, facilitating actin polymerization. IQGAP1 mutants that lack PIPKIγ or $PI4,5P_2$ binding lose the ability to control directional cell migration. Collectively, these data reveal a synergy between PIPKIγ and IQGAP1 in control of cell migration.

Introduction:

Cell migration is a highly orchestrated, multistep process requiring the establishment of polarity, the regulation of cytoskeleton dynamics and spatiotemporal signaling [50, 55]. Cell migration is initiated in response to extracellular stimuli, such as cytokines and signals from the extracellular matrix (ECM). These extracellular signals activate intracellular signaling cascades that promote changes in the cytoskeleton. A diverse array of proteins are implicated in these processes, but scaffold proteins that integrate signals from multiple structural and signaling molecules play pivotal roles in transmitting cellular information [15, 56]. Previous work has focused on how scaffold proteins coordinate different signals. However, the exact mechanism for how scaffold proteins themselves are targeted and activated remains largely unknown.

IQ motif containing GTPase activating protein 1 (IQGAP1) is a multidomain protein that regulates cytoskeletal dynamics, proliferation, adherens junction integrity and vesicular trafficking, by serving as a scaffold for key signals [147, 151, 163]. IQGAP1 targets to the leading edge, where it promotes actin polymerization through Rac1 and Cdc42 and their effectors, such as N-WASP and Dia1 [147, 157, 160, 161, 177]. IQGAP1 also controls microtubule (MT) behavior. IQGAP1 interacts with MT plus end regulators, CLIP-170 and adenomatous polyposis coli (APC), and recruits MTs to the leading edge membrane [153, 154]. By targeting MTs to the leading edge, IQGAP1 is believed to facilitate the polarized trafficking of protein to the migrating front [54, 151]. Yet, how IQGAP1 interacts with the leading edge membrane is largely undefined. A recent study has shown that $PI4,5P_2$ dependent microdomains are required for the recruitment of MTs to the plasma membrane (PM), and Cdc42, N-WASP and IQGAP1 are also required in this process [60]. However, the exact role for $PI4,5P_2$ in IQGAP1 regulation of the cytoskeleton at the plasma membrane (PM) is unknown.

At a molecular level, IQGAP1 is kept inactive through an autoinhibitory interaction between the GRD domain and RGCT domain [163]. This autoinhibition can be relieved by RhoGTPase binding to the GRD domain or phosphorylation on Ser1443 to activate IQGAP1 [162, 178]. In agreement with this model of activation, a mutant IQGAP1, defective in RhoGTPase binding on the GRD domain, induces multiple leading edges [154] and a phosphomimetic variant of IQGAP1 on Ser1443 stimulates neurite outgrowth [178].

PI4,5P_2 comprises approximately 1% of membrane phospholipids and is the most abundant phosphoinositide species at the plasma membrane (PM). Besides serving as a precursor for other lipid messengers, PI4,5P_2 exerts direct signaling roles by interacting with protein targets [21, 23]. Though PI4,5P_2-binding is often achieved by defined modules on proteins, including C2, pleckstrin homology (PH), and band 4.1/ezrin/radixin/moesin (FERM) domains, many PI4,5P_2-interacting proteins lack canonical modules and instead contain clusters of basic amino acids, known as polybasic motifs (PBMs) that bind PI4,5P_2 [179]. The interaction of PBMs with phosphoinositides is largely mediated by the positively charged residues in the PBM that interact with the phosphate head group. Therefore, these interactions in some cases can be promiscuous for phosphoinositides [5]. Recent advances in proteomic analyses have identified hundreds of putative PI4,5P_2 binding proteins, but most of them do not contain canonical modules [12, 13], thus many PBMs or atypical phosphoinositide binding motifs remain to be characterized.

PI4,5P_2 modulates the activity and targeting of cytoskeleton regulatory proteins, controlling cytoskeletal dynamics and, ultimately, migration [8, 180]. Although the roles for PI4,5P_2 in cytoskeleton regulation are extensively studied, the roles for PI4,5P_2-generating enzymes in this process are still emerging [7, 180]. In mammalian cells, PI4,5P_2 is primarily generated by type I PIP kinases (PIPKIs) and three isoforms, α, β and γ, are expressed in human with multiple isoforms [22]. For example, four different isoforms of PIPKIγ are expressed in human and each isoform shows a unique cellular distribution. PIPKIγi1 is the

most abundant isoform in most cell types and largely locates to the PM [181]. PIPKIγi2 is found at focal adhesions and cell-cell contacts [32, 41]. PIPKIγi4 is found largely in the nucleus, while PIPKIγi5 localizes to cell-cell contacts and intracellular compartments [26]. Often, protein-protein interactions recruit PIPKI isoforms to specific cellular regions, and many of these targeting proteins are themselves $PI4,5P_2$ effectors [21, 23]. For example, talin recruits PIPKIγi2 to focal adhesions, while the site-specific generation of $PI4,5P_2$ by PIPKIγi2 strengthens talin binding to β1-integrin [7].

PIPKIγ and IQGAP1 are implicated in cancer progression and metastasis [172, 182]. Overexpression of PIPKIγ in breast cancer was found to correlate with poor prognosis [182]. Loss of the PIPKIγi2 isoform from metastatic breast cancer cell lines reduces cell motility [33], but the role for other PIPKIγ isoforms and molecular mechanisms remain elusive. Similarly, loss of IQGAP1 in malignant breast epithelial cells reduces cell motility [183] and cell growth [184]. IQGAP1 overexpression is reported in cancers originating from many different tissues. IQGAP1 is shown to regulate the function of many oncoproteins. Notably, IQGAP1 is found at the invasive front of aggressive cancers [172] without knowing the underlined mechanism.

Here, we report IQGAP1 as a novel $PI4,5P_2$ effector that is tightly regulated by $PI4,5P_2$-generating enzyme PIPKIγ. PIPKIγ and IQGAP1 interact and function together in regulation of directional migration. Mechanistically, IQGAP1 requires PIPKIγ for targeting to the leading edge membrane of migrating cells. Also, IQGAP1 is activated specifically by $PI4,5P_2$, disrupting IQGAP1 autoinhibition to induce actin polymerization. Directional cell migration is dramatically attenuated in cells expressing IQGAP1 mutants that lack PIPKIγ or $PI4,5P_2$ interaction. Given that expression of both proteins is deregulated in cancers, this study will validate the PIPKIγ/IQGAP1 signaling nexus as a putative therapeutic target for early steps in cancer progression.

Results:

IQGAP1 and PIPKIγ interact: Interacting proteins often determine the function and intracellular targeting of PIPKIs [21]. To identify interacting proteins for PIPKIγ, i1 and i5 isoforms were inducibly expressed and immunoprecipitated (IP'ed) from MDCK cell lysates. The isolated complexes were separated by SDS-PAGE and the gels visualized by Coomassie staining. Then, protein bands were analyzed by mass spectrometry. IQGAP1 was identified to interact with the PIPKIγi1 and i5 complexes (Figure 2.01 A).

The interaction between PIPKIγ and IQGAP1 was confirmed in human cell lines. Endogenous proteins were IP'ed and association was examined by immunoblotting. IQGAP1 co-IP'ed with PIPKIγ, and vice versa, from HEK 293 or MDA-MB-231 cell lysates (Figure 2.01 B). The cellular location of the proteins was examined via immunostaining. DsRed-PIPKIγi1 co-localized with endogenous IQGAP1 at the periphery of MCF7 cells and to a lesser extent at a perinuclear compartment (Figure 2.01 C). To characterize binding, His-PIPKIγi1 and GST-IQGAP1 were expressed in E. coli, purified and in vitro binding was assessed. As shown in Figure 2.01 D, the binding was saturable and Scatchard analysis revealed the dissociation constant (K_d) for the interaction is ~175 nM, demonstrating that in vitro PIPKIγ directly interacts with IQGAP1 with a moderate affinity.

PIPKIγ interacts with the IQ domain: IQGAP1 integrates many signaling pathways by forming interactions through its calponin homology (CHD), WW, IQ, GAP-related (GRD) and RasGAP C-terminal (RGCT) domains [147]. To identify the PIPKIγ binding site on IQGAP1, we co-expressed Myc-IQGAP1 wild type (WT) or deletion mutants of each domain with HA-PIPKIγi1 in HEK 293 cells and performed an IP. Deletion of the IQ domain (ΔIQ) lost IQGAP1 co-IP with PIPKIγ (Figure 2.01 E) and in vitro the ΔIQ mutant also failed to interact with PIPKIγ (Figure 2.01 F). Further, the IQ domain alone was capable of interacting with

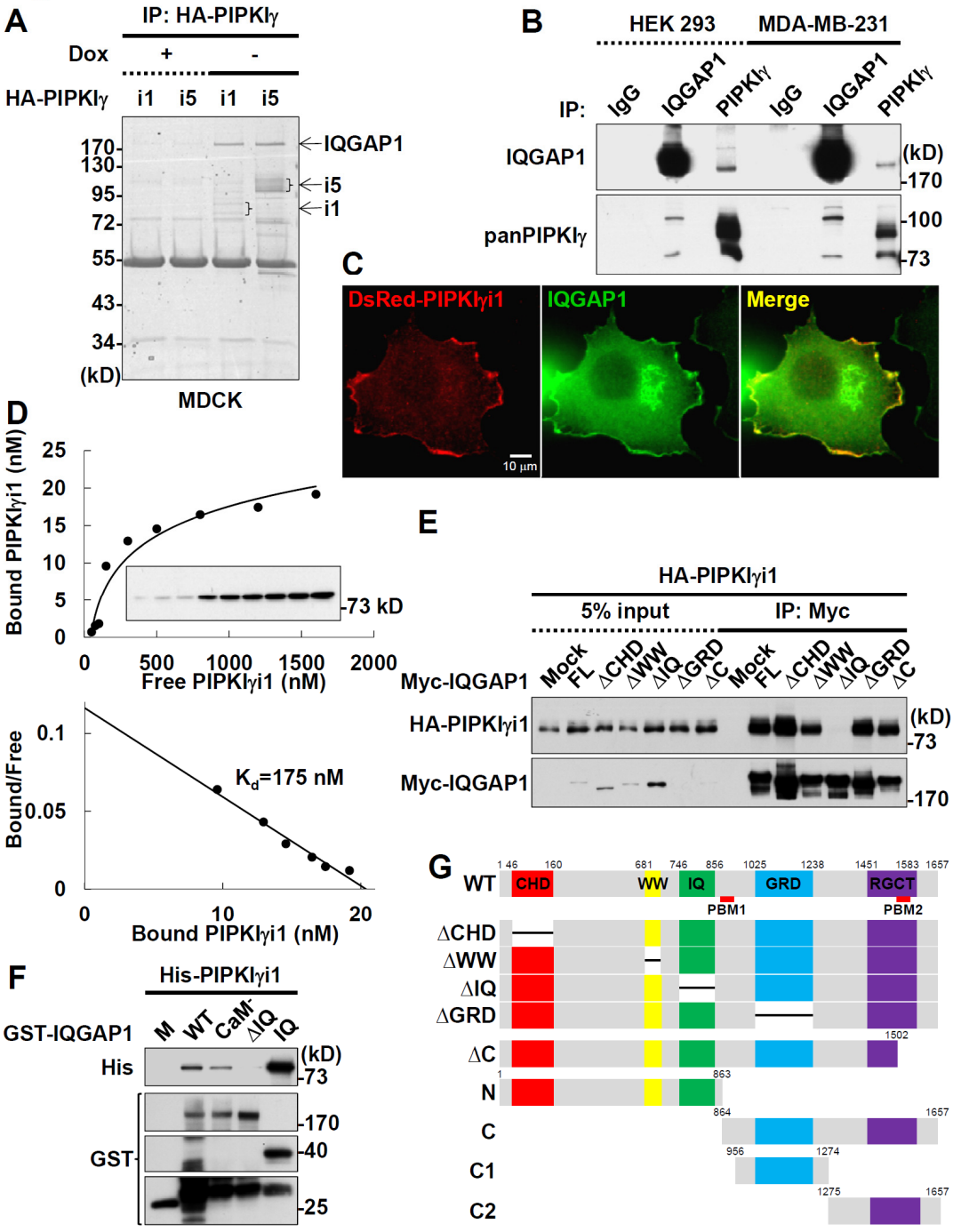
IQGAP1 (Figure 2.01 F). These data indicate that the IQ domain is both necessary and sufficient to interact with PIPKIγ.

The IQ domain is composed of four tandem IQ motifs. The CaM^- mutant which contains point mutations in the IQ motifs and abrogates calmodulin binding [185], bound PIPKIγ to a lesser extent than WT (Figure 2.01 F). Further, deletion or mutation of individual motifs reduced binding to PIPKIγ, compared to WT, and the combined mutation of multiple IQ motifs further reduced binding (Figure 2.01 F and data not shown). These data indicate that the intact IQ domain is required for the interaction with PIPKIγ. Further studies used the ΔIQ mutant to examine the functional importance of the PIPKIγ interaction.

Figure 2.01. PIPKIγ interacts with the IQ motif of IQGAP1.

(A) HA-PIPKIγi1 and i5 were expressed in tet-off MDCK cells, and an anti-HA antibody used to IP i1 and i5 containing complexes. Samples were resolved by SDS-PAGE and protein bands visualized by Coomassie staining. Dox, doxycycline. (B) PIPKIγ and IQGAP1 were separately IP'ed and association of the other protein examined by immunoblotting. IgG, isotype immunoglobulin control. (C) DsRed-PIPKIγi1 was transiently expressed in MCF-7 cells and endogenous IQGAP1 was immunostained. Cells were photographed under 600X magnification. (D) 50 pM GST-IQGAP1 was incubated with 5 to 1600 nM His-PIPKIγi1. Binding was detected by immunoblotting with an anti-His antibody (top). K_d was determined by standard Scatchard analysis (bottom). (E) Myc-IQGAP1 proteins were co-expressed with HA-PIPKIγi1 in HEK293 cells and proteins were IP'ed with an anti-Myc antibody. Associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody. (F) Purified GST-IQGAP1 proteins were incubated with His-PIPKIγi1. The associated protein complex was examined by immunoblotting with the indicated antibodies. Some degraded products of GST-IQGAP1 proteins were detected by immunoblotting with an anti-GST antibody. Data above are representative of at least four independent experiments. (G) Schematic representation of IQGAP1 domains and IQGAP1 constructs used for this study.

Figure 2.01


A
IP: HA-PIPKIγ
Dox
+
-
HA-PIPKIγ
i1
i5
i1
i5
170
130
95
72
55
43
34
(kD)
IQGAP1
i5
i1
MDCK
B
HEK 293
MDA-MB-231
IP:
IgG
IQGAP1
PIPKIγ
IgG
IQGAP1
PIPKIγ
IQGAP1
panPIPKIγ
(kD)
170
100
73
C
DsRed-PIPKIγi1
IQGAP1
Merge
10 μm
D
Bound PIPKIγi1 (nM)
25
20
15
10
5
0
0
500
1000
1500
2000
Free PIPKIγi1 (nM)
73 kD
Bound/Free
0.1
0.05
0
0
10
20
Bound PIPKIγi1 (nM)
Kd=175 nM
E
HA-PIPKIγi1
5% input
IP: Myc
Mock
FL
ΔCHD
ΔWW
ΔIQ
ΔGRD
ΔC
Mock
FL
ΔCHD
ΔWW
ΔIQ
ΔGRD
ΔC
Myc-IQGAP1
HA-PIPKIγi1
Myc-IQGAP1
(kD)
73
170
F
His-PIPKIγi1
GST-IQGAP1
M
WT
CaM-
ΔIQ
IQ
His
GST
(kD)
73
170
40
25
G
WT
CHD
WW
IQ
GRD
RGCT
PBM1
PBM2
ΔCHD
ΔWW
ΔIQ
ΔGRD
ΔC
N
C
C1
C2
1
46
160
681
746
856
1025
1238
1451
1583
1657
1502
863
864
956
1274
1275

Migration and lamellipodium formation require PIPKIγ: The role of PIPKIγi2 in migration is emerging [33, 186]. To further define a role of other PIPKIγ isoforms in regulation of migration, we stably knocked down PIPKIγ in MDA-MB-231 cells using two different shRNAs [33]. ShRNA#1 and #2 reduced total PIPKIγ (panPIPKIγ) expression ~75% and 90%, respectively. PIPKIγi2 expression was also slightly reduced (~24% and 36%, respectively), whereas i4 and i5 expression were not changed (Figure 2.02 B) as reported [187]. These data indicate that PIPKIγi1 is the predominant isoform in these cells [181]. By bright field microscopy, PIPKIγ knockdown cells were less spread than control cells with fewer protrusions (Figure 2.02 A). Serum-induced migration using a Transwell assay was significantly attenuated by PIPKIγ knockdown (Figure 2.02 B). These data indicate that PIPKIγ is required for proper spreading and migration.

Knockdown of PIPKIγi2 has a defined migration defect [33, 186], but PIPKIγi1 could not be knocked specifically as it is a splice variant with no unique coding sequence compared to the other isoforms. To explore the role of PIPKIγi1 and i2, we re-expressed these two isoforms to determine if they restore migration. ShRNA-resistant DsRed-PIPKIγ was stably re-expressed in PIPKIγ knockdown cells. Cells were then sorted to isolate cells with expression levels similar to endogenous PIPKIγ in control cells. Re-expression of PIPKIγi2 rescued migration (Figure 2.02 C), as reported [33]. Interestingly, PIPKIγi1 WT also rescued the migration whereas i1 kinase dead (KD) did not rescue, indicating that PIPKIγi1 or i2 are sufficient for serum-induced migration, and $PI4,5P_2$ synthesis is required for this process.

Migrating cells extend lamellipodia at the leading edge and persistent formation of lamellipodia is critical for directional migration [49]. To test how PIPKIγ regulates lamellipodium formation, a lamellipodial marker ARPC2 [161] was immunostained following initiation of migration by scratch-wounding confluent cells. 3 hours after scratching, ARPC2 localized at the periphery of protrusions in the control cells (Figure 2.02 D). In PIPKIγ

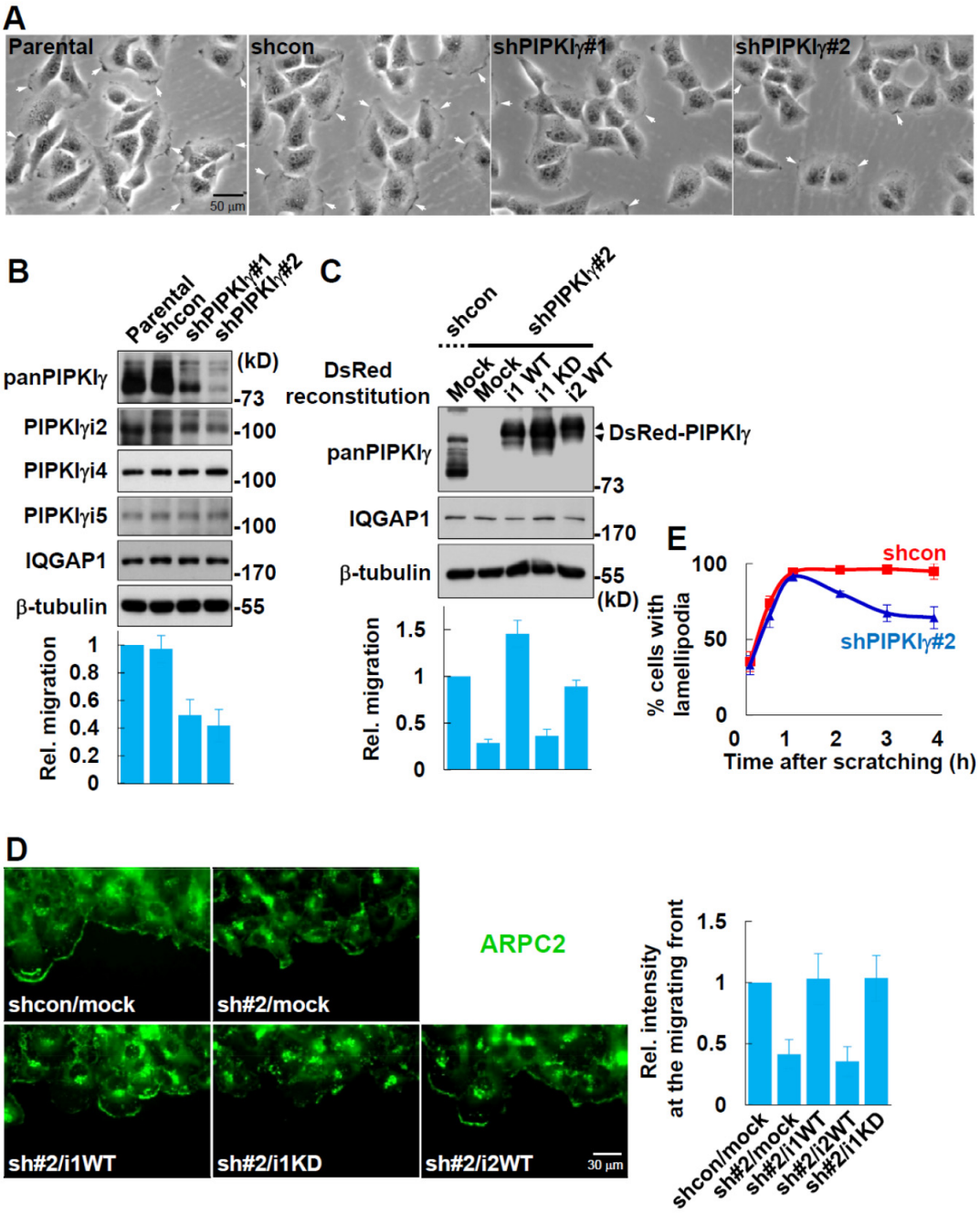
knockdown cells, formation of protrusions was retarded and ARPC2 no longer localized at the membrane extensions. PIPKIγi1 or i2 re-expression could recover lamellipodium formation, whereas PIPKIγi1 KD had no effect. Early protrusion formation was indistinguishable in different cells but persistent formation was diminished (Figure 2.02 E). This demonstrates that PIPKIγ by generation of $PI4,5P_2$ regulates persistent lamellipodium formation that is required for migration.

Figure 2.02. PIPKIγ is required for migration and lamellipodium formation.

(A) MDA-MB-231 breast cancer cells were infected with lentivirus expressing short hairpin (sh) RNAs against either human PIPKIγ or scrambled control. Infected cells were selected by a cell sorter (viral vector contains GFP coding sequence). Either parental or virus infected cells maintained in normal culture conditions were photographed under an inverted microscope at 200X magnification. White arrows indicate ruffle-like structures. (B) Either parental or virus infected cells were placed in the upper chamber of a Transwell and cells were allowed to migrate for 12-16 h towards 10% serum as a chemoattractant in the lower chamber. Cells were fixed and stained with a 0.5% crystal violet (CV) solution. CV-positive cells that had migrated across 3.0 μm pores were counted from photographs taken from at least five random fields (bottom). Expression levels of the endogenous proteins were analyzed by immunoblotting of cell lysates with isoform specific PIPKIγ antibodies (top). (C) ShRAN-resistant DsRed-tagged PIPKIγ isoforms were stably expressed in shPIPKIγ#2 cells. Cells expressing a similar amount of PIPKIγ compared to the control cells were isolated using a cell sorter. With these reconstituted cells, serum-induced chemotaxis was measured with a Transwell as described above (top). Protein expression was confirmed by immunoblotting against the indicated molecules (bottom). WT, wild type. KD, kinase dead. Data are shown as mean ± SD of four independent experiments. (D) The reconstituted cells allowed to migrate into a scratch wound were fixed after 3 hours and immunostained with the Arp2/3 complex component ARPC2. Images were taken at 400X magnification and the representative images are shown. (E) Either control or shPIPKIγ#2 cells were grown to confluence. Lawn of cells was scratched and boundaries between cells and cell-free space were photographed at 5, 30, 60, 120, 180 and 240 min after scratching. At least 200 cells were counted for disk-like protrusions. Data are shown as mean ± SD of four experiments.

The experiments described above were performed independently at least four times.

Figure 2.02


A
Parental
shcon
shPIPKIγ#1
shPIPKIγ#2
50 μm
B
Parental
shcon
shPIPKIγ#1
shPIPKIγ#2
panPIPKIγ
(kD)
-73
PIPKIγi2
-100
PIPKIγi4
-100
PIPKIγi5
-100
IQGAP1
-170
β-tubulin
-55
Rel. migration
1
0.8
0.6
0.4
0.2
0
C
shcon
shPIPKIγ#2
DsRed reconstitution
Mock
Mock
i1 WT
i1 KD
i2 WT
panPIPKIγ
DsRed-PIPKIγ
-73
IQGAP1
-170
β-tubulin
-55
(kD)
Rel. migration
1.5
1
0.5
0
E
% cells with lamellipodia
100
50
0
shcon
shPIPKIγ#2
0
1
2
3
4
Time after scratching (h)
D
shcon/mock
sh#2/mock
sh#2/i1WT
sh#2/i1KD
sh#2/i2WT
30 μm
ARPC2
Rel. intensity at the migrating front
1.5
1
0.5
0
shcon/mock
sh#2/mock
sh#2/i1WT
sh#2/i2WT
sh#2/i1KD

PIPKIγ and IQGAP1 interdependently control cell motility: Upon stimulation, IQGAP1 targets to the leading edge and recruits regulators of the cytoskeleton that control migration [54, 147]. As described above PIPKIγ also regulates migration [33]. *Pip5k1c*, a gene coding PIPKIγ in mice, knockout (KO) mice are embryonic lethal with migration defects of cardiovascular cell precursors [188], and cells from these mice have a defective association between the membrane and the cytoskeleton [189]. To investigate how PIPKIγ and IQGAP1 control cell motility, serum-induced cell motility was measured using a Transwell system. Individual knockdown of PIPKIγ or IQGAP1 significantly reduced both migration and invasion (Figure 2.03 A). Knockdown of both proteins dramatically reduced cell motility indicating a synergistic role. To better define the relationship of the two proteins, we overexpressed IQGAP1 that is reported to enhanced cell motility [183]. Overexpression of IQGAP1 in MDA-MB-231 cells increased cell motility over 3-fold whereas knockdown of PIPKIγ in IQGAP1 overexpressing cells reduced cell motility to the basal level. Consistently overexpression of PIPKIγi1 increased cell motility and this increase was inhibited by knockdown of IQGAP1 (Figure 2.03 B). Similar results were obtained in HeLa cells. Here, inducible expression of PIPKIγi1 increased cell motility and depletion of IQGAP1 under these conditions reduced motility to the basal level (Figure 2.03 C). Together these data indicate that PIPKIγ and IQGAP1 interdependently control cell motility.

The PIPKIγ-IQGAP1 interaction is required for migration: To investigate how PIPKIγ and IQGAP1 function together, we tested if their association is altered by stimuli that promote migration. Migration is initiated by a variety of extracellular stimuli, including chemokines or extracellular matrix (ECM) [55]. To define the pathway in which PIPKIγ and IQGAP1 function, cells were stimulated with type I collagen (COL) or serum and changes in the association were examined by IP. In response to either stimulus there was an increase in the panPIPKIγ-IQGAP1 complex, whereas the Rac1 interaction with IQGAP1 remained unchanged (Figure

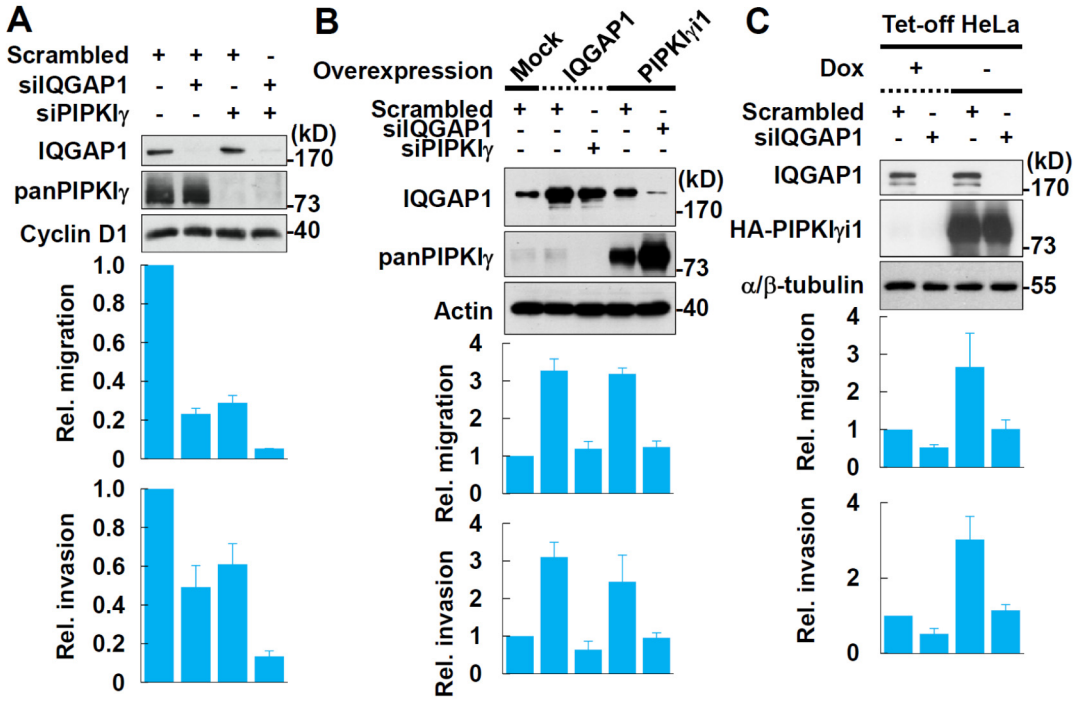
2.03 D). This demonstrates that the PIPKIγ interaction with IQGAP1 is enhanced by factors that stimulate migration. Furthermore, phosphorylation of Ser1441 and Ser1443 residues [162, 178] is required for the enhancement of interaction (Figure 2.04 C). Interestingly, the PIPKIγi2 interaction was unaffected, suggesting that migration enhances IQGAP1 interaction with the predominant isoform, PIPKIγi1 [181]. This is consistent with results indicating that PIPKIγi2 modulates cell migration by a different mechanism [33, 186].

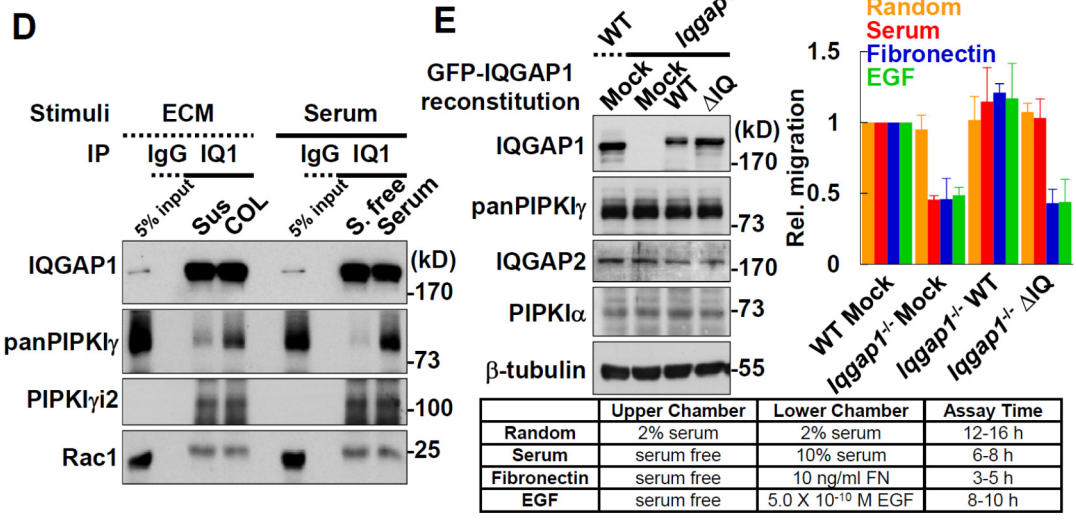
The IQGAP1 mutant that lacks interaction with PIPKIγ was examined to determine if this interaction is required for migration. For this, *Iqgap1* KO mouse embryonic fibroblasts (MEFs) [164] were reconstituted with WT or ΔIQ IQGAP1 and migration was examined in various conditions [190]. To avoid nonspecific effects from overexpression, we maintained IQGAP1 expression levels similar to the WT MEFs by the cell sorting method as above. *Iqgap1* KO MEFs showed >50% reduction in migration in response to serum, fibronectin or epidermal growth factor (EGF) stimuli. WT IQGAP1 fully rescued migration under all of these conditions, while the ΔIQ mutant showed no recovery of migration induced by fibronectin or EGF (Figure 2.03 E). This indicates that the PIPKIγ-IQGAP1 interaction is necessary for integrin- and EGF receptor-mediated migration. Intriguingly, the ΔIQ mutant still rescued serum-induced migration. Serum contains a collection of factors that induce migration and the contribution of each factor in PIPKIγ-regulated migration varies by cell types [186]. Collectively, the PIPKIγ-IQGAP1 interaction specifically regulates fibronectin- or EGF-induced migration in MEFs (Figure 2.04 D) indicating that the PIPKIγ-IQGAP1 nexus is regulated by these pathways.

Figure 2.03. PIPKIγ and IQGAP1 cooperate to regulate migration.

(A) MDA-MB-231 cells were transfected with the indicated siRNA for 48 h. Knockdown was confirmed by immunoblotting with the indicated antibodies (top). 10% serum-induced migration (middle) and invasion through 2 mg/ml Matrigel (bottom) were measured with a Transwell. (B) Cells were transfected with the indicated DNA and siRNA combinations for 24 h. Expression level was analyzed by immunoblotting with the indicated antibodies (top). Migration and invasion were measured as in (A) (bottom). (C) PIPKIγi1 was expressed in HeLa tet-off cells by removing doxycycline from media for 24 h. Protein expression and cell motility were measured as above. Data are shown as mean ± SD for four independent experiments. (D) Cells maintained in suspension were either plated on 10 ng/ml collagen I or kept in suspension for 30 m. Serum-starved cells were treated with or without 10% serum for 15 m. Endogenous IQGAP1 was IP'ed and associated PIPKIγ was analyzed by immunoblotting. Sus, suspension. COL, type I collagen. IQ1, IQGAP1. (E) *Iqgap1* KO MEFs were stably reconstituted with the indicated IQGAP1 proteins, and four different modes of migration were measured with a Transwell (top right). Protein expression was analyzed by immunoblotting (top left). Conditions used for treating Transwells (bottom). Data are shown as mean ± SD of four independent experiments. Data above are representative of at least four independent experiments.

Figure 2.03





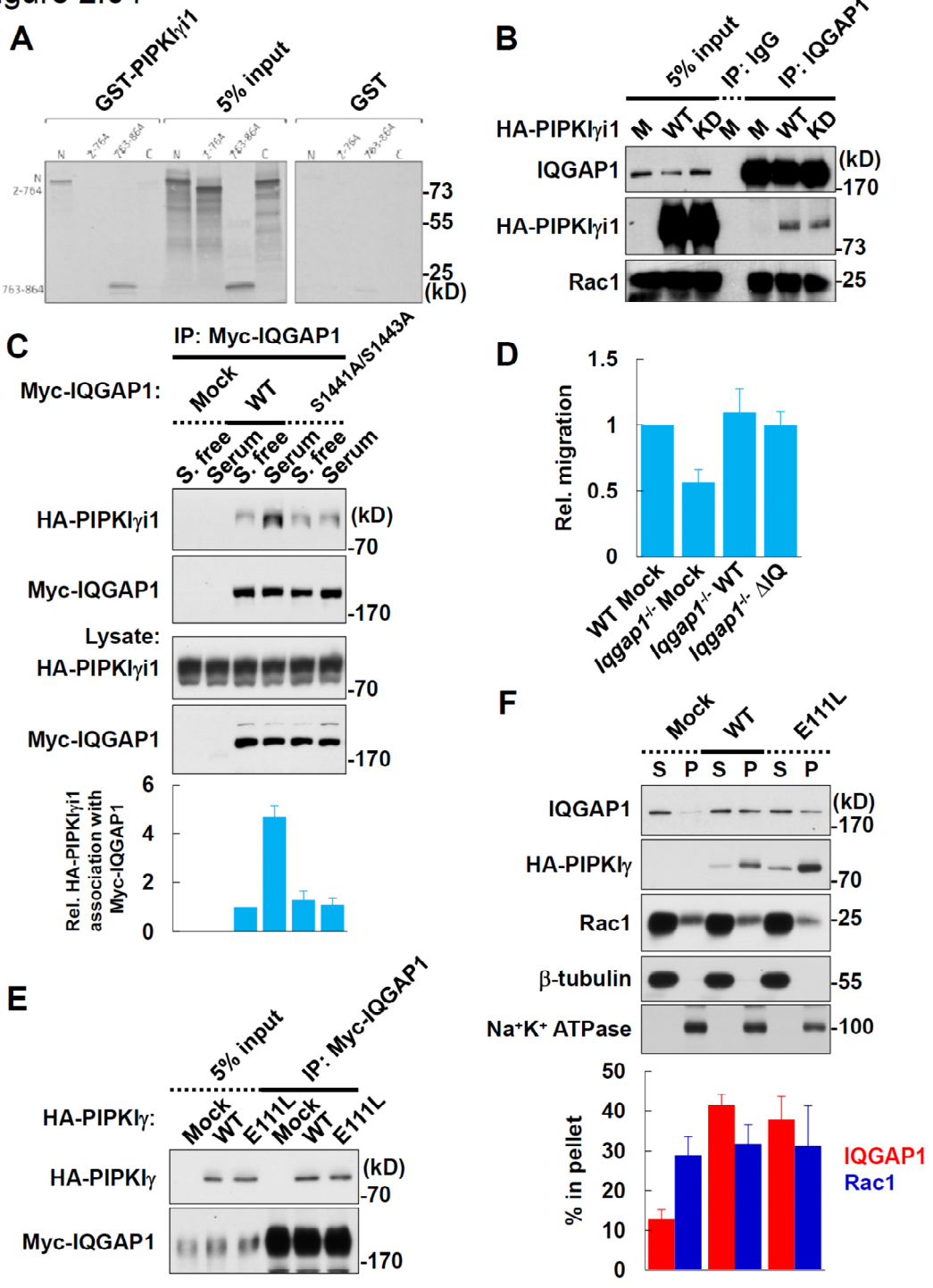
	Upper Chamber	Lower Chamber	Assay Time
Random	2% serum	2% serum	12-16 h
Serum	serum free	10% serum	6-8 h
Fibronectin	serum free	10 ng/ml FN	3-5 h
EGF	serum free	5.0 X 10^{-10} M EGF	8-10 h

Figure 2.04. PIPKIγ interacts with IQGAP1 regardless of kinase activity.

(A) Identification of the PIPKIγ binding site on IQGAP1. Equal amounts of [^{35}S]methionine-labeled IQGAP1-N, IQGAP1-(2-764), IQGAP1-(763-864) or IQGAP1-C were incubated with 4 μg GST-PIPKIγi1 or GST alone on glutathione beads. Complexes were washed, resolved by SDS-PAGE and processed by autoradiography. An aliquot of [^{35}S]methionine-labeled TNT product that was not subjected to chromatography was processed in parallel (Input). (B) Control vector or HA-tagged PIPKIγi1 wild type (WT) or kinase dead (KD) mutant was expressed with in MDA-MB-231 cells and endogenous IQGAP1 proteins were immunoprecipitated with an anti-IQGAP1 antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody. (C) HA-PIPKIγi1 was co-transfected with Myc-IQGAP1 WT or S1441S/S1443A mutant in MDA-MB-231 cells for 36 h. Then, cells were serum starved for 12 h before treating with 10% FBS for 30 m. Myc-IQGAP1 WT or mutant was immunoprecipitated with an anti-Myc antibody and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody (top). Data are shown as mean ± SD of three independent experiments (bottom). (D) The reconstituted MEFs were used for 5 μM lysophosphatidic acid induced cell migration using a Transwell. Data are shown as mean ± SD of three independent experiments. (E) Myc-IQGAP1 was co-transfected with HA-PIPKIγi1 WT or E111L mutant in MDA-MB-231 cells for 48 h. Cells were harvested and exogenous IQGAP1 was immunoprecipitated with an anti-Myc antibody and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody. (F) MDA-MB-231 cells were transfected with the indicated IQGAP1 proteins or mock control for 48 h. Cells were harvested with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. 10 μg of each protein was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S+P)

was calculated by quantifying the immunoblots (bottom). The experiments described above were performed independently at least four times.

Figure 2.04


A
GST-PIPKIγi1
5% input
GST
N
2-764
763-864
C
-73
-55
-25
(kD)
B
5% input
IP: IgG
IP: IQGAP1
HA-PIPKIγi1
M
WT
KD
IQGAP1
(kD)
-170
HA-PIPKIγi1
-73
Rac1
-25
C
IP: Myc-IQGAP1
Myc-IQGAP1:
Mock
WT
S1441A/S1443A
S. free
Serum
HA-PIPKIγi1
(kD)
-70
Myc-IQGAP1
-170
Lysate:
HA-PIPKIγi1
-70
Myc-IQGAP1
-170
Rel. HA-PIPKIγi1 association with Myc-IQGAP1
6
4
2
0
D
Rel. migration
1.5
1
0.5
0
WT Mock
Iqgap1-/- Mock
Iqgap1-/- WT
Iqgap1-/- ΔIQ
E
5% input
IP: Myc-IQGAP1
HA-PIPKIγ:
Mock
WT
E111L
HA-PIPKIγ
(kD)
-70
Myc-IQGAP1
-170
F
Mock
WT
E111L
S
P
IQGAP1
(kD)
-170
HA-PIPKIγ
-70
Rac1
-25
β-tubulin
-55
Na+K+ ATPase
-100
% in pellet
50
40
30
20
10
0
IQGAP1
Rac1

PIPKIγ controls IQGAP1 translocation to the leading edge membrane: At the onset of migration, many cytoskeleton regulatory proteins translocate to the leading edge membrane to mediate directional migration [7, 49, 191]. To further define how PIPKIγ and IQGAP1 regulate migration, we examined their targeting to the membrane by cell fractionation. Cells were plated on COL, then lysed and fractionated into membrane and cytosolic components [44]. In response to integrin activation, both PIPKIγ and IQGAP1 increased in the membrane fraction (Figure 2.05 A). Rac1 also increased in the membrane fraction as reported [191]. However, membrane proteins, such as calnexin, GM-130 and Na^+K^+ channel, remained unchanged (Figure 2.05 A).

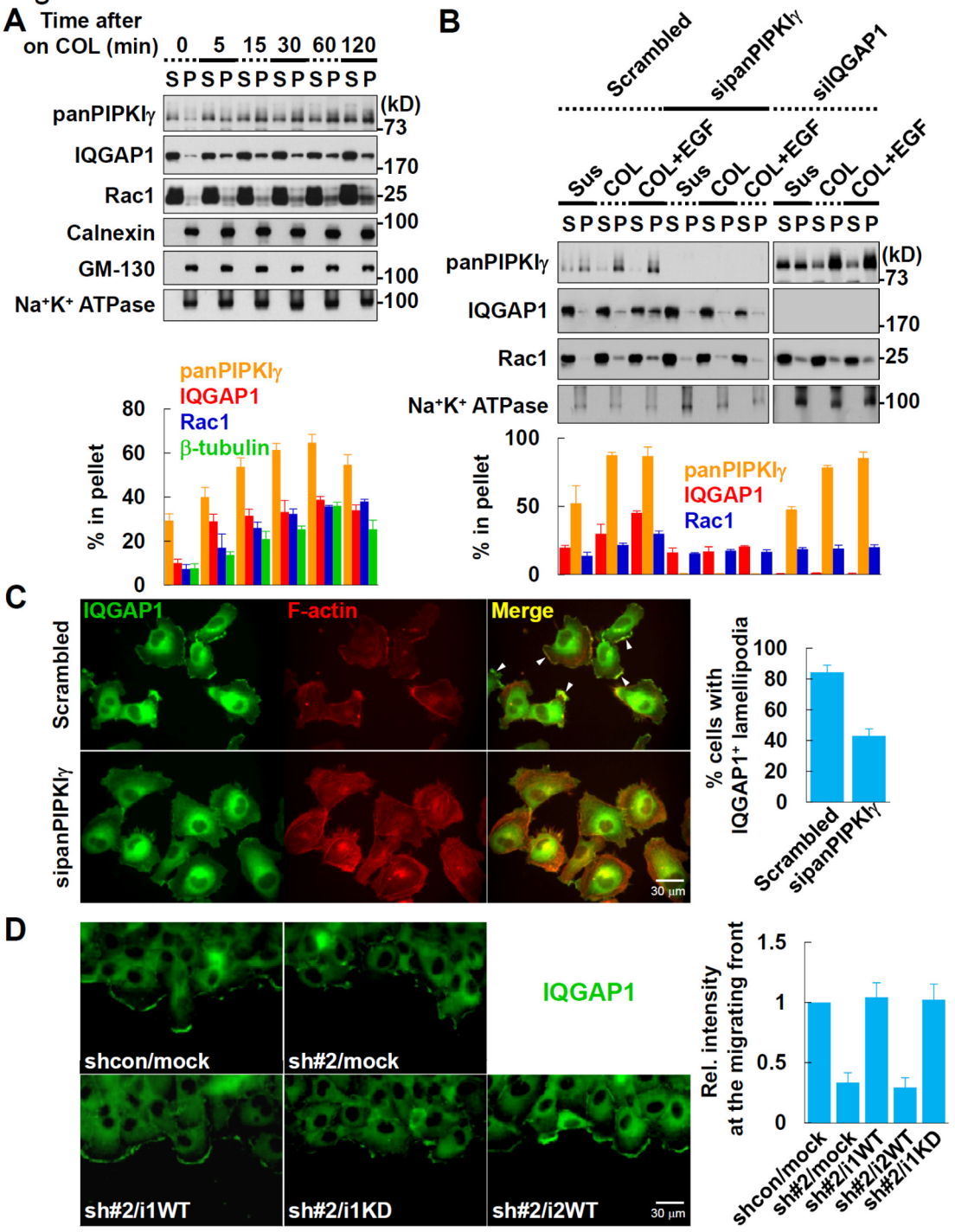
In response to receptor activation, IQGAP1 translocates to the leading edge membrane [150, 163]. Yet, the mechanism for IQGAP1 interaction with the membrane is largely unknown. To examine if PIPKIγ regulates IQGAP1 membrane targeting, PIPKIγ was knocked down using RNAi and cells were fractionated. Knockdown of PIPKIγ significantly reduced IQGAP1 in the membrane fraction upon COL and/or EGF stimulation (Figure 2.05 B). The knockdown of PIPKIγ also reduced the membrane content of Rac1, supporting reports that PIPKI and Rac1 interdependently control PM targeting [44, 75]. To test the sole contribution of PIPKIγ for the IQGAP1 targeting, we utilized a Rac1 binding defective mutant PIPKIγ (E111L) [75]. The mutant co-immunoprecipitated with IQGAP1 similar to wild type PIPKIγ (Figure 2.04 E) indicating that the Rac1 binding of PIPKIγ is not required for the PIPKIγ interaction with IQGAP1. Notably, the E111L mutant enhanced IQGAP1 association with the membrane fraction similar to wild type PIPKIγ (Figure 2.04 F). These data suggest that the IQGAP1 recruitment to the leading edge is largely regulated by PIPKIγ independent of Rac1. Knockdown of IQGAP1 reduced Rac1 in the membrane fraction, but had no effect on PIPKIγ accumulation in the membrane fraction.

To assess targeting in vivo, serum-starved cells were treated with EGF to induce lamellipodia formation [192] and IQGAP1 localization was observed by immunostaining. As shown in Figure 2.05 C, the number of PIPKIγ knockdown cells with IQGAP1-positive protrusions was reduced by >50% compared to the control cells. To assess PIPKIγ regulation of IQGAP1 localization in migrating cells, endogenous IQGAP1 was immunostained in cells migrating into the scratch wound. IQGAP1 nicely localized at the leading edge in the control cells, but in PIPKIγ knockdown cells the IQGAP1 staining at the cell periphery was significantly reduced (Figure 2.05 D). Reconstitution with either PIPKIγi1 or i2 WT, but not i1 KD, rescued IQGAP1 localization at the leading edge. The difference between WT and KD is not due to an improper interaction with IQGAP1 because the amount of PIPKIγi1 KD that co-IP'ed with IQGAP1 was indistinguishable from that of WT (Figure 2.04). Taken together, these results demonstrate that PIPKIγ and generation of $PI4,5P_2$ are required for IQGAP1 targeting to the leading edge membrane in response to migratory signals.

Figure 2.05. PIPKIγ regulates IQGAP1 targeting to the leading edge membrane.

(A) MDA-MB-231 cells maintained in suspension were plated on 10 ng/ml COL for the indicated times. Cells were lysed with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. 10 μg of each protein was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S+P) was calculated by quantifying the immunoblots (bottom). S, supernatant. P, pellet. (B) After transient knockdown with the indicated siRNA, cells were treated as (A) in the presence or absence of 50 ng/ml EGF for 30 m. Cells were fractionated and analyzed as above. (C) Serum starved control or PIPKIγ knockdown cells were treated with 20 ng/ml EGF for 1 h. Cells were fixed and stained for IQGAP1 and F-actin. Cells were photographed at 400X magnification. For quantification, at least 300 cells were counted. White arrowheads indicate IQGAP1-positive lamellipodia. Data are shown as mean ± SD of three independent experiments. (D) Cells grown to confluence were wounded and fixed 3 h later, followed by immunostaining for IQGAP1. Cells were photographed at 400X magnification. Intensity of fluorescent signal at the migrating front was measured from at least 10 different images of each condition and quantified using ImageJ software. All the experiments described above were performed independently at least three times.

Figure 2.05


A
Time after on COL (min)
0 5 15 30 60 120
S P S P S P S P S P S P
panPIPKIγ
IQGAP1
Rac1
Calnexin
GM-130
Na+K+ ATPase
(kD)
73
170
25
100
100
100
panPIPKIγ
IQGAP1
Rac1
β-tubulin
% in pellet
80
60
40
20
0
B
Scrambled
sipanPIPKIγ
siIQGAP1
Sus
COL
COL+EGF
S P S P S P S P S P S P S P S P S
panPIPKIγ
IQGAP1
Rac1
Na+K+ ATPase
(kD)
73
170
25
100
% in pellet
100
50
0
panPIPKIγ
IQGAP1
Rac1
C
Scrambled
sipanPIPKIγ
IQGAP1
F-actin
Merge
30 μm
% cells with IQGAP1+ lamellipodia
100
80
60
40
20
0
Scrambled
sipanPIPKIγ
D
shcon/mock
sh#2/mock
sh#2/i1WT
sh#2/i1KD
sh#2/i2WT
IQGAP1
30 μm
Rel. intensity at the migrating front
1.5
1
0.5
0
shcon/mock
sh#2/mock
sh#2/i1WT
sh#2/i2WT
sh#2/i1KD

IQGAP1 interacts with PI4,5P$_2$ through a polybasic motif: Signaling specificity of PI4,5P$_2$ can be defined by interaction of PIPKIs with PI4,5P$_2$ effectors [21, 23]. There is emerging evidence that PIPKIγ controls the cytoskeleton by interacting with cytoskeleton regulatory proteins, which are PI4,5P$_2$ effectors, such as talin [32] and trafficking components [33, 40]. Because PIPKIγ associated with IQGAP1 physically (Figure 2.01) and functionally (Figure 2.03 and 2.04), we hypothesized that IQGAP1 could be a PI4,5P$_2$ effector. Consistent with this hypothesis, two independent proteomic analyses suggest that IQGAP1 interacts with PI4,5P$_2$ [12, 13]. To understand how IQGAP1 interacts with PI4,5P$_2$, their cellular distributions were examined by immunostaining. PH domain of phospholipase Cδ1 (PLCδ1) has been extensively used to probe cellular PI4,5P$_2$ [97, 193, 194] but excessive expression limits PI4,5P$_2$ binding protein targeting to the plasma membrane [193]. Thus, we titrated the GFP-PLCδ1-PH expression and analyzed endogenous IQGAP1 localization (Figure 2.06). In the optimal amount of expression, endogenous IQGAP1 partially co-localized with GFP-PLCδ1-PH (Figure 2.07 B), indicating that both IQGAP1 and PI4,5P$_2$ are present at regions of the PM containing PI4,5P$_2$. To define PI4,5P$_2$ binding, liposomes were synthesized containing membrane lipids (57.5% of phosphatidylcholine, 20% of phosphatidylethanolamine and 20% of phosphatylserine in molar ratio) and 2.5% PI4,5P$_2$. A co-sedimentation assay was used to define the PI4,5P$_2$ binding site on IQGAP1. IQGAP1-N or -C (Figure 2.01 G) were examined and only IQGAP1-C co-sedimented with PI4,5P$_2$-liposomes indicating that PI4,5P$_2$ binds to the C-terminal half (Figure 2.07 B).

A lysine cluster mediates the IQGAP1 interaction with PI4,5P$_2$: IQGAP1 does not contain known PI4,5P$_2$ binding modules, but we found at least two potential PBMs within AA 921-970 and 1491-1560, named PBM1 and PBM2, respectively (Figure 2.01 G). Deletion of PBM2 dramatically reduced IQGAP1 interaction with PI4,5P$_2$-liposomes whereas deletion of PBM1 had little effect indicating that IQGAP1 interacts with PI4,5P$_2$ through PBM2 (Figure 2.07 C). To define a putative PI4,5P$_2$ binding site on PBM2, human IQGAP1, 2 and 3

sequences were aligned with IQGAP sequences from multiple species. As shown in Figure 2.07 D, the sequence alignment identified a lysine residue, marked by an asterisk, which is conserved in PBM2. Around this lysine, there are other conserved basic residues, highlighted in red. We mutated these residues to alanines as illustrated in Figure 2.07 E and tested the impact on $PI4,5P_2$-liposome binding. Mutating two or four lysine residues had little effect whereas mutating all six residues (termed AA3) eliminated IQGAP1 binding to the $PI4,5P_2$-liposomes.

To examine phospholipid binding specificity, a lipid overlay assay was performed. IQGAP1 WT and -C bound to multiple phosphoinositides but not other phospholipids (Figure 2.08 A). To better define IQGAP1 phosphoinositide binding, the IQGAP1-C fragment was used in liposome sedimentation assays, with liposomes containing 5% phosphoinositide [195]. In this assay, $PI3,4P_2$, $PI3,5P_2$ and $PI3,4,5P_3$ bound with a higher affinity than PI3P, PI4P, PI5P and $PI4,5P_2$ (Figure 2.07 F). Although the apparent affinity for other bis- and tris-phosphate species is up to 7-fold higher than $PI4,5P_2$, $PI4,5P_2$ is estimated to be present in the PM at a concentration 20 to 100-fold higher than other phosphoinositide species [195], indicating that $PI4,5P_2$ is the major in vivo ligand for IQGAP1. The AA3 mutation reduced binding to $PI3,4P_2$, $PI3,5P_2$ and $PI3,4,5P_3$ but not mono-phosphate species. Strikingly, the AA3 mutant lost binding to $PI4,5P_2$. The combined data indicate that IQGAP1-C has multiple distinct phosphoinositide binding sites [174] and the lysine cluster mutated in AA3 defines a specific $PI4,5P_2$ binding site.

Figure 2.06. PIPKIγ- and PI4,5P_2-binding of IQGAP1 are required for directionally persistent migration.

(A) The reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy at 400X. To locate cells expressing GFP-positive IQGAP1 proteins, cells were first photographed under a fluorescent channel. Immediately after, cells were imaged every 5 min for 3 h to generate the movies shown in videos 1-3. (B) MDA-MB-231 cells were transfected with mock control or increasing amount of GFP-PLCδ1-PH DNA for 4h. Cells plated on collagen I for 1h were fixed and immunostained with endogenous IQGAP1. Cells were photographed at 400X magnification. All the experiments described above were performed independently at least three times.

Figure 2.06

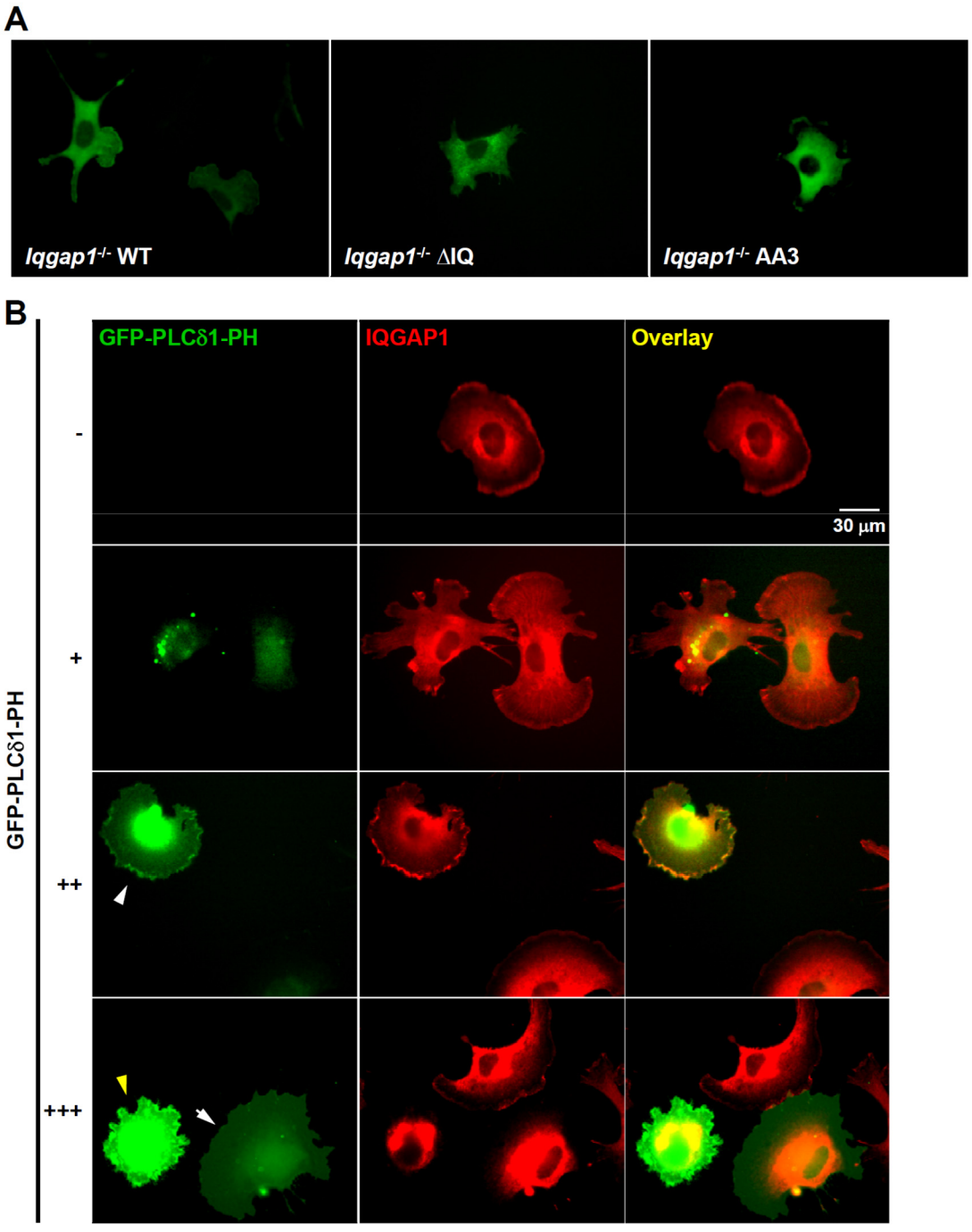

A
Iqgap1-/- WT
Iqgap1-/- ΔIQ
Iqgap1-/- AA3
B
GFP-PLCδ1-PH
IQGAP1
Overlay
30 μm
GFP-PLCδ1-PH
-
+
++
+++

Figure 2.07. IQGAP1 interacts with the phosphoinositides through a polybasic motif.

(A) GFP-PLCδ1-PH was transiently expressed in MDA-MB-231 cells and endogenous IQGAP1 was immunostained. Cells were photographed at 600X magnification. (B) 2.5% PI4,5P_2-liposomes were incubated with 0.5 μM GST-IQGAP1-N or -C for 10 m. Liposome-bound IQGAP1 was pelleted by centrifugation. Equal volume of the supernatant and the pellet were resolved by SDS-PAGE and IQGAP1 in each fraction was analyzed by immunoblotting with an anti-GST antibody. (C) GST-tagged WT or deletion mutants were used for a sedimentation assay with 2.5% PI4,5P_2-liposomes. (D) Amino acid sequence alignment of the PBM2 region among IQGAPs from the indicated species. (E) Selected lysine residues were mutated to alanines to generate a series of AA mutants (top). Binding of WT and the AA mutants to 5% PI4,5P_2-liposomes were tested (bottom). (F) Binding of GST-tagged WT and the AA3 mutant to 5 μM of 5% phosphoinositide-liposomes were tested. Samples were analyzed as above and liposome-bound proteins were detected by immunoblotting with anti-GST antibody. Immunoblots were quantified and the graph is shown as mean ± SD of three independent experiments. All the experiments described above were performed independently at least four times.

Figure 2.07

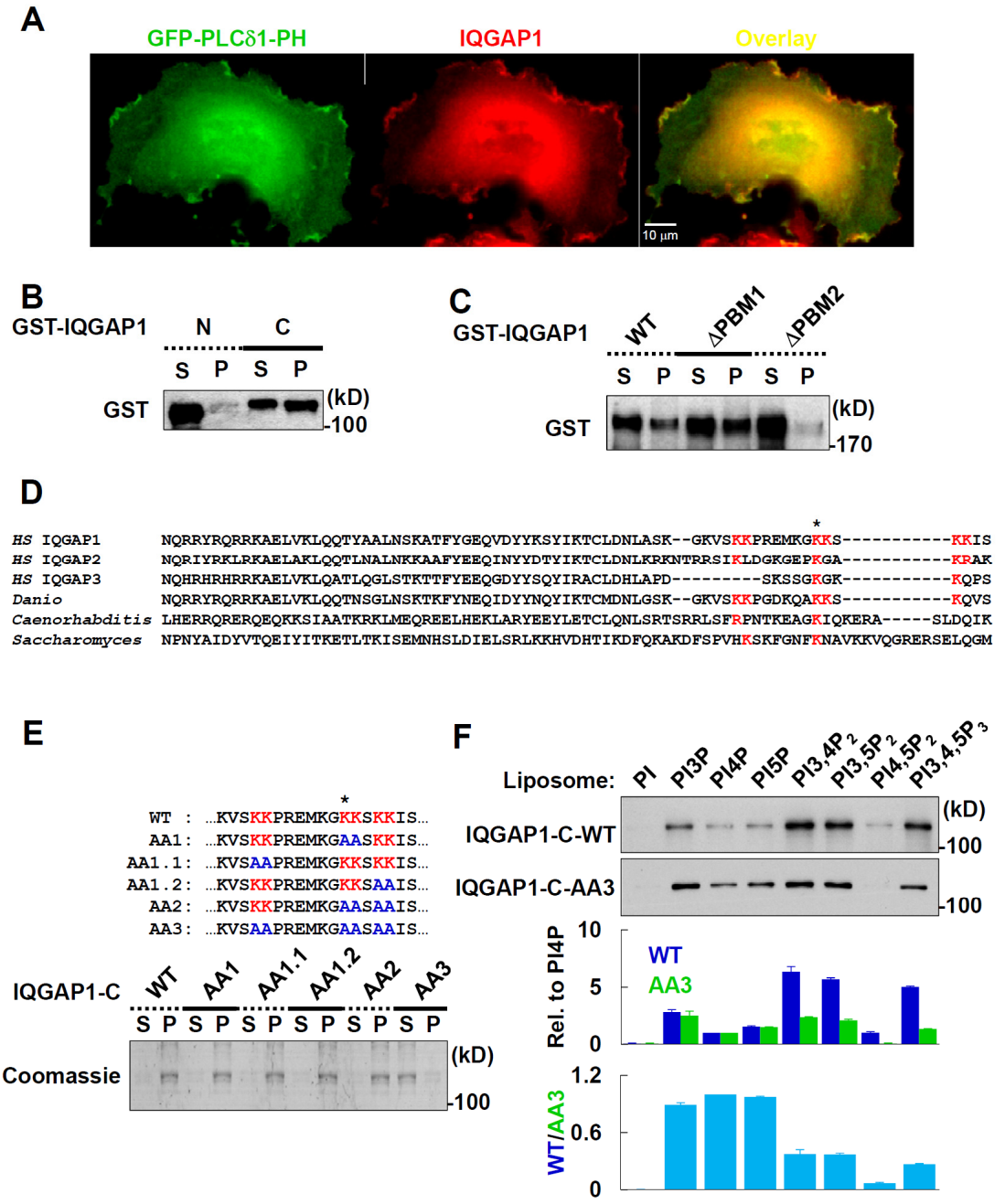
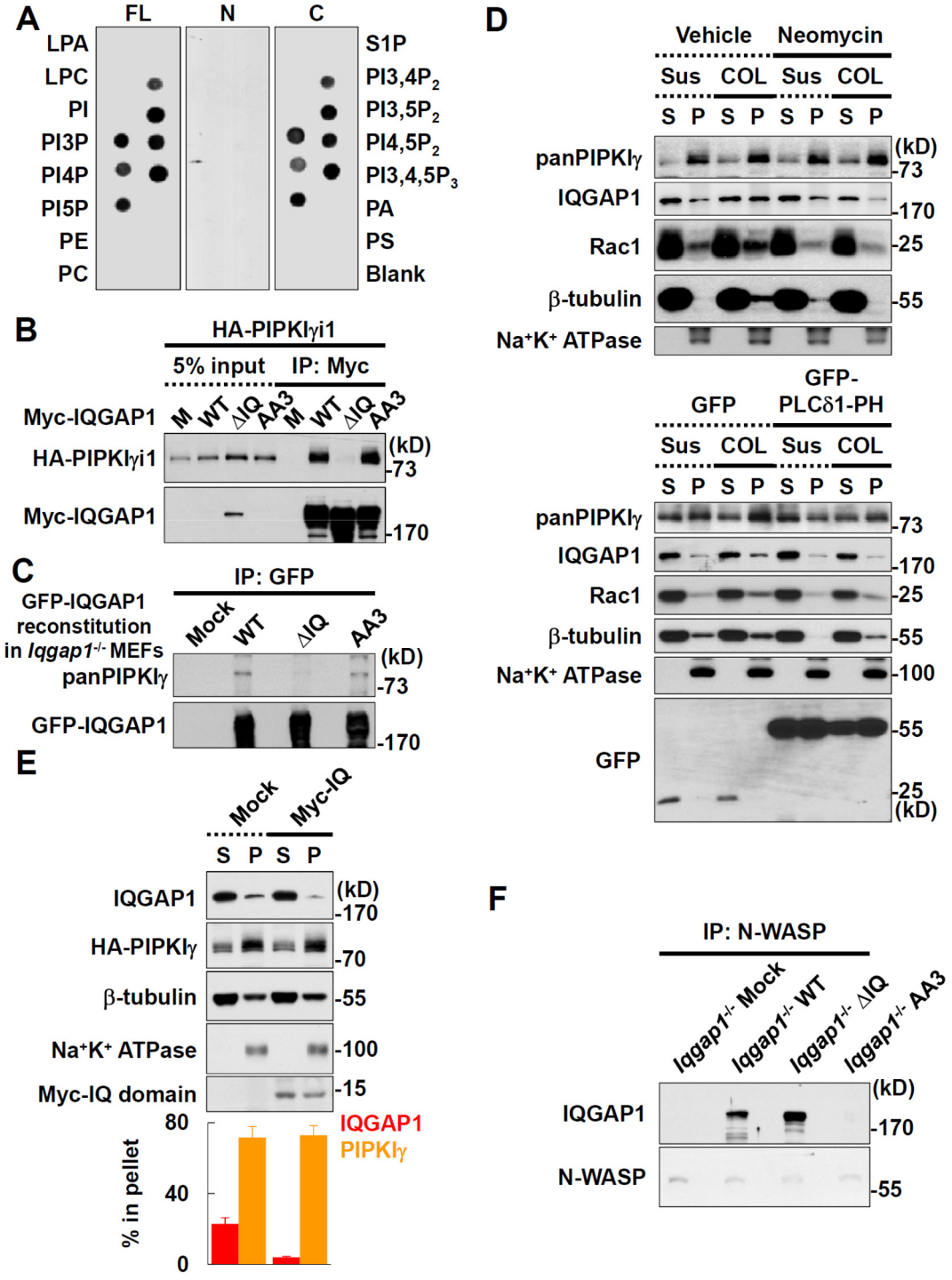

A
GFP-PLCδ1-PH
IQGAP1
Overlay
10 μm
B
GST-IQGAP1
N
C
S P S P
GST
(kD)
-100
C
GST-IQGAP1
WT
ΔPBM1
ΔPBM2
S P S P S P
GST
(kD)
-170
D
HS IQGAP1 NQRRYRQRRKAELVKLQQTYAALNSKATFYGEQVDYYKSYIKTCLDNLASK--GKVSKKPREMKGKKS----------KKIS
HS IQGAP2 NQRIYRKLRKAELAKLQQTLNALNKKAAFYEEQINYYDTYIKTCLDNLKRKNTRRSIKLDGKGEPKGA----------KRAK
HS IQGAP3 NQHRHRHRRKAELVKLQATLQGLSTKTTFYEEQGDYYSQYIRACLDHLAPD---------SKSSGKGK----------KQPS
Danio NQRRYRQRRKAELVKLQQTNSGLNSKTKFYNEQIDYYNQYIKTCMDNLGSK--GKVSKKPGDKQAKKS----------KQVS
Caenorhabditis LHERRQRERQEQKKSIAATKRKLMEQREELHEKLARYEEYLETCLQNLSRTSRRLSFRPNTKEAGKIQKERA-----SLDQIK
Saccharomyces NPNYAIDYVTQEIYITKETLTKISEMNHSLDIELSRLKKHVDHTIKDFQKAKDFSPVHKSKFGNFKNAVKKVQGRERSELQGM
E
WT : ...KVSKKPREMKGKKSKKIS...
AA1: ...KVSKKPREMKGAASKKIS...
AA1.1: ...KVSAAPREMKGKKSKKIS...
AA1.2: ...KVSKKPREMKGKKSAAIS...
AA2: ...KVSKKPREMKGAASAAIS...
AA3: ...KVSAAPREMKGAASAAIS...
IQGAP1-C
WT AA1 AA1.1 AA1.2 AA2 AA3
S P S P S P S P S P S P
Coomassie
(kD)
-100
F
Liposome:
PI PI3P PI4P PI5P PI3,4P2 PI3,5P2 PI4,5P2 PI3,4,5P3
IQGAP1-C-WT
IQGAP1-C-AA3
(kD)
-100
-100
Rel. to PI4P
10
5
0
WT
AA3
WT/AA3
1.2
0.6
0

Figure 2.08. IQGAP1 interacts with $PI4,5P_2$ and sequestration of $PI4,5P_2$ blocks IQGAP1 targeting to membrane.

(A) 0.01 μM of GST-tagged IQGAP1 full length (FL), N- or C-terminal half was incubated with Pip Strips (Eschelon Bioscience) for 1 h at room temperature and bound proteins were detected by immunoblotting with an anti-GST antibody. LPA, lysophosphatidic acid. LPC, lysophosphocholine. PE, phosphatidylethanolamine. PC, phosphatidylcholine. S1P, sphingosine 1-phosphate. PA, phosphatidic acid. PS, phosphatidylserine. (B) Myc-tagged IQGAP1 wild type or mutants was co-expressed with HA-tagged PIPKIγi1 in HEK293 cells and exogenous IQGAP1 proteins were immunoprecipitated with an anti-Myc antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody. (C) IQGAP1 proteins from the reconstituted MEFs were immunoprecipitated with an anti-GFP antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγ was analyzed by immunoblotting with an anti-PIPKIγ antibody. (D) Before plating, MDA-MB-231 cells were treated with either vehicle or 1 mM neomycin (Calbiochem) for 10 min, or transfected with either vector control or GFP-PLCδ1-PH for 24 h. Cells were plated on 10 ng/ml collagen I-coated culture dish for 1 h and similar fractionation assay was performed as Fig. 3 A. Equal amount of proteins (10 μg each) were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. (E) MDA-MB-231 cells were transfected with Myc-IQ domain alone or mock control for 48 h. Cells were harvested with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. 10 μg of each protein was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S+P) was calculated by quantifying the immunoblots (bottom). Data are shown as mean ± SD of three independent experiments. (F) Cell lysates from reconstituted MEFs were used for immunoprecipitating endogenous N-WASP. Immunoprecipitates were resolved by SDS-

PAGE and the associated IQGAP1 was analyzed by immunoblotting with an anti-IQGAP1 antibody. All the experiments described above were performed independently at least three times.

Figure 2.08


A
FL
N
C
LPA
LPC
PI
PI3P
PI4P
PI5P
PE
PC
S1P
PI3,4P2
PI3,5P2
PI4,5P2
PI3,4,5P3
PA
PS
Blank
B
HA-PIPKIγi1
5% input
IP: Myc
Myc-IQGAP1
M WT ΔIQ AA3 M WT ΔIQ AA3
HA-PIPKIγi1
Myc-IQGAP1
(kD)
-73
-170
C
IP: GFP
GFP-IQGAP1 reconstitution in Iqgap1-/- MEFs
Mock WT ΔIQ AA3
panPIPKIγ
GFP-IQGAP1
(kD)
-73
-170
D
Vehicle
Neomycin
Sus COL Sus COL
S P S P S P S P
panPIPKIγ
IQGAP1
Rac1
β-tubulin
Na+K+ ATPase
(kD)
-73
-170
-25
-55
GFP
GFP-PLCδ1-PH
Sus COL Sus COL
S P S P S P S P
panPIPKIγ
IQGAP1
Rac1
β-tubulin
Na+K+ ATPase
GFP
-73
-170
-25
-55
-100
-55
-25
(kD)
E
Mock
Myc-IQ
S P S P
IQGAP1
HA-PIPKIγ
β-tubulin
Na+K+ ATPase
Myc-IQ domain
(kD)
-170
-70
-55
-100
-15
% in pellet
80
40
0
IQGAP1
PIPKIγ
F
IP: N-WASP
Iqgap1-/- Mock
Iqgap1-/- WT
Iqgap1-/- ΔIQ
Iqgap1-/- AA3
IQGAP1
N-WASP
(kD)
-170
-55

The IQGAP1 PI4,5P_2 binding mutant exhibited multiple leading edges and loss of migration: To determine how PI4,5P_2 binding modulates IQGAP1 function, the AA3 mutant was expressed in *Iqgap1* KO MEFs and the cell morphology was examined. When plated on a stiff substratum (glass or plastic) coated with COL, fibronectin or gelatin all types of cells indistinguishably highly spread and formed massive stress fibers (data not shown). Cytoskeleton organization and cell shape are greatly influenced by substrate stiffness [196], therefore cells were plated on pliant gelatin gel and cell morphology was observed by staining F-actin. Three distinct cell morphologies were observed compared to the star-shaped cells (type 1 morphology) that were predominant in WT MEFs. *Iqgap1* KO resulted in an increase in the number of cells with a single leading edge (type 2). Reconstitution of IQGAP1 WT partially recovered shapes of WT MEFs, whereas the ΔIQ mutant had a limited effect. Interestingly, the number of cells with multiple leading edges (type 3) was increased in the AA3 reconstituted cells (Figure 2.09 A). To closely examine localization of the reconstituted proteins, IQGAP1 was immunostained. WT IQGAP1 localized at the leading edge where active actin polymerization occurs. The ΔIQ mutant was largely cytoplasmic and failed to localize at the leading edge (Figure 2.09 B, arrowhead), supporting the results in Figure 2.05 indicating that the interaction with PIPKIγ controls IQGAP1 targeting.

The AA3 reconstituted cells formed multiple leading edges and the AA3 mutant localized at these sites (Figure 2.09 B). Consistent with this morphological phenotype, the AA3 reconstituted cells did not rescue haptotactic migration (Figure 2.09 C). The functional defects of AA3 were not due to a change in interaction with PIPKIγ as co-IP of the AA3 mutant with PIPKIγ was indistinguishable from that of WT IQGAP1 (Figure 2.08 B and C). Rather, the defects result from the loss of directional persistence (Figure 2.09 D and 2.06 A). This indicates that the IQGAP1 interaction with PIPKIγ is required for IQGAP1 targeting to the leading edge, but PI4,5P_2 binding is required for IQGAP1's role in normal membrane protrusions (lamellipodia formation) and migration.

Figure 2.09. PI4,5P_2-binding of IQGAP1 is important for cell morphology and migration.

For both A and B, *Iqgap1* KO MEFs, reconstituted with the indicated proteins, were plated on 0.2% gelatin gel for 3 h. Fixed cells were stained for IQGAP1 and F-actin. Cells were photographed at 600X magnification. (A) At least 300 cells were counted for each condition and categorized based on cell morphology (left). The graph is shown as mean of three independent experiments (right bottom). Expression levels of the proteins were analyzed by immunoblotting with antibodies against the indicated molecules (right top). (B) IQGAP1 and F-actin staining. Arrowhead indicates the lamellipodium that is deficient of the ΔIQ mutant. (C) With the reconstituted MEFs, fibronectin-induce haptotaxis was measured as described in Figure 2.03 E. (D) Reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy. Images were collected every minute for 6 h at 100X magnification and combined into a time-lapse movie. The migration path of six individual cells was then traced and plotted on a grid, with the origin of each cell placed in the center of the grid. All the experiments described above were performed independently at least three times.

Figure 2.09

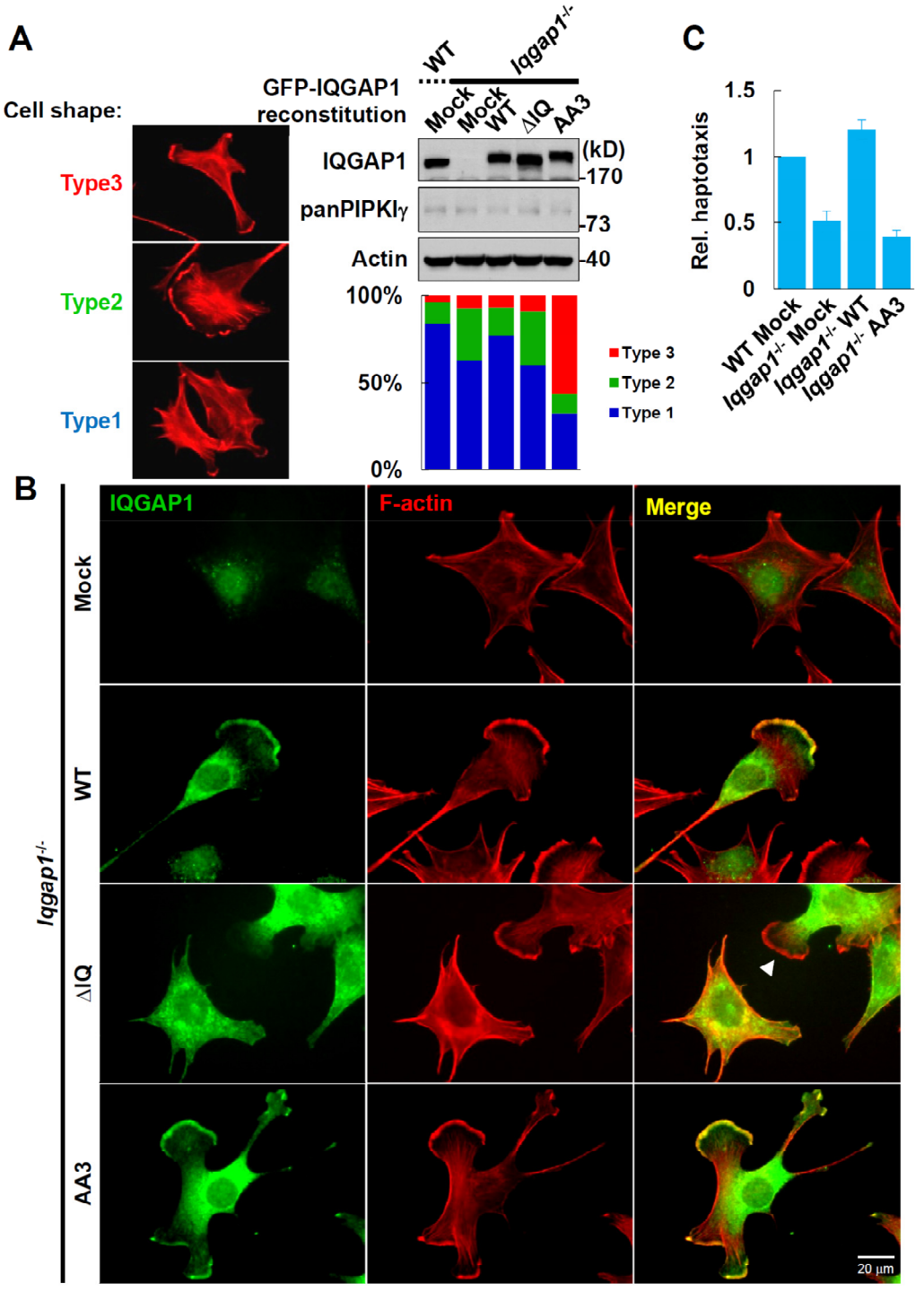
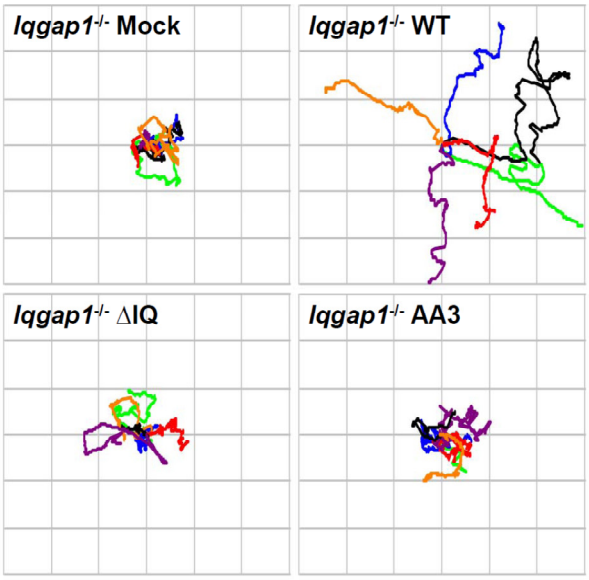

A
Cell shape:
Type3
Type2
Type1
GFP-IQGAP1 reconstitution
WT
Iqgap1-/-
Mock
Mock
WT
ΔIQ
AA3
IQGAP1
panPIPKIγ
Actin
(kD)
170
73
40
100%
50%
0%
Type 3
Type 2
Type 1
C
Rel. haptotaxis
1.5
1
0.5
0
WT Mock
Iqgap1-/- Mock
Iqgap1-/- WT
Iqgap1-/- AA3
B
IQGAP1
F-actin
Merge
Iqgap1-/-
Mock
WT
ΔIQ
AA3
20 μm

Figure 2.09 (cont.)

D



IQGAP1-PI4,5P$_2$ interaction regulates actin polymerization: Knockdown of PIPKIγ reduced IQGAP1 targeting to the leading edge membrane. Also, in knockdown cells actin polymerization at the leading edge, indicated by strong F-actin staining, was lost and stress fiber formation was increased (Figure 2.05 C and 2.10 A), signifying that PIPKIγ controls actin polymerization at the leading edge by regulating IQGAP1 targeting. However, the AA3 mutant is capable of interacting with PIPKIγ and localizes at the leading edge membrane, but forms multiple leading edges (Figure 2.09 B). These data suggest that PIPKIγ regulates activity of IQGAP1 required for persistent formation of a single leading edge.

IQGAP1 folds into an inactive conformation through an intramolecular interaction between the GRD and the RGCT domains [163]. RhoGTPase binding to the GRD or phosphorylation of Ser1443 disrupts autoinhibition and activates IQGAP1 [162]. We identified a PI4,5P$_2$ binding PBM within the RGCT domain close to Ser1443, suggesting that PI4,5P$_2$-binding to this PBM may open the inactive conformation [157, 161]. To test this hypothesis, we examined how phosphoinositides affect binding between the GRD and the RGCT domains. For this analysis His-C2 was incubated with immobilized GST-C1 (Figure 2.01 G) in the presence or absence of phosphoinositide-liposomes. In the absence of liposomes, C1 bound to C2 as reported [162]. Intriguingly, the binding was dramatically decreased in the presence of PI4,5P$_2$-liposomes, while other phosphoinositides or phosphatidylinositol had no significant effect. Introduction of the AA3 mutation in the C2 fragment eliminated the effect of PI4,5P$_2$ on the C1-C2 binding (Figure 2.10 B and Figure 2.11 C). Although the AA3 IQGAP1-C interacts with other phosphoinositide species, it lacks PI4,5P$_2$ binding (Figure 2.07 F). This indicates that there are multiple phosphoinositide binding sites in IQGAP1-C [174], but only PI4,5P$_2$ binding to the PBM modulates the activation of IQGAP1 as indicated by a loss of the C1-C2 interaction.

The C-terminal fragment of IQGAP1 (AA 746-1657) enhances actin polymerization by activating N-WASP [161]. Using this system, the influence of phosphoinositides in IQGAP1-

mediated actin polymerization was assessed. Since the actin polymerization activity of N-WASP is also regulated by $PI4,5P_2$, a N-WASP-ΔB mutant, which lacks the $PI4,5P_2$-responsive element [81], was used for this assay. Addition of $PI4,5P_2$-liposomes had no effect while addition of IQGAP1-C enhanced actin polymerization as shown [161]. Introduction of $PI4,5P_2$-liposomes in combination with WT IQGAP1-C significantly enhanced actin polymerization activity, whereas $PI4,5P_2$ had a limited effect on actin polymerization by the AA3 mutant (Figure 2.10 C). Strikingly, stimulation of actin polymerization was highly specific for $PI4,5P_2$ (Figure 2.11 A-D).

Figure 2.10. Phosphoinositide binding regulates IQGAP1 function in actin polymerization.

(A) Control or PIPKIγ knockdown MDA-MB-231 cells were grown on cover glass for 24 h. Cells were fixed and endogenous IQGAP1 and F-actin were stained. Cells were photographed at 600X magnification. (B) 0.05 μM of His-C2 WT or AA3 mutant were incubated with 1 μM of GST-C1 immobilized on glutathione beads in the absence or presence of the indicated phosphoinositide-liposomes (2 μM) for 10 m. Liposome-bound proteins were detected by immunoblotting with an anti-His antibody. Immunoblots were quantified and the graph is shown as mean ± SD of three independent experiments. (C) Actin polymerization in the presence of the indicated combinations of GST-IQGAP1-C (50 nM) or 5% PI4,5P_2-liposomes (2 μM). The experiments described above were performed independently at least four times.

Figure 2.10

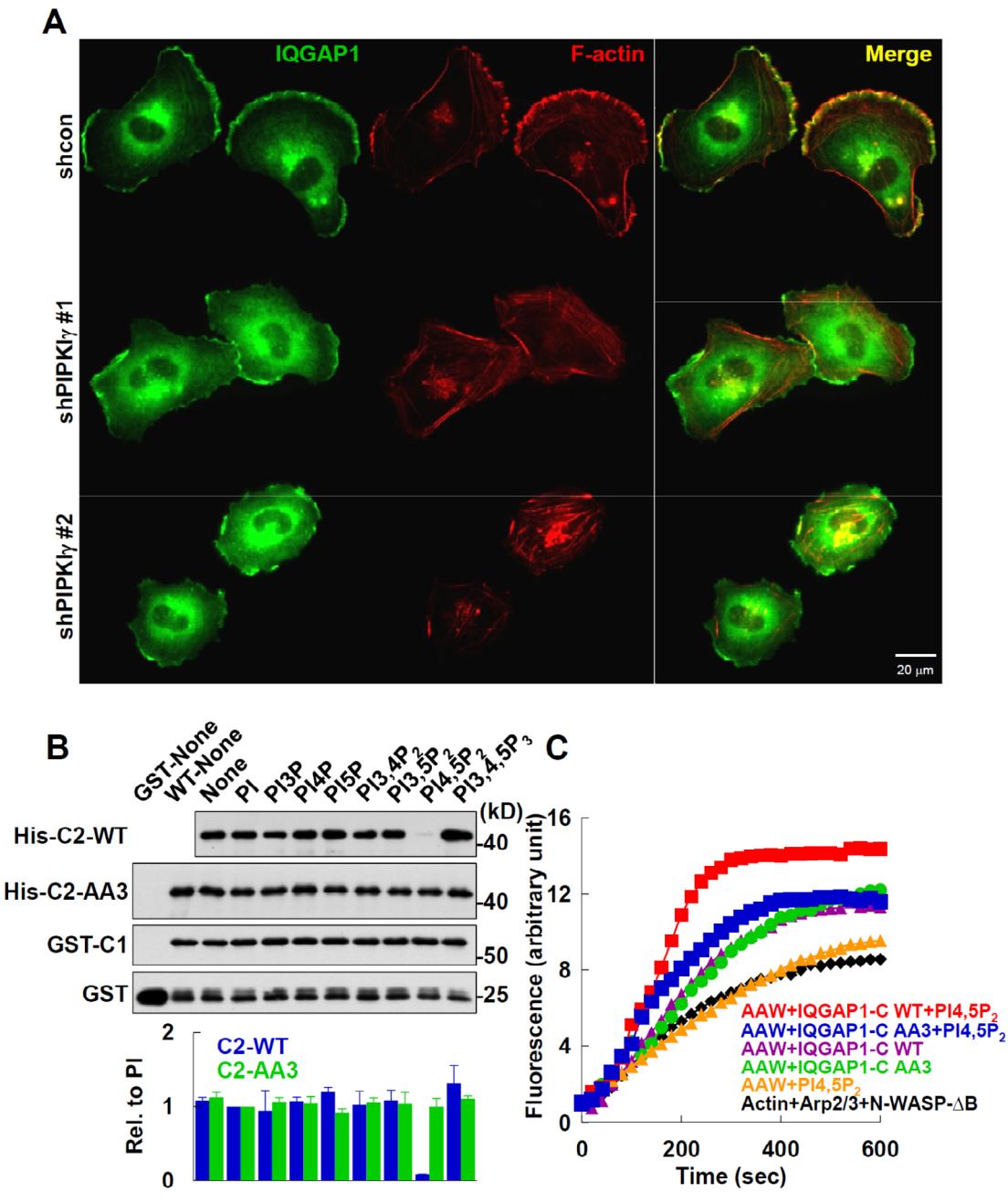
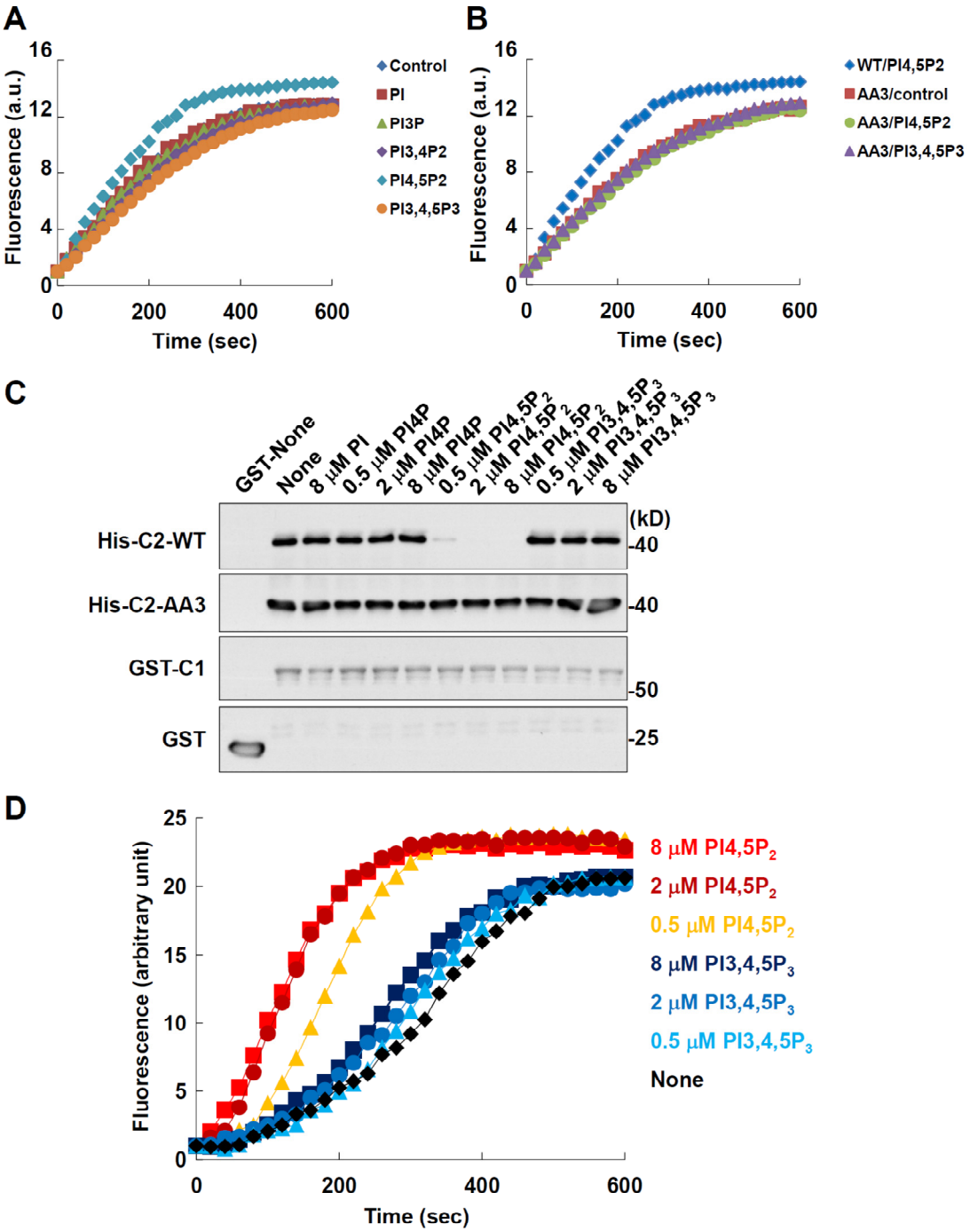

A
IQGAP1
F-actin
Merge
shcon
shPIPKIγ #1
shPIPKIγ #2
20 μm
B
GST-None
WT-None
None
PI
PI3P
PI4P
PI5P
PI3,4P2
PI3,5P2
PI4,5P2
PI3,4,5P3
(kD)
His-C2-WT
His-C2-AA3
GST-C1
GST
-40
-40
-50
-25
C2-WT
C2-AA3
Rel. to PI
2
1
0
C
Fluorescence (arbitrary unit)
16
12
8
4
0
0
200
400
600
Time (sec)
AAW+IQGAP1-C WT+PI4,5P2
AAW+IQGAP1-C AA3+PI4,5P2
AAW+IQGAP1-C WT
AAW+IQGAP1-C AA3
AAW+PI4,5P2
Actin+Arp2/3+N-WASP-ΔB

Figure 2.11. Enhancement of actin polymerization is specific to $PI4,5P_2$.

(A and B) Actin polymerization (1.5 μM of pyrene-conjugated G-actin, 12.5 nM of Arp2/3 comple and 40 nM of N-WASP-ΔB) in the presence of the indicated combinations of GST-IQGAP1-C (50 nM) or 5% phosphoinositide-liposomes (2 μM). (C) 0.1 μM of His-C2 WT or AA3 mutant were incubated with 1 μM of GST-C1 immobilized on glutathione beads in the absence or presence of the indicated phosphoinositide-liposomes for 10 m. Liposome-bound proteins were detected by immunoblotting with an anti-His antibody. (D) Actin polymerization was performed in the presence of 50 nM GST-IQGAP1-C with the indicated liposomes. All the experiments described above were performed independently at least three times.

Figure 2.11


A
Fluorescence (a.u.)
Time (sec)
Control
PI
PI3P
PI3,4P2
PI4,5P2
PI3,4,5P3
B
Fluorescence (a.u.)
Time (sec)
WT/PI4,5P2
AA3/control
AA3/PI4,5P2
AA3/PI3,4,5P3
C
GST-None
None
8 μM PI
0.5 μM PI4P
2 μM PI4P
8 μM PI4P
0.5 μM PI4,5P2
2 μM PI4,5P2
8 μM PI4,5P2
0.5 μM PI3,4,5P3
2 μM PI3,4,5P3
8 μM PI3,4,5P3
(kD)
His-C2-WT
His-C2-AA3
GST-C1
GST
-40
-40
-50
-25
D
Fluorescence (arbitrary unit)
Time (sec)
8 μM PI4,5P2
2 μM PI4,5P2
0.5 μM PI4,5P2
8 μM PI3,4,5P3
2 μM PI3,4,5P3
0.5 μM PI3,4,5P3
None

Discussion:

Here, we define a novel mechanism for how PIPKIγ and IQGAP1 function together as a signaling nexus to regulate migration (Figure 2.12). In polarized epithelial cells, IQGAP1 is largely localized at the cell-cell contacts [53, 153, 158]. In directionally migrating cells, IQGAP1 translocates to the leading edge and facilitates actin polymerization. In detail, in response to receptor signaling, PIPKIγ associates with IQGAP1 and recruits IQGAP1 to the leading edge membrane. There, generation of $PI4,5P_2$ by PIPKIγ activates IQGAP1, as $PI4,5P_2$-binding to a PBM relieves autoinhibition between the RGD and RGCT domains. This allows the RGCT domain to recruit N-WASP and the Arp2/3 complex to facilitate actin polymerization (Figure 2.08 E) [163]. Overall, extracellular stimuli control the spatiotemporal activation of the PIPKIγ/IQGAP1 nexus to regulate actin polymerization required for persistent formation of lamellipodia and migration.

All PIPKIγ isoforms have the potential to interact with IQGAP1 (Figure 2.01 A) and this suggests that IQGAP1 may mediate isoform-specific functions at different compartments. For example, IQGAP1 is found in the nucleus and ectopic expression of IQGAP1 enhances transcriptional activity of β-catenin [172]. Similarly, PIPKIγ also modulates β-catenin-mediated transcriptional co-activation [197]. IQGAP1 associates with the exocyst complex and regulates cancer cell invasion a function also regulated by PIPKIγi2 [173]. Here, we demonstrate that receptor signaling stimulates the recruitment of IQGAP1 to the leading edge through an interaction with PIPKIγ, likely the PIPKIγi1 isoform (Figure 2.03 D). PIPKIγi2 isoform plays an analogous role by interaction with talin, linking the trafficking of integrin-containing vesicles to talin rich adhesions [33].

The C-terminal half of IQGAP1 (IQGAP1-C) binds to different phosphoinositide species with a varying binding affinity (Figure 2.07 F). A recent study shows that very C-terminus of IQGAP1 (AA 1559-1657) forms a pseudo C2 domain fold and binds to class I

phosphoinositide 3-kinase products, PI3,4P_2 and PI3,4,5P_3 [174]. According to the solved structure Lys^{1562} and Lys^{1604} are important for ligand recognition. Here we define a distinct PI4,5P_2-binding site at Lys^{1546}, Lys^{1547}, Lys^{1554}, Lys^{1555}, Lys^{1557} and Lys^{1558} (Figure 2.07). These data indicate that there could be multiple phosphoinositide binding sites on IQGAP1-C. Consistent with this possibility, the IQGAP1-C1 interaction with IQGAP1-C2 is specifically inhibited by PI4,5P_2, while mutating the six lysine residues blocks the inhibition (Figure 2.10 B). Further work is necessary to define other phosphoinositide binding sites on IQGAP1. These works will give us mechanistic insights into how IQGAP1 is found at the intracellular compartments where different phosphoinositide species are predominant [97, 151].

PIPKIγ regulates IQGAP1 targeting to the leading edge and this event requires PI4,5P_2 generation (Figure 2.05). IQGAP1 is widely believed to target to the PM by association with Rac1 and Cdc42 [153, 154, 163]. Rac1 and Cdc42 contain PBMs near the C termini and these PBMs contribute to membrane targeting [6, 191]. This raises the possibility that PI4,5P_2 controls IQGAP1 targeting to the PM by indirectly regulating Rac1 targeting. Consistently, sequestration of cellular PI4,5P_2 either by neomycin treatment [198] or PLCδ1-PH expression [193] blocks both Rac1 and IQGAP1 translocation to membrane in response to integrin activation (Figure 2.08 D). To examine the sole contribution of the PI4,5P_2-binding for IQGAP1 targeting, we generated and expressed a PI4,5P_2-binding defective mutant in *Iqgap1*$^{-/-}$ MEFs. The PI4,5P_2-binding defective mutant still localizes to the PM, while the PIPKIγ-binding defective (ΔIQ) mutant is largely cytosolic (Figure 2.09 B). These data indicate that the physical interaction between the two proteins is more important than PI4,5P_2-binding for IQGAP1 PM targeting.

Cells expressing the PI4,5P_2 binding mutant (AA3) form multiple leading edges, suggesting that PI4,5P_2 regulation of IQGAP1 is important for maintaining polarity and leading edge integrity (Figure 2.09 B). These cells exhibit perpetual formation and retraction of leading edges but display little movement (Figure 2.09 D). Consistent with this observation,

IQGAP1 is suggested to maintain polarity of migrating cells through local capture of MTs at the leading edge by interaction with MT regulators [54]. The interaction sites for these proteins are within the RGCT domain, which also contains the $PI4,5P_2$ binding site [147]. We envision that the autoinhibitory interaction between the GRD and RGCT domains may also block MT recruitment, and $PI4,5P_2$ binding may relieve this (Figure 2.05 A). In this model, the AA3 mutant may remain inactive at the leading edge and fail to recruit MTs, which would result in loss of cell polarity. Alternatively, multiple leading edges could be induced by perturbation of actin dynamics. In support of this possibility, cells display multiple leading edges after manipulation of certain actin regulatory proteins. For example, multiple leading edges also form in Cdc42 KO dendritic cells [199] and in Vero cells after expression of an IQGAP1 mutant that is defective in Rac1/Cdc42 binding [154].

Finally, multiple reports suggest roles for both PIPKIγ and IQGAP1 in cancer metastasis [152, 172, 182]. The current findings define a molecular mechanism of how these two proteins interact and function together in migration and invasion, and potentially other processes required for cancer progression.

Materials and Methods:

Cell culture and constructs: MDA-MB-231, HEK 293, MCF-7 and MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco). MDCK and HeLa tet-off cells were cultured as previously described [41] and induction of transgene was achieved by removing of doxycycline from media for 24 h. The constructs used for this work are described previously [161, 164, 195, 200].

Stable cell line generation: To generate stable MDA-MB-231 cell lines, cells were transfected with vectors expressing DsRed-PIPKIγ isoforms using Lipofectamine 2000 (Invitrogen) and selected with 1.2 mg/ml Geneticin (Gibco) for 15 days, and further selected for DsRed expression using cell sorter. Cells expressing the transgene at a level similar to the endogenous level of PIPKIγ were used for experiments. For generation of stable in MEFs, cells were infected with retrovirus for 24 h. Then, cells expressing GFP-IQGAP1 were first selected for GFP expression, and then further sorted by expression level.

Antibodies and siRNAs: Monoclonal antibodies against IQGAP1, β-tubulin, Myc-tag, Na^+K^+ ATPase, GST-tag, His-tag (Millipore), α/β-tubulin, cyclin D1 (Cell Signaling Technology), Rac1, calnexin, GM-130 (BD Biosciences), HA-tag (Covence Biotechnology), actin (MP Biomedicals) and polyclonal antibody against IQGAP2 (Santa Cruz Biotechnology) were used for this study. Polyclonal and monoclonal antibodies against total and specific isoforms of PIPKIγ were produced as described previously [26]. Pooled siRNAs against PIPKIγ was obtained from Dharmacon and IQGAP1 from Santa Cruz Biotechnology.

IP and immunoblotting: Cells were lysed in a buffer containing 1% Brij58, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na_3VO_4, 1 mM Na_2MoO_4 and protease inhibitors. Protein concentration of lysates was measured by the BCA method (Pierce) and equal amounts of protein were used for further analysis. For IP, 0.5 to 1 mg of proteins were incubated with 1 μg of antibodies at 4°C for 8 h and then incubated with a 50%

slurry of Protein G Sepharose (GE Life Sciences) for another 2 h. After washing 5X with lysis buffer, the protein complex was eluted with SDS sample buffer. For immunoblotting, 10 to 20 μg of proteins loaded. After developing immunoblots, the film was scanned using a transmitted light scanner (resolution = 600 dpi). Protein bands were quantified using ImageJ, and statistical analysis of the data was performed with Microsoft Excel. The statistical analysis was performed using data from at least three independent experiments.

***In vitro* binding assay**: Recombinant proteins were expressed in BL21 E. coli strain. GST-tagged proteins were then purified with GST Sepharose 4B (GE Life Sciences) and His-tagged proteins were purified with His-Bind Resin (Novagen). GST-tagged proteins were incubated with glutathione beads before binding assays. The binding assay was performed in the lysis buffer used for IP by adding 10 nM to 5 μM of His-tagged proteins and 20 μl of GST-tagged protein bound glutathione beads. After incubation for 1h at 25°C, unbound proteins were washed out and the protein complex was analyzed by immunoblotting. For the binding assay with liposomes, analysis was performed for 10 m at 25°C without detergent (150 mM KCl, 50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and protease inhibitors) to maintain the integrity of liposomes.

Transwell motility assay: Motility assays were performed with a Transwell (Corning) as before [190]. Briefly, an equal number of cells were loaded on the upper chamber and cells migrated toward attractants were fixed with 4% paraformaldehyde followed by staining with 0.5% crystal violet. Cells were counted from photographs taken from at least five random fields with a Nikon Eclipse TE2000U at 200X resolution. Statistical analysis was performed with Microsoft Excel, using data from at least three independent experiments. A Transwell with 3.0 μm pores was used for migration assay and 8.0 μm pores for invasion assay.

Subcellular fractionation assay: Cells were lysed in a hypotonic lysis buffer [191] for 10 m. Then cell lysates were homogenized with 15 strokes of a Dounce homogenizer.

Homogenates were centrifuged at 700 g for 3 m to pellet nuclei and intact cells. The supernatants were spun at 100,000 g for 30 m at 4°C to sediment particulates. The cytosol-containing supernatant was removed and the crude membrane pellet was gently washed with the lysis buffer. Protein concentration was determined in the membrane and cytosolic fractions. Equal amounts of protein were resolved by SDS-PAGE and further analyzed by immunoblotting.

Fluorescence microscopy: Glass coverslips were coated with 10 ng/ml COL, fibronectin, gelatin or 10% serum before seeding cells. For Figure 2.09, coverslips were coated as previously [173]. Cells were grown on coverslips placed inside 6-well plates until experimental manipulation. Coverslips were washed twice in 37°C PBS, and then fixed with 4% paraformaldehyde, followed by permeabilization with 0.5% Triton X-100 in PBS. The cells were then blocked for 1 h at 25°C in 3% BSA. Primary antibody incubation was performed at 4°C for 12 h, while incubation with fluorophore-conjugated secondary antibodies occurred at 37°C for 45 m. Fluorescence microscopy was performed using a 60x plan-fluor objective on a Nikon Eclipse TE2000U equipped with a Photometrics CoolSNAP ES CCD camera. Images were captured using MetaMorph v6.3 (Molecular Devices). Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

Liposome sedimentation assay: Liposomes were prepared as previously described [195]. Dried lipids were resuspended with a buffer containing 150 mM KCl, 50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 300 mM sucrose. After bath sonication for 20 m, the rehydrated lipids were subjected to at least 5 cycles of freezing and thawing and extruded through a 0.1 μm filter with a lipid extruder (Avanti). Liposome co-sedimentation assay was performed by mixing 0.5 μM of proteins with 2.5 μM of liposomes in the buffer without sucrose. After 10 m incubation at 25°C, samples were centrifuged at 100,000 g for 30 m at

4°C. Pellets were gently washed and resuspended in SDS sample buffer for a final volume equal to the supernatant. Samples were resolved by SDS-PAGE and proteins were detected by either Coomassie staining or immunoblotting.

Live cell imaging: Delta TPG dish (Fisher Scientific) were coated with a gelatin gel as previously [173]. Cells were seeded at a density of $1.0X10^4$ cells/dish in L15 culture medium and placed in a temperature-controlled chamber of a Nikon Eclipse TE2000U. Time-lapse recording started 3 h after cell plating. Images were collected every 30 or 60 s for over 5 h with a Photometrics CoolSNAP ES CCD camera (Roper Scientific) operated by Metamorph image analysis software (Molecular Devices). Analyses of collected images including tracking the migration path of individual cells and generation of movies were performed with Metamorph.

Actin polymerization assay: Actin polymerization assay was performed as before [161]. Pyrene-conjugated G-actin (Cytoskeleton) was prepared according to the manufacturer's instructions. 12.5 nM of Arp2/3 complex and 40 nM of N-WASP-ΔB in the presence of GST-IQGAP1-C (50 nM) and/or 5% phosphoinositide-liposomes (2 μM) were incubated for 5 m before the addition of 1.5 μM of pyrene-conjugated G-actin stock. Fluorescence was read immediately after the addition of actin using a PC1 photon counting spectrofluorometer (ISS) set on kinetic mode to read every 20 s for the duration of the assay. PC1 setting was as followed: Excitation, 365 nm; emission 407 mm. Obtained fluorescence density was converted to arbitrary units.

Chapter 3

The Roles of Alpha Isoform of Type I PIP Kinase and IQGAP1 in The PI3K-Akt pathway

Chapter Summary:

Phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) serves as a substrate for class I phosphoinositide 3-kinases (PI3Ks) for $PI3,4,5P_3$ synthesis. Although the PI3K signaling pathway has been the subject of intensive study over the past two decades, investigation of the direct role for $PI4,5P_2$ synthesis in this process has been largely neglected. A prevailing assumption is that activated class I PI3Ks readily utilize the abundant $PI4,5P_2$ pool in the plasma membrane. However, recent studies suggest that the majority of $PI4,5P_2$ is sequestered by $PI4,5P_2$ binding proteins, protecting $PI4,5P_2$ from enzymatic turnover. Thus, class I PI3Ks might require *de novo* synthesis of $PI4,5P_2$ for efficient $PI3,4,5P_3$ production. In this study, we reveal that class I PI3Ks and PIPKIα, a $PI4,5P_2$ generating enzyme, are spatially organized by IQGAP1, a scaffold protein. In the IQGAP1-PIPKIα-PI3K complex $PI4,5P_2$ generated by PIPKIα is channeled to PI3K for $PI3,4,5P_3$ synthesis. Also, the organization is stimulated by integrin and growth factor receptor signaling. Moreover, blockade of the IQGAP1 interaction with the phosphoinositide kinases inhibits $PI3,4,5P_3$ synthesis and Akt activation. Collectively, our current study demonstrates an active role for $PI4,5P_2$ synthesis in the PI3K signaling.

Introduction:

Phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) is a lipid messenger regulating nearly every aspect of cellular function [7, 21, 97, 201]. $PI4,5P_2$ serves as a substrate for phospholipases and phosphoinositide 3-kinases (PI3Ks) to generate other messengers [202, 203], or $PI4,5P_2$ directly binds to its effector proteins and regulates their targeting and activities [21, 97]. To mediate these diverse processes, $PI4,5P_2$ is present at a relatively high concentration, with total cellular concentration of approximately 50 μM and concentration on the inner leaflet of the plasma membrane is estimated to be 5 mM, where the majority of $PI4,5P_2$ is found in the cell [2, 16]. The majority of the $PI4,5P_2$ pool is maintained by phosphorylation at the 5 hydroxyl of the myo-inositol ring of PI4P by type I phosphatidylinositol phosphate kinases (PIPKIs). In human, there are three PIPKI isoforms (α, β and γ) with multiple splice variants [21, 22]. Despite of its abundance, sustained downstream signaling emanating from $PI4,5P_2$ requires continuous phosphorylation of PI4P by PIPKIs [204-207] suggesting that functionally available $PI4,5P_2$ is present in a limited concentration in the cell.

Stimulation of receptor tyrosine kinases, G protein-coupled receptors and integrins leads to downstream activation of class I PI3Ks, which utilize $PI4,5P_2$ as a substrate to generate $PI3,4,5P_3$ [208]. Although class I PI3Ks catalyze phosphorylation of $PI4,5P_2$ at the 3 hydroxyl *in vitro* [209] and *in vivo* [2, 16, 210], however the cellular source of $PI4,5P_2$ used as substrate remains unstudied. An assumption is that $PI4,5P_2$ is available in excess for class I PI3Ks to synthesize $PI3,4,5P_3$, with cellular $PI4,5P_2$ concentration at least two orders of magnitude higher than that of $PI3,4,5P_3$ [2, 210]. This assumption is challenged by a recent finding that the majority of cellular $PI4,5P_2$ is sequestered from enzymatic modification and effector binding by a vast array of $PI4,5P_2$ binding proteins [5], suggesting that *de novo* synthesis of $PI4,5P_2$ by PIPKIs is required for efficient $PI3,4,5P_3$ generation by class I PI3Ks. In line with this possibility, in *Dictyostelium*, depletion of a PIPKI, that is responsible for approximately 90% of cellular $PI4,5P_2$ generation, attenuates Akt activity, a

readout of class I PI3K activity [140]. In human keratinocytes, knockdown of PIPKIα also reduces extracellular calcium-induced Akt activation [141]. In this particular study, knockdown of PIPKIα reduced approximately 40% of global PI4,5P_2 levels, but the reduction of PI3,4,5P_3 levels and Akt activation is much greater (up to approximately 90% reduction) suggesting that PI4,5P_2 and PI3,4,5P_3 synthesis might be locally organized and that only a specific pool of PI4,5P_2 (but not all) is available and responsible for PI3,4,5P_3 generation. The local organization of PI4,5P_2 and PI3,4,5P_3 synthesis is further supported by immunostaining analyses in migrating leukocytes [31]. PIPKIα and PIPKIγ are colocalized with PI3,4,5P_3 at the leading edges, suggesting that PI4,5P_2 synthesis by PIPKIs is coupled to PI3,4,5P_3 localization at the leading edges of migrating cell. However, the physical association of PIPKIs with class I PI3Ks remains untested.

IQ motif containing GTPase activating protein 1 (IQGAP1) is a multidomain protein that scaffolds multiple signaling pathways to regulate a plethora of cell functions, including migration, survival, proliferation and trafficking [150, 151, 163]. The scaffold role of IQGAP1 in the mitogen activated protein kinase (MAPK) pathway is best characterized. MAPK pathway components, such as Ras, Raf, MEK and Erk, directly interact with IQGAP1, which brings these kinases in close proximity to facilitate their sequential phosphorylation, activation and signaling [164-167]. In this way, IQGAP1 mediates MAPK pathway activation downstream of growth factor receptors in many different cell types [169, 170]. The scaffold role of IQGAP1 is not limited to the MAPK pathway. For example, roles for IQGAP1 in phosphoinositide, Wnt and integrin signaling were recently identified [10, 175, 211]. How IQGAP1 has such versatile roles in cells remains elusive. Notably, an unbiased quantitative study reveals that the messenger RNA and protein copy number of IQGAP1 is at least two orders of magnitude higher than that of its interacting proteins [149]. This suggests that IQGAP1 might be present in many discrete signaling complexes in a cell and even more IQGAP1-regulated signaling pathways are remained to be characterized. Although it is

shown that IQGAP1 is required for Akt activation in several stress conditions [171, 175], the role of IQGAP1 in the PI3K-Akt pathway is largely unknown.

In this study, we reveal that IQGAP1 interacts with PIPKIα and class I PI3K subunits, forming a complex containing two phosphoinositide kinases. Complex formation is enhanced by growth factor receptor and integrin activation, with the integrin-mediated pathway having a more profound effect. The two phosphoinositide kinases are brought in close proximity by IQGAP1, which allows for $PI4,5P_2$ produced from PIPKIα to be channeled to PI3K for $PI3,4,5P_3$ synthesis. Furthermore, blockade of IQGAP1 interaction with either PIPKIα or PI3K by cell permeable peptides inhibits $PI3,4,5P_3$ synthesis and Akt activation in breast cancer cells. Collectively, our data strongly suggest that the majority of $PI3,4,5P_3$ production requires spatiotemporal organization of PIPKIα and PI3K by IQGAP1 downstream of receptor activation.

Results:

PIPKIα and IQGAP1 are required for Akt activation: Previously we reported that type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) regulates directional cell migration by controlling IQGAP1 targeting and activity [10]. IQGAP1 interacts with numerous proteins that participate in diverse signaling pathways [150, 151, 172]. Potentially, IQGAP1 is able to function in multiple signaling pathways due to its abundance. A recent study showed that the IQGAP1 protein copy number is at least 100 times higher than other signaling proteins [149]. In line with this, recent studies have revealed that IQGAP1 is implicated in previously unidentified signaling pathways [167, 211]. To further investigate the roles of IQGAP1 with PIPKs, IQGAP1 was immunoprecipitated from Hs578T breast cancer cell lysates and associated proteins were analyzed by immunoblotting. PIPKIγ [10] and IQGAP2 [212] are coimmunoprecipitated with IQGAP1 as previously shown. Further, PIPKIα also coimmunoprecipitated with IQGAP1, with more PIPKIα associated with IQGAP1 than PIPKIγ (Figure 3.01 A). To directly compare binding affinity, equal amounts of recombinant PIPKs were used for *in vitro* binding assays. As shown in Figure 3.01 B, PIPKIα directly binds to IQGAP1 and PIPKIα has at least 10 fold higher binding affinity for IQGAP1 than PIPKIγ. This is also true *in vivo*, as approximately 8.5 times more PIPKIα is coimmunoprecipitated with IQGAP1 than PIPKIγ (Figure 3.01 C). Interestingly, class I PI3K subunits p110α, p110β and p85 are also coimmunoprecipitated with IQGAP1 as well as a class I PI3K downstream molecule Akt (Figure 3.01 A). These data indicate that IQGAP1 might have a role in the PI3K-Akt pathway.

To examine if IQGAPs and PIPKIs have roles in PI3K-Akt signaling, proteins were knocked down by transiently transfecting small interfering RNAs (siRNAs) in Hs578 breast cancer cells and Akt activation was measured by immunoblotting phosphorylated Akt as a readout for the PI3K-Akt pathway [202, 213]. Knockdown of IQGAP1, but not IQGAP2, significantly reduced Akt phosphorylation. Amongst the three PIPKI isoforms, only PIPKIα

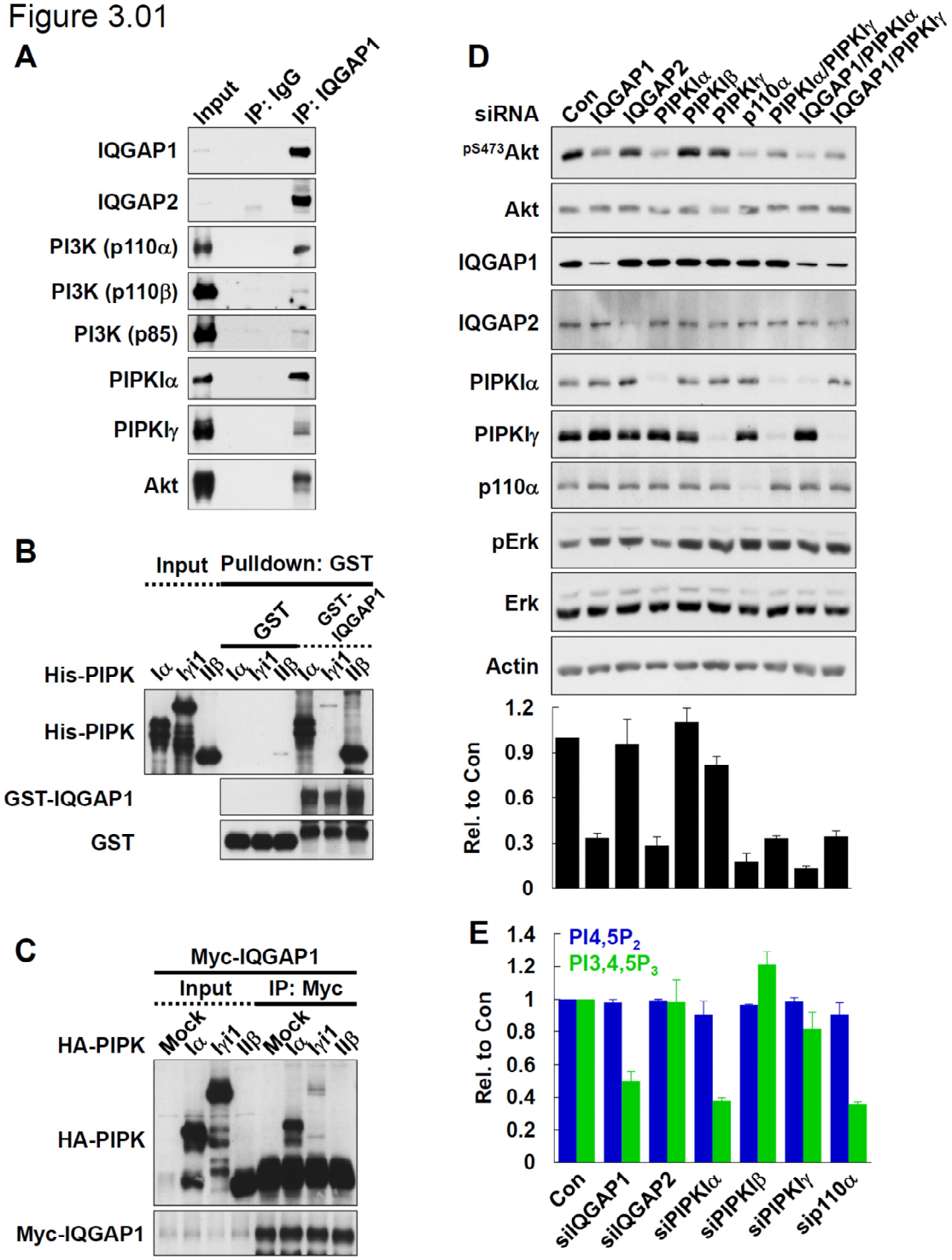
knockdown significantly reduced Akt phosphorylation. Double knockdown of IQGAP1 and PIPKIα further reduced Akt phosphorylation that is comparable to knockdown of PI3K subunit p110α (Figure 3.01 D).

Knockdown of PIPKIα might reduce cellular $PI4,5P_2$ levels, diminishing its availability as substrate for class I PI3Ks to produce $PI3,4,5P_3$ that is required for Akt phosphorylation. In *Dictyostelium*, it was demonstrated that depletion of a PIPKI reduces approximately 90% of cellular $PI4,5P_2$ levels and results in attenuation of Akt phosphorylation [140]. To directly measure how IQGAPs and PIPKs regulate cellular $PI4,5P_2$ and $PI3,4,5P_3$ levels, lipids from the same number of the siRNA transfected cells were extracted and used for a quantitative assay (see Materials and Methods). As shown in Figure 3.01 E, knockdown of IQGAP1 and PIPKIα reduced cellular $PI3,4,5P_3$ levels approximately 50% and 60%, respectively. These data indicate that the attenuation of Akt phosphorylation by knockdown of IQGAP1 and PIPKIα is likely resulted from defective $PI3,4,5P_3$ synthesis. However, global $PI4,5P_2$ levels remain unchanged ruling out the possibility that diminished Akt phosphorylation by knockdown of IQGAP1 and PIPKIα is due to defective global $PI4,5P_2$ synthesis (Figure 3.01 E). This is consistent with previous studies that in mammalian cells unlike in *Dictyostelium* depletion of individual PIPKI isoforms has no significant impact on global $PI4,5P_2$ levels [41, 45].

Figure 3.01. PIPKIα and IQGAP1 are required for the PI3K-Akt pathway.

(A) Endogenous IQGAP1 was immunoprecipitated from Hs578T breast cancer cell lysates and the associated protein complex was analyzed by immunoblotting with the indicated antibodies. Isotype immunoglobulin (IgG) was used as a control. (B) 1 μM His-tagged PIPK isoforms were incubated with 1 μM GST alone or GST-tagged IQGAP1 immobilized on glutathione beads. PIPK isoforms that are associated with GST-IQGAP1 were analyzed by immunoblotting with an anti-His antibody. (C) Myc-IQGAP1 and HA-PIPK isoforms were co-expressed in HEK293 cells. Myc-IQGAP1 was immunoprecipitated with an anti-Myc antibody and the associated PIPK isoforms were analyzed by immunoblotting with an anti-HA antibody. (D) Small interfering (si) RNAs against IQGAP1, IQGAP2, PIPKIα, PIPKIβ, PIPKIγ or PI3K p110α subunit were transfected singly or in the indicated combinations in Hs578T cells for 48 h. Non-targeting siRNA was used as a control. Cell lysates were analyzed by immunoblotting with the indicated antibodies (top). ^{pS473}Akt immunoblots were quantified and the graph is shown as mean ± SD of three independent experiments (bottom). (E) Hs578T cells were transfected with siRNAs against the indicated proteins for 48 h. Lipids were extracted from the cells and analyzed for $PI4,5P_2$ and $PI3,4,5P_3$ content using kits from Echelon Biosciences. The graph is shown as mean ± SD of three independent experiments. The experiments described above were performed independently at least four times.

Figure 3.01


A
Input
IP: IgG
IP: IQGAP1
IQGAP1
IQGAP2
PI3K (p110α)
PI3K (p110β)
PI3K (p85)
PIPKIα
PIPKIγ
Akt
B
Input
Pulldown: GST
GST
GST-IQGAP1
His-PIPK
Iα
Iγi1
IIβ
His-PIPK
GST-IQGAP1
GST
C
Myc-IQGAP1
Input
IP: Myc
HA-PIPK
Mock
Iα
Iγi1
IIβ
HA-PIPK
Myc-IQGAP1
D
siRNA
Con
IQGAP1
IQGAP2
PIPKIα
PIPKIβ
PIPKIγ
p110α
PIPKIα/PIPKIγ
IQGAP1/PIPKIα
IQGAP1/PIPKIγ
pS473Akt
Akt
IQGAP1
IQGAP2
PIPKIα
PIPKIγ
p110α
pErk
Erk
Actin
Rel. to Con
1.2
0.9
0.6
0.3
0
E
PI4,5P2
PI3,4,5P3
Rel. to Con
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Con
siIQGAP1
siIQGAP2
siPIPKIα
siPIPKIβ
siPIPKIγ
sip110α

IQGAP1 mediates a physical interaction between PIPKIα and PI3K: Next we further investigated how knockdown of IQGAP1 or PIPKIα attenuates Akt activation without compromising global $PI4,5P_2$ levels. Early studies estimated that cellular $PI4,5P_2$ levels are three orders of magnitude higher than that of $PI3,4,5P_3$ [2, 16] and this lead scientists to believe that $PI4,5P_2$ is passively utilized by class I PI3Ks for $PI3,4,5P_3$ generation. However, more recent studies suggest that the majority of cellular $PI4,5P_2$ is sequestered by a set of $PI4,5P_2$-binding proteins and, consequently, enzymatically inert [5, 21]. Thus, *de novo* synthesis of $PI4,5P_2$ might be required for PI3Ks to efficiently produce $PI3,4,5P_3$ at a specific time and location. This raises a possibility that local organization of PIPKIα and PI3K with IQGAP1 might be required for $PI3,4,5P_3$ generation and Akt activation. To test this possibility, we first examined their association by immunoprecipitation. Endogenous IQGAP1, PIPKIα and p110a subunit of class I PI3K were immunoprecipitated and the association was examined by immunoblotting. As shown in Figure 3.02 A, all three proteins coimmunoprecipitate with one another, indicating that IQGAP1, PIPKIα and p110α form a complex *in vivo*. Their subcellular localization was examined by immunostaining. As class I PI3K immunostaining with multiple commercially available antibodies produced diffuse signals throughout cells, $PI3,4,5P_3$ immunostaining was instead used to probe activated class I PI3K in cells [31]. As shown in Figure 3.02 B, PIPKIα and $PI3,4,5P_3$ colocalize with IQGAP1 at the plasma membrane (white arrow heads).

Next, how IQGAP1 regulates PIPKIα and PI3K association was tested in Hs578T breast cancer cells. Knockdown of IQGAP1 diminished p110α coimmunoprecipitation with PIPKIα indicating that IQGAP1 is necessary for the PIPKIα-PI3K interaction (Figure 3.02 C). However, neither knockdown of PIPKIα affects the IQGAP1-PI3K interaction (Figure 3.02 D) nor knockdown of p110α affects the IQGAP1-PIPKIα interaction (Figure 3.02 E). To test if IQGAP1 is sufficient to regulate the PIPKIα-PI3K interaction, IQGAP1 was overexpressed in HEK293 cells and the interaction was examined by immunoprecipitation. Overexpression of

IQGAP1 significantly increases PIPKIα coimmunoprecipitation with p110α (Figure 3.02 G) indicating that IQGAP1 is present in a limiting concentration in HEK293 cells for the PIPKIα-PI3K interaction. These data suggest that IQGAP1 mediates an interaction between PIPKIα and PI3K *in vivo*.

Next it was examined how IQGAP1 controls the PIPKIα-PI3K interaction *in vitro* using purified recombinant proteins (Figure 3.02 H). At first, as shown in Figure 3.03 A and B, IQGAP1-N fragment but not IQGAP-C fragment directly binds to class I PI3K. However, PIPKIα does not directly bind to class I PI3K. To test how IQGAP1 regulates the two phosphoinositide kinases' interaction, class I PI3K was incubated with PIPKIα in the presence of increasing amounts of IQGAP1. Then, PIPKIα was pulled down and the associated IQGAP1 and class I PI3K were examined by immunoblotting. In increasing amount of IQGAP1-N fragment, the amount of IQGAP1-N fragment associated with PIPKIα is increased and then saturated. In these conditions, PI3K subunits associated with PIPKIα are also increased and then saturated. As PIPKIα and PI3K are not directly bind (Figure 3.03 A), these date indicate that IQGAP1 mediates a physical interaction between PIPKIα and PI3K *in vitro*.

Figure 3.02. IQGAP1 mediates a physical interaction between PIPKIα and PI3K *in vitro* and *in vivo*.

(A) Endogenous IQGAP1, PIPKIα and p110α were immunoprecipitated from Hs578T cell lysates. Isotype immunoglobulin (IgG) was used as a control. Associated proteins were analyzed by immunoblotting with the indicated antibodies. (B) MDA-MB-231 cells grown on a slide glass were fixed and immunostained with endogenous molecules. Cells were photographed under 400X magnification. White arrow heads indicate regions that the three molecules are colocalized in the plasma membrane. (C) Hs578T cells were transfected with non-targeting or IQGAP1 siRNA for 48 h. PIPKIα was immunoprecipitated and associated p110α was analyzed by immunoblotting. (D) Hs578T cells were transfected with non-targeting or PIPKIα siRNA for 48 h. IQGAP1 was immunoprecipitated and associated p110α was analyzed by immunoblotting. (E) Hs578T cells were transfected with non-targeting or p110α siRNA for 48 h. IQGAP1 was immunoprecipitated and associated PIPKIα was analyzed by immunoblotting. (F) HEK293 cells were transfected with empty vector (Mock) or Myc-IQGAP1 for 48 h. P110α was immunoprecipitated and associated PIPKIα was analyzed by immunoblotting. (G) Immunoblots for (F) were quantified and the graph is shown as mean ± SD of three independent experiments. (H) 1 μM PIPKIα was incubated with 3 μM PI3K in the presence of 0-10 μM GST-IQGAP1-N. PIPKIα was pulled down with an anti- PIPKIα antibody immobilized on beads and associated proteins were analyzed by immunoblotting with the indicated antibodies. The experiments described above were performed independently at least four times.

Figure 3.02

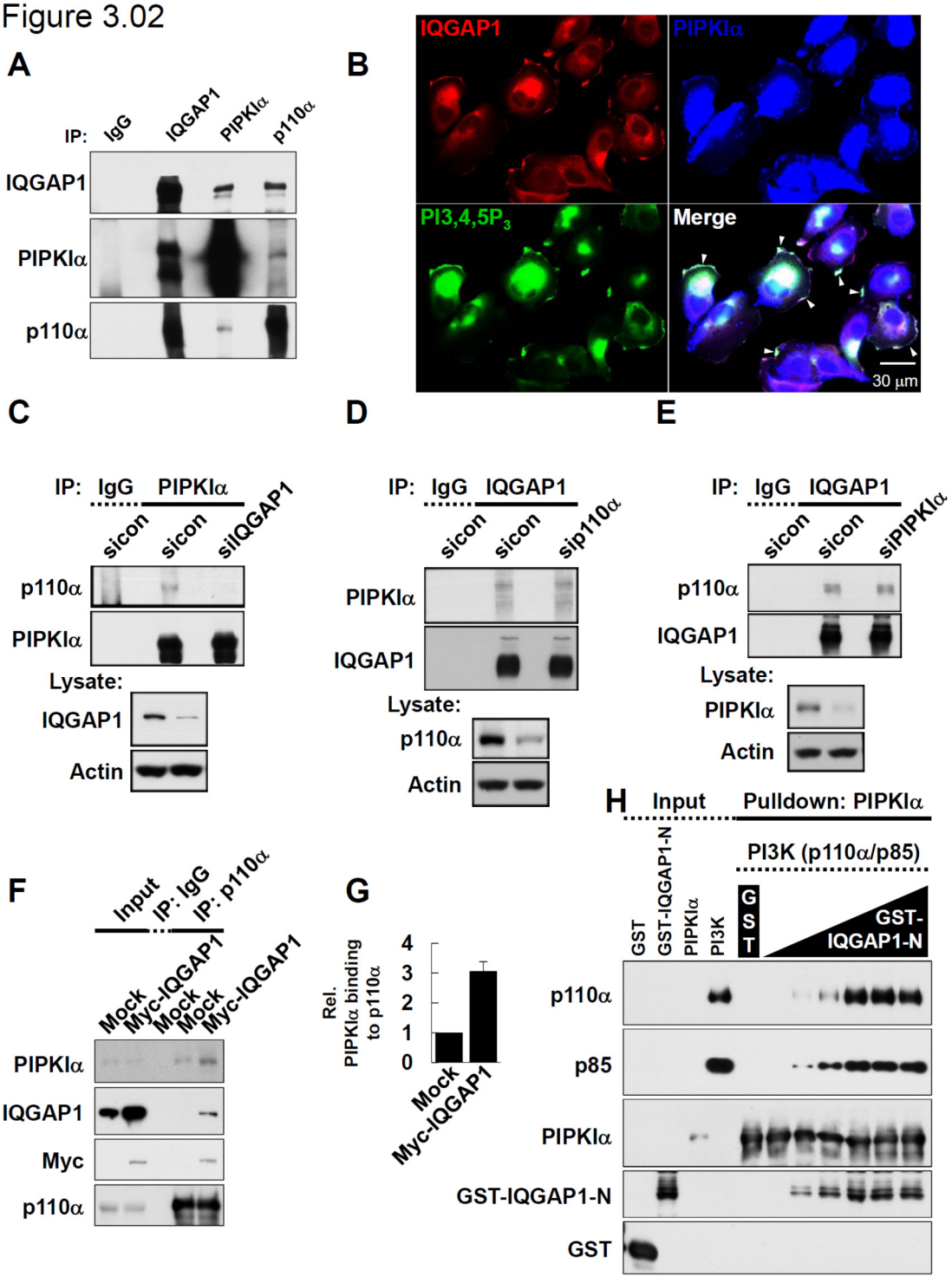
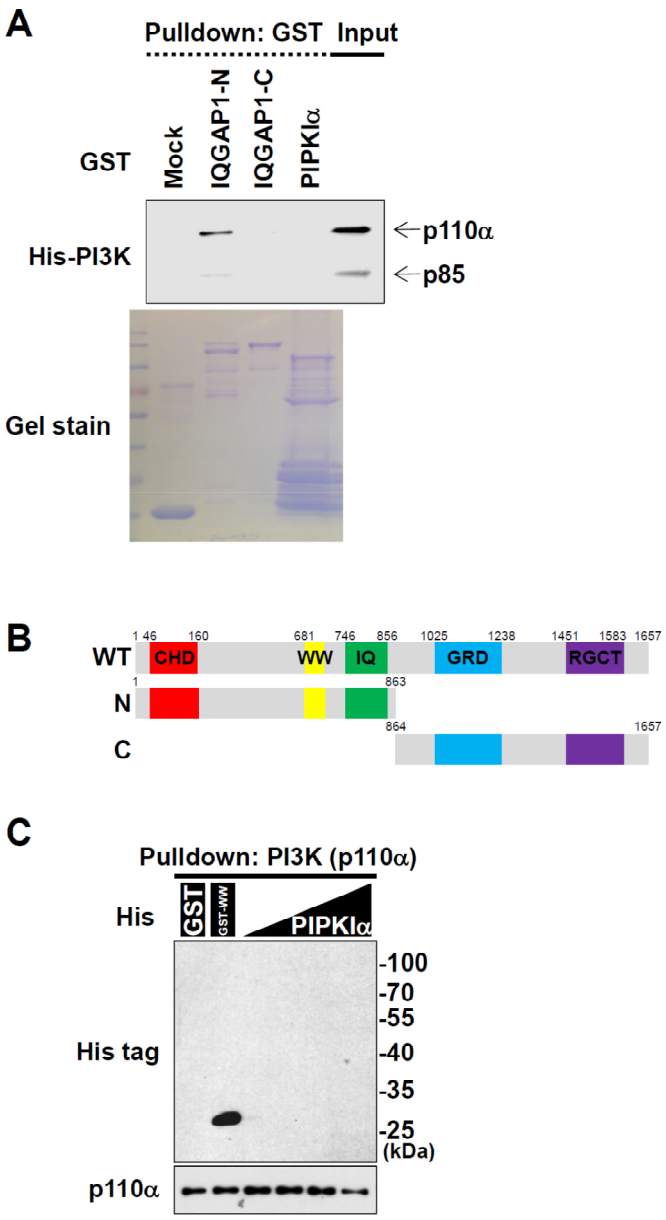

A
IP: IgG IQGAP1 PIPKIα p110α
IQGAP1
PIPKIα
p110α
B
IQGAP1
PIPKIα
PI3,4,5P3
Merge
30 μm
C
IP: IgG PIPKIα
sicon sicon siIQGAP1
p110α
PIPKIα
Lysate:
IQGAP1
Actin
D
IP: IgG IQGAP1
sicon sicon sip110α
PIPKIα
IQGAP1
Lysate:
p110α
Actin
E
IP: IgG IQGAP1
sicon sicon siPIPKIα
p110α
IQGAP1
Lysate:
PIPKIα
Actin
F
Input IP: IgG IP: p110α
Mock Myc-IQGAP1 Mock Mock Myc-IQGAP1
PIPKIα
IQGAP1
Myc
p110α
G
Rel. PIPKIα binding to p110α
4
3
2
1
0
Mock
Myc-IQGAP1
H
Input Pulldown: PIPKIα
PI3K (p110α/p85)
GST GST-IQGAP1-N PIPKIα PI3K
G S T
GST-IQGAP1-N
p110α
p85
PIPKIα
GST-IQGAP1-N
GST

Figure 3.03. PIPKIα and PI3K do not directly interact.

(A) GST-tagged IQGAP1 fragments and PIPKIα immobilized on glutathione beads were incubated with His-PI3K. Associated PI3K was analyzed by immunoblotting with an anti-His antibody. IQGAP1-N fragment directly binds to PI3K, whereas IQGAP1-C fragment or PIPKIα does not. (B) Schematic representation of IQGAP1-N and –C fragments used for *in vitro* binding assays. (C) His-tagged GST alone, GST-WW domain and PIPKIα were incubated with untagged PI3K. PI3K was immunoprecipitated with a p110α antibody and the associated proteins were analyzed by immunoblotting with an anti-His antibody.

Figure 3.03


A
Pulldown: GST
Input
GST
Mock
IQGAP1-N
IQGAP1-C
PIPKIα
His-PI3K
p110α
p85
Gel stain
B
WT
CHD
WW
IQ
GRD
RGCT
1
46
160
681
746
856
1025
1238
1451
1583
1657
N
1
863
C
864
1657
C
Pulldown: PI3K (p110α)
His
GST
GST-WW
PIPKIα
His tag
p110α
-100
-70
-55
-40
-35
-25
(kDa)

PI4,5P_2 produced by PIPKIα is channeled to PI3K for PI3,4,5P_3 generation: PIPKα and PI3K are spatially organized in a complex by IQGAP1 and this might coordinate enzymatic activities of the two phosphoinositide kinases. PIPKIα generates PI4,5P_2 from PI4P and class I PI3K generates PI3,4,5P_3 using PI4,5P_2 as substrate. To test how IQGAP1 mediates enzymatic reactions of PIPKα and PI3K, *in vitro* kinase assays were performed using PI4P as a substrate and the amounts of PI4,5P_2 and PI3,4,5P_3 generated were measured. As shown in Figure 3.04 A, PIPKIα alone or in combination with IQGAP1 fragments produced a negligible amount of PI3,4,5P_3, whereas PIPKIα in combination with PI3K produced detectable amount of PI3,4,5P_3, suggesting that the *in vitro* kinase assay system is functioning. In these conditions, addition of IQGAP1-N fragment significantly enhanced PI3,4,5P_3 production (approximately 4.1-fold), whereas IQGAP1-C fragment had no additional effect. These data indicate that IQGAP1-N fragment facilitates PI3,4,5P_3 production from PI4P by physically linking PIPKIα and PI3K. Interestingly, PI4,5P_2 production was significantly reduced by addition of IQGAP1-N fragment suggesting that PI4,5P_2 generated by PIPKIα is utilized by PI3K for PI3,4,5P_3 generation. As IQGAP1-N fragment does not enhance enzyme activity of PIPKIα (compare PI4,5P_2 levels in PIPKIα column to PIPKIα+IQGAP1-N column in Figure 3.04 A) and PI3K (Figure 3.04 B), these data collectively indicate that *in vitro* PI4,5P_2 produced by PIPKIα is channeled to PI3K for PI3,4,5P_3 generation in a complex with IQGAP1.

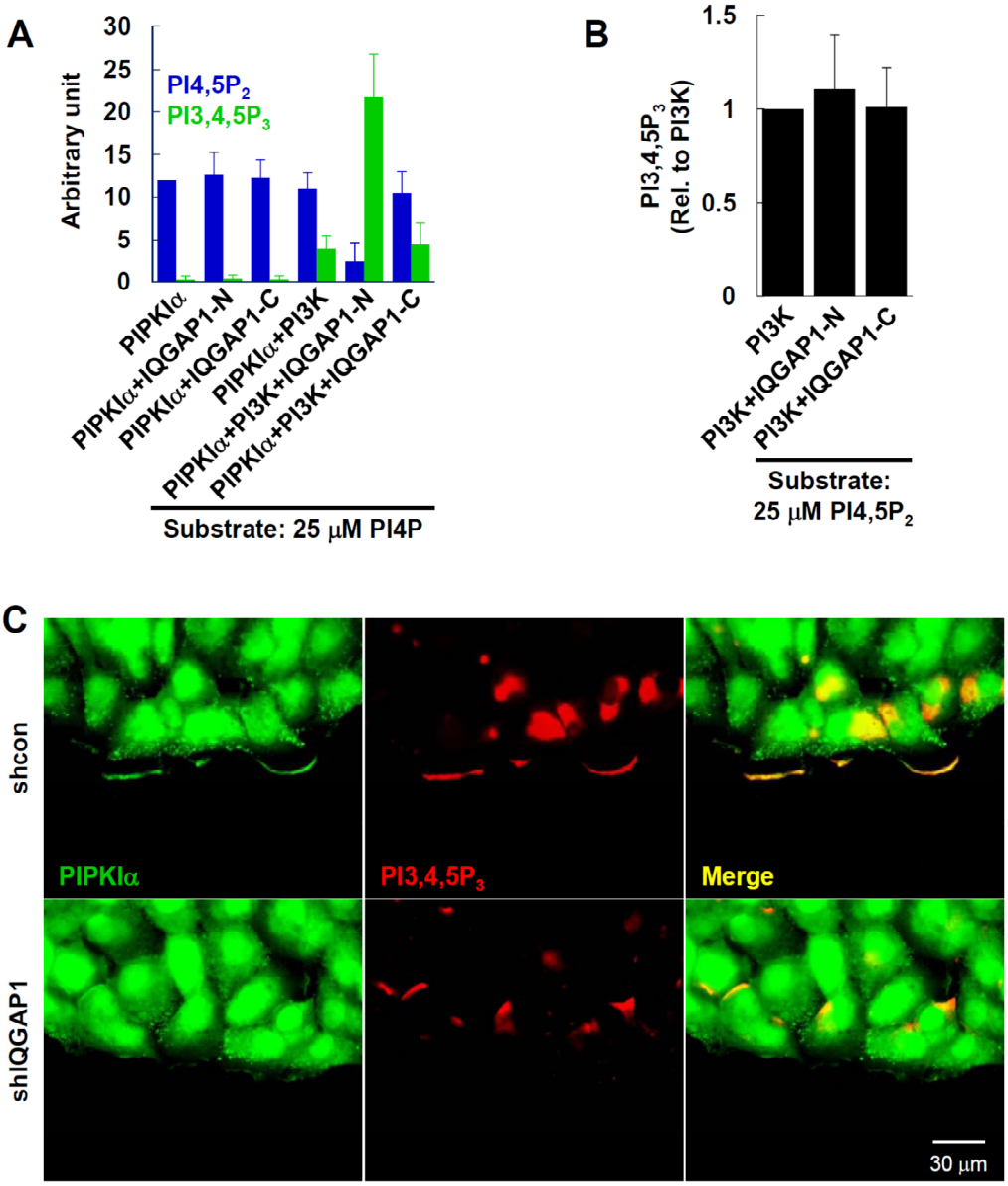
To test whether IQGAP1 mediates PI4,5P_2 generation coupling to PI3,4,5P_3 generation *in vivo*, an immunostaining approach was employed. PI4,5P_2 and PI3,4,5P_3 co-staining would be ideal for this purpose, but we found that co-staining is technically impossible as previously reported [31], whereas co-staining of PIPKIα and PI3,4,5P_3 provides discrete signals. As class I PI3K and PI3,4,5P_3 are reportedly confined to the leading edges of migrating cells [214], cells migrating into scratch wounds were fixed for immunostaining PIPKIα and PI3,4,5P_3. 3 h after scratching, control cells form larger

protrusions into the wounds, whereas IQGAP1 knockdown cells have much smaller protrusions (Figure 3.04 C). PIPKIα colocalizes with $PI3,4,5P_3$ at the leading edges in control cells. However, in IQGAP1 depleted cells, there is a dramatic reduction of PIPKIα signals at the leading edges, indicating IQGAP1 regulates PIPKIα localization at the leading edges. Interestingly, $PI3,4,5P_3$ signals at the leading edges are almost lost by IQGAP1 knockdown, whereas signals elsewhere are intact. As PI3K and $PI3,4,5P_3$ are indispensible for lamellipodium formation [49, 55, 143, 215], the smaller protrusions in IQGAP1 knockdown cells could be the result of defective PI3K activation at the leading edges. These data indicate that *de novo* $PI4,5P_2$ synthesis by PIPKIα colocalizes with $PI3,4,5P_3$ at the leading edges of migrating cells.

Figure 3.04. $PI4,5P_2$ produced by PIPKIα is channeled to PI3K for $PI3,4,5P_3$ generation.

(A) 0.02 μM PIPKIα, PI3K and IQGAP1-N or-C were mixed in the indicated combinations and used for *in vitro* kinase assays with 25 μM PI4P as a substrate. $PI4,5P_2$ and $PI3,4,5P_3$ generated were measured by kits from Echelon Biosciences. The graph is shown as mean ± SD of four independent experiments. (B) $PI3,4,5P_3$ generation from 25 μM $PI4,5P_2$ by PI3K and IQGAP1 fragments was measured. The graph is shown as mean ± SD of three independent experiments. (C) Cells expressing empty vector (shcon) or shRNA against IQGAP1 (shIQGAP1) were grown to confluence. Cells were wounded and fixed 3 h later, followed by immunostaining for PIPKIα and $PI3,4,5P_3$. Cells were photographed at 400X magnification. All the experiments described above were performed independently at least three times.

Figure 3.04


A
Arbitrary unit
30
25
20
15
10
5
0
PI4,5P2
PI3,4,5P3
PIPKIα
PIPKIα+IQGAP1-N
PIPKIα+IQGAP1-C
PIPKIα+PI3K
PIPKIα+PI3K+IQGAP1-N
PIPKIα+PI3K+IQGAP1-C
Substrate: 25 μM PI4P
B
PI3,4,5P3 (Rel. to PI3K)
1.5
1
0.5
0
PI3K
PI3K+IQGAP1-N
PI3K+IQGAP1-C
Substrate: 25 μM PI4,5P2
C
shcon
shIQGAP1
PIPKIα
PI3,4,5P3
Merge
30 μm

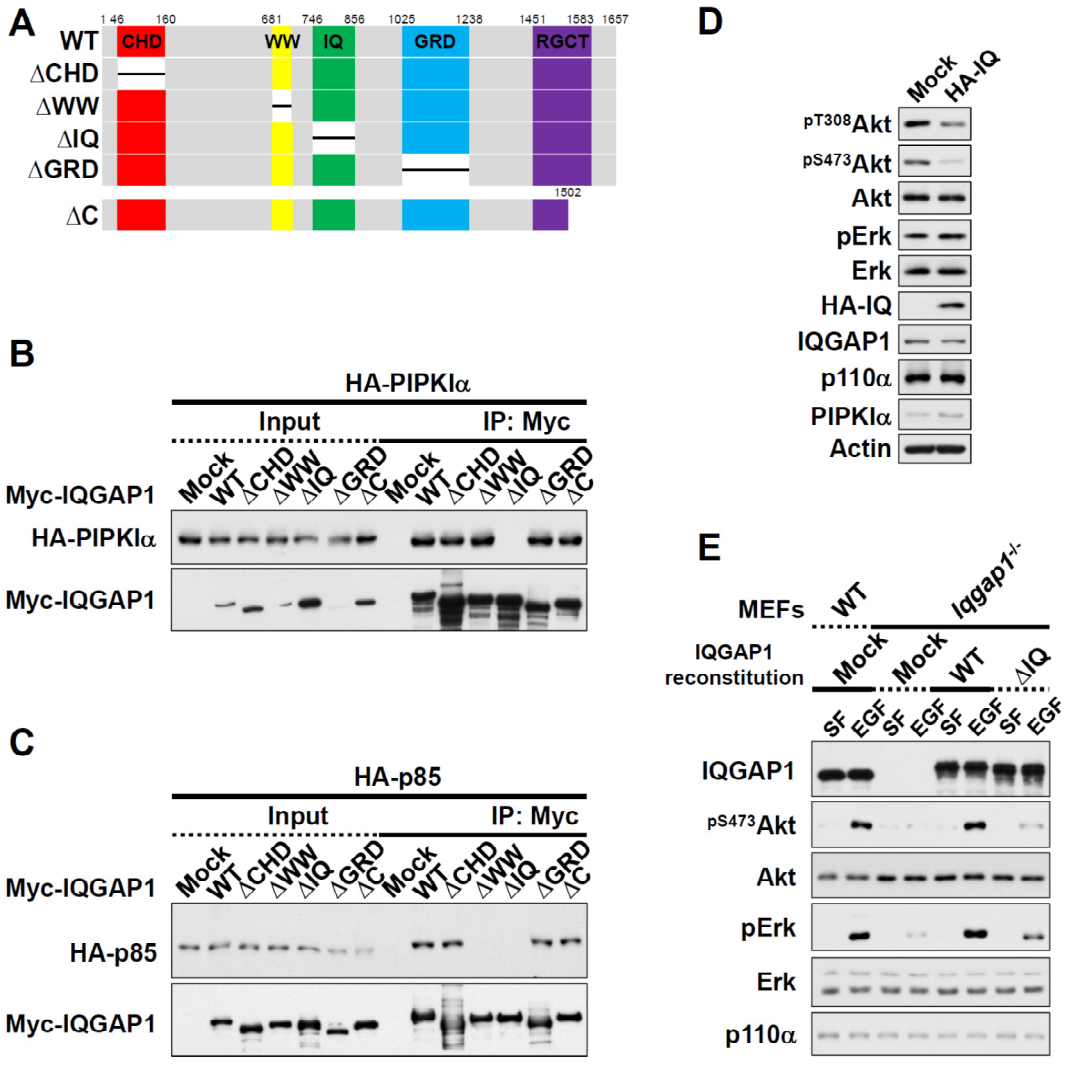
The WW and IQ domains mediate IQGAP1 interactions with PIPKIα and PI3K: We next tested the PIPKIα and class I PI3K binding sites on IQGAP1. IQGAP1 contains five different domains that mediate protein-protein interactions (Figure 3.05 A) [150, 152, 172]. Wild type or truncation mutants of each domain were used to define the binding sites by immunoprecipitation. Deletion of the IQ domain dramatically reduced PIPKIα coimmunoprecipitation with IQGAP1 suggesting the IQ domain is required for the interaction (Figure 3.05 B). Also, the IQ domain alone is sufficient to interact with PIPKIα ([10] and data not shown). For IQGAP1 interaction with class I PI3K, the WW and IQ domain are required (Figure 3.05 C) and the WW domain is sufficient to interact with PI3K (Figure 3.03 B).

As the IQ domain is responsible for IQGAP1 interaction with PIPKIα and class I PI3K, we reasoned that ectopic expression of the IQ domain alone might inhibit the PI3K-Akt pathway by competition with endogenous IQGAP1 for PIPKIα and class I PI3K binding. To test this, the IQ domain was stably expressed in Hs578T breast cancer cells and Akt activation was measured by immunoblotting phosphorylated Akt at Thr308 and Ser473 residues [213]. As shown in Figure 3.05 D, overexpression of the IQ domain reduced Akt phosphorylation suggesting that PIPKIα and PI3K interaction with IQGAP1 is required for Akt activation. Consistently, the IQ domain is required for EGF-stimulated Akt activation (Figure 3.05 E).

Figure 3.05. The WW and IQ domains are required for IQGAP1 interaction with PIPKIα and PI3K.

(A) Schematic representation of IQGAP1 domains and IQGAP1 constructs used. (B) HA-PIPKIα and Myc-IQGAP1 truncation constructs were co-transfected in HEK293 cells for 48 h. Myc-IQGAP1 proteins were immunoprecipitated with an anti-Myc antibody and associated PIPKIα was analyzed by immunoblotting with an anti-HA antibody. (C) HA-p85 subunit of PI3K and Myc-IQGAP1 truncation constructs were co-transfected in HEK293 cells for 48 h. Myc-IQGAP1 proteins were immunoprecipitated with an anti-Myc antibody and associated p85 was analyzed by immunoblotting with an anti-HA antibody. (D) Empty vector (Mock) and HA-IQ domain was stably expressed in Hs578T cells. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) *Iqgap1* knockout mouse embryonic fibroblasts (MEFs) were reconstituted with human wild type or the ΔIQ mutant. Confluent cells were treated with 10 ng/ml EGF or vehicle control for 30 m. SF, serum free. Cell lysates were examined by immunoblotting with the indicated antibodies.

Figure 3.05


A
WT
ΔCHD
ΔWW
ΔIQ
ΔGRD
ΔC
CHD
WW
IQ
GRD
RGCT
1502
B
HA-PIPKIα
Input
IP: Myc
Mock
WT
ΔCHD
ΔWW
ΔIQ
ΔGRD
ΔC
Myc-IQGAP1
HA-PIPKIα
Myc-IQGAP1
C
HA-p85
Input
IP: Myc
Myc-IQGAP1
HA-p85
Myc-IQGAP1
D
Mock
HA-IQ
pT308Akt
pS473Akt
Akt
pErk
Erk
HA-IQ
IQGAP1
p110α
PIPKIα
Actin
E
MEFs
WT
Iqgap1-/-
IQGAP1 reconstitution
Mock
Mock
WT
ΔIQ
SF
EGF
IQGAP1
pS473Akt
Akt
pErk
Erk
p110α

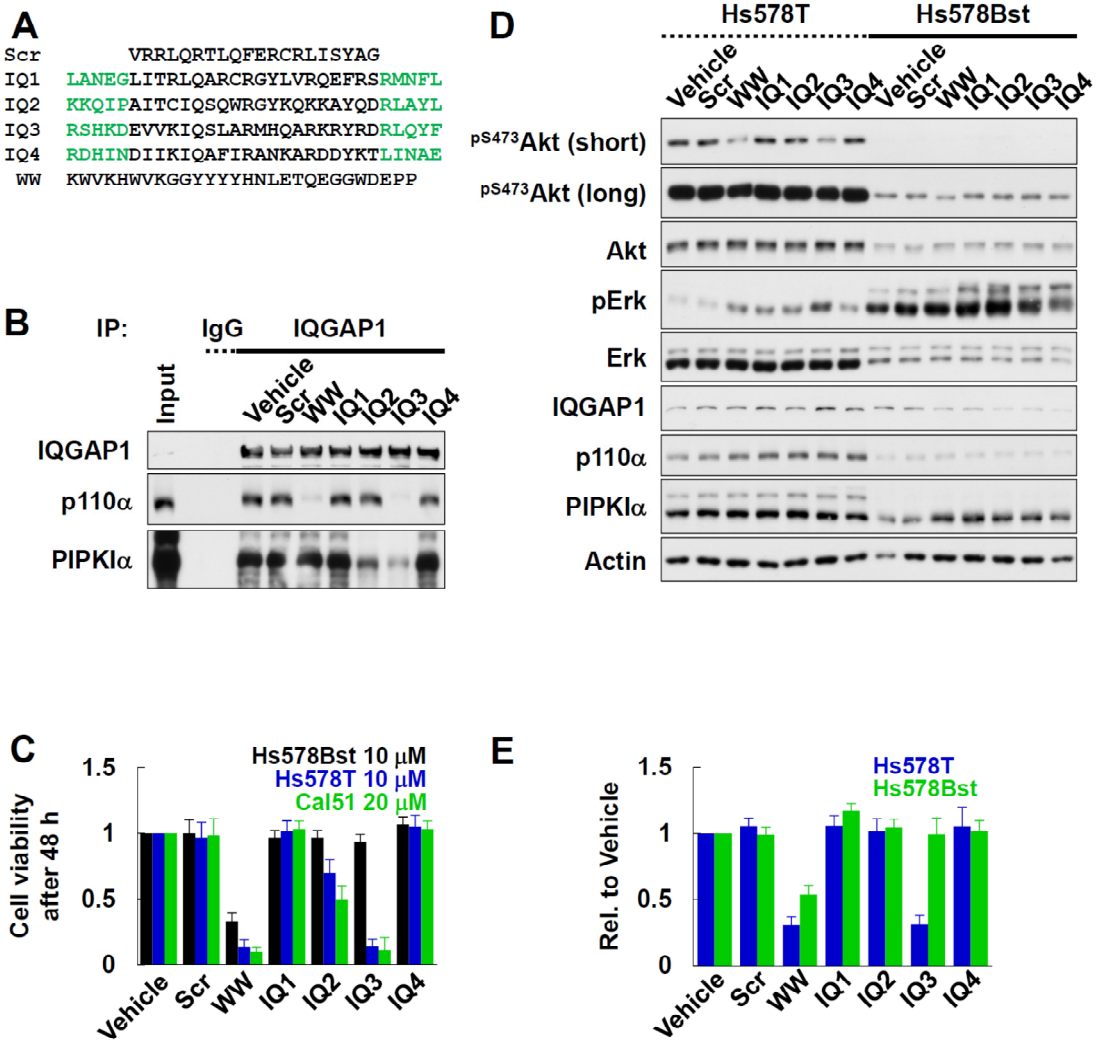
Cell permeable peptides corresponding to the WW domain and IQ motifs of IQGAP1 inhibit the PI3K-Akt pathway: The WW and IQ domains of IQGAP1 consist of approximately 32 and 120 amino acids, respectively [150]. The IQ domain of IQGAP1 contains 4 structurally conserved IQ motifs [185]. To narrow down the PIPKIα and class I PI3K binding sites on the IQ domain, peptides corresponding to each IQ motif (20 amino acids in length) and the WW domain (28 amino acids) were synthesized with the goal of blocking endogenous binding of PIPKIα and class I PI3K (Figure 3.06 A). The peptides are cell and tissue permeable, by fusing eight arginine residues at the N-termini, and this approach was validated previously [216]. Hs578T cells were treated with these peptides, and PIPKIα and class I PI3K interaction with IQGAP1 was tested by immunoprecipitation (Figure 3.06 B). The WW peptide reduced p110α coimmunoprecipitation with IQGAP1 confirming the immunoprecipitation result with the WW domain delition mutant in Figure 3.05 C. The PIPKIα coimmunoprecipitation with IQGAP1 was largely reduced by the IQ3 peptide and slightly by the IQ2 peptide (Figure 3.06 B). The PI3K-Akt pathway regulates cell survival in many cell types [202, 208, 217]. The function of the peptides in cell survival and Akt activation were tested. 10 to 20 μM peptides were treated in three different breast cell lines for 48 h and viable cell number was counted (Figure 3.06 C). Hs578T and Cal51 are triple negative breast cancer cell lines and [218] and Hs578Bst cell line, a counterpart of Hs578T, is derived from normal breast tissue of a breast cancer patient [219]. The WW peptide dramatically suppressed cell viability in the all three cell lines tested, whereas the IQ3 peptide selectively induced cell death in the two triple negative cell lines. The IQ2 peptide also had a minor effect on cell survival (Figure 3.06 C). To test the peptides target the PI3K-Akt pathway, two Hs578 counterpart cells were treated with 10 μM peptides for 24 h and Akt phosphorylation was examined by immunoblotting (Figure 3.06 D). The WW peptide significantly reduced Akt phosphorylation in both cell lines, whereas the IQ3 peptides only significantly reduced Akt phosphorylation in Hs578T cell line (Figure 3.06 E). MAPK pathway

is also important in regulation of cell survival and proliferation [220, 221] and IQGAP1 is implicated in the pathway [147, 150, 166, 216]. However, treatment of the peptides did not reduce Erk phosphorylation signifying a specific role of the peptides in the PI3K-Akt pathway.

Figure 3.06. Cell permeable peptides corresponding to the WW domain and IQ motifs target the PI3K-Akt pathway.

(A) The WW domain and IQ motif amino acid sequences. 28 amino acids from the WW domain and 20 amino acids (in black) from each IQ motifs were used in the cell permeable peptides. Scrambled (Scr) peptide has scrambled sequence of the IQ1 peptide. (B) Confluent Hs578T cells were incubated with the indicated peptides or vehicle control for 24 h. IQGAP1 was immunoprecipitated and associated PIPKIα and p110α were analyzed by immunoblotting. (C) Confluent Hs578Bst, Hs578T and Cal51 cells were incubated with the peptides in the indicated concentration for 48 h. Trypan blue negative viable cell numbers were counted using a hemocytometer. The graph is shown as mean ± SD of three independent experiments. (D) Confluent Hs578Bst and Hs578T cells were incubated with the peptides for 24 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) ^{pS473}Akt immunoblots were quantified and the graph is shown as mean ± SD of three independent experiments. The experiments described above were performed independently at least four times.

Figure 3.06


A
Scr VRRLQRTLQFERCRLISYAG
IQ1 LANEGLITRLQARCRGYLVRQEFRSRMNFL
IQ2 KKQIPAITCIQSQWRGYKQKKAYQDRLAYL
IQ3 RSHKDEVVKIQSLARMHQARKRYRDRLQYF
IQ4 RDHINDIIKIQAFIRANKARDDYKTLINAE
WW KWVKHWVKGGYYYYHNLETQEGGWDEPP
B
IP:
IgG
IQGAP1
Input
Vehicle
Scr
WW
IQ1
IQ2
IQ3
IQ4
IQGAP1
p110α
PIPKIα
D
Hs578T
Hs578Bst
Vehicle
Scr
WW
IQ1
IQ2
IQ3
IQ4
pS473Akt (short)
pS473Akt (long)
Akt
pErk
Erk
IQGAP1
p110α
PIPKIα
Actin
C
Cell viability after 48 h
1.5
1
0.5
0
Hs578Bst 10 μM
Hs578T 10 μM
Cal51 20 μM
Vehicle
Scr
WW
IQ1
IQ2
IQ3
IQ4
E
Rel. to Vehicle
1.5
1
0.5
0
Hs578T
Hs578Bst
Vehicle
Scr
WW
IQ1
IQ2
IQ3
IQ4

Discussion:

In the present study, we reveal that $PI4,5P_2$ generation is linked to $PI3,4,5P_3$ generation by spatially organizing $PI4,5P_2$ and $PI3,4,5P_3$ producing enzymes in a complex via the scaffold IQGAP1. In response to receptor activation, such as integrins and growth factor receptors, PIPKIα, class I PI3K and IQGAP1 translocate to the plasma membrane [10, 44, 202, 222]. There, PIPKIα and class I PI3K are tethered in a complex by binding to the WW and IQ domains of IQGAP1. Then $PI4,5P_2$ generated by PIPKIα is channeled to PI3K for efficient $PI3,4,5P_3$ production. $PI3,4,5P_3$ lipid product in turn activates signaling molecules such as PDK1 and Akt and regulates cell survival, proliferation, migration and metabolism (Figure 3.07).

The PI3K-Akt pathway is activated by membrane receptors such as G protein-coupled receptors, integrins and growth factor receptors [202, 208, 213, 223]. As shown in Figure 3.08, PIPKIα and IQGAP1 are required for activation of the PI3K-Akt pathway downstream of both integrins and growth factor receptors. Importantly, knockdown of PIPKIα and IQGAP1 has a more profound effect on integrin-mediated Akt activation compared to growth factor-mediated (Figure 3.08 A-D), suggesting that the PIPKIα-PI3K-IQGAP1 signaling complex preferentially function downstream of integrin signaling. This is supported by the observation that there is more efficient formation of the PIPKIα-PI3K-IQGAP1 signaling complex by integrin signaling compared to growth factor stimulation (Figure 3.08 E-F). Integrins and growth factors can activate distinct signaling pathways to mediate these differential effects. Integrin clustering induces autophosphorylation of focal adhesion kinase (FAK) at Tyr397, which recruits class I PI3K to the activated integrin sites on the plasma membrane [222]. As IQGAP1 interacts with FAK [175], it is possible that IQGAP1 recruits PIPKIα and PI3K to the sites of integrin activation. Consistently, targeting of PIPKI isoforms to focal adhesions is reported [32].

Since its initial discovery, a plethora of upstream regulators and downstream effectors of class I PI3K are identified [202, 208, 217]. Although PIPKIs generate PI4,5P_2, a substrate for PI3K for PI3,4,5P_3 synthesis, the roles of PIPKIs in PI3,4,5P_3 synthesis have received little attention. According to early studies, cellular PI4,5P_2 concentration was estimated to be three orders of magnitude higher than that of PI3,4,5P_3 [2, 16]. This lead scientists to believe that, in cells, PI3K readily utilizes abundant PI4,5P_2 pool for PI3,4,5,P_3 generation. However, this notion is challenged by discoveries of PI4,5P_2 sequestering proteins. These proteins are present in a comparable concentration of PI4,5P_2 and bind to PI4,5P_2 with relatively strong affinity (the dissociation constants are within 1-100 nM range) [5]. Thus, the majority of cellular PI4,5P_2 is sequestered from enzymatic modification, and *de novo* synthesis of PI4,5P_2 is required for use as substrate. For example, the sustained production of diacylglycerol and inositol 1,4,5-trisphosphate from breakdown of PI4,5P_2 by PLC requires continuous phosphorylation of phosphatidylinositol to PI4P by PI4K and PI4P to PI4,5P_2 by PIPKI, as enzymatically labile PI4,5P_2 is present in a limited concentration in cells [205-207]. This link between PI4,5P_2 generation by PIPKI and its enzymatic breakdown by PLC is further supported by the physical association of the two enzymes in a complex [186]. However, the physical and functional association of PIPKI and class I PI3K has not been addressed. In this study, we found that PIPKIα forms a complex with class I PI3K via IQGAP1 (Figure 3.01-3.03). Importantly, the physical association assures that PI4,5P_2 produced by PIPKIα is channeled to class I PI3K for PI3,4,5P_3 synthesis (Figure 3.04).

Spatial and temporal organization of catalytically sequential enzymes provides efficient means for signal synthesis and transmission [15]. Often, this organization is mediated by scaffold proteins. IQGAP1 was previously characterized as a scaffold for MAPK pathway. IQGAP1 directly binds to MAPK components Ras, Raf, MEK and Erk and regulates growth factor receptor signaling [164-166, 169, 170, 224]. In this study, we revealed that PIPKIα binds to the IQ domain and class I PI3K binds to the WW and IQ domains. This

raises the possibility that PIPKIα competes with PI3K for the IQ domain binding. However, our observations demonstrate that knockdown of class I PI3K does not alter PIPKIα binding to IQGAP1, nor knockdown of PIPKIα alters PI3K binding to IQGAP1 (Figure 3.02). Also, overexpression of PIPKIα does not alter class I PI3K binding to IQGAP1 (data not shown). Collectively, these data suggest that PIPKIα and class I PI3K are present in a complex with IQGAP1 by binding to the IQ and WW domains, respectively. Another remaining question is whether PIPKIα and class I PI3K are close enough to channel $PI4,5P_2$ in the IQGAP1-mediated complex. Although the WW domain and IQ3 motif of the IQ domain, which is responsible for the PIPKIα binding, are around 90 amino acids apart in the primary structure, the distance in the tertiary structure is unknown as the structure of the IQGAP1 WW and IQ domains are unsolved. However, whether it is close enough for the $PI4,5P_2$ channeling could be inferred from MAPK pathway. It is known that MEK directly binds to the IQ domain and Erk binds to the WW domain of IQGAP1 [165, 166]. As IQGAP1 mediates MEK phosphorylation on Erk [166, 169, 216], the proximity of PIPIKα and class I PI3K in complex with IQGAP1 may enable sequential phosphorylation of PI4P to $PI3,4,5P_3$ (Figure 3.04).

Alteration of the PI3K-Akt pathway is linked to many human diseases including cancer [225, 226]. IQGAP1 is also implicated in many cancers [152, 172] and overexpression of IQGAP1 in breast tumor tissues and breast cancer cell lines is related to enhanced tumorigenesis [173, 184]. The PIPKIα gene is amplified in a subset of breast cancer cell lines [227, 228]. To test the physiological relevance of the PIPKα-PI3K-IQGAP1 complex in breast cancer, specific peptide inhibitors were designed to block the formation of this complex. As shown in Figure 3.06, the WW and IQ3 peptides effectively block Akt activation and breast cancer cell proliferation. Notably, unlike the WW peptide, the IQ3 peptide selectively kills breast cancer cells likely by attenuating Akt activation. This selectivity could in part result from the accelerated activation of the PI3K-Akt pathway in the tested breast cancer cells. Often, cancer cells are addicted to a certain oncogenic pathway

for their survival and blockade of the pathway induces cancer cell death [229-231]. Another interesting observation is that the peptides only exert their effect when cells are confluent in the tissue culture dish. Subconfluent cells grow indistinguishably in the absence or presence of the peptides (data not shown). Although the PI3K-Akt pathway has roles in cell proliferation [208, 225], under the conditions tested, its major role might be to maintain cell survival. Signaling pathways that govern cell proliferation such as MAPK pathway might overcome the need for PI3K-Akt when cancer cells are rapidly growing at subconfluent density (note that Erk phosphorylation is not affected by the peptide in Figure 3.06). When cells reach confluence, proliferation might be suppressed, and blockade of the PI3K-Akt pathway might have a profound effect in cancer cell survival.

In summary, in this study we revealed a previously unrecognized mechanism for $PI3,4,5P_3$ generation by class I PI3K. This is achieved by spatiotemporal regulation via a complex scaffolded by IQGAP1, where class I PI3K efficiently generates $PI3,4,5P_3$ using $PI4,5P_2$ channeled from PIPKIα. Also, we provide evidence that blockade of this signaling platform could be used for therapeutics, such as cancers that have altered PI3K-Akt signaling.

Figure 3.07. Model of IQGAP1-mediated phosphoinositide synthesis channeling

IQGAP1 connects PIPKIα and class I PI3K through interactions with its IQ and WW domains respectively. In this complex, $PI4,5P_2$ produced by PIPKIα can be used directly by class I PI3K for $PI3,4,5P_3$ generation.

Figure 3.07

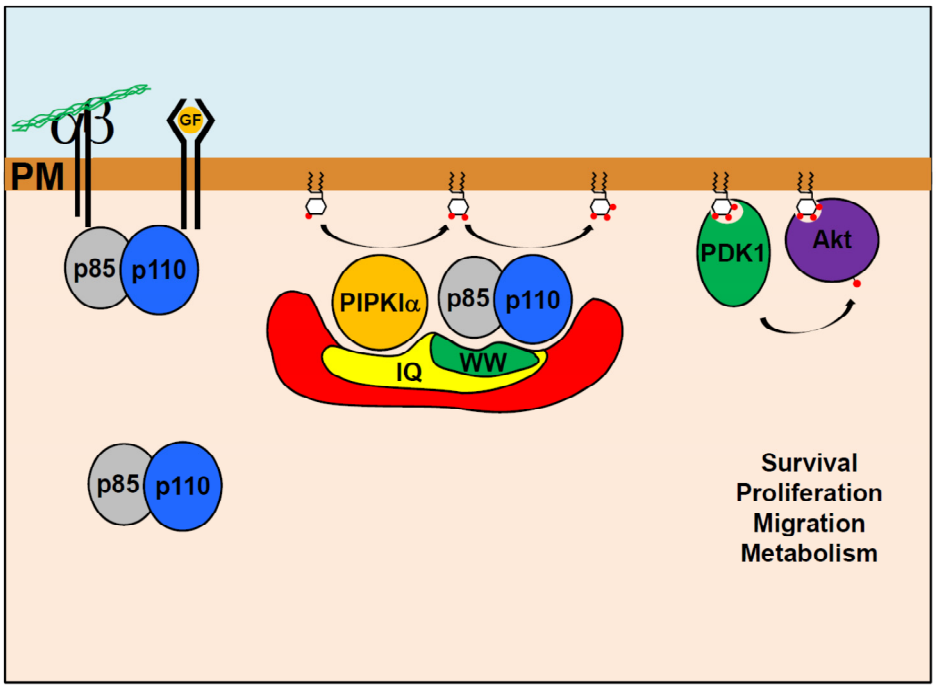
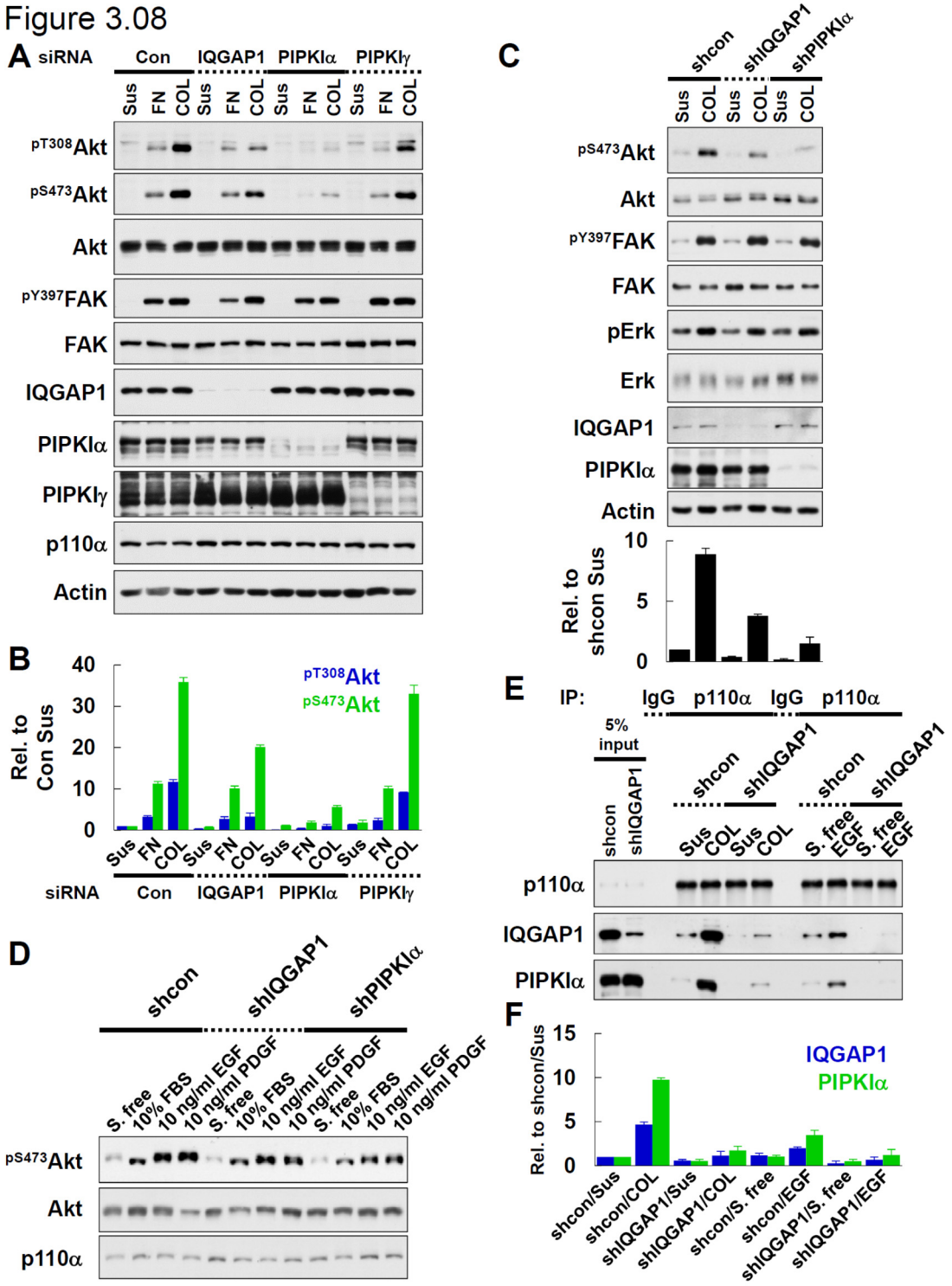

PM
GF
p85
p110
PIPKIα
p85
p110
IQ
WW
PDK1
Akt
p85
p110
Survival
Proliferation
Migration
Metabolism

Figure 3.08. IQGAP1 and PIPIKα are required for Akt activation downstream of integrin receptor.

(A) MDA-MB-231 cells were transiently transfected with the indicated siRNAs for 48 h. Cells were plated on 10 μg/ml fibronectin (FN)- or collagen (COL)-coated dishes, or maintained in suspension (Sus) for 45 m. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (B) Immunoblots for (A) were quantified and the graph is shown as mean ± SD of three independent experiments. (C) IQGAP1 and PIPKIα expression were stably knocked down in Hs578T cells. Cells were plated on 10 μg/ml collagen (COL)-coated dishes or maintained in suspension (Sus) for 1 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies (top). Immunoblots were quantified and the graph is shown as mean ± SD of four independent experiments (bottom). (D) Serum starved Hs578T stable cell lines were treated with indicated cytokines or remained in serum free media (S. free) for 30 m. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) MDA-MB-231 cells stably expressing the indicated shRNAs were as above. PI3K p110α was immunoprecipitated and associated IQGAP1 and PIPKIa were analyzed by immunoblotting. 100 ng/ml EGF was used for these experiments. The experiments described above were performed independently at least four times.

Figure 3.08


A
siRNA
Con
IQGAP1
PIPKIα
PIPKIγ
Sus
FN
COL
pT308Akt
pS473Akt
Akt
pY397FAK
FAK
IQGAP1
PIPKIα
PIPKIγ
p110α
Actin
B
Rel. to Con Sus
pT308Akt
pS473Akt
siRNA
C
shcon
shIQGAP1
shPIPKIα
pS473Akt
Akt
pY397FAK
FAK
pErk
Erk
IQGAP1
PIPKIα
Actin
Rel. to shcon Sus
D
shcon
shIQGAP1
shPIPKIα
S. free
10% FBS
10 ng/ml EGF
10 ng/ml PDGF
pS473Akt
Akt
p110α
E
IP:
IgG
p110α
5% input
shcon
shIQGAP1
S. free
EGF
p110α
IQGAP1
PIPKIα
F
Rel. to shcon/Sus
IQGAP1
PIPKIα
shcon/Sus
shcon/COL
shIQGAP1/Sus
shIQGAP1/COL
shcon/S. free
shcon/EGF
shIQGAP1/S. free
shIQGAP1/EGF

Materials and Methods:

Cell culture and constructs: MDA-MB-231, HEK 293, Hs578Bst, Hs578T, Cal51 and MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco). The constructs used for this work are described previously [10, 25].

Stable cell line generation: To generate stable MDA-MB-231 and Hs578T cell lines expressing shRNAs against human IQGAP1, PIPKIα or vector control, pLL3.7 vector-based lentiviral delivery system was used. pLL3.7 lentiviral vector was cloned to express shRNAs against IQGAP1 (either 5'-GGAAAGCUCUGGCAAUUUAUU-3' or 5'-GAACGUGGCUUAUGAGUACUU-3') [173] and PIPKIα (either 5'-AAGTTGGAGCACTCTTGG-3' or 5'-CACATTATCCCTACCTTA-3'). After infection, cells were passaged at least 5 times before sorting GFP-positive cells in a cell sorter to select cells expressing shRNAs as pLL3.7 vector also express GFP bicistronically [33]. For generation of stable in MEFs, cells were infected with retrovirus for 24 h. Then, cells expressing GFP-IQGAP1 were first selected for GFP expression, and then further sorted by expression level.

Antibodies and siRNAs: Monoclonal antibodies against IQGAP1, IQGAP2, p110α, p85, FAK, Myc-tag, GST-tag, His-tag (Millipore), Akt, ^{pT308}Akt, ^{pS473}Akt, p110α, p110β, p85, Erk, pErk (Cell Signaling Technology), HA-tag (Covence Biotechnology), ^{pY397}FAK (Invitrogen), actin (MP Biomedicals), PI3,4,5P_3 (Echelon Biosciences) and polyclonal antibody against IQGAP1 and IQGAP2 (Santa Cruz Biotechnology) were used for this study. Polyclonal and monoclonal antibodies against total and PIPKIα and PIPKIγ were produced as described previously [26, 43]. Pooled siRNAs used in the study were obtained from Dharmacon.

IP and immunoblotting: Cells were lysed in a buffer containing 1% Brij58, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na_3VO_4, 1 mM Na_2MoO_4 and protease inhibitors. Protein concentration of lysates was measured by the BCA method

(Pierce) and equal amounts of protein were used for further analysis. For IP, 0.5 to 1 mg of proteins were incubated with 1 μg of antibodies at 4°C for 8 h and then incubated with a 50% slurry of Protein G Sepharose (GE Life Sciences) for another 2 h. After washing 5X with lysis buffer, the protein complex was eluted with SDS sample buffer. For immunoblotting, 5 to 20 μg of proteins loaded. After developing immunoblots, the film was scanned using a transmitted light scanner (resolution = 600 dpi). Protein bands were quantified using ImageJ, and statistical analysis of the data was performed with Microsoft Excel. The statistical analysis was performed using data from at least three independent experiments.

***In vitro* binding assay**: Recombinant proteins were expressed in BL21 *E. coli* strain. GST-tagged proteins were then purified with GST Sepharose 4B (GE Life Sciences) and His-tagged proteins were purified with His-Bind Resin (Novagen). Recombinant PI3K (p110α and p85α) proteins were purchased from Echelon Biosciences. GST-tagged proteins were incubated with glutathione beads before binding assays. The binding assay was performed in the lysis buffer used for IP by adding 10 nM to 5 μM of His-tagged proteins and 20 μl of GST-tagged protein bound glutathione beads. After incubation for 1h at 25°C, unbound proteins were washed out and the protein complex was analyzed by immunoblotting. For the triple binding assay with recombinant GST-IQGAP1, His-PIPKIα and PI3K (His-p110α and untagged p85α), PIPKIα was pulled down with an anti-PIPKIα antibody pre-bound on Protein G Sepharose beads.

Immunofluorescence microscopy: Glass coverslips were coated with 10 ng/ml collagen, fibronectin or 10% serum before seeding cells. Cells were grown on coverslips placed inside 6-well plates until experimental manipulation. Immunostaining was performed as previously described [30, 31] with modifications. Briefly, cells grown in medium were rapidly fixed by adding equal volume of 8% paraformaldehyde and 0.5% glutaraldehyde to medium for 15 m at room temperature. After 30 m wash with PBS containing 50 mM NH_4Cl, cells were

permeabilized and blocked with a solution of buffer A (20 mM PIPES, pH 6.8, 135 mM NaCl, 5 mM KCl) containing 0.5% saponin and 5 volume % FBS for 45 m at room temperature. Primary antibodies were incubated in a solution of buffer A containing 0.1% saponin and 5 volume % of FBS for 12 h at 4°C. 2-4 μg/ml primary antibodies were used. After 30 m wash with buffer A, fluorophore-conjugated secondary antibodies were incubated in a solution of buffer A containing 0.1% saponin and 5 volume % of FBS for 1 h at room temperature. Then, cells were washed with buffer A for 45 m at room temperature before post-fixation with 2% paraformaldehyde and 0.125% glutaraldehyde % for 10 m at room temperature. Coverslips were washed 5 times with PBS containing 50 mM NH_4Cl and once with distilled water. Fluorescence microscopy was performed using a 40 or 60x plan-fluor objective on a Nikon Eclipse TE2000U equipped with a Photometrics CoolSNAP ES CCD camera. Images were captured using MetaMorph v6.3 (Molecular Devices). Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

Cellular phosphoinositide measurement: Lipid extraction and cellular phosphoinositide measurement were performed a kit Echelon Biosciences according to manufacturer's instructions. Briefly, cells were lysed in icecold trichloroacetic acid (TCA, 0.5 M). Pellets were washed in 5%TCA/1 mM EDTA and neutral lipids were extracted with MeOH:$CHCl_3$ (2:1) and discarded. Acidic lipids were extracted with MeOH:$CHCl_3$:HCl (80:40:1), recovered by phase-split, dried and resuspended. PI4,5P_2 and PI3,4,5P_3 levels were quantified by competitive ELISA (Echelon Biosciences).

***In vitro* phosphoinositide kinase assay**: *In vitro* phosphoinositide kinase assays were performed as described [25, 232] with modification. Briefly, 0.02-0.1 μM of recombinant IQGAP1, PIPKIα and PI3K were incubated with 250 μM phosphoinositide liposomes in a kinase buffer (50 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM EDTA). The liposomes containing 10 molar % of phosphoinositides were generated as described [10]. Kinase

reaction was initiated by adding 20 μM ATP and incubated for 30 m at 25°C. 50 μl reaction was terminated by adding 100 μl 1M HCl and 200 μl $CHCl_3$:MeOH (1:1). Extracted lipids were dried and PI4,5P_2 and PI3,4,5P_3 levels were quantified by competitive ELISA (Echelon Biosciences) as above.

Peptide production and treatment in cells: Octo-arginine conjugated WW and IQ motifs peptides were synthesized by Genscript. Peptides were resuspended in water to a 2.5–5 mM working solution. Peptide was added directly into the medium of tissue culture at the concentrations indicated.

Chapter 4

Conclusions and Future Directions

Conclusions and Significance:

As depicted in the central hypothesis (Figure 1.01), the localization and function of PIPKIs are regulated by their interactors. In an effort to reveal previously unknown interactors of PIPKIγ, PIPKIγ immunoprecipitates were analyzed by a proteomic method, and IQGAP1 was identified as a novel interactor (Figure 2.01). Interestingly IQGAP1 interacts not only with PIPKIγ, but also PIPKIα with a seemingly tighter binding affinity (Figure 3.01). IQGAP1 interaction with PIPKIγ recruits IQGAP1 to leading edges of migrating cells (Figure 2.09). Although IQGAP1 binding to other proteins, including Rac1, Cdc42 and mDia [153, 154, 157], regulate IQGAP1 localization at the leading edges, the data presented in Figure 2.05 and 2.09 clearly show that the PIPKIγ interaction is also required for IQGAP1 targeting to the leading edge. Furthermore, IQGAP1 is a novel $PI4,5P_2$ effector in regulation of cell migration. IQGAP1 binds to $PI4,5P_2$ through a newly defined polybasic motif (Figure 2.07) and IQGAP1 binding to $PI4,5P_2$ relieves the autoinhibitory intramolecular interaction between the GRD and RGCT domains (Figure 2.10 and 2.11). Consequently, IQGAP1 binding to $PI4,5P_2$ regulates *de novo* actin polymerization at the leading edges by recruiting N-WASP and Arp2/3 complex through the relieved RGCT domain (Figure 2.10).

IQGAP1 binding to PIPKIα has a different cellular role. By fractionation analysis, PIPKIα does not regulate IQGAP1 targeting to membrane compartments (data not shown), signifying a specific role of PIPKIγ in the IQGAP1 membrane targeting. Instead, IQGAP1 interaction with PIPKIα regulates the PI3K-Akt pathway. Knockdown of PIPKIα but not PIPKIβ or PIPKIγ reduces $PI3,4,5P_3$ levels and Akt activation without compromising cellular $PI4,5P_2$ levels (Figure 3.01). IQGAP1 mediates PIPKIα interaction with class I PI3K *in vitro* and *in vivo* (Figure 3.02 and 3.03). This physical organization of PIPKIα and class I PI3K by IQGAP1 enables that $PI4,5P_2$ generated by PIPKIα is channeled to class I PI3K for

$PI3,4,5P_3$ synthesis (Figure 3.04). Furthermore, blockade of IQGAP1 interaction with PIPKIα and class I PI3K reduces Akt activation and cell survival (Figure 3.05 and 3.06).

Although in proteomic analyses hundreds of putative $PI4,5P_2$ binding proteins are identified [12, 14], only a small portion have been functionally studied. In the current investigation, we reveal how $PI4,5P_2$ binding of IQGAP1 regulates IQGAP1 function in cell migration. Importantly, IQGAP1 is spatiotemporally organized with a $PI4,5P_2$ generating enzyme PIPKIγ and this organization assures $PI4,5P_2$ signal is produced only when and where it is needed for regulating cell migration. $PI4,5P_2$ is an indispensible regulator of cell migration [8] and aberrant $PI4,5P_2$ signaling in cell migration is linked to human diseases such as cancer [24, 233]. As IQGAP1 is a master regulator of cell migration [50, 55, 56], this study reveals a fundamental mechanism in cell migration, and blockade of the PIPKIγ-IQGAP1 signaling platform could be used as therapeutics for human diseases.

The interaction of PIPKIα with class I PI3K was an unexpected observation. Although PIPKIs generate $PI4,5P_2$, a substrate for class I PI3Ks, the roles of PIPKIs in the PI3K-Akt signaling have been underestimated. In this study, we showed that $PI4,5P_2$ generation is directly linked to $PI3,4,5P_3$ generation by physical and functional association of the two sequential phosphoinositide kinases in a complex by the scaffold IQGAP1. Importantly, the complex formation is enhanced by extracellular stimuli such as integrin and growth factor receptor activation ensuring $PI3,4,5P_3$ signal is only produced in the right place at the right time. Alteration in the PI3K-Akt pathway is linked to numerous human diseases such as cancer, diabetes and autoimmune diseases [217, 225, 234]. In this study, we provide evidence that blockade of this signaling platform has potential use in therapeutics for diseases where PI3K-Akt signaling is altered, such as in cancers.

Future Directions:

The discovery of IQGAP1 interaction with PIPKIs may have numerous implications in cellular physiology. This study covers the roles in cell migration and the PI3K-Akt signaling. Additional roles of IQGAP1 that might have with PIPKIs will be discussed below.

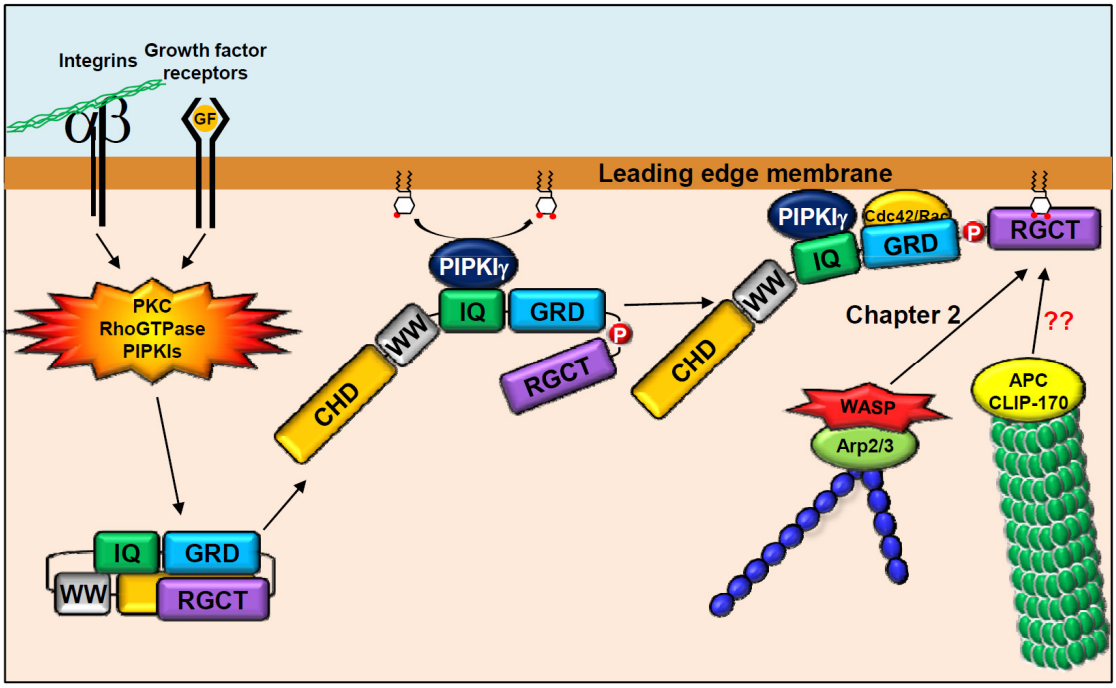
Does IQGAP1 regulate cell migration by controlling microtubule recruitment to the leading edges?: Migrating cells are polarized, with an asymmetric distribution of the cytoskeleton and signaling molecules. Microtubules (MTs) maintain polarization in migrating cells by providing tracks for vesicle trafficking of key molecules, such as integrins, to the migrating front [54, 56]. There is evidence that IQGAP1 recruits MTs to the leading edge membrane by association with the MT-binding proteins, CLIP-170 and APC [153, 154]. Also, the IQGAP1/MT contact sites at the plasma membrane are enriched in $PI4,5P_2$ [60]. Further, we have shown that PIPKIγi2 is required for cell polarization during cell migration [33]. Thus, it will be important to define the role of PIPKIγ in IQGAP1 modulation of MT organization and integrin trafficking to the leading edge (Figure 4.01).

Consistently, cells expressing the $PI4,5P_2$ binding defective IQGAP1 AA3 mutant form multiple leading edges, suggesting that the $PI4,5P_2$ regulation of IQGAP1 is important for maintaining polarity (Figure 2.09). These cells exhibit perpetual formation and retraction of leading edges but display little movement (data not shown). IQGAP1 is suggested to maintain polarity of migrating cells through local capture of MTs at the leading edge by interaction with MT plus tip proteins, CLIP-170 and APC [54, 55]. The interaction sites for these proteins are within the RGCT domain [153, 154]. We envision that the autoinhibitory interaction between the GRD and RGCT domains may also block MT recruitment, and $PI4,5P_2$ binding to the RGCT domain may relieve this. To test this, PIPKIγ and $PI4,5P_2$ binding mutants will be used for testing MT and integrin recruitment to the leading edges. Immunostaining IQGAP1 (wild type vs. the mutants), tubulin and β1-integrin in the cells migrating into scratch wounds will be applicable for this purpose as previously shown [10, 33].

Figure 4.01. A proposed mechanism of IQGAP1 in regulation of cell migration.

Integrin or growth factor receptor activation stimulates regulators of IQGAP1, including PKC, Rho family GTPases and PIPKIs. In unstimulated conditions, the N-terminus of IQGAP1 forms an intramolecular interaction with the C-terminus. Phosphorylation at Ser1441 and Ser1443 inhibits this intramolecular interaction exposing the IQ domain for PIPKIγ interaction. Phosphorylation also partially relieves the intramolecular interaction between the GRD and RGCT domains. PIPKIγ mediates IQGAP1 recruitment to the leading edge membrane, where PIPKIγ generates $PI4,5P_2$ from PI4P. $PI4,5P_2$ then binds to the RGCT domain, relieving the autoinhibitory interaction between the GRD and RGCT domains. Binding of active Cdc42 or Rac1 to the GRD domain also induces this process. The relieved RGCT domain recruits microtubules via an interaction with APC or CLIP-170, and facilitates actin polymerization by activating N-WASP.

Figure 4.01


Integrins
Growth factor receptors
GF
Leading edge membrane
PKC
RhoGTPase
PIPKIs
IQ
GRD
WW
RGCT
PIPKIγ
CHD
P
Cdc42/Rac
Chapter 2
??
WASP
Arp2/3
APC
CLIP-170

Does the PIPKIγ-IQGAP1 interaction control cell migration?: The PIPKIγ interaction site on IQGAP1 is in the IQ domain (Figure 2.01). In this study, we used the IQ domain deletion mutant to test whether the PIPKIγ-IQGAP1 interaction is important for regulating cell migration. Besides PIPKIγ, PIPKIα (Figure 3.01), MEK [166], Raf [164] and calmodulin [185] also bind on the IQ domain, and they also might have roles in cell migration. Therefore, the IQ domain deletion mutant may have broad effects due to loss of binding to other IQ domain interacting proteins besides PIPKIγ. It will be necessary to narrow down the PIPKIγ-binding site on the IQ domain. For this, the IQ motif peptides used in Figure 3.06 would have value. Using these cell permeable peptides, inhibition of the PIPKIγ-IQGAP1 interaction, or IQGAP1 interaction with other proteins, will be tested by immunoprecipitation or *in vitro* binding assay with recombinant proteins. Also, collagen- or EGF-induced cell migration will be measured by Transwell in the absence or presence of the peptides.

Mechanism of compaction-dependent effect of the cell permeable peptides: Cell permeable peptides derived from the WW domain and IQ motifs exert their effects in cell proliferation and Akt activation only in cells grown to confluence in tissue culture dish (Figure 3.06 and data not shown). MAPK and Akt pathways regulate cell survival and proliferation in many cell types [213, 220]. Interestingly, recent studies indicate that the two pathways compensate for each other in transformed cells, thus blocking one pathway is insufficient for inhibiting cancer cell survival and proliferation [235]. Upon compaction, cell proliferation and MAPK activity are largely inhibited [236, 237]. Interestingly, the PI3K-Akt pathway remains active in confluent cells (Figure 3.06) suggesting that the PI3K-Akt pathway regulates survival and proliferation of confluent cells. Consistently, inhibition of the PI3K-Akt activity by the peptides drastically induces cell death in compacted cells (Figure 3.06). To support the PI3K-Akt pathway-dependent cell death induced by these peptides, a constitutively active Akt will be transfected and it will be determined if cell death by the peptides is inhibited. Also, the compaction-dependent effect of the peptides will be tested using MAPK inhibitors. In

sub-confluent conditions, MAPK pathway will be blocked by inhibitors and whether the peptides induce cell death will be tested.

An alternative explanation for the compaction-dependent effect of the peptides comes from the study of IQGAP3. IQGAP3 is an IQGAP1 paralogue that also regulates cell proliferation by Ras/Erk pathway [237], suggesting a redundant role of IQGAP1 and IQGAP3 in cell proliferation. Notably, IQGAP3 expression is dramatically suppressed in confluent cells, whereas IQGAP1 expression remains intact [237]. This implies that IQGAP3 attenuates the peptides' effect in sub-confluent conditions by compromising IQGAP1 function. The peptides unlikely target IQGAP3 as IQGAP3 WW and IQ domains have low similarity (less than 50% similarity) with those of IQGAP1. To test this possibility, IQGAP3 expression will be knocked down and the effect of peptides will be examined in sub-confluent conditions.

It will be also interesting to test the effect of peptides in xenograft model to gain *in vivo* relevance for our study. Human carcinoma cells (Hs578T, Cal51 and other carcinoma cells) will be xenografted in immunocompromised mice, and the effect of the peptides on tumor growth and metastasis will be examined.

Knockout animal: Mice with class I PI3K genes deleted are embryonic lethal signifying their essential roles in development [238-241]. *Pip5k1b*$^{-/-}$ (a mouse orthologue of human *PIP5K1a* gene) and *Iqgap1*$^{-/-}$ knockout mice, however, are viable and have no profound phenotypic alterations [189, 216, 242, 243]. Cells derived from *Iqgap1*$^{-/-}$ knockout mice have defective Akt activity (Figure 3.05 and [171, 175]), but how the PI3K-Akt pathway is affected by PIPKIα gene knockout has not been reported. As simultaneous knockdown of IQGAP1 and PIPKIα further reduces Akt activity compared to single knockdown (Figure 3.01), it will be interesting to test how IQGAP1 and PIPKIα double knockout impacts the PI3K-Akt pathway in mice. The double knockout mice will be generated by crossing single knockout mice

(*Pip5k1b*$^{-/-}$ x *Iqgap1*$^{-/-}$) and their development will monitored. If the double knockout mice are embryonic lethal as class I PI3K knockout mice, it will further support our hypothesis. If the double knockout mice are viable, their responses to stress conditions that are related to the PI3K-Akt pathway such as insulin sensitivity and cancer progression will be tested.

Literature Cited:

[1] M.A. Lemmon, Membrane recognition by phospholipid-binding domains, Nat Rev Mol Cell Biol 9 (2008) 99-111.

[2] L.R. Stephens, T.R. Jackson, P.T. Hawkins, Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system?, Biochim Biophys Acta 1179 (1993) 27-75.

[3] R.A. Anderson, V.T. Marchesi, Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide, Nature 318 (1985) 295-298.

[4] B.C. Suh, B. Hille, Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate, Curr Opin Neurobiol 15 (2005) 370-378.

[5] S. McLaughlin, D. Murray, Plasma membrane phosphoinositide organization by protein electrostatics, Nature 438 (2005) 605-611.

[6] W.D. Heo, T. Inoue, W.S. Park, M.L. Kim, B.O. Park, T.J. Wandless, T. Meyer, PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane, Science 314 (2006) 1458-1461.

[7] K. Ling, N.J. Schill, M.P. Wagoner, Y. Sun, R.A. Anderson, Movin' on up: the role of PtdIns(4,5)P(2) in cell migration, Trends Cell Biol 16 (2006) 276-284.

[8] H.L. Yin, P.A. Janmey, Phosphoinositide regulation of the actin cytoskeleton, Annu Rev Physiol 65 (2003) 761-789.

[9] C.P. Downes, A. Gray, J.M. Lucocq, Probing phosphoinositide functions in signaling and membrane trafficking, Trends Cell Biol 15 (2005) 259-268.

[10] S. Choi, N. Thapa, A.C. Hedman, Z. Li, D.B. Sacks, R.A. Anderson, IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration, EMBO J 32 (2013) 2617-2630.

[11] C.A. Barlow, R.S. Laishram, R.A. Anderson, Nuclear phosphoinositides: a signaling enigma wrapped in a compartmental conundrum, Trends Cell Biol 20 (2012) 25-35.

[12] B. Catimel, C. Schieber, M. Condron, H. Patsiouras, L. Connolly, J. Catimel, E.C. Nice, A.W. Burgess, A.B. Holmes, The PI(3,5)P2 and PI(4,5)P2 interactomes, J Proteome Res 7 (2008) 5295-5313.

[13] M.J. Dixon, A. Gray, F.M. Boisvert, M. Agacan, N.A. Morrice, R. Gourlay, N.R. Leslie, C.P. Downes, I.H. Batty, A screen for novel phosphoinositide 3-kinase effector proteins, Mol Cell Proteomics 10 (2011) M110 003178.

[14] A.E. Lewis, L. Sommer, M.O. Arntzen, Y. Strahm, N.A. Morrice, N. Divecha, C.S. D'Santos, Identification of nuclear phosphatidylinositol 4,5-bisphosphate-interacting proteins by neomycin extraction, Mol Cell Proteomics 10 (2011) M110 003376.

[15] M.C. Good, J.G. Zalatan, W.A. Lim, Scaffold proteins: hubs for controlling the flow of cellular information, Science 332 (2011) 680-686.

[16] L.R. Stephens, K.T. Hughes, R.F. Irvine, Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils, Nature 351 (1991) 33-39.

[17] R.H. Insall, O.D. Weiner, PIP3, PIP2, and cell movement--similar messages, different meanings?, Dev Cell 1 (2001) 743-747.

[18] M.P. Sheetz, J.E. Sable, H.G. Dobereiner, Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics, Annu Rev Biophys Biomol Struct 35 (2006) 417-434.

[19] H. Kalwa, T. Michel, The MARCKS protein plays a critical role in phosphatidylinositol 4,5-bisphosphate metabolism and directed cell movement in vascular endothelial cells, J Biol Chem 286 (2010) 2320-2330.

[20] J. Vaaraniemi, R. Palovuori, V.P. Lehto, S. Eskelinen, Translocation of MARCKS and reorganization of the cytoskeleton by PMA correlates with the ion selectivity, the confluence, and transformation state of kidney epithelial cell lines, J Cell Physiol 181 (1999) 83-95.
[21] J.N. Heck, D.L. Mellman, K. Ling, Y. Sun, M.P. Wagoner, N.J. Schill, R.A. Anderson, A conspicuous connection: structure defines function for the phosphatidylinositol-phosphate kinase family, Crit Rev Biochem Mol Biol 42 (2007) 15-39.
[22] I. van den Bout, N. Divecha, PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions, J Cell Sci 122 (2009) 3837-3850.
[23] R.A. Anderson, I.V. Boronenkov, S.D. Doughman, J. Kunz, J.C. Loijens, Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes, J Biol Chem 274 (1999) 9907-9910.
[24] T. Sasaki, S. Takasuga, J. Sasaki, S. Kofuji, S. Eguchi, M. Yamazaki, A. Suzuki, Mammalian phosphoinositide kinases and phosphatases, Prog Lipid Res 48 (2009) 307-343.
[25] J.C. Loijens, R.A. Anderson, Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family, J Biol Chem 271 (1996) 32937-32943.
[26] N.J. Schill, R.A. Anderson, Two novel phosphatidylinositol-4-phosphate 5-kinase type Igamma splice variants expressed in human cells display distinctive cellular targeting, Biochem J 422 (2009) 473-482.
[27] Y. Sun, N. Thapa, A.C. Hedman, R.A. Anderson, Phosphatidylinositol 4,5-bisphosphate: targeted production and signaling, Bioessays 35 (2013) 513-522.
[28] P. Varnai, T. Balla, Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools, J Cell Biol 143 (1998) 501-510.
[29] S.A. Watt, G. Kular, I.N. Fleming, C.P. Downes, J.M. Lucocq, Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1, Biochem J 363 (2002) 657-666.
[30] G.R. Hammond, G. Schiavo, R.F. Irvine, Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P(2), Biochem J 422 (2009) 23-35.
[31] V.P. Sharma, V. DesMarais, C. Sumners, G. Shaw, A. Narang, Immunostaining evidence for PI(4,5)P2 localization at the leading edge of chemoattractant-stimulated HL-60 cells, J Leukoc Biol 84 (2008) 440-447.
[32] K. Ling, R.L. Doughman, A.J. Firestone, M.W. Bunce, R.A. Anderson, Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions, Nature 420 (2002) 89-93.
[33] N. Thapa, Y. Sun, M. Schramp, S. Choi, K. Ling, R.A. Anderson, Phosphoinositide signaling regulates the exocyst complex and polarized integrin trafficking in directionally migrating cells, Dev Cell 22 (2012) 116-130.
[34] Y. Sun, A.C. Hedman, X. Tan, N.J. Schill, R.A. Anderson, Endosomal type Igamma PIP 5-kinase controls EGF receptor lysosomal sorting, Dev Cell 25 (2013) 144-155.
[35] G.R. Hammond, M.P. Machner, T. Balla, A novel probe for phosphatidylinositol 4-phosphate reveals multiple pools beyond the Golgi, J Cell Biol 205 (2014) 113-126.

[36] M.L. Giudici, P.C. Emson, R.F. Irvine, A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isoform gamma, Biochem J 379 (2004) 489-496.
[37] Y. Xia, R.F. Irvine, M.L. Giudici, Phosphatidylinositol 4-phosphate 5-kinase Igamma_v6, a new splice variant found in rodents and humans, Biochem Biophys Res Commun 411 (2011) 416-420.
[38] M.L. Giudici, K. Lee, R. Lim, R.F. Irvine, The intracellular localisation and mobility of Type Igamma phosphatidylinositol 4P 5-kinase splice variants, FEBS Lett 580 (2006) 6933-6937.
[39] G. Di Paolo, L. Pellegrini, K. Letinic, G. Cestra, R. Zoncu, S. Voronov, S. Chang, J. Guo, M.R. Wenk, P. De Camilli, Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin, Nature 420 (2002) 85-89.
[40] S.F. Bairstow, K. Ling, X. Su, A.J. Firestone, C. Carbonara, R.A. Anderson, Type Igamma661 phosphatidylinositol phosphate kinase directly interacts with AP2 and regulates endocytosis, J Biol Chem 281 (2006) 20632-20642.
[41] K. Ling, S.F. Bairstow, C. Carbonara, D.A. Turbin, D.G. Huntsman, R.A. Anderson, Type I gamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin, J Cell Biol 176 (2007) 343-353.
[42] N.J. Schill, A.C. Hedman, S. Choi, R.A. Anderson, Isoform 5 of PIPKIgamma regulates the endosomal trafficking and degradation of E-cadherin, J Cell Sci 127 (2014) 2189-2203.
[43] R.L. Doughman, A.J. Firestone, M.L. Wojtasiak, M.W. Bunce, R.A. Anderson, Membrane ruffling requires coordination between type Ialpha phosphatidylinositol phosphate kinase and Rac signaling, J Biol Chem 278 (2003) 23036-23045.
[44] W.T. Chao, A.C. Daquinag, F. Ashcroft, J. Kunz, Type I PIPK-alpha regulates directed cell migration by modulating Rac1 plasma membrane targeting and activation, J Cell Biol 190 (2010) 247-262.
[45] Y.S. Mao, M. Yamaga, X. Zhu, Y. Wei, H.Q. Sun, J. Wang, M. Yun, Y. Wang, G. Di Paolo, M. Bennett, I. Mellman, C.S. Abrams, P. De Camilli, C.Y. Lu, H.L. Yin, Essential and unique roles of PIP5K-gamma and -alpha in Fcgamma receptor-mediated phagocytosis, J Cell Biol 184 (2009) 281-296.
[46] L. Mathis, S. Wernimont, S. Affentranger, A. Huttenlocher, V. Niggli, Determinants of phosphatidylinositol-4-phosphate 5-kinase type Igamma90 uropod location in T-lymphocytes and its role in uropod formation, PeerJ 1 (2013) e131.
[47] M.A. Lokuta, M.A. Senetar, D.A. Bennin, P.A. Nuzzi, K.T. Chan, V.L. Ott, A. Huttenlocher, Type Igamma PIP kinase is a novel uropod component that regulates rear retraction during neutrophil chemotaxis, Mol Biol Cell 18 (2007) 5069-5080.
[48] R.A. Lacalle, R.M. Peregil, J.P. Albar, E. Merino, A.C. Martinez, I. Merida, S. Manes, Type I phosphatidylinositol 4-phosphate 5-kinase controls neutrophil polarity and directional movement, J Cell Biol 179 (2007) 1539-1553.
[49] A.J. Ridley, Life at the leading edge, Cell 145 (2011) 1012-1022.
[50] J.T. Parsons, A.R. Horwitz, M.A. Schwartz, Cell adhesion: integrating cytoskeletal dynamics and cellular tension, Nat Rev Mol Cell Biol 11 (2010) 633-643.
[51] R.J. Petrie, A.D. Doyle, K.M. Yamada, Random versus directionally persistent cell migration, Nat Rev Mol Cell Biol 10 (2009) 538-549.

[52] R.H. Adams, K. Alitalo, Molecular regulation of angiogenesis and lymphangiogenesis, Nat Rev Mol Cell Biol 8 (2007) 464-478.
[53] J. Noritake, T. Watanabe, K. Sato, S. Wang, K. Kaibuchi, IQGAP1: a key regulator of adhesion and migration, J Cell Sci 118 (2005) 2085-2092.
[54] T. Watanabe, J. Noritake, K. Kaibuchi, Regulation of microtubules in cell migration, Trends Cell Biol 15 (2005) 76-83.
[55] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704-1709.
[56] O.C. Rodriguez, A.W. Schaefer, C.A. Mandato, P. Forscher, W.M. Bement, C.M. Waterman-Storer, Conserved microtubule-actin interactions in cell movement and morphogenesis, Nat Cell Biol 5 (2003) 599-609.
[57] J.H. Hartwig, G.M. Bokoch, C.L. Carpenter, P.A. Janmey, L.A. Taylor, A. Toker, T.P. Stossel, Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets, Cell 82 (1995) 643-653.
[58] K.F. Tolias, J.H. Hartwig, H. Ishihara, Y. Shibasaki, L.C. Cantley, C.L. Carpenter, Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly, Curr Biol 10 (2000) 153-156.
[59] Y. Shibasaki, H. Ishihara, N. Kizuki, T. Asano, Y. Oka, Y. Yazaki, Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase in vivo, J Biol Chem 272 (1997) 7578-7581.
[60] T. Golub, P. Caroni, PI(4,5)P2-dependent microdomain assemblies capture microtubules to promote and control leading edge motility, J Cell Biol 169 (2005) 151-165.
[61] N. Thapa, R.A. Anderson, PIP2 signaling, an integrator of cell polarity and vesicle trafficking in directionally migrating cells, Cell Adh Migr 6 (2013) 409-412.
[62] P. Pinon, J. Parssinen, P. Vazquez, M. Bachmann, R. Rahikainen, M.C. Jacquier, L. Azizi, J.A. Maatta, M. Bastmeyer, V.P. Hytonen, B. Wehrle-Haller, Talin-bound NPLY motif recruits integrin-signaling adapters to regulate cell spreading and mechanosensing, J Cell Biol 205 (2014) 265-281.
[63] A.C. Kalli, I.D. Campbell, M.S. Sansom, Conformational changes in talin on binding to anionic phospholipid membranes facilitate signaling by integrin transmembrane helices, PLoS Comput Biol 9 (2013) e1003316.
[64] K.R. Legate, S. Takahashi, N. Bonakdar, B. Fabry, D. Boettiger, R. Zent, R. Fassler, Integrin adhesion and force coupling are independently regulated by localized PtdIns(4,5)2 synthesis, EMBO J 30 (2011) 4539-4553.
[65] P.R. Elliott, B.T. Goult, P.M. Kopp, N. Bate, J.G. Grossmann, G.C. Roberts, D.R. Critchley, I.L. Barsukov, The Structure of the talin head reveals a novel extended conformation of the FERM domain, Structure 18 (2010) 1289-1299.
[66] N.J. Anthis, K.L. Wegener, F. Ye, C. Kim, B.T. Goult, E.D. Lowe, I. Vakonakis, N. Bate, D.R. Critchley, M.H. Ginsberg, I.D. Campbell, The structure of an integrin/talin complex reveals the basis of inside-out signal transduction, EMBO J 28 (2009) 3623-3632.
[67] E. Goksoy, Y.Q. Ma, X. Wang, X. Kong, D. Perera, E.F. Plow, J. Qin, Structural basis for the autoinhibition of talin in regulating integrin activation, Mol Cell 31 (2008) 124-133.

[68] A.C. Kalli, K.L. Wegener, B.T. Goult, N.J. Anthis, I.D. Campbell, M.S. Sansom, The structure of the talin/integrin complex at a lipid bilayer: an NMR and MD simulation study, Structure 18 (2010) 1280-1288.
[69] K. Kwiatkowska, One lipid, multiple functions: how various pools of PI(4,5)P(2) are created in the plasma membrane, Cell Mol Life Sci 67 (2010) 3927-3946.
[70] F. Saltel, E. Mortier, V.P. Hytonen, M.C. Jacquier, P. Zimmermann, V. Vogel, W. Liu, B. Wehrle-Haller, New PI(4,5)P2- and membrane proximal integrin-binding motifs in the talin head control beta3-integrin clustering, J Cell Biol 187 (2009) 715-731.
[71] C. Cluzel, F. Saltel, J. Lussi, F. Paulhe, B.A. Imhof, B. Wehrle-Haller, The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells, J Cell Biol 171 (2005) 383-392.
[72] K. Ling, R.L. Doughman, V.V. Iyer, A.J. Firestone, S.F. Bairstow, D.F. Mosher, M.D. Schaller, R.A. Anderson, Tyrosine phosphorylation of type Igamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch, J Cell Biol 163 (2003) 1339-1349.
[73] G.R. Hammond, M.J. Fischer, K.E. Anderson, J. Holdich, A. Koteci, T. Balla, R.F. Irvine, PI4P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity, Science 337 (2012) 727-730.
[74] P.A. Weernink, K. Meletiadis, S. Hommeltenberg, M. Hinz, H. Ishihara, M. Schmidt, K.H. Jakobs, Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42, J Biol Chem 279 (2004) 7840-7849.
[75] J.R. Halstead, N.E. Savaskan, I. van den Bout, F. Van Horck, A. Hajdo-Milasinovic, M. Snell, W.J. Keune, J.P. Ten Klooster, P.L. Hordijk, N. Divecha, Rac controls PIP5K localisation and PtdIns(4,5)P synthesis, which modulates vinculin localisation and neurite dynamics, J Cell Sci 123 (2010) 3535-3546.
[76] M. Yamazaki, H. Miyazaki, H. Watanabe, T. Sasaki, T. Maehama, M.A. Frohman, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling, J Biol Chem 277 (2002) 17226-17230.
[77] F.P. van Horck, E. Lavazais, B.J. Eickholt, W.H. Moolenaar, N. Divecha, Essential role of type I(alpha) phosphatidylinositol 4-phosphate 5-kinase in neurite remodeling, Curr Biol 12 (2002) 241-245.
[78] D.J. Hernandez-Deviez, M.G. Roth, J.E. Casanova, J.M. Wilson, ARNO and ARF6 regulate axonal elongation and branching through downstream activation of phosphatidylinositol 4-phosphate 5-kinase alpha, Mol Biol Cell 15 (2004) 111-120.
[79] C. Bakolitsa, D.M. Cohen, L.A. Bankston, A.A. Bobkov, G.W. Cadwell, L. Jennings, D.R. Critchley, S.W. Craig, R.C. Liddington, Structural basis for vinculin activation at sites of cell adhesion, Nature 430 (2004) 583-586.
[80] I. Chandrasekar, T.E. Stradal, M.R. Holt, F. Entschladen, B.M. Jockusch, W.H. Ziegler, Vinculin acts as a sensor in lipid regulation of adhesion-site turnover, J Cell Sci 118 (2005) 1461-1472.
[81] R. Rohatgi, H.Y. Ho, M.W. Kirschner, Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate, J Cell Biol 150 (2000) 1299-1310.
[82] A.L. Rozelle, L.M. Machesky, M. Yamamoto, M.H. Driessens, R.H. Insall, M.G. Roth, K. Luby-Phelps, G. Marriott, A. Hall, H.L. Yin, Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3, Curr Biol 10 (2000) 311-320.
[83] V. Niggli, Regulation of protein activities by phosphoinositide phosphates, Annu Rev Cell Dev Biol 21 (2005) 57-79.

[84] I. Liepina, C. Czaplewski, P. Janmey, A. Liwo, Molecular dynamics study of a gelsolin-derived peptide binding to a lipid bilayer containing phosphatidylinositol 4,5-bisphosphate, Biopolymers 71 (2003) 49-70.
[85] T.Y. El Sayegh, P.D. Arora, K. Ling, C. Laschinger, P.A. Janmey, R.A. Anderson, C.A. McCulloch, Phosphatidylinositol-4,5 bisphosphate produced by PIP5KIgamma regulates gelsolin, actin assembly, and adhesion strength of N-cadherin junctions, Mol Biol Cell 18 (2007) 3026-3038.
[86] M.W. Chan, P.D. Arora, P. Bozavikov, C.A. McCulloch, FAK, PIP5KIgamma and gelsolin cooperatively mediate force-induced expression of alpha-smooth muscle actin, J Cell Sci 122 (2009) 2769-2781.
[87] S.K. Mitra, D.A. Hanson, D.D. Schlaepfer, Focal adhesion kinase: in command and control of cell motility, Nat Rev Mol Cell Biol 6 (2005) 56-68.
[88] N. Thapa, S. Choi, A. Hedman, X. Tan, R.A. Anderson, Phosphatidylinositol phosphate 5-kinase Igammai2 in association with Src controls anchorage-independent growth of tumor cells, J Biol Chem 288 (2013) 34707-34718.
[89] N.M. Levinson, M.A. Seeliger, P.A. Cole, J. Kuriyan, Structural basis for the recognition of c-Src by its inactivator Csk, Cell 134 (2008) 124-134.
[90] S.R. Hubbard, Src autoinhibition: let us count the ways, Nat Struct Biol 6 (1999) 711-714.
[91] C.T. Sigal, W. Zhou, C.A. Buser, S. McLaughlin, M.D. Resh, Amino-terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids, Proc Natl Acad Sci U S A 91 (1994) 12253-12257.
[92] S. Manes, G. Fuentes, R.M. Peregil, A.M. Rojas, R.A. Lacalle, An isoform-specific PDZ-binding motif targets type I PIP5 kinase beta to the uropod and controls polarization of neutrophil-like HL60 cells, FASEB J 24 (2010) 3381-3392.
[93] K. Takahashi, T. Sasaki, A. Mammoto, K. Takaishi, T. Kameyama, S. Tsukita, Y. Takai, Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein, J Biol Chem 272 (1997) 23371-23375.
[94] S.J. Franco, A. Huttenlocher, Regulating cell migration: calpains make the cut, J Cell Sci 118 (2005) 3829-3838.
[95] T.C. Saido, M. Shibata, T. Takenawa, H. Murofushi, K. Suzuki, Positive regulation of mu-calpain action by polyphosphoinositides, J Biol Chem 267 (1992) 24585-24590.
[96] H.J. McCrea, P. De Camilli, Mutations in phosphoinositide metabolizing enzymes and human disease, Physiology (Bethesda) 24 (2009) 8-16.
[97] G. Di Paolo, P. De Camilli, Phosphoinositides in cell regulation and membrane dynamics, Nature 443 (2006) 651-657.
[98] Y.J. Kim, M.L. Hernandez, T. Balla, Inositol lipid regulation of lipid transfer in specialized membrane domains, Trends Cell Biol 23 (2013) 270-278.
[99] Y.J. Kim, M.L. Guzman-Hernandez, T. Balla, A highly dynamic ER-derived phosphatidylinositol-synthesizing organelle supplies phosphoinositides to cellular membranes, Dev Cell 21 (2011) 813-824.
[100] D.J. Owen, B.M. Collins, P.R. Evans, Adaptors for clathrin coats: structure and function, Annu Rev Cell Dev Biol 20 (2004) 153-191.
[101] Y.J. Wang, J. Wang, H.Q. Sun, M. Martinez, Y.X. Sun, E. Macia, T. Kirchhausen, J.P. Albanesi, M.G. Roth, H.L. Yin, Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi, Cell 114 (2003) 299-310.

[102] I.G. Mills, G.J. Praefcke, Y. Vallis, B.J. Peter, L.E. Olesen, J.L. Gallop, P.J. Butler, P.R. Evans, H.T. McMahon, EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking, J Cell Biol 160 (2003) 213-222.
[103] J. Wang, H.Q. Sun, E. Macia, T. Kirchhausen, H. Watson, J.S. Bonifacino, H.L. Yin, PI4P promotes the recruitment of the GGA adaptor proteins to the trans-Golgi network and regulates their recognition of the ubiquitin sorting signal, Mol Biol Cell 18 (2007) 2646-2655.
[104] L. Demmel, M. Gravert, E. Ercan, B. Habermann, T. Muller-Reichert, V. Kukhtina, V. Haucke, T. Baust, M. Sohrmann, Y. Kalaidzidis, C. Klose, M. Beck, M. Peter, C. Walch-Solimena, The clathrin adaptor Gga2p is a phosphatidylinositol 4-phosphate effector at the Golgi exit, Mol Biol Cell 19 (2008) 1991-2002.
[105] A. Godi, P. Pertile, R. Meyers, P. Marra, G. Di Tullio, C. Iurisci, A. Luini, D. Corda, M.A. De Matteis, ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex, Nat Cell Biol 1 (1999) 280-287.
[106] K. Ling, S.F. Bairstow, C. Carbonara, D.A. Turbin, D.G. Huntsman, R.A. Anderson, Type I{gamma} phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with {micro}1B adaptin, J Cell Biol 176 (2007) 343-353.
[107] T. Itoh, S. Koshiba, T. Kigawa, A. Kikuchi, S. Yokoyama, T. Takenawa, Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis, Science 291 (2001) 1047-1051.
[108] T. Itoh, T. Takenawa, Regulation of endocytosis by phosphatidylinositol 4,5-bisphosphate and ENTH proteins, Curr Top Microbiol Immunol 282 (2004) 31-47.
[109] M.G. Ford, I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, H.T. McMahon, Curvature of clathrin-coated pits driven by epsin, Nature 419 (2002) 361-366.
[110] D. Padron, Y.J. Wang, M. Yamamoto, H. Yin, M.G. Roth, Phosphatidylinositol phosphate 5-kinase Ibeta recruits AP-2 to the plasma membrane and regulates rates of constitutive endocytosis, J Cell Biol 162 (2003) 693-701.
[111] A.E. Engqvist-Goldstein, D.G. Drubin, Actin assembly and endocytosis: from yeast to mammals, Annu Rev Cell Dev Biol 19 (2003) 287-332.
[112] J. Zheng, S.M. Cahill, M.A. Lemmon, D. Fushman, J. Schlessinger, D. Cowburn, Identification of the binding site for acidic phospholipids on the pH domain of dynamin: implications for stimulation of GTPase activity, J Mol Biol 255 (1996) 14-21.
[113] K. Letinic, R. Sebastian, D. Toomre, P. Rakic, Exocyst is involved in polarized cell migration and cerebral cortical development, Proc Natl Acad Sci U S A 106 (2009) 11342-11347.
[114] J. Liu, W. Guo, The exocyst complex in exocytosis and cell migration, Protoplasma 249 (2012) 587-597.
[115] X. Xiong, Q. Xu, Y. Huang, R.D. Singh, R. Anderson, E. Leof, J. Hu, K. Ling, An association between type Igamma PI4P 5-kinase and Exo70 directs E-cadherin clustering and epithelial polarization, Mol Biol Cell 23 (2012) 87-98.
[116] X. Zhang, K. Orlando, B. He, F. Xi, J. Zhang, A. Zajac, W. Guo, Membrane association and functional regulation of Sec3 by phospholipids and Cdc42, J Cell Biol 180 (2008) 145-158.

[117] J. Liu, X. Zuo, P. Yue, W. Guo, Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells, Mol Biol Cell 18 (2007) 4483-4492.
[118] K.S. Spiczka, C. Yeaman, Ral-regulated interaction between Sec5 and paxillin targets Exocyst to focal complexes during cell migration, J Cell Sci 121 (2008) 2880-2891.
[119] P. Wan, D. Wang, J. Luo, D. Chu, H. Wang, L. Zhang, J. Chen, Guidance receptor promotes the asymmetric distribution of exocyst and recycling endosome during collective cell migration, Development 140 (2013) 4797-4806.
[120] M. Pourcher, M. Santambrogio, N. Thazar, A.M. Thierry, I. Fobis-Loisy, C. Miege, Y. Jaillais, T. Gaude, Analyses of sorting nexins reveal distinct retromer-subcomplex functions in development and protein sorting in Arabidopsis thaliana, Plant Cell 22 3980-3991.
[121] M. Verges, Retromer and sorting nexins in development, Front Biosci 12 (2007) 3825-3851.
[122] D.G. Schwarz, C.T. Griffin, E.A. Schneider, D. Yee, T. Magnuson, Genetic analysis of sorting nexins 1 and 2 reveals a redundant and essential function in mice, Mol Biol Cell 13 (2002) 3588-3600.
[123] M. Harterink, F. Port, M.J. Lorenowicz, I.J. McGough, M. Silhankova, M.C. Betist, J.R. van Weering, R.G. van Heesbeen, T.C. Middelkoop, K. Basler, P.J. Cullen, H.C. Korswagen, A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion, Nat Cell Biol.
[124] K.W. Yoo, E.H. Kim, S.H. Jung, M. Rhee, B.K. Koo, K.J. Yoon, Y.Y. Kong, C.H. Kim, Snx5, as a Mind bomb-binding protein, is expressed in hematopoietic and endothelial precursor cells in zebrafish, FEBS Lett 580 (2006) 4409-4416.
[125] C.A. Worby, J.E. Dixon, Sorting out the cellular functions of sorting nexins, Nat Rev Mol Cell Biol 3 (2002) 919-931.
[126] J.G. Carlton, P.J. Cullen, Sorting nexins, Curr Biol 15 (2005) R819-820.
[127] J. Carlton, M. Bujny, A. Rutherford, P. Cullen, Sorting nexins--unifying trends and new perspectives, Traffic 6 (2005) 75-82.
[128] L.F. Seet, W. Hong, The Phox (PX) domain proteins and membrane traffic, Biochim Biophys Acta 1761 (2006) 878-896.
[129] L.M. Koharudin, W. Furey, H. Liu, Y.J. Liu, A.M. Gronenborn, The phox domain of sorting nexin 5 lacks phosphatidylinositol 3-phosphate (PtdIns(3)P) specificity and preferentially binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), J Biol Chem 284 (2009) 23697-23707.
[130] O. Pylypenko, R. Lundmark, E. Rasmuson, S.R. Carlsson, A. Rak, The PX-BAR membrane-remodeling unit of sorting nexin 9, EMBO J 26 (2007) 4788-4800.
[131] Y. Sun, A.C. Hedman, X. Tan, R.A. Anderson, An unexpected role for PI4,5P 2 in EGF receptor endosomal trafficking, Cell Cycle 12 (2013) 1991-1992.
[132] C. Raiborg, B. Bremnes, A. Mehlum, D.J. Gillooly, A. D'Arrigo, E. Stang, H. Stenmark, FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes, J Cell Sci 114 (2001) 2255-2263.
[133] W.M. Henne, N.J. Buchkovich, S.D. Emr, The ESCRT pathway, Dev Cell 21 77-91.
[134] T. Slagsvold, R. Aasland, S. Hirano, K.G. Bache, C. Raiborg, D. Trambaiolo, S. Wakatsuki, H. Stenmark, Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain, J Biol Chem 280 (2005) 19600-19606.

[135] N. Shin, N. Ahn, B. Chang-Ileto, J. Park, K. Takei, S.G. Ahn, S.A. Kim, G. Di Paolo, S. Chang, SNX9 regulates tubular invagination of the plasma membrane through interaction with actin cytoskeleton and dynamin 2, J Cell Sci 121 (2008) 1252-1263.
[136] K. Nishikawa, A. Toker, K. Wong, P.A. Marignani, F.J. Johannes, L.C. Cantley, Association of protein kinase Cmu with type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase, J Biol Chem 273 (1998) 23126-23133.
[137] D.H. Jones, J.B. Morris, C.P. Morgan, H. Kondo, R.F. Irvine, S. Cockcroft, Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment, J Biol Chem 275 (2000) 13962-13966.
[138] A. Balla, G. Tuymetova, A. Tsiomenko, P. Varnai, T. Balla, A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: studies with the PH domains of the oxysterol binding protein and FAPP1, Mol Biol Cell 16 (2005) 1282-1295.
[139] Y. Aikawa, T.F. Martin, ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis, J Cell Biol 162 (2003) 647-659.
[140] L. Fets, J.M. Nichols, R.R. Kay, A PIP5 kinase essential for efficient chemotactic signaling, Curr Biol 24 (2014) 415-421.
[141] Z. Xie, S.M. Chang, S.D. Pennypacker, E.Y. Liao, D.D. Bikle, Phosphatidylinositol-4-phosphate 5-kinase 1alpha mediates extracellular calcium-induced keratinocyte differentiation, Mol Biol Cell 20 (2009) 1695-1704.
[142] P. Devreotes, C. Janetopoulos, Eukaryotic chemotaxis: distinctions between directional sensing and polarization, J Biol Chem 278 (2003) 20445-20448.
[143] M. Iijima, Y.E. Huang, P. Devreotes, Temporal and spatial regulation of chemotaxis, Dev Cell 3 (2002) 469-478.
[144] S. Funamoto, R. Meili, S. Lee, L. Parry, R.A. Firtel, Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis, Cell 109 (2002) 611-623.
[145] K.M. Chu, S. Minogue, J.J. Hsuan, M.G. Waugh, Differential effects of the phosphatidylinositol 4-kinases, PI4KIIalpha and PI4KIIIbeta, on Akt activation and apoptosis, Cell Death Dis 1 (2010) e106.
[146] M.A. De Matteis, C. Wilson, G. D'Angelo, Phosphatidylinositol-4-phosphate: the Golgi and beyond, Bioessays 35 (2013) 612-622.
[147] M.D. Brown, D.B. Sacks, IQGAP1 in cellular signaling: bridging the GAP, Trends Cell Biol 16 (2006) 242-249.
[148] L. Weissbach, J. Settleman, M.F. Kalady, A.J. Snijders, A.E. Murthy, Y.X. Yan, A. Bernards, Identification of a human rasGAP-related protein containing calmodulin-binding motifs, J Biol Chem 269 (1994) 20517-20521.
[149] B. Schwanhausser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, M. Selbach, Global quantification of mammalian gene expression control, Nature 473 (2011) 337-342.
[150] C.D. White, H.H. Erdemir, D.B. Sacks, IQGAP1 and its binding proteins control diverse biological functions, Cell Signal 24 (2012) 826-834.
[151] M. Osman, An emerging role for IQGAP1 in regulating protein traffic, ScientificWorldJournal 10 (2010) 944-953.
[152] C.D. White, M.D. Brown, D.B. Sacks, IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis, FEBS Lett 583 (2009) 1817-1824.

[153] T. Watanabe, S. Wang, J. Noritake, K. Sato, M. Fukata, M. Takefuji, M. Nakagawa, N. Izumi, T. Akiyama, K. Kaibuchi, Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration, Dev Cell 7 (2004) 871-883.
[154] M. Fukata, T. Watanabe, J. Noritake, M. Nakagawa, M. Yamaga, S. Kuroda, Y. Matsuura, A. Iwamatsu, F. Perez, K. Kaibuchi, Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170, Cell 109 (2002) 873-885.
[155] S. Kuroda, M. Fukata, K. Kobayashi, M. Nakafuku, N. Nomura, A. Iwamatsu, K. Kaibuchi, Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1, J Biol Chem 271 (1996) 23363-23367.
[156] L.B. Bensenor, H.M. Kan, N. Wang, H. Wallrabe, L.A. Davidson, Y. Cai, D.A. Schafer, G.S. Bloom, IQGAP1 regulates cell motility by linking growth factor signaling to actin assembly, J Cell Sci 120 (2007) 658-669.
[157] D.T. Brandt, S. Marion, G. Griffiths, T. Watanabe, K. Kaibuchi, R. Grosse, Dia1 and IQGAP1 interact in cell migration and phagocytic cup formation, J Cell Biol 178 (2007) 193-200.
[158] M. Fukata, M. Nakagawa, N. Itoh, A. Kawajiri, M. Yamaga, S. Kuroda, K. Kaibuchi, Involvement of IQGAP1, an effector of Rac1 and Cdc42 GTPases, in cell-cell dissociation during cell scattering, Mol Cell Biol 21 (2001) 2165-2183.
[159] M.J. Hart, M.G. Callow, B. Souza, P. Polakis, IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs, EMBO J 15 (1996) 2997-3005.
[160] Y.D. Ho, J.L. Joyal, Z. Li, D.B. Sacks, IQGAP1 integrates Ca2+/calmodulin and Cdc42 signaling, J Biol Chem 274 (1999) 464-470.
[161] C. Le Clainche, D. Schlaepfer, A. Ferrari, M. Klingauf, K. Grohmanova, A. Veligodskiy, D. Didry, D. Le, C. Egile, M.F. Carlier, R. Kroschewski, IQGAP1 stimulates actin assembly through the N-WASP-Arp2/3 pathway, J Biol Chem 282 (2007) 426-435.
[162] K. Grohmanova, D. Schlaepfer, D. Hess, P. Gutierrez, M. Beck, R. Kroschewski, Phosphorylation of IQGAP1 modulates its binding to Cdc42, revealing a new type of rho-GTPase regulator, J Biol Chem 279 (2004) 48495-48504.
[163] D.T. Brandt, R. Grosse, Get to grips: steering local actin dynamics with IQGAPs, EMBO Rep 8 (2007) 1019-1023.
[164] J.G. Ren, Z. Li, D.B. Sacks, IQGAP1 modulates activation of B-Raf, Proc Natl Acad Sci U S A 104 (2007) 10465-10469.
[165] M. Roy, Z. Li, D.B. Sacks, IQGAP1 binds ERK2 and modulates its activity, J Biol Chem 279 (2004) 17329-17337.
[166] M. Roy, Z. Li, D.B. Sacks, IQGAP1 is a scaffold for mitogen-activated protein kinase signaling, Mol Cell Biol 25 (2005) 7940-7952.
[167] H. Matsunaga, K. Kubota, T. Inoue, F. Isono, O. Ando, IQGAP1 selectively interacts with K-Ras but not with H-Ras and modulates K-Ras function, Biochem Biophys Res Commun 444 (2014) 360-364.
[168] M.J. Smith, W.R. Hardy, G.Y. Li, M. Goudreault, S. Hersch, P. Metalnikov, A. Starostine, T. Pawson, M. Ikura, The PTB domain of ShcA couples receptor activation to the cytoskeletal regulator IQGAP1, EMBO J 29 (2010) 884-896.
[169] D.E. McNulty, Z. Li, C.D. White, D.B. Sacks, R.S. Annan, MAPK scaffold IQGAP1 binds the EGF receptor and modulates its activation, J Biol Chem 286 (2011) 15010-15021.

[170] C.D. White, Z. Li, D.A. Dillon, D.B. Sacks, IQGAP1 protein binds human epidermal growth factor receptor 2 (HER2) and modulates trastuzumab resistance, J Biol Chem 286 (2011) 29734-29747.
[171] M. Sbroggio, D. Carnevale, A. Bertero, G. Cifelli, E. De Blasio, G. Mascio, E. Hirsch, W.F. Bahou, E. Turco, L. Silengo, M. Brancaccio, G. Lembo, G. Tarone, IQGAP1 regulates ERK1/2 and AKT signalling in the heart and sustains functional remodelling upon pressure overload, Cardiovasc Res 91 (2011) 456-464.
[172] M. Johnson, M. Sharma, B.R. Henderson, IQGAP1 regulation and roles in cancer, Cell Signal 21 (2009) 1471-1478.
[173] M. Sakurai-Yageta, C. Recchi, G. Le Dez, J.B. Sibarita, L. Daviet, J. Camonis, C. D'Souza-Schorey, P. Chavrier, The interaction of IQGAP1 with the exocyst complex is required for tumor cell invasion downstream of Cdc42 and RhoA, J Cell Biol 181 (2008) 985-998.
[174] M.J. Dixon, A. Gray, M. Schenning, M. Agacan, W. Tempel, Y. Tong, L. Nedyalkova, H.W. Park, N.R. Leslie, D.M. van Aalten, C.P. Downes, I.H. Batty, IQGAP Proteins Reveal an Atypical Phosphoinositide (aPI) Binding Domain with a Pseudo C2 Domain Fold, J Biol Chem 287 (2012) 22483-22496.
[175] M. Sbroggio, A. Bertero, S. Velasco, F. Fusella, E. De Blasio, W.F. Bahou, L. Silengo, E. Turco, M. Brancaccio, G. Tarone, ERK1/2 activation in heart is controlled by melusin, focal adhesion kinase and the scaffold protein IQGAP1, J Cell Sci 124 (2011) 3515-3524.
[176] Y.K. Tekletsadik, R. Sonn, M.A. Osman, A conserved role of IQGAP1 in regulating TOR complex 1, J Cell Sci 125 (2012) 2041-2052.
[177] R. Li, B. Debreceni, B. Jia, Y. Gao, G. Tigyi, Y. Zheng, Localization of the PAK1-, WASP-, and IQGAP1-specifying regions of Cdc42, J Biol Chem 274 (1999) 29648-29654.
[178] Z. Li, D.E. McNulty, K.J. Marler, L. Lim, C. Hall, R.S. Annan, D.B. Sacks, IQGAP1 promotes neurite outgrowth in a phosphorylation-dependent manner, J Biol Chem 280 (2005) 13871-13878.
[179] S. McLaughlin, J. Wang, A. Gambhir, D. Murray, PIP(2) and proteins: interactions, organization, and information flow, Annu Rev Biophys Biomol Struct 31 (2002) 151-175.
[180] L. Zhang, Y.S. Mao, P.A. Janmey, H.L. Yin, Phosphatidylinositol 4, 5 bisphosphate and the actin cytoskeleton, Subcell Biochem 59 (2012) 177-215.
[181] Y.S. Mao, H.L. Yin, Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases, Pflugers Arch 455 (2007) 5-18.
[182] Y. Sun, D.A. Turbin, K. Ling, N. Thapa, S. Leung, D.G. Huntsman, R.A. Anderson, Type I gamma phosphatidylinositol phosphate kinase modulates invasion and proliferation and its expression correlates with poor prognosis in breast cancer, Breast Cancer Res 12 (2010) R6.
[183] J.M. Mataraza, M.W. Briggs, Z. Li, A. Entwistle, A.J. Ridley, D.B. Sacks, IQGAP1 promotes cell motility and invasion, J Biol Chem 278 (2003) 41237-41245.
[184] L. Jadeski, J.M. Mataraza, H.W. Jeong, Z. Li, D.B. Sacks, IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells, J Biol Chem 283 (2008) 1008-1017.
[185] Z. Li, D.B. Sacks, Elucidation of the interaction of calmodulin with the IQ motifs of IQGAP1, J Biol Chem 278 (2003) 4347-4352.

[186] Y. Sun, K. Ling, M.P. Wagoner, R.A. Anderson, Type I gamma phosphatidylinositol phosphate kinase is required for EGF-stimulated directional cell migration, J Cell Biol 178 (2007) 297-308.
[187] Y.J. Wang, W.H. Li, J. Wang, K. Xu, P. Dong, X. Luo, H.L. Yin, Critical role of PIP5KI{gamma}87 in InsP3-mediated Ca(2+) signaling, J Cell Biol 167 (2004) 1005-1010.
[188] Y. Wang, L. Lian, J.A. Golden, E.E. Morrisey, C.S. Abrams, PIP5KI gamma is required for cardiovascular and neuronal development, Proc Natl Acad Sci U S A 104 (2007) 11748-11753.
[189] Y. Wang, R.I. Litvinov, X. Chen, T.L. Bach, L. Lian, B.G. Petrich, S.J. Monkley, Y. Kanaho, D.R. Critchley, T. Sasaki, M.J. Birnbaum, J.W. Weisel, J. Hartwig, C.S. Abrams, Loss of PIP5KIgamma, unlike other PIP5KI isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes, J Clin Invest (2008).
[190] P.J. Keely, Ras and Rho protein induction of motility and invasion in T47D breast adenocarcinoma cells, Methods Enzymol 333 (2001) 256-266.
[191] M.A. Del Pozo, W.B. Kiosses, N.B. Alderson, N. Meller, K.M. Hahn, M.A. Schwartz, Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI, Nat Cell Biol 4 (2002) 232-239.
[192] M. Baumgartner, A.L. Sillman, E.M. Blackwood, J. Srivastava, N. Madson, J.W. Schilling, J.H. Wright, D.L. Barber, The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors, Proc Natl Acad Sci U S A 103 (2006) 13391-13396.
[193] D. Raucher, T. Stauffer, W. Chen, K. Shen, S. Guo, J.D. York, M.P. Sheetz, T. Meyer, Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion, Cell 100 (2000) 221-228.
[194] M.P. Czech, PIP2 and PIP3: complex roles at the cell surface, Cell 100 (2000) 603-606.
[195] V. Papayannopoulos, C. Co, K.E. Prehoda, S. Snapper, J. Taunton, W.A. Lim, A polybasic motif allows N-WASP to act as a sensor of PIP(2) density, Mol Cell 17 (2005) 181-191.
[196] J. Solon, I. Levental, K. Sengupta, P.C. Georges, P.A. Janmey, Fibroblast adaptation and stiffness matching to soft elastic substrates, Biophys J 93 (2007) 4453-4461.
[197] M. Schramp, N. Thapa, J. Heck, R. Anderson, PIPKIgamma regulates beta-catenin transcriptional activity downstream of growth factor receptor signaling, Cancer Res 71 (2011) 1282-1291.
[198] E. Gabev, J. Kasianowicz, T. Abbott, S. McLaughlin, Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2), Biochim Biophys Acta 979 (1989) 105-112.
[199] T. Lammermann, J. Renkawitz, X. Wu, K. Hirsch, C. Brakebusch, M. Sixt, Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration, Blood 113 (2009) 5703-5710.
[200] S.Y. Sokol, Z. Li, D.B. Sacks, The effect of IQGAP1 on Xenopus embryonic ectoderm requires Cdc42, J Biol Chem 276 (2001) 48425-48430.
[201] M. Schramp, A. Hedman, W. Li, X. Tan, R. Anderson, PIP Kinases from the Cell Membrane to the Nucleus, Subcell Biochem 58 (2012) 25-59.
[202] L.C. Cantley, The phosphoinositide 3-kinase pathway, Science 296 (2002) 1655-1657.
[203] G. Kadamur, E.M. Ross, Mammalian phospholipase C, Annu Rev Physiol 75 (2013) 127-154.

[204] L. Cocco, M.A. M., R.S. Gilmour, A. Ognibene, F.A. Manzoli, R.F. Irvine, Rapid changes in phospholipid metabolism in the nuclei of Swiss 3T3 cells induced by treatment of the cells with insulin-like growth factor I, Biochem Biophys Res Commun 154 (1988) 1266-1272.
[205] J.A. Creba, C.P. Downes, P.T. Hawkins, G. Brewster, R.H. Michell, C.J. Kirk, Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca2+-mobilizing hormones, Biochem J 212 (1983) 733-747.
[206] A. Balla, Y.J. Kim, P. Varnai, Z. Szentpetery, Z. Knight, K.M. Shokat, T. Balla, Maintenance of hormone-sensitive phosphoinositide pools in the plasma membrane requires phosphatidylinositol 4-kinase IIIalpha, Mol Biol Cell 19 (2008) 711-721.
[207] S. Nakanishi, K.J. Catt, T. Balla, A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids, Proc Natl Acad Sci U S A 92 (1995) 5317-5321.
[208] B. Vanhaesebroeck, J. Guillermet-Guibert, M. Graupera, B. Bilanges, The emerging mechanisms of isoform-specific PI3K signalling, Nat Rev Mol Cell Biol 11 (2010) 329-341.
[209] K.R. Auger, L.A. Serunian, S.P. Soltoff, P. Libby, L.C. Cantley, PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells, Cell 57 (1989) 167-175.
[210] P.T. Hawkins, T.R. Jackson, L.R. Stephens, Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase, Nature 358 (1992) 157-159.
[211] K.S. Carmon, X. Gong, J. Yi, A. Thomas, Q. Liu, RSPO-LGR4 functions via IQGAP1 to potentiate Wnt signaling, Proc Natl Acad Sci U S A 111 (2014) E1221-1229.
[212] V.A. Schmidt, C.S. Chiariello, E. Capilla, F. Miller, W.F. Bahou, Development of hepatocellular carcinoma in Iqgap2-deficient mice is IQGAP1 dependent, Mol Cell Biol 28 (2008) 1489-1502.
[213] E. Fayard, L.A. Tintignac, A. Baudry, B.A. Hemmings, Protein kinase B/Akt at a glance, J Cell Sci 118 (2005) 5675-5678.
[214] Y.E. Huang, M. Iijima, C.A. Parent, S. Funamoto, R.A. Firtel, P. Devreotes, Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells, Mol Biol Cell 14 (2003) 1913-1922.
[215] C. Jimenez, R.A. Portela, M. Mellado, J.M. Rodriguez-Frade, J. Collard, A. Serrano, A.C. Martinez, J. Avila, A.C. Carrera, Role of the PI3K regulatory subunit in the control of actin organization and cell migration, J Cell Biol 151 (2000) 249-262.
[216] K.L. Jameson, P.K. Mazur, A.M. Zehnder, J. Zhang, B. Zarnegar, J. Sage, P.A. Khavari, IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase-driven tumors, Nat Med 19 (2013) 626-630.
[217] J.A. Engelman, J. Luo, L.C. Cantley, The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism, Nat Rev Genet 7 (2006) 606-619.
[218] B.D. Lehmann, J.A. Bauer, X. Chen, M.E. Sanders, A.B. Chakravarthy, Y. Shyr, J.A. Pietenpol, Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, J Clin Invest 121 (2011) 2750-2767.
[219] A.J. Hackett, H.S. Smith, E.L. Springer, R.B. Owens, W.A. Nelson-Rees, J.L. Riggs, M.B. Gardner, Two syngeneic cell lines from human breast tissue: the aneuploid

mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines, J Natl Cancer Inst 58 (1977) 1795-1806.
[220] P.J. Roberts, C.J. Der, Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer, Oncogene 26 (2007) 3291-3310.
[221] J.S. Sebolt-Leopold, R. Herrera, Targeting the mitogen-activated protein kinase cascade to treat cancer, Nat Rev Cancer 4 (2004) 937-947.
[222] H.C. Chen, P.A. Appeddu, H. Isoda, J.L. Guan, Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase, J Biol Chem 271 (1996) 26329-26334.
[223] R.J. Cain, A.J. Ridley, Phosphoinositide 3-kinases in cell migration, Biol Cell 101 (2009) 13-29.
[224] M. Yamaoka-Tojo, M. Ushio-Fukai, L. Hilenski, S.I. Dikalov, Y.E. Chen, T. Tojo, T. Fukai, M. Fujimoto, N.A. Patrushev, N. Wang, C.D. Kontos, G.S. Bloom, R.W. Alexander, IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species--dependent endothelial migration and proliferation, Circ Res 95 (2004) 276-283.
[225] J.A. Engelman, Targeting PI3K signalling in cancer: opportunities, challenges and limitations, Nat Rev Cancer 9 (2009) 550-562.
[226] J. Luo, B.D. Manning, L.C. Cantley, Targeting the PI3K-Akt pathway in human cancer: rationale and promise, Cancer Cell 4 (2003) 257-262.
[227] J. Kao, K. Salari, M. Bocanegra, Y.L. Choi, L. Girard, J. Gandhi, K.A. Kwei, T. Hernandez-Boussard, P. Wang, A.F. Gazdar, J.D. Minna, J.R. Pollack, Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery, PLoS One 4 (2009) e6146.
[228] T. Kroll, L. Odyvanova, J.H. Clement, C. Platzer, A. Naumann, N. Marr, K. Hoffken, S. Wolfl, Molecular characterization of breast cancer cell lines by expression profiling, J Cancer Res Clin Oncol 128 (2002) 125-134.
[229] I.B. Weinstein, A. Joe, Oncogene addiction, Cancer Res 68 (2008) 3077-3080; discussion 3080.
[230] S.V. Sharma, J. Settleman, Oncogene addiction: setting the stage for molecularly targeted cancer therapy, Genes Dev 21 (2007) 3214-3231.
[231] A. Hubner, A. Jaeschke, R.J. Davis, Oncogene addiction: role of signal attenuation, Dev Cell 11 (2006) 752-754.
[232] C.E. Bazenet, R.A. Anderson, Phosphatidylinositol-4-phosphate 5-kinases from human erythrocytes, Methods Enzymol 209 (1992) 189-202.
[233] J.R. Halstead, K. Jalink, N. Divecha, An emerging role for PtdIns(4,5)P2-mediated signalling in human disease, Trends Pharmacol Sci 26 (2005) 654-660.
[234] J.G. Foster, M.D. Blunt, E. Carter, S.G. Ward, Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies, Pharmacol Rev 64 (2012) 1027-1054.
[235] M.C. Mendoza, E.E. Er, J. Blenis, The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation, Trends Biochem Sci 36 (2011) 320-328.
[236] F. Vinals, J. Pouyssegur, Confluence of vascular endothelial cells induces cell cycle exit by inhibiting p42/p44 mitogen-activated protein kinase activity, Mol Cell Biol 19 (1999) 2763-2772.
[237] H. Nojima, M. Adachi, T. Matsui, K. Okawa, S. Tsukita, IQGAP3 regulates cell proliferation through the Ras/ERK signalling cascade, Nat Cell Biol 10 (2008) 971-978.

[238] E. Lelievre, P.M. Bourbon, L.J. Duan, R.L. Nussbaum, G.H. Fong, Deficiency in the p110alpha subunit of PI3K results in diminished Tie2 expression and Tie2(-/-)-like vascular defects in mice, Blood 105 (2005) 3935-3938.
[239] K. Ueki, C.M. Yballe, S.M. Brachmann, D. Vicent, J.M. Watt, C.R. Kahn, L.C. Cantley, Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase, Proc Natl Acad Sci U S A 99 (2002) 419-424.
[240] L. Bi, I. Okabe, D.J. Bernard, R.L. Nussbaum, Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase, Mamm Genome 13 (2002) 169-172.
[241] L. Bi, I. Okabe, D.J. Bernard, A. Wynshaw-Boris, R.L. Nussbaum, Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase, J Biol Chem 274 (1999) 10963-10968.
[242] Y. Wang, X. Chen, L. Lian, T. Tang, T.J. Stalker, T. Sasaki, Y. Kanaho, L.F. Brass, J.K. Choi, J.H. Hartwig, C.S. Abrams, Loss of PIP5KIbeta demonstrates that PIP5KI isoform-specific PIP2 synthesis is required for IP3 formation, Proc Natl Acad Sci U S A 105 (2008) 14064-14069.
[243] H. Hasegawa, J. Noguchi, M. Yamashita, R. Okada, R. Sugimoto, M. Furuya, T. Unoki, Y. Funakoshi, T. Baba, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase is indispensable for mouse spermatogenesis, Biol Reprod 86 (2012) 136, 131-112.