THE REGULATION OF PREX2 BY PHOSPHORYLATION

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ABSTRACT

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Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3)-dependent RAC exchanger 2 (PREX2) is a guanine nucleotide exchange factor (GEF) for the Ras-related C3 botulinum toxin substrate 1 (RAC1) GTPase. As a GEF, PREX2 facilitates the exchange of GDP for GTP on RAC1. GTP bound RAC1 then activates its downstream effectors, including p21-activated kinases (PAK). PREX2, RAC1, and PAK kinases all have key roles within the insulin signaling pathway. The insulin receptor is a tyrosine kinase that phosphorylates the insulin receptor substrate (IRS) family of adaptor proteins, leading to the activation of phosphatidylinositide 3-kinase (PI3K) and the generation of PI(3,4,5)P3. PI(3,4,5)P3 then activates numerous downstream signaling proteins, including AKT and RAC1, to regulate several important cellular processes, such as glucose metabolism and cell proliferation. In addition to being a RAC1 GEF, PREX2 affects the insulin signaling pathway by inhibiting the lipid phosphatase activity of phosphatase and tensin homolog (PTEN), which dephosphorylates PI(3,4,5)P3 to antagonize PI3K. PREX2 is also important in cancer, which is likely a consequence of both its role as a RAC1 GEF and as a PTEN inhibitor.

PREX2 GEF activity is activated by PI(3,4,5)P3 and by Gβγ, which is a heterodimer that is released after GPCR activation. However, PREX2 regulation within specific signaling pathways is poorly understood. This thesis aims to understand the

regulation of PREX2 downstream of ligand binding to receptors on the cell surface, with a focus on insulin. This is achieved by studying the phosphorylation of PREX2 after insulin stimulation and by characterizing protein-protein interactions involving PREX2 and key proteins in the insulin signaling pathway.

Herein, we identified PI(3,4,5)P3-dependent phosphorylation events on PREX2 that occur downstream of insulin stimulation. Phosphorylation of PREX2 also occurred downstream of Gβγ, suggesting that phosphorylation was associated with the activation of PREX2 GEF activity. Interestingly, phosphorylation of PREX2 reduced GEF activity towards RAC1 and a phospho-mimicking mutation of PREX2 at an insulin-mediated phosphorylation site reduced cancer cell invasion. Phosphorylation of PREX2 also decreased PREX2 binding to the cellular membrane, PI(3,4,5)P3, and Gβγ, providing a mechanism for reduced GEF activity. These data suggested that phosphorylation was part of a negative feedback circuit to decrease the RAC1 signal, which led to the identification of the PAK kinases as mediators of PREX2 phosphorylation. Importantly, insulin-induced phosphorylation of PREX2 was delayed compared to AKT, which is consistent with a model where PREX2 phosphorylation by PAK occurs after activation of PREX2 to attenuate its function. Altogether, we propose that second messengers activate the PREX2-RAC1 signal, which sets in motion a cascade whereby PAK kinases phosphorylate and negatively regulate PREX2 to decrease RAC1 activation. This type of regulation would allow for transient activation of the PREX2-RAC1 signal. We then asked whether PAK phosphorylation of PREX2 was altered in cancer. To do this, we analyzed four recurrent somatic PREX2 tumor mutations, R155W, R297C, R299Q, and R363Q. Interestingly, all four mutants had reduced insulin and PAK1 dependent

phosphorylation, and R297C had lower levels of phosphorylation induced by PI3K activating tumor mutants. This suggests that tumors might be mutating PREX2 in order to avoid PAK mediated negative regulation of RAC1.

Lastly, we characterized PREX2 interactions with proteins that are critical for insulin signaling, with a focus on the interaction between the PREX2 pleckstrin homology (PH) domain and PTEN. PREX2 inhibition of PTEN is mediated by the PH domain, and we discovered that the $\beta_3\beta_4$ loop of the PH domain was required for binding of the isolated PH domain to PTEN. We also found that PREX2 co-immunoprecipitates with other insulin related proteins, including the p85 regulatory subunit of PI3K, IRS4, and the insulin receptor.

Taken together, the studies in this thesis solidify the role of PREX2 in insulin signaling by showing that PREX2 GEF activity is tightly regulated by insulin and PAK-induced phosphorylation and also by characterizing PREX2 interactions with critical insulin related proteins. Further, this PAK dependent negative regulatory circuit downstream of both PI(3,4,5)P3 and Gβγ activation of PREX2 could have impacts in many aspects of biology given the roles that PREX2 and RAC1 have in critical cellular functions such as cell motility and glucose metabolism, and in diseases such as cancer and diabetes.

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Dedication

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CHAPTER 1: Introduction

The binding of ligands to receptors on the cell membrane regulates intracellular signaling pathways, allowing cells to receive and process signals from the extracellular environment. Receptor activation oftentimes modulates the levels of second messengers within the cell. Second messengers, such as phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) and Gβγ, initiate signal transduction cascades that carry out fundamental cellular processes. Both PI(3,4,5)P3 and Gβγ stimulate the guanine nucleotide exchange factor (GEF) PI(3,4,5)P3-dependent RAC exchanger 2 (PREX2), resulting in the activation of the small GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1). RAC1 has profound effects on both cell motility downstream of numerous ligand activated receptors, and glucose metabolism downstream of the insulin receptor. Herein, we study how PREX2 function, and therefore RAC activity, is regulated downstream of second messengers, with a focus on the phosphorylation of PREX2 after insulin receptor activation.

PREX2: FUNCTION AND REGULATION WITH INSIGHTS FROM PREX1

PREX2 discovery and structure

PREX2 is a GEF of the small GTPase RAC1. GEFs such as PREX2 bind to RAC1 and facilitate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), and while in the GTP bound state, RAC1 is able to bind many effectors and carry out various cellular functions. For example, GTP bound RAC1 activates the WASP-family verprolin-homologous proteins (WAVE) regulatory complex, p21-activated kinase 1 (PAK1), the NADPH oxidase complex, and the p110β catalytic subunit of phosphatidylinositide 3-kinase (PI3K) (Abo et al., 1991; Chen et al., 2010; Fritsch et al.,

2013; Manser et al., 1994). PREX2 is located on chromosome 8, q13.2, and was identified simultaneously by two groups based on its homology to the founding member of the PREX family of GEFs, PREX1. A splice variant, PREX2b, is missing a large portion of the C-terminal region (Donald et al., 2004; Rosenfeldt et al., 2004). PREX1 was discovered in neutrophils during a search for a PI(3,4,5)P3 and Gβγ-stimulated RAC GEF regulating the formation of reactive oxygen species (Welch et al., 2002). In addition to RAC1, PREX1 can activate a number of other RAS (Rat sarcoma) homolog (RHO) family GTPases *in vitro*, including RAC2, RAC3, RHOG, and cell division control protein 42 homolog (CDC42). However, there is not yet any *in vivo* evidence that PREX1 can activate CDC42 (Damoulakis et al., 2014; Welch et al., 2002). It is possible that PREX2 has GEF activity towards RHO GTPases other than RAC1, however this has not been reported. PREX2 shares 59% sequence homology with PREX1, and like PREX1, PREX2 is a multi-domain protein containing the tandem DH (Dbl homology) and PH (Pleckstrin homology) domains, two DEP (Dishevelled, Egl-10, and Pleckstrin) domains, two PDZ (post synaptic density protein, disc large, and zonula occludens-1) domains, and a C-terminal region with homology to inositol polyphosphate-4-phosphatase (IP4P) (Figure 1.1) (Donald et al., 2004; Rosenfeldt et al., 2004). The DH domain is the functional GEF domain for Dbl family RHO GEFs, and the PH domain is often important in targeting the GEF to certain locations within the cell, and can

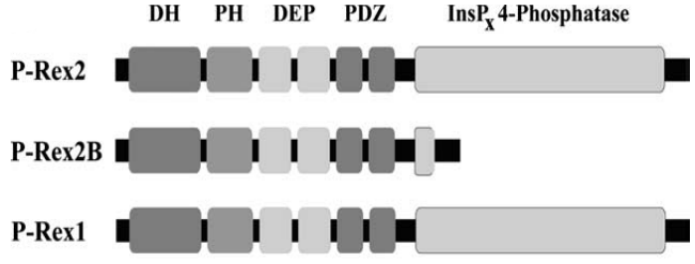


Figure 1.1 – PREX domain structure (Donald et al., 2004)

sometimes affect GEF activity of the DH domain (Schmidt and Hall, 2002). In addition, the IP4P domain of PREX1 has no reported phosphatase activity (Welch et al., 2002). PREX2 mRNA is widely expressed, with high expression in the skeletal muscle and heart, and no expression in blood leukocytes, which interestingly is where PREX1 mRNA expression is strongest (Donald et al., 2004; Rosenfeldt et al., 2004). PREX2 protein expression is more restricted, and is highest in the brain and lung, with weaker expression in the liver and adipose tissue (Donald et al., 2008; Hodakoski et al., 2014). Within the brain, PREX2 is most strongly expressed in the cerebellum, which is consistent with the reported deficiencies in Purkinje cell morphology and motor coordination in PREX2$^{-/-}$ mice (Donald et al., 2008).

PREX2 and PREX1 GEF activity is activated by the second messengers PI(3,4,5)P3 and Gβγ. PI(3,4,5)P3 is generated by PI3K, which can be activated downstream of receptor tyrosine kinase activation and also by Gβγ, which is a dimer composed of the beta and gamma subunits of heterotrimeric G proteins that is released upon activation of G-protein coupled receptors (GPCRs). PREX2 can be directly activated by both PI(3,4,5)P3 and Gβγ *in vitro*, and expression of both PI3K and Gβγ synergistically activates PREX2 *in vivo* (Donald et al., 2004; Rosenfeldt et al., 2004).

The involvement of PREX proteins in PI(3,4,5)P3 and Gβγ dependent RAC activation

PI(3,4,5)P3 and Gβγ levels at the membrane are regulated by numerous ligand-activated receptors, and PREX regulation of RAC1 has been studied in many of these contexts. Both PREX2 and PREX1 have roles downstream of insulin receptor activation, a pathway that is a focus of this thesis, and the roles of PREX proteins in insulin signaling will be described in more detail in a later section (Balamatsias et al., 2011; Fine

et al., 2009; Hodakoski et al., 2014). Expression of both PREX1 and PREX2 increase the level of platelet derived growth factor (PDGF) stimulated RAC activity in porcine aortic endothelial (PAE) cells (Donald et al., 2004; Welch et al., 2002). In addition, PDGFRβ knockdown in immortalized human fibroblasts blocks PREX1 driven invasive migration (Campbell et al., 2013). PREX2b also regulates RAC1 activity and cell migration during the stimulation of endothelial cells with sphingosine-1-phosphate (Li et al., 2005b). In PC-12 cells, knockdown of PREX1 impairs nerve growth factor mediated migration, and in cultured cortical neurons the expression of a dominant negative PREX1 mutant blocks brain derived neurotrophic factor and epidermal growth factor (EGF) driven migration (Yoshizawa et al., 2005). In breast cancer cells, PREX1 knockdown prevents RAC activation after stimulation by neuregulin, EGF, and transforming growth factor-α, all of which are ligands that activate epidermal growth factor receptor (EGFR) family members (Sosa et al., 2010). Both PREX2 and PREX1 can also bind to mammalian target of rapamycin (mTOR), and PREX1 knockdown reduces leucine stimulated RAC activation and cell migration (Hernandez-Negrete et al., 2007). Further, lipopolysaccharide (LPS) or tumor necrosis factor α (TNFα) primed neutrophils from PREX1$^{-/-}$ mice have significantly lower N-Formylmethionine leucyl-phenylalanine (fMLP) stimulated ROS formation. Given that LPS and TNFα are known to activate PI3K, this demonstrates how both PI(3,4,5)P3 and Gβγ inputs are important for this effect. RAC2 activation following fMLP treatment is also reduced in both PREX1$^{-/-}$ and PREX1 mutant mouse neutrophils (Dong et al., 2005; Welch et al., 2005). In addition, PREX1 expression increases fMLP dependent reactive oxygen species formation in COS cells (Nie et al., 2010).

Regulation of PREX1 by phosphorylation

There are no published reports of PREX2 phosphorylation, however many groups have reported phosphorylation events on PREX1. Given the similarities previously established in the regulation of these two proteins, an understanding of PREX1 phosphorylation may help inform how we study the regulation of PREX2. The first PREX1 phosphorylation event identified was shown to be mediated by cAMP-dependent protein kinase A (PKA). This study showed that *in vitro* incubation of PREX1 with purified PKA decreases Gβγ-induced PREX1 GEF activity. Further, when PREX1 is dephosphorylated with λ phosphatase, there is an increase in Gβγ-induced GEF activity (Mayeenuddin and Garrison, 2006). PKA phosphorylation of PREX1 blocks an intramolecular interaction between the IP4P and the second DEP/first PDZ domain. This intramolecular interaction is important for binding of PREX1 to Gβγ, Gβγ-induced GEF activity, and PREX1 dependent lamellipodia formation. This provides a potential mechanism explaining how PKA mediated phosphorylation events negatively regulate GEF activity (Mayeenuddin and Garrison, 2006; Urano et al., 2008).

PREX1 is also stoichiometrically phosphorylated downstream of the ErbB receptor tyrosine kinase family ligand, neuregulin (NRG). Interestingly, this study showed that while neuregulin causes an increase in phosphorylation at both S605 and S1169, it also reduces phosphorylation at S315 and S319 within the PH domain. S1169 phosphorylation is also induced by IGF-1 in MCF-7 and T-47D breast cancer cells, by PDGF in HS578T breast cancer cells, and by fibroblast growth factor (FGF) in MCF7 cells. Interestingly, EGF is unable to stimulate S1169 phosphorylation in MCF7 cells, suggesting that this

phosphorylation only occurs downstream of specific growth factors (Montero et al., 2011; Montero et al., 2013).

Both PREX2 and PREX1 contain a highly conserved consensus PP1α binding site in the IP4P domain. The PP1α consensus binding sequence has been defined as [RK]-X(0,1)-[VI]-~~P~~-[FW], where X is any residue and ~~P~~ any residue but proline. PREX2 was identified as a PP1α binding partner in a screen for proteins that contain this consensus sequence, however this study did not show whether PP1α could actually dephosphorylate PREX2 (Hendrickx et al., 2009). A later study then showed that PP1α binds to and dephosphorylates PREX1, and that this dephosphorylation activates the GEF activity of PREX1 both *in vitro* and *in vivo*. Using mass spectrometry, they identified S1165 as a PP1α sensitive phosphorylation site, and showed that an alanine mutation at this residue mimics PP1α dephosphorylation and activates GEF activity of the protein (Barber et al., 2012).

Taken together, it appears that phosphorylation of PREX1 is important for its function, and often decreases PREX1 GEF activity. These studies present the possibility that PREX2 could be regulated by phosphorylation as well.

Regulation of PREX1 membrane binding

Localization to the cell membrane, where both PI(3,4,5)P3 and Gβγ are produced, is an important step in the activation of PREX1. PI3K activity and Gβγ synergistically increase the amount of PREX1 at the membrane, and membrane bound PREX1 has a higher basal GEF activity towards RAC2 (Barber et al., 2007; Zhao et al., 2007). Further, it appears that phosphorylation of PREX1 regulates membrane binding. Surface plasmon resonance experiments showed that PREX1 that has been phosphorylated *in*

vitro by PKA has reduced binding to Gβγ (Urano et al., 2008). Additionally, when human neutrophils are treated with the cAMP-elevating agent forskolin and the cAMP analog 6-Bnz-cAMP, both of which activate PKA, GPCR mediated translocation of PREX1 to the membrane is reduced. This effect is rescued with concurrent inhibition of PKA using the small molecule H89 (Zhao et al., 2007).

RAC FAMILY GTPASES

RAC structure and regulation of activity

Thus far, PREX2 has only been shown to have GEF activity towards RAC1, a member of the RHO family small GTPases. RAC1 and RAC2 were first discovered based on their homology to previously discovered members of the RAS and RHO families of GTPases. RAC1 is ubiquitously expressed, while RAC2 is primarily expressed in hematopoietic cells (Didsbury et al., 1989). A third family member, RAC3, is expressed in multiple tissues, with strong expression in the brain (Haataja et al., 1997). Lastly, RAC1b, a splice variant of RAC1, is most strongly expressed in the colon (Jordan et al., 1999).

The first known function of RAC1 and RAC2 was in the activation of an NADPH oxidase in response to bacterial infection in phagocytes, and this function requires GTP loaded RAC (Abo et al., 1991; Knaus et al., 1991). This finding was critical in that it demonstrates how small GTPases like RAC must be in the GTP bound state to bind many of its effectors. The cycling of RAC between the inactive GDP bound state and an active GTP bound state is tightly regulated through interactions with other proteins in the cell. GTPase activating proteins (GAPs) increase the GTPase activity of RAC, accelerating the hydrolysis of GTP to GDP, promoting the inactive, GDP bound state. In addition,

guanine nucleotide dissociation inhibitors (GDIs) decrease RAC activation by sequestering inactive RAC in the cytosol. As described previously with regards to PREX2 function, GEFs bind to RAC and facilitate the release of GDP once it has been hydrolyzed, leading to the loading of GTP (Cherfils and Zeghouf, 2013). Like other small GTPases, RAC structure consists of a central β-sheet made up of six strands, surrounded by α-helicies (six in the case of RAC1) (Hirshberg et al., 1997). Additionally, there are two regions of RAC1 called switch I and switch II that are critical for binding of the guanine nucleoside, and magnesium binding to this switch region is important for stable nucleoside loading. GEFs that activate RHO GTPases, including RAC, contain a catalytic DH domain, which binds to the switch I and II regions of RAC and alters their conformation to disrupt magnesium binding and allow for release of the nucleoside. Given that GTP is present at much higher concentrations in the cell, this results in GTP loading and activation of RAC (Vetter and Wittinghofer, 2001; Worthylake et al., 2000). Interestingly, RAC1b contains an additional 19 amino acids after the switch II region, and appears to be more active in cells and less sensitive to inactivation by GDIs (Matos et al., 2003).

RAC1 regulation of actin polymerization

RAC1 activation causes profound effects on the reorganization of the cytoskeleton. Activated RAC1 stimulates lamellipodia formation and focal adhesion complexes, making RAC1 a critical regulator of cell migration and cell adhesion (Nobes and Hall, 1995; Ridley et al., 1992). Many of the RAC1 dependent functions in cell motility stem from the ability of RAC1 to regulate actin polymerization. Actin polymerization is mediated in part by the ability of GTP bound RAC to stimulate actin polymerization by

binding to the WAVE regulatory complex (WRC). The WRC is a multi-protein complex consisting of WAVE1, SRA1 (specifically RAC1-associated protein 1), NAP1 (NCK-associated protein 1), ABI1 or 2 (ABL interactor 1 or 2) and HSPC300 (Eden et al., 2002). While in this complex, WAVE1 is inactive and unable to nucleate actin; however, crystallographic studies have revealed that RAC1 relieves an inhibitory interaction between WAVE1 and SRA1 that occludes the WAVE VCA motif (verprolin-homology, central and acidic regions), which is responsible for the activation of actin polymerization. The binding of GTP bound RAC1 to the complex allows the WAVE1 VCA motif to bind the ARP2/3 complex, leading to actin polymerization (Chen et al., 2010; Eden et al., 2002; Ismail et al., 2009).

RAC1 regulation of PAK kinases

Another major effector of GTP bound RAC1 is the PAK family of kinases. The PAK family is comprised of Group I (PAK1-3) and Group II (PAK4-6) (Abo et al., 1998; Dan et al., 2002; Manser et al., 1994; Yang et al., 2001). All members of the PAK family contain a GTPase binding domain (PBD) near the N-terminus, and a conserved kinase domain on the C-terminus of the protein. Group I PAKs, the more extensively studied of the two groups, contain an auto-inhibitory domain (AID), which overlaps with the PBD. Group I PAKs are thought to form homodimers that keep the kinases in an inactive state. Upon binding to GTP bound RAC1 or CDC42, the homodimer is disrupted, allowing an activating autophosphorylation event in the activation loop at T423 on PAK, resulting in an active kinase (Lei et al., 2000). The activation of Group II PAKs is not as well understood. PAK4 has been the most extensively studied, and its mode of activation is different from Group I PAKs. Instead of being activated by phosphorylation, PAK4 is

constitutively phosphorylated at S474, and its kinase activity appears to be autoinhibited by residues near the N-terminus of the protein that bind to the kinase domain. While it is known that CDC42 can bind to PAK4, it is unclear to what extent RHO GTPases can activate PAK4 kinase activity (Baskaran et al., 2012; Ha et al., 2012; Wang et al., 2013).

A large number of substrates have been identified for PAK kinases resulting in a multitude of cellular functions (Figure 1.2). One such function is to help carry out the RAC1 driven cytoskeletal reorganization that occurs during lamellipodia formation.

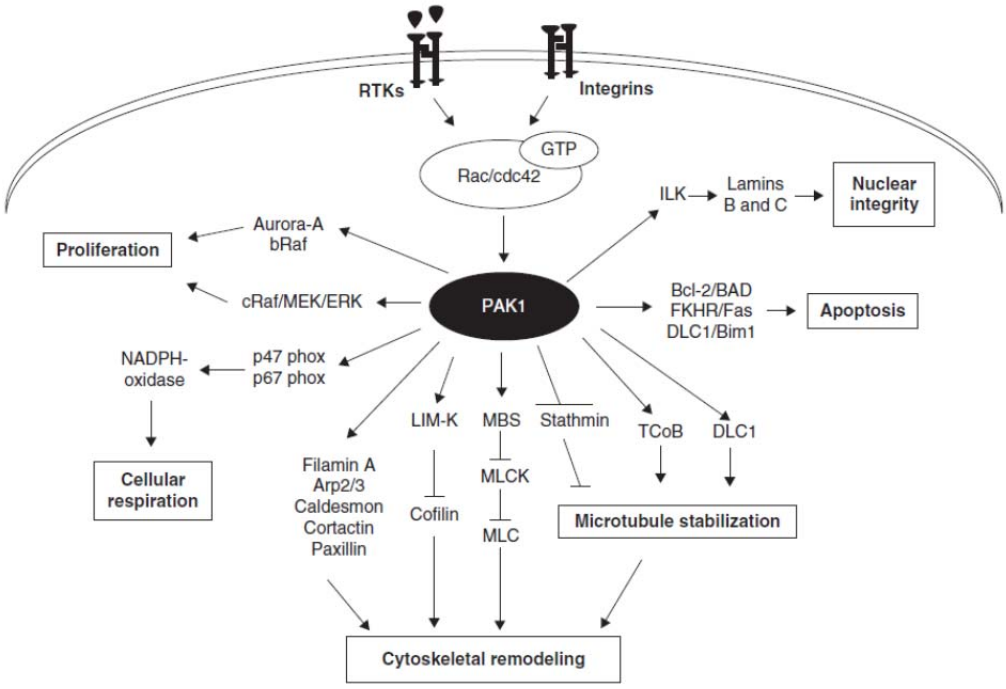


Figure 1.2 – PAK substrates and downstream effects (Kichina et al., 2010)

Phosphorylation of myosin light chain kinase (MLCK) by PAK1 reduces stress fibers in the cell, decreasing rigidity and promoting cell movement (Sanders et al., 1999). Both PAK1 and PAK4 can also phosphorylate LIM kinsase (LIMK) (Dan et al., 2001; Edwards et al., 1999). Once phosphorylated, LIMK phosphorylates ADF/cofilin and prevents the ability of ADF/cofilin to carry out its normal function in promoting

depolymerization of actin filaments (Arber et al., 1998). PAKs also have a role in cell survival by preventing apoptosis. PAK1 and PAK5 phosphorylation of RAF1 causes RAF1 to translocate to the mitochondria where it phosphorylates the pro-apoptotic factor, BCL-2 antagonist of cell death (BAD), blocking the ability of BAD to promote apoptosis (Jin et al., 2005; Wu et al., 2008). Furthermore, PAK1, PAK4, and PAK5 can directly phosphorylate and inhibit BAD (Cotteret et al., 2003; Gnesutta et al., 2001; Ye et al., 2011). In addition, PAK activation by GTPases is important for cellular proliferation. For example, PAK phosphorylation of RAF1 and MEK1 is important for effective propagation of the RAS-MAPK-ERK signaling pathway, which regulates proliferation and the cell cycle (King et al., 1998; Slack-Davis et al., 2003; Sun et al., 2000; Tang et al., 1997; Tran and Frost, 2003).

Activation of RAC1 in a polarized cell

RAC1 dependent actin polymerization is critical for the chemotaxis of a cell towards a chemoattractant, and as previously mentioned, PREX1 and PREX2 have defined roles in the context of chemoattractant driven cell migration. During chemotaxis, a concentration gradient of chemoattractant leads to the establishment of a leading edge and lagging edge of a cell, with both of these poles being molecularly different. Chemoattractants often bind to GPCRs, which are uniformly distributed on the cellular membrane, and activate numerous intracellular pathways to cause cell migration, including the activation of PI3K to modulate PI(3,4,5)P3 levels (Servant et al., 1999; Xiao et al., 1997). Growth factors that bind receptor tyrosine kinases (RTKs), like PDGF, can also serve as chemoattractants (Grotendorst et al., 1982). Given that PREX proteins are activated by both RTKs and GPCRs, they are in a unique position to affect

chemoattractant driven RAC1 activation. One of the key consequences of receptor activation by chemoattractants is an increase in PI(3,4,5)P3 at the leading edge of the cell, allowing for activation of specific signals at the leading edge, including RAC1 dependent actin polymerization and lamellipodia formation. In *Dictyostelium,* neutrophils, and mouse fibroblasts, it was shown that proteins with PI(3,4,5)P3 binding PH domains localize to the leading edge, and abrogation of PI3K activity blocks this effect (Funamoto et al., 2001; Haugh et al., 2000; Meili et al., 1999; Servant et al., 2000)**.** Further, in *Dictyostelium*, gradients of the chemoattractant cAMP cause translocation of PI3K isoforms to the leading edge of the cell, with a concurrent localization of PTEN to the trailing edge, setting up a gradient of PI(3,4,5)P3 concentrations on the plasma membrane (Funamoto et al., 2002; Iijima and Devreotes, 2002). However, follow up studies have indicated that the critical role of PI(3,4,5)P3 in chemotaxis is not so straightforward, and is likely cell type and context dependent (Ferguson et al., 2007; Hoeller and Kay, 2007; Loovers et al., 2006)

RAC1 activation by PI(3,4,5)P3 at the leading edge results in cell protrusions due to its roles in actin dependent lamellipodia formation and cell adhesion, two critical processes for cell motility (Das et al., 2000; Han et al., 1998; Shinohara et al., 2002; Welch et al., 2002). PREX2, in addition to a number of other RAC GEFs, contain PH domains that can bind membrane lipids such as PI(3,4,5)P3, and these GEFs can potentially regulate RAC1 at the leading edge. In contexts where PI(3,4,5)P3 is important for cell polarity and chemotaxis, PREX2 is likely to have an important role.

RAC and cancer

RAC has a role in many oncogenic cellular processes, such as cell migration, invasion, and proliferation, and there are some indications that it has a role in tumorigenesis. Increases in RAC1 expression have been reported in breast and colorectal cancers, as well as in leukemia (Fritz et al., 1999; Schnelzer et al., 2000; Wang et al., 2009). This has been supported by publically available data on the cBio portal showing that RAC1 expression is elevated in many different cancer types, including breast, lung, stomach and uterine cancer (Cerami et al., 2012; Gao et al., 2013). Analysis of mutations in a cohort of melanomas showed that a P29S mutation in RAC1 occurred in 9% of sun-exposed melanomas. This mutant displays increased binding to PAK1 and spontaneous loading of GTP in the absence of a GEF (Davis et al., 2013; Krauthammer et al., 2012). Additionally, the splice variant of RAC1, RAC1b, has increased expression in colon, breast, and lung tumors (Jordan et al., 1999; Schnelzer et al., 2000; Zhou et al., 2013)**.** Furthermore, members of the PAK family of kinases, especially PAK1 and PAK4, are often overexpressed in many different types of cancer, including breast and ovarian cancer. In addition, PAK kinases frequently have higher levels of phosphorylation in cancer, suggesting they have increased levels of kinase activity (King et al., 2014)

PREX2 and cancer

GEFs that activate RAC1, including PREX2, are frequently altered in cancer. PREX2 expression is increased in many different tumor types, and PREX2 is one of the most frequently mutated GEFs across all cancer types, with especially high rates of mutation in melanoma (Cerami et al., 2012; Fine et al., 2009; Gao et al., 2013). One particular study sequenced 25 melanomas and found that after NRAS and BRAF, PREX2

was one of the most significantly mutated genes in that cohort. They also showed that certain PREX2 tumor mutations have functional consequences. The truncation mutants K278*, E824*, and Q1430*, in addition to the missense mutation G844D, cooperate with NRAS expression to increase tumorigenesis in a melanocyte xenograft model (Berger et al., 2012). Further, melanoma tumor mutants G844D and P498S, and the pancreatic tumor mutant V432M are able to evade inhibition of GEF activity by PTEN (Mense et al., 2015). Additionally, PREX2 expression is higher in breast cancers with PI3K activating mutations, and PREX2 cooperates with these PI3K mutations to form colonies in soft agar (Fine et al., 2009). PREX2 is also the target of a microRNA that is down regulated in metastatic neuroblastomas and gastric cancer (Chen et al., 2013; Guo et al., 2014). PREX1 expression is also increased in cancer, including breast and prostate cancer, as well as melanoma (Lindsay et al., 2011; Qin et al., 2009; Sosa et al., 2010).

REGULATION OF SECOND MESSENGERS THAT ACTIVATE PREX2

The second messengers Gβγ and PI(3,4,5)P3 both activate PREX2 GEF activity. They are also integral for the activation of many intracellular signals after ligand binding to receptors on the membrane. The regulation of Gβγ and PI(3,4,5)P3 has been extensively studied and will be outlined in this section.

Gβγ

GPCR structure and activation

Gβγ is a dimer of β and γ subunits that is regulated by GPCRs. There are estimated to be over 800 different GPCRs encoded in the human genome with a wide

range of functions (Fredriksson et al., 2003). GPCRs have a common structure including seven transmembrane domains that are connected by three extracellular and three intracellular loops. Additionally, the intracellular C-terminus and extracellular N-terminus of GPCRs can vary greatly in length and composition. In the absence of an agonist binding to a GPCR, Gα-GDP, Gβ, and Gγ form an inactive heterotrimer. Upon agonist binding, the GPCR acts as a GEF to facilitate the exchange of GDP for GTP on Gα, and this causes dissociation of Gα-GTP and the Gβγ heterodimer from the receptor (Kobilka, 2007).

Gβγ regulation of intracellular signaling pathways

Early studies on GPCRs made it clear that dissociation of Gα-GTP from Gβγ allows Gα isoforms to regulate various intracellular signals, including the activation ($G_{\alpha s}$) and inhibition ($G_{\alpha i}$) of adenylyl cyclase to regulate cAMP levels, and the activation of phospholipase C ($G_{\alpha q/11}$), which hydrolyzes PtdIns to regulate protein kinase C (PKC) and intracellular calcium levels. It was also originally thought that Gβγ could be a negative regulator of the heterotrimeric complex (Gilman, 1987). However, the discovery that purified Gβγ activates potassium channels in the heart showed that this signaling heterodimer could independently activate downstream signals upon GPCR activation (Logothetis et al., 1987). This was confirmed by many studies showing that Gβγ activates other signaling molecules, such as adenylyl cyclase, phospholipase C-β2, voltage-gated calcium channels, and importantly for this thesis, PI3K (Camps et al., 1992; Ikeda, 1996; Stephens et al., 1994; Tang and Gilman, 1991). In humans, there are five Gβ subunits, $G\beta_{1\text{-}5}$, where $G\beta_{1\text{-}4}$ share a high level of sequence homology, while $G\beta_5$ does not. In addition, there are 12 Gγ subunits, $G\gamma_{1\text{-}5,\ 7\text{-}13}$. Gγ isoforms are not as well

conserved as Gβ isoforms, and much of the diversity in Gγ appears to exist on the outward facing residues of the protein as opposed to the region of the protein that is bound to Gβ. This diversity of exposed residues potentially allows for specificity for different Gβγ targets (Khan et al., 2013). While a role for PREX2 has not yet been identified downstream of any specific GPCRs or GPCR ligands, Gβγ expression can activate PREX2 GEF activity in insect cells. Further, PREX2b, the PREX2 splice variant, has a role in RAC1 activation downstream of the GPCR ligand sphingosine-1-phosphate (Li et al., 2005b). In addition, PREX1 has been characterized downstream of ligand induced GPCR activation, as highlighted above.

PI(3,4,5)P3

The intracellular levels of PI(3,4,5)P3 are tightly regulated by the lipid kinase PI3K and the lipid phosphatase PTEN. Here, the regulation of PI3K and PTEN will be discussed.

PI3K

Prior to the identification of PI3K, it was known that kinase activity towards phosphoinositol (PtdIns) co-purified with gene products from cancer-inducing viruses, and that this lipid kinase activity was important for the transformation of cells (Kaplan et al., 1986; Macara et al., 1984; Sugimoto et al., 1984; Whitman et al., 1985). The inositol kinases responsible for this activity were later called PI3Ks based on the discovery that they phosphorylate PtdIns on the 3-OH position of the inositol ring, leading to the identification of this critical class of second messengers (Whitman et al., 1988). Importantly, several groups began to study signals that altered the activity of PI3Ks or the cellular levels of 3-OH phosphorylated lipids. Early studies identified two contexts

during which PI3K is activated: (1) after receptor tyrosine kinase activation in response to PDGF or insulin treatment and (2) after GPCR activation by fMLP (Auger et al., 1989; Ruderman et al., 1990; Traynor-Kaplan et al., 1989). Analysis of lipids in the cell after cell stimulation showed that PI(4,5)P2 was the most likely substrate for PI3Ks, and PI(3,4,5)P3 was established as a very important second messenger (Hawkins et al., 1992; Stephens et al., 1991).

Three classes of PI3K proteins have been identified which differ in both structure and substrate preference. Class I PI3Ks are subdivided into two groups, Class IA and IB, and they function to phosphorylate PI(4,5)P2 to generate PI(3,4,5)P3. Class IA PI3Ks exist as a heterodimer consisting of a p85 regulatory subunit (p85α, p55α, p50α, p85β, or p55γ) and a p110 catalytic subunit (p110α, p110β, or p110δ). Class IA p110 catalytic subunits contain an N-terminal p85 binding domain, a RAS binding domain (RBD), a C2 domain, a helical domain, and a lipid kinase domain, while the p85 isoforms contain a p110 binding domain flanked by two SH2 domains (Escobedo et al., 1991; Hiles et al., 1992; Hu et al., 1993; Otsu et al., 1991; Skolnik et al., 1991; Vanhaesebroeck et al., 1997). Class IB PI3Ks have either a p87 or p101 regulatory subunit in addition to a p110γ catalytic subunit, which lacks the N-terminal p85-binding domain found on Class IA PI3Ks (Stephens et al., 1997; Stoyanov et al., 1995; Suire et al., 2005; Voigt et al., 2006). Class II PI3Ks contain one of three catalytic subunit isoforms, PI3K‑C2α, PI3K‑C2β, or PI3K‑C2γ, which phosphorylate PtdIns at the three position, with a preference for PtdIns and PtdIns4P. Lastly, there is only one member of Class III PI3Ks, called vacuolar protein-sorting defective 34 (Vps34), which can phosphorylate PtdIns to generate

PtdIns3P (Vanhaesebroeck et al., 2010). This thesis involves signaling events downstream of PI(3,4,5)P3 and therefore will focus on the Class I PI3Ks.

PH domains have been shown to bind to phospholipids, and a small group of these lipid binding PH domains are able to specifically bind the PI3K products, PI(3,4)P2 and PI(3,4,5)P3. The dynamic regulation of PI(3,4)P2 and PI(3,4,5)P3 upon PI3K activation allows for very specific regulation of both localization and activation of certain PH domain containing proteins in response to specific signals (Lemmon, 2008). For example, the kinase AKT, a well-studied downstream effector of PI3K signaling, contains a PI(3,4,5)P3 binding PH domain that is critical for its function (Manning and Cantley, 2007).

AKT

AKT was originally discovered as the homolog to a rodent viral oncogene (Bellacosa et al., 1991). Since then, two additional isoforms of the protein have been identified, AKT2 and AKT3 (Jones et al., 1991; Nakatani et al., 1999). The presence of a PH domain on the N-terminus of AKT suggested that lipids may regulate its kinase activity. It was then established that AKT kinase activity is increased in a PI3K dependent manner following insulin receptor activation (Cross et al., 1995; Kohn et al., 1995). After PI3K stimulation, AKT is phosphorylated at T308 and S473, events that are important for activation of its kinase activity (Alessi et al., 1996). AKT is phosphorylated by another PI(3,4,5)P3 regulated kinase, PDK1, at T308, and also by mammalian target of rapamycin complex 2 (mTORC2), which is activated by RTKs, at S473 (Alessi et al., 1997; Sarbassov et al., 2005; Stokoe et al., 1997). AKT activation leads to many

physiological outputs, including proliferation, cell survival, glucose uptake, and metabolism (Manning and Cantley, 2007).

PI3K regulation by RTKs, Gβγ, and small GTPases

Activation of Class I PI3Ks can be achieved through a variety of mechanisms. Activation can occur though the binding of SH2 domains on the p85 regulatory subunit of Class IA PI3Ks to phosphorylated tyrosine pYxxM motifs on growth factor receptors or adaptor molecules such as insulin receptor substrate (IRS) family proteins. In the basal state, p85 inhibits the lipid kinase activity of p110, and this inhibition is alleviated upon binding of p85 to these phosphorylated tyrosine residues (Auger et al., 1989; Backer et al., 1992; Carpenter et al., 1993; Panayotou et al., 1992). In addition to localizing p110 to the membrane and regulating its activation, regulatory p85 subunits also stabilize p110, which is critical for PI3K activity in the cell (Yu et al., 1998). Additionally, Gβγ can regulate Class I PI3Ks. While most of the Class IA PI3Ks are only regulated by receptor tyrosine kinases, PI3K that includes p110β is synergistically activated by Gβγ and tyrosine phosphorylated proteins (Kurosu et al., 1997). The Class IB PI3Ks are regulated by Gβγ; however they are not regulated by phosphorylated tyrosine residues on RTKs or their adaptor proteins. This is not surprising given that the Class IB regulatory subunits p87 and p101 do not have SH2 domains (Stephens et al., 1997; Stoyanov et al., 1995).

PI3Ks can also be regulated by small GTPases. RAS cooperates with tyrosine-phosphorylated peptides to activate the p85-p110α dimer, and ablation of the RBD in p110α results in decreased RAS driven tumorigenesis (Gupta et al., 2007; Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). p110δ is also stimulated by RAS, however only by R-RAS and TC21 (also called R-RAS2) (Rodriguez-Viciana et al.,

2004; Vanhaesebroeck et al., 1997). RAS can also cooperate with Gβγ to activate the Class IB PI3K p110γ, and neutrophils harboring a mutation that blocks the RAS-p110γ interaction have reduced ability to migrate towards GPCR-activating chemoattractants, a phenotype also seen in p110γ$^{-/-}$ neutrophils (Pacold et al., 2000; Suire et al., 2006). Interestingly, p110β is not activated by RAS, but it is activated by RAC1 and CDC42, and this is mediated by its RBD domain (Fritsch et al., 2013).

PTEN

PI3K generation of PI(3,4,5)P3 is antagonized by the protein and lipid phosphatase PTEN. Loss of heterozygosity on chromosome 10q23 occurs at a high rate in many human cancers, and three different groups identified PTEN as a candidate tumor suppressor that mapped to this region (Li and Sun, 1998; Li et al., 1997; Steck et al., 1997). PTEN contains an N-terminal PIP(4,5)P2 binding domain, followed by the catalytic phosphatase domain, a C2 domain, and a C-terminal tail that contains a PDZ binding domain (Lee et al., 1999). It was established that PTEN contains the signature protein tyrosine phosphatase motif, HCxxGxxR, and that PTEN weakly de-phosphorylates serine, threonine, and tyrosine peptide substrates, with a preference for acidic substrates (Myers et al., 1998). In addition, PTEN reduces tyrosine phosphorylation of focal adhesion kinase both *in vivo* and *in vitro* (Tamura et al., 1998). However, the observation that PTEN is a poor protein phosphatase, and that it prefers very acidic substrates led to the discovery that PTEN is primarily a lipid phosphatase, dephosphorylating PI(3,4,5)P3 at the three position to generate PIP(4,5)P2. This is further supported by the fact that PTEN decreases the cellular levels of PI(3,4,5)P3 (Maehama and Dixon, 1998). These findings suggested that PTEN could counteract the effects of

PI3K and its downstream targets, such as AKT. PTEN$^{-/-}$ mouse embryonic fibroblasts (MEFs) have elevated levels of AKT phosphorylation and increased AKT kinase activation. Re-expression of PTEN in PTEN$^{-/-}$ MEFs also increases their sensitivity to apoptosis, and this is rescued by constitutively active AKT (Stambolic et al., 1998). Expression of exogenous PTEN in breast cancer cells results in both decreased proliferation and increased apoptosis, and the increase in apoptosis can be rescued by myristolated AKT, which goes to the membrane to be activated in the absence of PI(3,4,5)P3 (Li et al., 1998). These studies confirmed that PTEN lipid phosphatase activity is important in cells to counteract PI3K activation and the downstream effects of PI(3,4,5)P3.

PTEN and cancer

The role of PTEN as a tumor suppressor is well documented and is supported by data from human tumors and mouse models. Germline mutations in PTEN are strongly linked to human disease in which patients are predisposed to developing cancer, such as Cowden's syndrome, where PTEN mutations occur in nearly 80% of patients (Liaw et al., 1997; Marsh et al., 1998). Furthermore, as discussed earlier, PTEN was discovered because it is located in a region that is commonly deleted in cancer (Li et al., 1997; Steck et al., 1997). For example, loss of heterozygosity of the PTEN locus was reported in 70% of glioblastomas and 40% of invasive breast cancers (Singh et al., 1998; Wang et al., 1997). PTEN is also often somatically mutated in cancer. Currently, publically available data shows over 1200 PTEN mutations identified in a large variety of tumor types (Cerami et al., 2012; Gao et al., 2013). PTEN mutations are particularly common in

endometrial cancer, where a recent study found that PTEN was mutated in 62% of cases (Cancer Genome Atlas Research et al., 2013).

Studies in mice have also strongly supported a role for PTEN as a tumor suppressor. PTEN knockout mice are embryonic lethal; however, PTEN was found to be a haploinsufficient tumor suppressor, meaning that PTEN can contribute to tumor formation even in the presence of a functional PTEN allele. $PTEN^{-/-}$ mice develop tumors in many tissues, such as colon, breast, prostate, and thyroid (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). Tissue specific knockouts of PTEN have been powerful tools to study tumor formation in the absence of PTEN. For example, knocking out PTEN in the breast leads to tumors within 2 months, and knocking out PTEN in the prostate resulted in invasive tumors within 3 weeks (Backman et al., 2004; Li et al., 2002; Wang et al., 2003). Further, studies in mice have also demonstrated that small changes in PTEN dose can alter the rate at which tumors form (Alimonti et al., 2010; Trotman et al., 2003).

Regulation of PTEN phosphatase activity

PTEN is extensively regulated by both post-translational modifications and protein-protein interactions. Phosphorylation of PTEN occurs primarily in the C-terminal tail of PTEN (C-tail). It has been shown that GSK3 can phosphorylate PTEN at S362 and T366 and casein kinase 2 (CK2) phosphorylates PTEN primarily at S370 and S385, in addition to S380, T382, and T383 (Al-Khouri et al., 2005; Miller et al., 2002; Torres and Pulido, 2001; Vazquez et al., 2000). It appears that phosphorylation on the C-tail of PTEN affects an intramolecular interaction to regulate PTEN function. Phosphorylation at S380, T382, T383, and S385 promotes binding of the C-tail to the catalytic region of

PTEN, favoring the "closed" conformation, while dephosphorylated PTEN favors an "open" conformation. The phosphorylated, closed form of PTEN has decreased overall function as it cannot efficiently bind the membrane where it dephosphorylates PI(3,4,5)P3 (Rahdar et al., 2009). PTEN also functions to block cell migration, and phosphorylation at T383 reduces the ability of PTEN to inhibit cell migration (Raftopoulou et al., 2004). On the other hand, the dephosphorylated, open conformation of PTEN can bind to the membrane more effectively and is more active, but is also less stable (Vazquez et al., 2000). Additionally, tyrosine phosphorylation of PTEN by SRC kinase decreases its phosphatase activity, while the SRC family kinase RAK increases PTEN stability (Lu et al., 2003; Yim et al., 2009). Multiple phosphorylation events by RHO-associated protein kinase (ROCK) appear to increase PTEN phosphatase activity (Li et al., 2005a). Lastly, studies have indicated that PTEN protein phosphatase activity can auto-dephosphorylate residues on its own C-tail to regulate its lipid phosphatase activity (Tibarewal et al., 2012; Zhang et al., 2012). In addition to phosphorylation, PTEN can be regulated by other forms of post translational modification, including oxidation, acetylation, SUMOylation, or ubiquitylation (Hopkins et al., 2014).

Interactions with other proteins affect PTEN function through a variety of different mechanisms. Some PTEN interactors, such as RAK kinase and melanocortin-1 receptor (MC1R), have been shown to increase PTEN stability by disrupting PTEN ubiquitylation and proteosomal degradation (Cao et al., 2013; Yim et al., 2009). PTEN membrane localization is also regulated by protein interactions. MAGI proteins, β-arrestins, Na+/H+ exchanger regulatory factor (NHERF), neutral endopeptidase (NEP), and the PI3K regulatory subunit p85 all promote PTEN membrane localization where it

can be part of important signaling complexes (Chagpar et al., 2010; Kotelevets et al., 2005; Lima-Fernandes et al., 2011; Sumitomo et al., 2004; Takahashi et al., 2006; Tolkacheva et al., 2001; Wu et al., 2000a; Wu et al., 2000b). In contrast, cytosolic α-mannosidase 2C1 (MAN2C1) inhibits PTEN phosphatase activity, and it was speculated it might prevent PTEN membrane binding (He et al., 2011).

PREX2 inhibition of PTEN phosphatase activity

Previous work from our lab has shown that PTEN also interacts with PREX2, resulting in inhibition of PTEN phosphatase activity. *In vitro,* PREX2 inhibits PTEN phosphatase activity in a non-competitive manner with the substrate PI(3,4,5)P3. In PTEN deficient U87MG glioblastoma cells, PTEN expression causes an expected decrease in AKT phosphorylation, and PREX2 expression rescues this effect, suggesting that this inhibition occurs in cells. PREX2 knockdown by shRNA decreases the rate of proliferation in MCF7 breast cancer cells, which contain PTEN, however this effect is not seen in BT549 cells, a PTEN-null breast cancer cell line (Fine et al., 2009). Taken together, these data suggest that PREX2 can bind to PTEN and inhibit its ability to dephosphorylate PI(3,4,5)P3 and antagonize PI3K signaling in cells.

Mechanism of PTEN inhibition by PREX2

Further work to characterize the mechanism of PREX2 mediated inhibition of PTEN showed that phosphorylation on the C- tail of PTEN at S380, T382, and T383 is required for inhibition of PTEN phosphatase activity. When either wild type PTEN or PTEN S380A/T382A/T383A was expressed and immunoprecipitated from PTEN null U87MG cells, only in the context of wild type PTEN did PREX2 expression reduce phosphatase activity. Further, the PH domain of PREX2 is the primary domain needed

for inhibition. Full length PREX2 or PREX2 DHPH domain does not inhibit bacterial GST PTEN, while purified PREX2 PH domain can. Given that bacterial GST PTEN is not phosphorylated, this suggests that phosphorylation of PTEN is not required for inhibition by the PH domain. In addition, the mechanism of binding between PREX2 and PTEN was analyzed. In pull-down experiments using GST PTEN, *in vitro* translated PREX2 PH domain binds to the phosphatase and C2 domains of PTEN; however, in cells

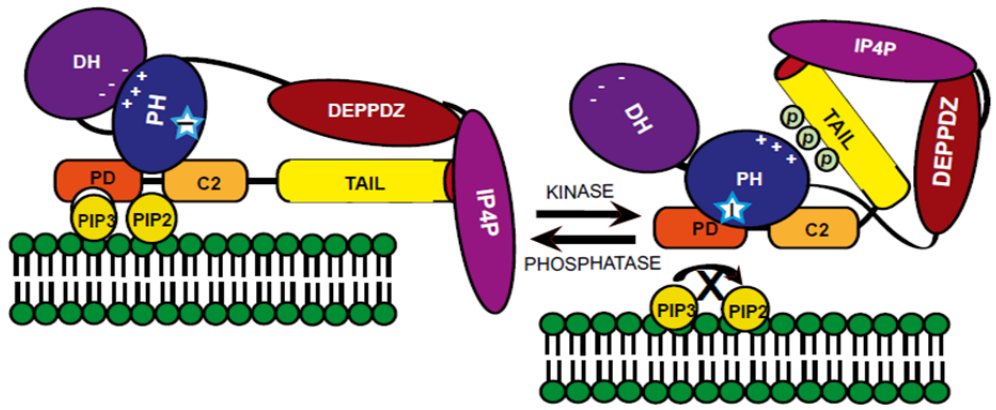


Figure 1.3 – Model of PH domain mediated inhibition of PTEN phosphatase activity by PREX2 The PREX2 DH domain blocks PH domain mediated inhibition of PTEN when PTEN is not phosphorylated. The DH domain senses PTEN phosphorylation, revealing the region of the PREX2 PH domain that can inhibit PTEN (Hodakoski et al., 2014).

this interaction is relatively weak. Further, the C-terminal IP4P domain of PREX2 binds strongly to the PDZ binding domain at the C-terminus of PTEN. The proposed model is that this high affinity interaction involving the PDZ binding domain of PTEN and the IP4P domain of PREX2 initiates binding between the two proteins (Figure 1.3). This then puts the DHPH domain of PREX2 in close proximity to the phosphatase and C2 domains of PTEN, resulting in the inhibition of PTEN. Further, when PTEN is not phosphorylated, the DH domain of PREX2 potentially binds to the PH domain and prevents it from inhibiting PTEN. When PTEN is phosphorylated, the DH domain can

sense the phosphorylation state of PTEN, which changes the conformation of PREX2 so that the PH domain is revealed and can inhibit PTEN phosphatase activity (Hodakoski et al., 2014). This is the first report of PH domain mediated inhibition of PTEN. Given the high number of mammalian proteins that contain PH domains, PH domain mediated inhibition of PTEN could represent a common mechanism of regulation, an exciting possibility given the importance of PTEN in many physiological processes.

PTEN inhibition of PREX2 GEF activity

Studies from our lab showed that the interaction between PREX2 and PTEN also results in inhibition of PREX2 GEF activity (Mense et al., 2015). This provides a potential mechanism for the previously characterized role of PTEN in reducing cell migration (Raftopoulou et al., 2004; Tamura et al., 1998). PTEN inhibits PREX2 GEF activity *in vitro* and also blocks PREX2 driven cell invasion and migration, and these effects do not require the phosphatase activity of PTEN. Further, $PTEN^{-/-}$ MEFs show increased invasion, and this effect is ablated in $PTEN^{-/-}$ $PREX2^{-/-}$ MEFs, suggesting that PREX2 is required for the effect of PTEN on invasion (Mense et al., 2015).

INSULIN SIGNALING

In this thesis, we focus on PI(3,4,5)P3-mediated regulation of PREX2 in the insulin signaling pathway. As will be discussed below, PREX2 has an important role in insulin signaling and insulin-mediated glucose metabolism, which is likely a result of both RAC1 activation and PTEN inhibition.

Overview of insulin signaling

Insulin is a hormone that is produced in the β-cells of the pancreas in response to the high levels of carbohydrates and amino acids in the blood. Insulin then acts on multiple organs within the body, and a primary function of insulin is to reduce blood glucose levels. The molecular mechanisms by which insulin achieves this effect will be discussed in this section.

Insulin was first purified from pancreatic extracts to treat diabetic patients by Frederick Banting and Charles Best in 1922 (Banting et al., 1922). In 1950 it was shown that insulin regulates blood glucose levels by increasing the permeability of cellular membranes for glucose, and it was later discovered that insulin exerts its effects by binding to the insulin receptor on the cell membrane (Freychet et al., 1971; Levine et al., 1950). Characterization of the insulin receptor revealed that it is a tyrosine kinase consisting of two extracellular α subunits and two cytoplasmic β subunits (Ebina et al., 1985; Ullrich et al., 1985). Binding of insulin to the α subunits results in autophosphorylation of the β subunits, which activates the tyrosine kinase activity of the receptor (Avruch et al., 1982; Kasuga et al., 1982a; Kasuga et al., 1982b; Petruzzelli et al., 1982; Van Obberghen and Kowalski, 1982). A key group of proteins that are phosphorylated by the insulin receptor belong to the IRS family (White et al., 1985).

Once phosphorylated, IRS proteins can then bind the p85 regulatory subunit of PI3K to activate PI3K and increase PI(3,4,5)P3 at the membrane, activating important downstream effectors, including AKT and RAC1, to carry out insulin dependent physiological outputs (Figure 1.4) (Backer et al., 1992; Ruderman et al., 1990).

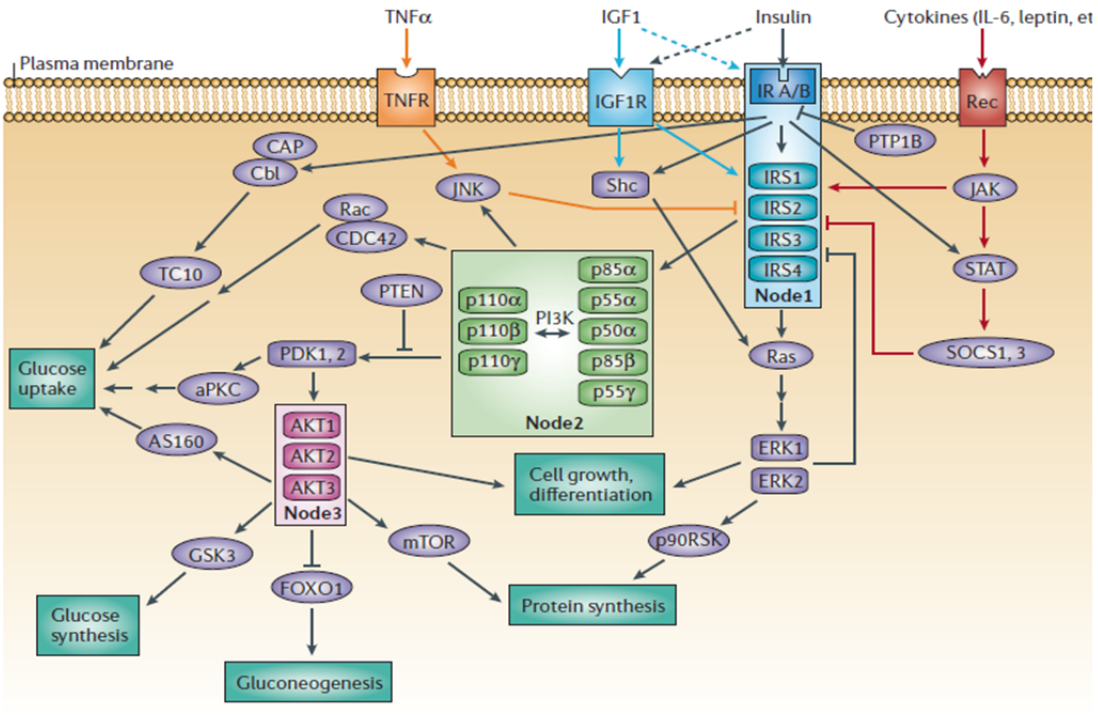


Figure 1.4 – Intracellular insulin signaling and physiological effects (Taniguchi et al., 2006)

Role of AKT in glucose metabolism

AKT has a critical role in regulating glucose levels in the blood in response to insulin stimulation. In cultured 3T3-L1 adipocytes, siRNA knockdown of AKT1 or AKT2 reduces glucose uptake, with AKT2 having a stronger effect. Further, knockdown of both AKT1 and AKT2 together significantly reduces GLUT4 levels at the membrane (Jiang et al., 2003). On the other hand, overexpression of a constitutively active form of AKT in 3T3-L1 adipocytes increases glucose uptake and GLUT4 at the membrane (Kohn et al., 1996). $AKT2^{-/-}$ mice are diabetic as they have higher blood glucose levels during resting state and during a glucose tolerance test. Also, glucose uptake is significantly reduced in isolated muscle from $AKT2^{-/-}$ mice when compared to wild type counterparts (Cho et al., 2001a).

A number of AKT substrates that are important for glucose regulation have been identified. AKT increases glucose storage through phosphorylation of GSK3, a kinase that phosphorylates and inhibits glycogen synthase. Phosphorylation by AKT inhibits GSK3 kinase activity, leading to an increase in the synthesis of the glucose storage polymer glycogen (Cross et al., 1995). AKT also decreases glucose production in the liver through phosphorylation of forkhead box O (FOXO) proteins. Phosphorylation of FOXO by AKT prevents their translocation to the nucleus where they carry out their function as transcription factors (Brownawell et al., 2001; Brunet et al., 1999). FOXO transcription factors increase the expression of genes that promote gluconeogenesis in the liver, and exclusion from the nucleus after AKT phosphorylation results in a reduction in liver glucose production (Gross et al., 2008). AKT also phosphorylates a number of substrates to increase insulin regulated glucose uptake in muscle and adipose tissue,

specifically affecting the transport of the main glucose transporter, GLUT4, to the membrane. In a basal state, GLUT4 is often sequestered in various intracellular membrane structures, and upon insulin binding, rates of both endocytosis and exocytosis are regulated to increase levels of GLUT4 at the membrane. Insulin activation of AKT leads to phosphorylation and inhibition of a RAB GAP called AKT substrate of 160 kDa (AS160), which results in increased GLUT4 translocation to the membrane (Kane et al., 2002; Sano et al., 2003). AKT2 also phosphorylates RGC2, which is a GAP for the GTPase RALA. RALA is a component of GLUT4 storage vesicles (GSVs), and active GTP-bound RALA is critical for the recognition of GSVs by the exocyst complex on the membrane. Insulin-stimulated phosphorylation of RGC2 by AKT2 blocks the RGC2 inhibition of RALA activity, promoting targeting of GSVs to exocysts (Chen et al., 2007; Chen et al., 2011). Lastly, AKT2 phosphorylates the 138kDa C2-domain containing phosphoprotein (CDP138), shown to affect the fusion step of GSVs with the membrane resulting in GLUT4 receptors being available for insulin binding (Xie et al., 2011).

The role of RAC GTPases and PAK kinases in glucose uptake

RAC1 has also been identified as an important mediator for glucose uptake in skeletal muscle cells, and this effect depends on PI3K generation of PI(3,4,5)P3. It is thought that RAC1 does not play a significant role in glucose uptake in adipocytes, where another protein, TC10, regulates glucose uptake in a PI3K independent manner (JeBailey et al., 2004). However, a study in mouse 3T3-L1 adipocytes showed that PREX1 is important for glucose uptake, and that this occurs through RAC1, suggesting a potential role for RAC1 in adipocytes (Balamatsias et al., 2011). In rat muscle cells, RAC1 is quickly activated following insulin stimulation, and blocking RAC1 function, either by

siRNA or expression of a dominant negative form of the protein, reduces GLUT4 translocation to the cell membrane, while constitutively active RAC1 has the opposite effect (Chiu et al., 2013; JeBailey et al., 2007; Khayat et al., 2000; Ueda et al., 2008). Further, in muscle specific RAC1 knockout mice, there is deficient glucose uptake in the muscle and higher levels of glucose in the blood during glucose tolerance test (Sylow et al., 2013; Ueda et al., 2010).

The major RAC1 output that is critical for glucose uptake is the stimulation of actin polymerization, which is necessary for GSVs to bring GLUT4 to the membrane. Insulin treatment causes actin remodeling, and when the actin network is disrupted by pharmacologic agents, such as cytochalasin D, GLUT4 is no longer able to translocate to the membrane (Tong et al., 2001; Tsakiridis et al., 1994; Wang et al., 1998). In rat muscle cells, both the expression of dominant negative RAC1 and the knockdown of RAC1 using siRNA block insulin-induced actin remodeling (JeBailey et al., 2007; Khayat et al., 2000). In addition, the established effector of RAC1 activation that is required for actin polymerization, ARP2/3, is necessary for GLUT4 translocation to the membrane after insulin binding (Chiu et al., 2010). These studies show that actin rearrangement downstream of RAC1 activation is important for glucose uptake.

In addition, the RAC1-GTP activated kinase PAK1 is an important mediator of glucose uptake downstream of RAC1 activation. PAK1 is activated after insulin stimulation, an effect that requires PI3K activity (Tsakiridis et al., 1996). Further studies in mice revealed that GLUT4 membrane translocation following insulin stimulation in skeletal muscle of $PAK1^{-/-}$ mice is defective compared to wild type littermates (Wang et al., 2011). Insulin dependent GLUT4 translocation, glucose uptake, and actin remodeling

is also reduced in L6 rat myoblasts after pharmacological inhibition of PAK1 (Tunduguru et al., 2014). In addition, the actin-regulating protein cofilin, which is downstream of the PAK1 substrate LIMK, is required for the actin changes necessary for GLUT4 translocation (Chiu et al., 2010).

Role of PREX2 in insulin signaling and glucose metabolism

Given the dual roles of PREX2 as a RAC1 GEF and a PTEN inhibitor, and the importance of RAC and PI3K signaling for propagation of the insulin signal, our lab hypothesized that PREX2 would be important for insulin signaling, which was confirmed in the analysis of PREX2$^{-/-}$ mice. Following insulin treatment, there is a reduction in the stimulation of phosphorylation of AKT and the phosphorylation of its downstream targets GSK3β and FOXO1/3 in both the adipose and liver tissue of the PREX2$^{-/-}$ mice. In livers of wild type mice, PTEN phosphatase activity is reduced following insulin treatment, and this effect is not observed in the PREX2$^{-/-}$ mice, suggesting that PREX2 is altering PTEN function in the mouse. Additionally, PREX2$^{-/-}$ MEFs show reduced insulin and IGF1 stimulated phosphorylation of AKT, GSK3β, and FOXO1/3 up to at least 60 minutes after stimulation. Interestingly, signaling downstream of EGF and PDGF stimulation is unaffected in the PREX2$^{-/-}$ MEFs, suggesting that the role of PREX2 in growth factor signaling may be specific for insulin and IGF1. This possibility is strengthened by the fact that PREX2 interacts with the phosphorylated insulin receptor, but not with the phosphorylated EGF receptor (Hodakoski et al., 2014).

PREX2 loss in mice also leads to increased glucose in the blood after glucose or insulin injection (Hodakoski et al., 2014). These data show a clear role of PREX2 in insulin mediated glucose uptake, however the mechanism for this effect is less certain.

Both AKT and RAC1 are especially important for glucose metabolism, and given the dual roles of PREX2 as an inhibitor of PTEN and an activator of RAC1, it is likely that both functions are involved in PREX2 mediated glucose uptake. To date, there is no data describing a mechanism for PREX2 regulation downstream of insulin receptor activation. It is unclear whether there are additional modes of regulation for PREX2 other than simply increasing levels of PI(3,4,5)P3 at the membrane, leading to activation of PREX2. It is likely that PREX2 function downstream of insulin is modified by other signaling molecules through protein-protein interactions or post-translational modifications, a possibility thoroughly investigated in this thesis.

Mechanisms of insulin resistance

Defective insulin signaling in multiple tissues results in insulin resistance, which is an underlying cause of the widespread disease Type 2 diabetes. Disruption of the insulin signaling pathway at many levels has been shown to contribute to insulin resistance. IRS proteins appear to be particularly important. IRS1 mutations identified in a cohort of highly insulin resistant patients leads to reduced insulin signaling, and both IRS1 and IRS2 knockout mice are insulin resistant (Araki et al., 1994; Tamemoto et al., 1994; Whitehead et al., 1998; Withers et al., 1998). Further, AKT2 knockout mice are insulin resistant, and this appears to be specific for this isoform given that this phenotype is not seen in mice lacking AKT1 (Cho et al., 2001a; Cho et al., 2001b). AKT activity is also reduced in skeletal muscle tissue from patients with diabetes, and a family with inherited diabetes carried a mutation in AKT2 that disrupts kinase activity *in vitro* and reduces insulin signaling in cells (George et al., 2004; Krook et al., 1998). FOXO transcription factors, which produce glucose in the liver, have also been linked to insulin

resistance in mice. Mice expressing constitutively active FOXO1 that cannot be inhibited by AKT develop insulin resistance, while the loss of one FOXO1 allele restores insulin sensitivity in mice that are heterozygotic for the insulin receptor (Nakae et al., 2002). Further, PTEN activity is associated with changes in insulin sensitivity in humans and in mice, which is not surprising given the importance of PTEN in regulating PI(3,4,5)P3. Patients with PTEN mutations are reported to have lower measures of insulin resistance (Pal et al., 2012). In mice, PTEN heterozygosity restores β-cell function and insulin sensitivity that is reduced in $IRS2^{-/-}$ mice (Kushner et al., 2005). Adipose specific $PTEN^{-/-}$ mice show increased insulin sensitivity, and muscle specific knockout of PTEN protects mice from insulin resistance after being fed a high fat diet (Komazawa et al., 2004; Wijesekara et al., 2005). In addition, activation of RAC1, a critical mediator of glucose uptake in the insulin pathway, can have reduced expression in diabetic patients (Sylow et al., 2013; Sylow et al., 2014).

Much of the recent research on diabetes and insulin resistance has focused on trying to determine the actual cause of reductions in insulin signaling that can lead to disease. Given that the single biggest risk factor for diabetes is obesity, the link between lipids and insulin resistance has been explored. Infusion of free fatty acids into healthy human subjects can reduce glucose metabolism and insulin signaling in muscle (Dresner et al., 1999; Roden et al., 1996). Metabolism of high levels of intracellular lipids results in increased amounts of lipid intermediates, and some of these intermediates may cause insulin resistance. For example, increases in diacylglycerol (DAG) levels following free fatty acid infusion have been strongly linked to insulin resistance. Increases in intracellular DAG are associated with a concurrent increase in PKCθ kinase activity,

leading to IRS1 serine phosphorylation and a decrease in insulin signaling and glucose uptake in muscle (Yu et al., 2002). Furthermore, fatty acids can bind and activate GPCRs, such as GPR41 and GPR120, to increase glucose uptake in mouse adipose and muscle cells (Han et al., 2014; Oh et al., 2010). It is possible that perturbations in these fatty acid-stimulated pathways, or desensitization or downregulation of these receptors caused by high plasma fatty acid levels, could also be contributing to insulin resistance. In addition, contexts of cellular stress such as the unfolded protein response and inflammation are also associated with insulin resistance (Samuel and Shulman, 2012). Given that PREX2 has an established role in insulin signaling and glucose uptake, it is possible that PREX2 is important in the development in insulin resistance. This is supported by the finding that PREX2 protein expression is lower in adipose tissue from insulin resistant human subjects (Hodakoski et al., 2014).

THE NEED FOR A BETTER UNDERSTANDING OF PREX2 REGULATION

The two known roles of PREX2 in cellular signaling, as a PI(3,4,5)P3 and Gβγ dependent RAC GEF and an inhibitor of PTEN phosphatase activity, give PREX2 the potential to impact many physiological processes. However, very little is known about the mechanism of PREX2 regulation downstream of ligand activation of membrane receptors. Given the complex regulation of other RHO GEFs by post-translational modifications or through interactions with other proteins, we hypothesized that PREX2 is also regulated by similar mechanisms (Rossman et al., 2005). Further, a more complete understanding of PREX2 regulation will potentially lead to novel ways to target and modulate RAC1 activity or PTEN phosphatase activity in the widespread diseases in

which they play a role, such as cancer and diabetes. Here, we characterize the regulation of PREX2 by phosphorylation and protein-protein interactions, with a focus on the insulin signaling pathway, where PREX2 has an established role.

CHAPTER 2: PREX2 is phosphorylated downstream of insulin stimulation

BACKGROUND AND SIGNIFICANCE

PREX2 can be directly activated by both PI(3,4,5)P3 and Gβγ *in vitro*, and expression of both PI3K and Gβγ synergistically activates PREX2 *in vivo* (Donald et al., 2004; Rosenfeldt et al., 2004). PI(3,4,5)P3 is generated by PI3K following receptor tyrosine kinase and GPCR activation and Gβγ is released after GPCR activation. The levels of these second messengers are regulated by numerous ligand-activated receptors and PREX proteins have been studied in many of these contexts. One particular context where PREX2 plays an important role is in the insulin-signaling pathway. The insulin receptor is a receptor tyrosine kinase that stimulates PI3K, activating RAC1 and AKT, both of which are critical for regulating glucose metabolism in many tissues (Alessi et al., 1996; JeBailey et al., 2004; Khayat et al., 2000; Ruderman et al., 1990; Taniguchi et al., 2006). In mice, PREX2 loss leads to increased glucose in the blood after glucose or insulin injection. Further, in liver and adipose tissue, PREX2 knockout reduces the phosphorylation of AKT and its substrates after insulin stimulation (Hodakoski et al., 2014). These phenotypes are likely the result of both PREX2 GEF activity towards RAC1 and PREX2 inhibition of PTEN (Fine et al., 2009).

Despite the known roles of PREX2 in insulin signaling, very little is known about PREX2 regulation downstream of insulin receptor activation. Though there are no reports of PREX2 phosphorylation, we hypothesized that phosphorylation is likely to be important for PREX2 regulation and function, especially given that phosphorylation can regulate the function of PREX1 and other RAC GEFS, such as VAV and T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1) (Aghazadeh et al., 2000; Fleming et al., 1999; Fleming et al., 1997; Lopez-Lago et al., 2000; Mayeenuddin and Garrison,

2006; Montero et al., 2011; Montero et al., 2013; Urano et al., 2008). In this chapter, we explore the possibility of PREX2 regulation by phosphorylation downstream of insulin receptor activation.

RESULTS

PREX2 is phosphorylated downstream of insulin receptor activation by a PI3K dependent mechanism

Given the established role of PREX2 in insulin signaling, we tested the effect of insulin treatment on post-translational modifications of PREX2. When V5 PREX2 was expressed in HEK293 cells that were treated with insulin, enrichment of a slower migrating species of PREX2 was detected relative to starved cells after gel electrophoresis. Treatment of V5 PREX2 with Lambda phosphatase, a non-specific protein phosphatase, eliminated the insulin-induced upper band, suggesting that this mobility shift was caused by phosphorylation (Figure

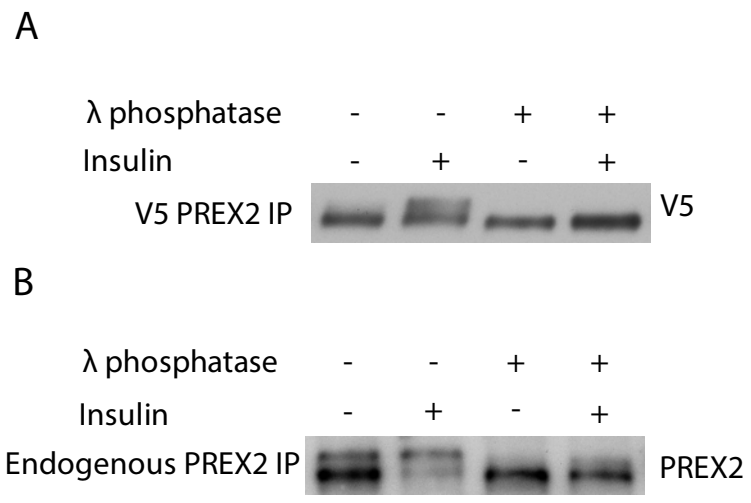


Figure 2.1 - PREX2 undergoes a Lambda phosphatase sensitive electrophoretic mobility shift in response to insulin A) Western blot analysis of V5 PREX2 that was isolated from starved or insulin treated (5μg/mL for 30 minutes) HEK293 cells and then treated with Lambda phosphatase. B) Western blot analysis of endogenous PREX2 that was immunoprecipitated from starved or insulin treated HEK293 cell lysates and then treated with Lambda phosphatase.

2.1A). When endogenous PREX2 was immunoprecipitated from insulin-treated HEK293 cells, a Lambda phosphatase sensitive electrophoretic mobility shift was also observed (Figure 2.1B). It is important to note that the endogenous PREX2 is almost completely shifted, suggesting that under physiologic conditions PREX2 is stoichiometrically phosphorylated by a signal activated by insulin.

To determine whether phosphorylation of PREX2 is specific to the insulin-signaling pathway, we analyzed the electrophoretic mobility shift downstream of IGF1 and EGF. IGF1 induced the phosphorylation of PREX2; however, treatment with EGF had no effect (Figure 2.2). While ERK signaling was stimulated by EGF in these cells,

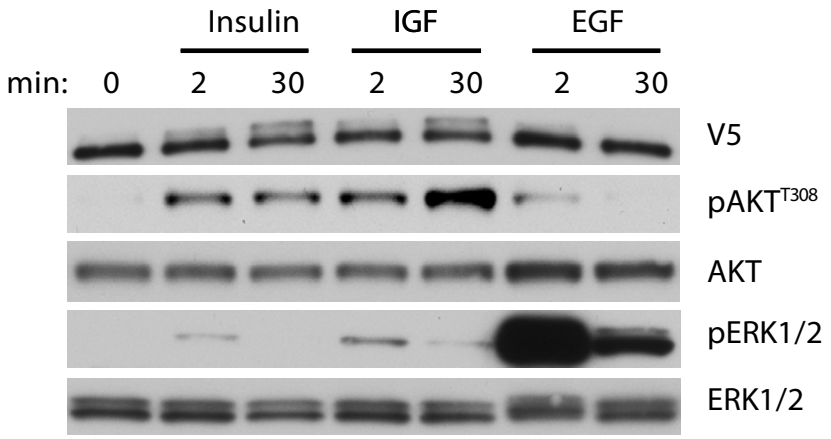


Figure 2.2 - PREX2 phosphorylation is induced by insulin and IGF1, but not EGF Western blot analysis of HEK293 cells expressing V5 PREX2, which were starved and treated with insulin, 50ng/mL IGF, or 50ng/mL EGF for the indicated times.

AKT signaling was induced only slightly, indicating that PI3K was probably not significantly activated under these conditions. This result suggested that phosphorylation of PREX2 could be PI3K dependent. We tested this hypothesis with the pan class I PI3K inhibitor GDC0941, and found that this drug eliminated insulin-induced phosphorylation of PREX2 (Figure 2.3A). PI3K generation of PI(3,4,5)P3 activates many kinases that

could phosphorylate PREX2. Using small molecule inhibitors, we tested whether AKT, PDK1, or mTORC1, three kinases activated by PI(3,4,5)P3 downstream of insulin

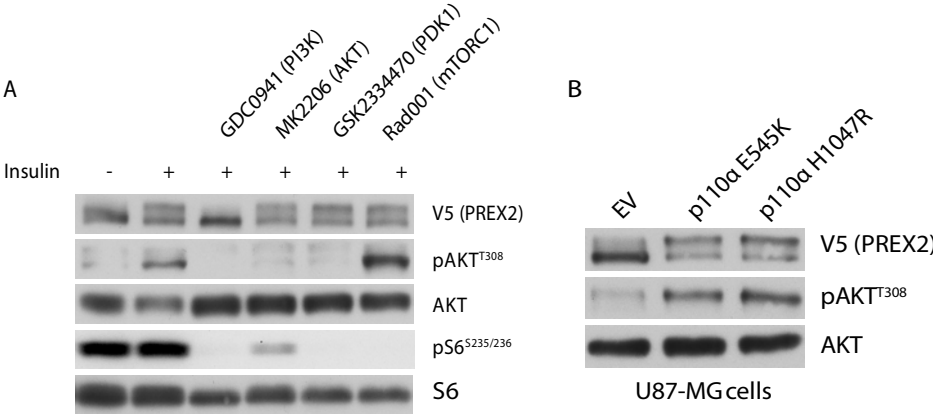


Figure 2.3 - PREX2 phosphorylation is PI3K dependent A) Western blot analysis of V5 PREX2 expressing HEK293 cells, which were starved and treated with DMSO or 500nM GDC0941, 500nM MK2206, 5μM GSK2334470, or 20nM Rad001 followed by treatment with insulin. B) Western blot analysis of starved U87MG cells expressing V5 PREX2, and either p110α E545K, or H1047R.

receptor activation, had a role in the phosphorylation of PREX2. These inhibitors had no effect on PREX2 phosphorylation, suggesting that PREX2 is either phosphorylated by PI3K directly, or by another kinase downstream of PI(3,4,5)P3. In PTEN-null U87MG glioblastoma cells, expression of two tumor hotspot mutants that constitutively activate the catalytic PI3K subunit p110α, E545K and H1047R, induced PREX2 phosphorylation, confirming that phosphorylation of PREX2 is mediated by PI3K (Figure 2.3B). These data show that PREX2 is phosphorylated after insulin stimulation through a PI3K dependent mechanism.

PREX2 is heavily phosphorylated, including at the insulin sensitive site, S1107

To identify PREX2 phosphorylation sites, we analyzed immunoprecipitated V5 PREX2 from HEK293 cells by mass spectrometry. PREX2 was purified from starved HEK293 cells that were treated with ether the PI3K inhibitor GDC0941, insulin alone, or insulin in combination with the PP1/PP2A inhibitor, calyculin A (Figure 2.4A). Mass spectrometry analysis revealed a total of 19 serine and threonine phosphorylation sites, 12 of which were only identified on PREX2 from stimulated cells (cells treated with insulin alone or in combination with calyculin A) (Figure 2.4B). Using the Scansite online tool under medium stringency we found that none of our phosphorylated residues fit a consensus motif of a kinase that is known to be activated by insulin and is included in the Scansite database, including AKT, GSK3β, PDK1, and ERK (Obenauer et al.,

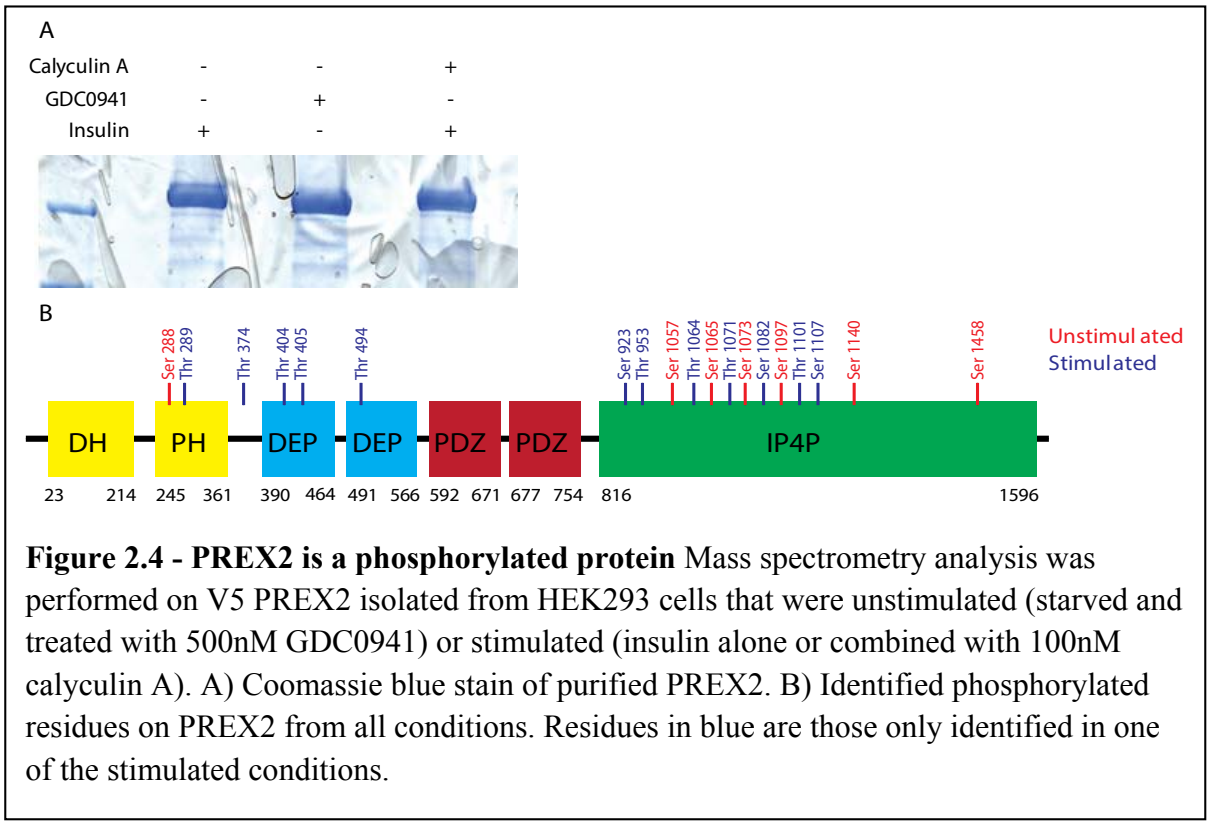


Figure 2.4 - PREX2 is a phosphorylated protein Mass spectrometry analysis was performed on V5 PREX2 isolated from HEK293 cells that were unstimulated (starved and treated with 500nM GDC0941) or stimulated (insulin alone or combined with 100nM calyculin A). A) Coomassie blue stain of purified PREX2. B) Identified phosphorylated residues on PREX2 from all conditions. Residues in blue are those only identified in one of the stimulated conditions.

2003).

To determine which of these phosphorylation events are regulated by insulin, we made alanine mutants at sites that were phosphorylated in the stimulated condition and determined the effect on the electrophoretic mobility of PREX2. We found that an alanine mutation at S1107 reduced enrichment of the slower migrating, phosphorylated form of PREX2 after insulin stimulation (Figure 2.5A). After developing an antibody to S1107, we confirmed that the antibody could not recognize PREX2 S1107A, demonstrating that the antibody is specific for the phosphorylated form of PREX2 at S1107 (Figure 2.5B). Importantly, phosphorylation of S1107 on V5 PREX2 was increased after insulin treatment and enriched in the slower migrating form of PREX2

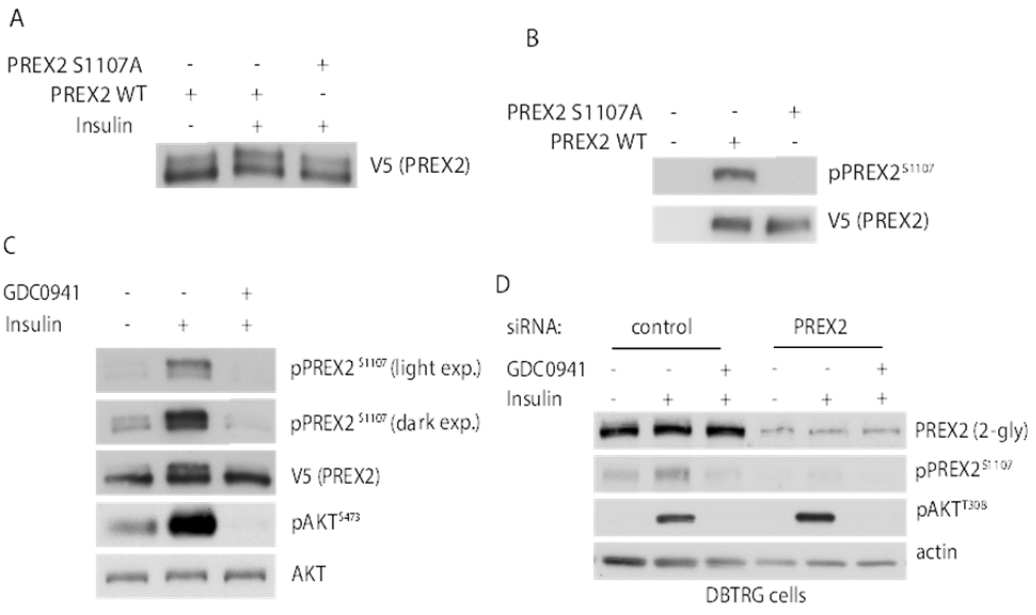


Figure 2.5 – S1107 is an insulin and PI3K dependent phosphorylation site A) Western blot analysis of HEK293 cells expressing either V5 PREX2 WT or S1107A that were starved or treated with insulin. B) Western blot analysis of HEK293 cells expressing either V5 PREX2 WT or S1107A. C) Western blot analysis of HEK293 cells expressing V5 PREX2 that were starved and then treated with DMSO or 500nM GDC0941 followed by treatment with insulin. D) Western blot analysis of DBTRG cells that were transiently transfected with control or PREX2 siRNA, and then starved and treated with DMSO or 500nM GDC0941 followed by insulin treatment.

(Figure 2.5C). S1107 phosphorylation was also eliminated by PI3K inhibition. These data show that S1107 is an insulin regulated phosphorylation site; however, it is important to note that the alanine mutation at this residue did not completely eliminate the upper band of PREX2, suggesting that other insulin sensitive phosphorylation sites exist. To confirm that endogenous PREX2 is phosphorylated at S1107, DBTRG glioblastoma cells, which contain high levels of endogenous PREX2, were treated with insulin and GDC0941 (Figure 2.5D) (Fine et al., 2009). As expected, phosphorylation at S1107 increased after insulin treatment and was reduced upon PI3K inhibition. DBTRG cells with PREX2 transiently knocked down by siRNA were used as controls to verify that the S1107 signal was specific to PREX2. Taken together, we identified S1107 as an insulin and PI3K dependent phosphorylation site, the first signal dependent phosphorylation event reported for PREX2.

Phosphorylation of PREX2 is regulated by the protein phosphatases PP1α and PP2A

As shown in Figure 2.4, the mass spectrometry analysis revealed only serine and threonine phosphorylation sites on PREX2, many of which were detected with a PP1 inhibitor. It has also been reported that PP1α binds a fragment of PREX2 that contains a consensus PP1α binding site (Barber et al., 2012; Hendrickx et al., 2009). These data led us to test the hypothesis that PP1α could dephosphorylate PREX2. First, we confirmed that PREX2 could bind to GST PP1α (Figure 2.6A). Additionally, the PREX2-PP1α interaction was eliminated by mutating two critical residues within the PP1α consensus binding site, V1085 and F1087, to alanine. Treatment of WT PREX2 with calyculin A stimulated phosphorylation of PREX2 even in the absence of insulin, and resulted in a maximally phosphorylated form of PREX2 (Figure 2.6B). Conversely, overexpression of

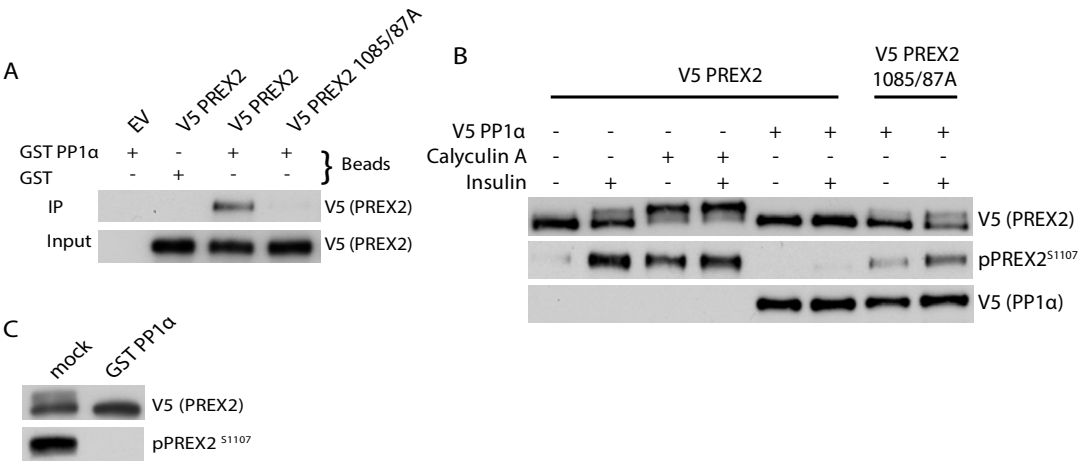


Figure 2.6 – PP1α can dephosphorylate PREX2 A) Western blot analysis of GST or GST PP1α pull downs from HEK293 cells expressing either V5 PREX2 WT or 1085/87A. B) Western blot analysis of HEK293 cells expressing V5 PREX2 WT or 1085/87A with or without co-expression of V5 PP1α. Cells were starved and then treated with DMSO or 100nM calyculin A, followed by treatment with insulin. C) The ability of GST PP1α to dephosphorylate V5 PREX2 isolated from HEK293 cells was assessed and analyzed by Western blot.

PP1α eliminated the insulin-induced phosphorylation of PREX2; however, the 1085/87A mutant, which cannot bind PP1α, was insensitive to PP1α dephosphorylation. To test whether PREX2 is a direct substrate of PP1α, we performed an *in vitro* phosphatase assay, and found that GST PP1α efficiently dephosphorylated PREX2 (Figure 2.6C). Further, S1107 phosphorylation was ablated by PP1α overexpression and by GST PP1α *in vitro*, showing that this is a PP1α sensitive phosphorylation event (Figure 2.6B, C).

Since the 1085/1087A mutant does not bind PP1α, it was surprising that it was not always in the maximally shifted form that is seen after calyculin A treatment (Figure 2.6B). We hypothesized that PP2A, which is also inhibited by calyculin A, is able to compensate for the absence of PP1α regulation by dephosphorylating PREX2. To probe this, we used okadaic acid, which inhibits both PP1α and PP2A but is much more potent

towards PP2A at low doses (Cohen et al., 1989). When treated with okadaic acid, phosphorylation of PREX2 was increased, suggesting that PP2A may have a role in PREX2 dephosphorylation (Figure 2.7A). The 1085/1087A mutant was phosphorylated more than wild type PREX2 at all doses, indicating that PP1α contributes to PREX2

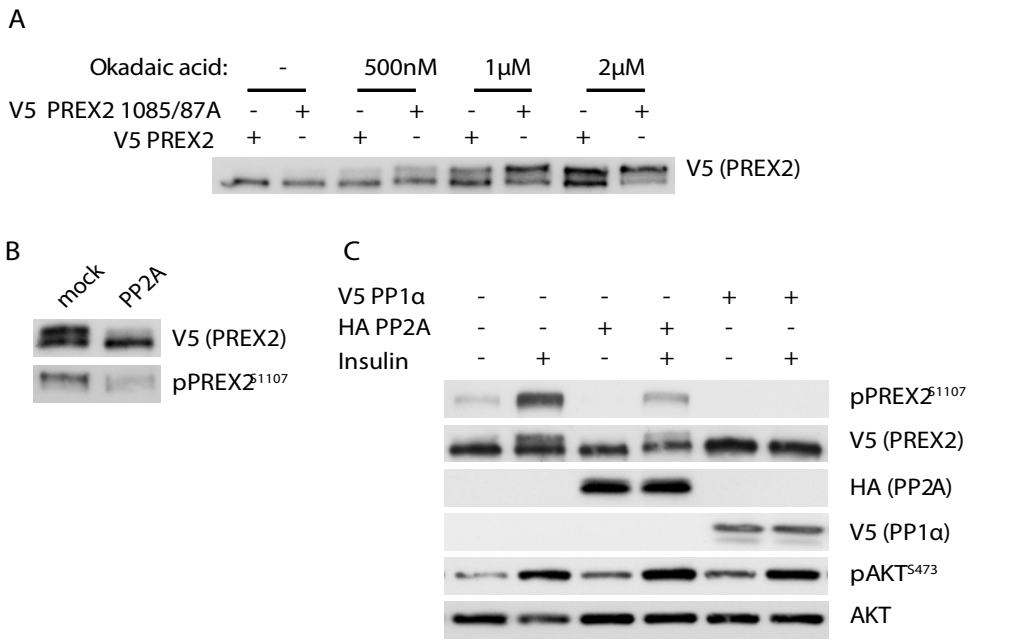


Figure 2.7 – PP2A can dephosphorylate PREX2 A) Western blot analysis of starved V5 PREX2 WT or 1085/87A expressing HEK293 cells that were treated with the indicated concentrations of okadaic acid for 30 minutes. B) The ability of purified PP2A to dephosphorylate V5 PREX2 isolated from HEK293 cells was assessed and analyzed by Western blot. C) Western blot analysis of HEK293 cells expressing V5 PREX2 with or without co-expression of V5-PP1α or HA-PP2A. Cells were starved and then treated with insulin.

dephosphorylation when PP2A is inhibited. We then performed an *in vitro* PP2A phosphatase assay to show that PP2A dephosphorylates PREX2 (Figure 2.7B). Lastly, overexpression of the PP2A catalytic subunit reduced phosphorylation of PREX2, including at S1107, although not nearly as efficiently as PP1α (Figure 2.7C). From this data, it is clear that phosphatases are important in regulating PREX2 phosphorylation, potentially acting in opposition of a kinase downstream of insulin stimulation.

PREX2 phosphorylation is dependent on binding to PI(3,4,5)P3

We next sought to understand how PREX2 phosphorylation was regulated after insulin stimulation to better inform our search for a PREX2 kinase. Our data from Figures 2.2 and 2.3 suggest that PREX2 is either phosphorylated by PI3K, or by a kinase downstream of PI(3,4,5)P3 generation. We found that expression of PTEN in U87MG cells eliminated PREX2 phosphorylation, and that the lipid phosphatase activity is required as the lipid phosphatase dead G129E mutant had no effect (Figure 2.8A). Since

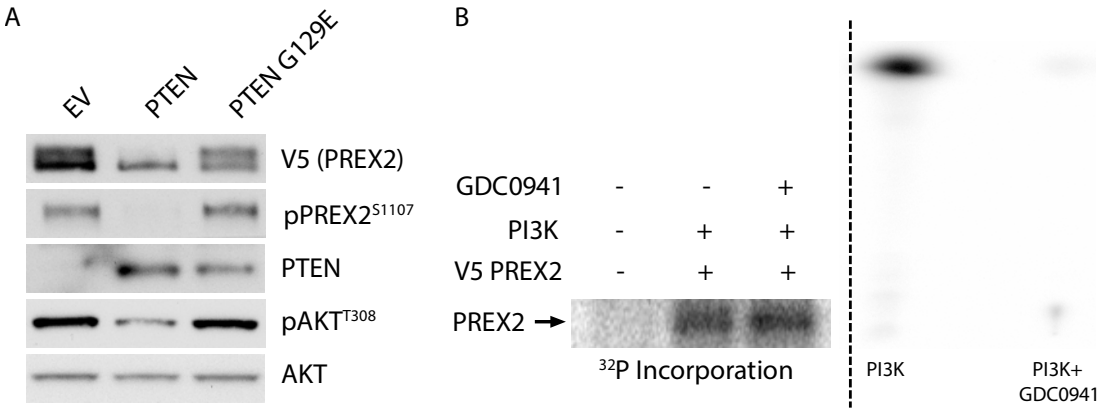


Figure 2.8 – PREX2 is not a PI3K substrate A) Western blot analysis of U87MG cells expressing V5 PREX2 and either PTEN WT or the lipid phosphatase dead mutant PTEN G129E. B) (Left) *In vitro* kinase assay with purified PI3K and V5 PREX2 isolated on V5 agarose beads, performed with or without GDC0941. Incorporation of [γ-^{32}P]-ATP was analyzed by gel electrophoresis. (Right) *In vitro* kinase assay with purified PI3K and phosphatidylinositol, performed with or without GDC0941. Incorporation of [γ-^{32}P]-ATP was analyzed by thin layer chromatography.

PTEN counteracts PI3K by reducing PI(3,4,5)P3 levels rather than directly regulating PI3K, this result demonstrated that PREX2 phosphorylation is dependent on a PI(3,4,5)P3 regulated kinase. This conclusion was supported by a kinase assay showing that there was no GDC0941 sensitive phosphorylation that occurred on PREX2 after

incubation with purified PI3K (Figure 2.8B). There did appear to be some baseline level of phosphorylation, but since PI3K inhibition had no effect, it is likely coming from a kinase that co-precipitates with PREX2, and not PI3K.

To further support the idea that phosphorylation of PREX2 is PI(3,4,5)P3 dependent, we blocked PI(3,4,5)P3 binding to PREX2 by removing the lipid binding PH domain, and then determined the effect on PREX2 phosphorylation. A construct of PREX2 lacking the DH and PH domains, hereafter called ΔDHPH, was overexpressed in HEK293 cells, and was unable to bind to PI(3,4,5)P3 beads or be phosphorylated at S1107, suggesting that PI(3,4,5)P3 binding is important for PREX2 phosphorylation (Figure 2.9A, B). To analyze the effect of PI(3,4,5)P3 binding in the full-length PREX2 protein, we attempted to disrupt PI(3,4,5)P3 binding with point mutations in the PH domain of PREX2. Previous studies have identified a PI(3,4,5)P3 consensus binding sequence within PH domains which consists of a lysine at the second to last position in the PH domain β_1 strand and an arginine or lysine at the second and

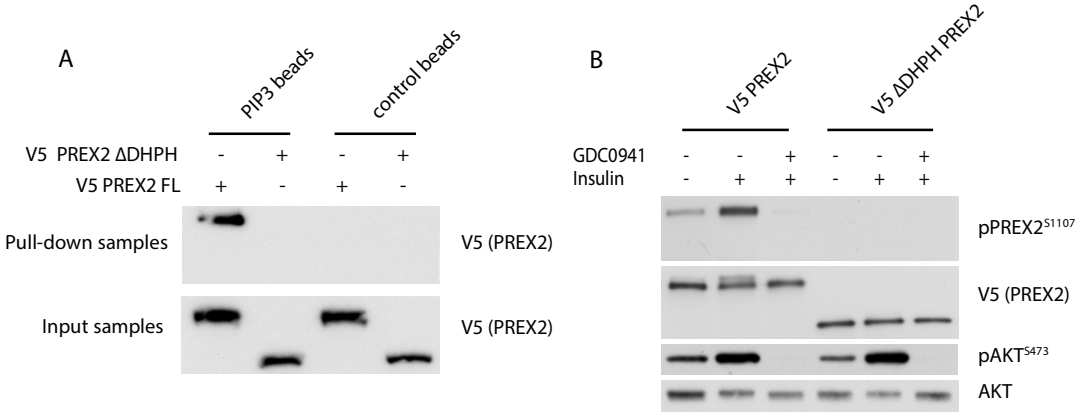


Figure 2.9 – PI(3,4,5)P3 binding to a region in the DHPH domain of PREX2 is required for PREX2 phosphorylation A) Western blot analysis of PI(3,4,5)P3 bead pull downs from HEK293 cells expressing V5 PREX2 or V5 PREX2 ΔDHPH. B) Western blot analysis of V5 PREX2 or V5 PREX2 ΔDHPH expressing HEK293 cells that were starved and then treated with DMSO or 500nM GDC0941 followed by treatment with insulin.

fourth position from the beginning of the β_2 strand (Isakoff et al., 1998; Park et al., 2008). Currently, there is no crystal structure of PREX2; however, we created a model of the PREX2 PH domain based on homology to known PH domain structures, and the potential residues that comprise the common PH domain secondary structures were identified (Figure 2.10A, B). The $\beta_1\beta_2$ loop of PREX2 from our model was aligned with the $\beta_1\beta_2$

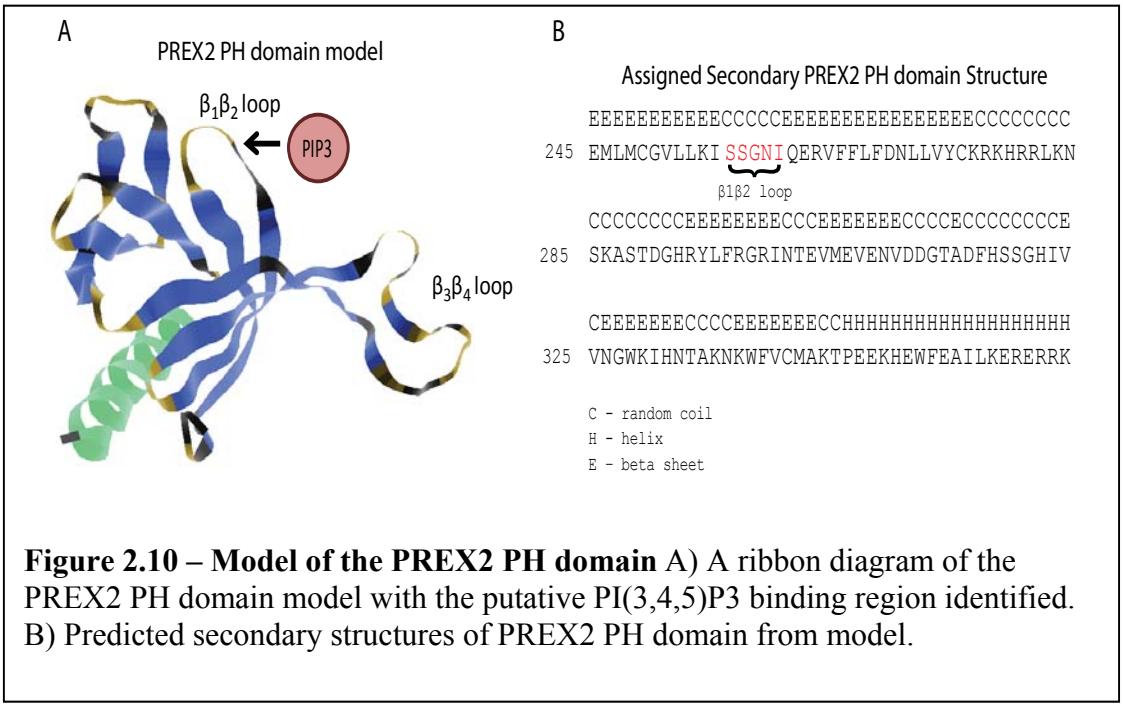


Figure 2.10 – Model of the PREX2 PH domain A) A ribbon diagram of the PREX2 PH domain model with the putative PI(3,4,5)P3 binding region identified. B) Predicted secondary structures of PREX2 PH domain from model.

loop of two well-known PI(3,4,5)P3 binding proteins, AKT1 and PDK1. Critical residues for binding of AKT1 and PDK1 to PI(3,4,5)P3 have been identified, and these residues are highlighted in Figure 2.11A (Komander et al., 2004; Thomas et al., 2002). According to our model, PREX2 contains the conserved lysine in the β_1 strand (K254), and has an arginine at the third position of the β_2 strand (R263), and we hypothesized that these two residues could be critical for PREX2 binding to PI(3,4,5)P3. Indeed, mutation of either K254 or R263 to glutamate was sufficient to completely block the binding of PREX2 to PI(3,4,5)P3 beads (Figure 2.11A). Given that PI(3,4,5)P3 activates PREX2 GEF activity, we tested whether blocking PI(3,4,5)P3 binding to the $\beta_1\beta_2$ loop affected PREX2

function. To do this, we measured the ability of PREX2 WT and PREX2 R263E to stimulate RAC1 loading of radiolabeled GTPγS ([^{35}S]GTPγS), a non-hydrolyzable analog of GTP. We found that the R263E mutation eliminated PI(3,4,5)P3 mediated stimulation of PREX2 GEF activity (Figure 2.11B). Importantly, both of these mutations also resulted in significantly reduced phosphorylation of PREX2 after insulin treatment (Figure 2.11C). These data confirm that insulin dependent phosphorylation of PREX2 requires PI(3,4,5)P3 binding to the PH domain and that K254 and R263 surrounding the $\beta_1\beta_2$ loop are necessary for this interaction, and thus are critical for phosphorylation.

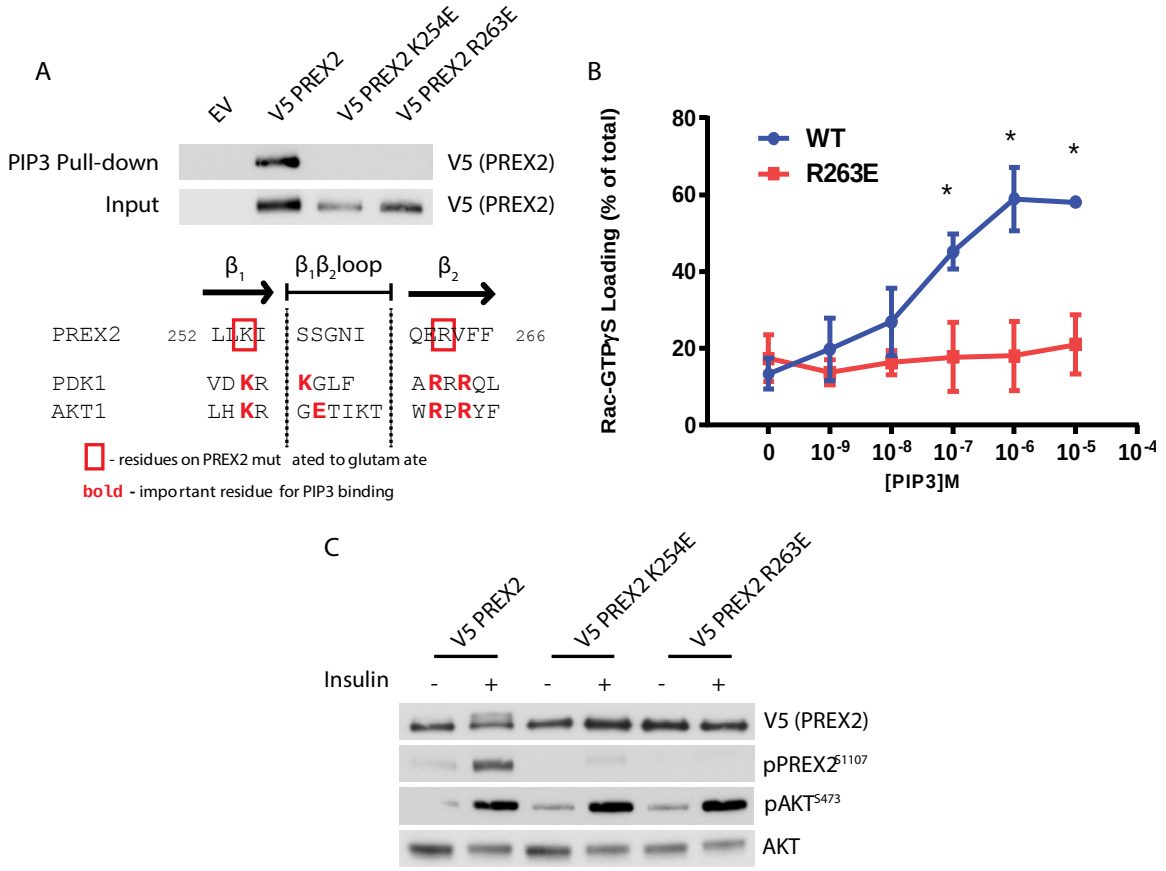


Figure 2.11 – PI(3,4,5)P3 binding to PREX2 and insulin-induced PREX2 phosphorylation requires the $\beta_1\beta_2$ loop of the PREX2 PH domain A) (Top) Western blot analysis of PI(3,4,5)P3 pull downs from HEK293 cells expressing either V5 PREX2 WT, K254E, or R263E. (Bottom) Diagram showing the $\beta_1\beta_2$ loop region of the PREX2 PH domain model aligned with the same region on PDK1 and AKT1. B) V5 PREX2 WT or R263E was purified from HEK293 cells and the ability of increasing doses of PI(3,4,5)P3 to stimulate PREX2 GEF activity towards GST RAC1 was assessed in an *in vitro* GEF assay. Data are mean ± standard deviation (SD) for at least two experiments with samples done in duplicate at each PI(3,4,5)P3 concentration, and * represents $p < 0.001$ by t-test. C) Western blot analysis of starved or insulin treated HEK293 cells expressing V5 PREX2 WT, K254E, or R263E.

Gβγ stimulates phosphorylation of PREX2

Given that PREX2 is activated by PI(3,4,5)P3 and requires PI(3,4,5)P3 for phosphorylation, we hypothesized that phosphorylation of PREX2 may be associated with activation of PREX2 GEF activity. The second messenger Gβγ also activates PREX2, so we tested the effect of Gβγ on the phosphorylation of PREX2. We found that overexpression of Gβγ induced the phosphorylation of PREX2 in starved cells, further suggesting a link between PREX2 activation and phosphorylation (Figure 2.12A). Phosphorylation of PREX2 at S1107 was also increased by Gβγ. Further, Gβγ-induced phosphorylation of PREX2 was only mildly sensitive to PI3K inhibition by GDC0941 at

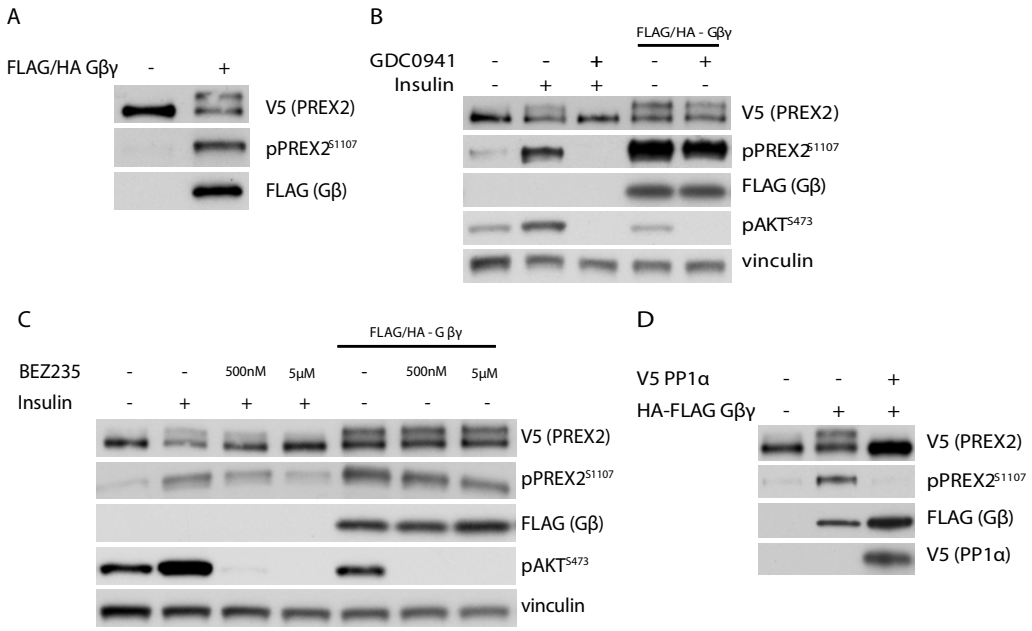


Figure 2.12 – Gβγ stimulates phosphorylation of PREX2 A) Western blot analysis of starved HEK293 cells where V5 PREX2 was expressed with or without FLAG/HA Gβγ. B) Western blot analysis of HEK293 cells where V5 PREX2 was expressed with or without FLAG/HA Gβγ. Cells were then starved and treated with DMSO or 500nM GDC0941 followed by treatment with insulin. C) Western blot analysis of HEK293 cells where V5 PREX2 was expressed with or without FLAG/HA Gβγ. Cells were then starved and treated with DMSO or either 500nM or 5μM BEZ235 followed by treatment with insulin. D) Western blot analysis of starved HEK293 cells expressing V5 PREX2 with FLAG/HA Gβγ and V5 PP1α.

a concentration where insulin-induced phosphorylation of PREX2 and AKT was completely eliminated, indicating that phosphorylation mediated by Gβγ occurs primarily through a PI3K independent mechanism (Figure 2.12B). Given that Gβγ activates PI3Kγ and PI3Kβ independently of insulin receptor activation, and GDC0941 has a relatively high IC50 towards PI3Kγ, we tested a more effective PI3Kγ inhibitor, BEZ235 (Maira et al., 2008). This drug had a minimal effect on Gβγ-induced phosphorylation of PREX2 at very high doses, while ablating insulin dependent phosphorylation (Figure 2.12C). The mild effect of PI3K inhibitors on Gβγ-induced phosphorylation of PREX2 could be a result of eliminating the basal PI(3,4,5)P3 dependent phosphorylation that exists in starved cells, or there could be some cross-talk between Gβγ and certain PI3K isoforms. Further, as was the case with insulin dependent phosphorylation, PP1α expression eliminated phosphorylation of PREX2 that was stimulated by Gβγ (Figure 2.12D). From these data, it is clear that increasing the levels of intracellular Gβγ is another mechanism to stimulate the phosphorylation of PREX2.

DISCUSSION

PREX2 is a PI(3,4,5)P3 and Gβγ dependent RAC GEF with established roles in the insulin signaling pathway (Donald et al., 2004; Hodakoski et al., 2014; Rosenfeldt et al., 2004). In this chapter, we describe the first reported instance of phosphorylation events occurring on PREX2. Our data show that PREX2 was phosphorylated after insulin receptor activation through a PI3K dependent mechanism. While phosphorylation of PREX2 was not regulated by the PI(3,4,5)P3 regulated kinases AKT, PDK1, and mTORC1, it required intact PREX2 binding to PI(3,4,5)P3. PREX2 phosphorylation was

also stimulated by Gβγ overexpression, suggesting phosphorylation of PREX2 occurs in the context of PREX2 GEF activation.

Using a mass spectrometry approach, we identified many residues on PREX2 that were phosphorylated, some of which were only phosphorylated in stimulated conditions. After generating an antibody to PREX2 that was phosphorylated at S1107, S1107 was revealed as an insulin and Gβγ-induced phosphorylation site. While we followed up on S1107, our data would suggest that there are more sites that are induced by insulin and Gβγ. For example, when S1107 was mutated to alanine so that it could no longer be phosphorylated, there was a reduction in the insulin-induced upper band of PREX2, but it was not completely eliminated. In addition, as shown in Figure 2.5C, while S1107 was significantly enriched in the upper band of PREX2 compared to the total protein, the lower band was also phosphorylated to a small degree. If S1107 were the sole site responsible for the shift, then only the upper band should be phosphorylated. A better understanding of how PREX2 phosphorylation is regulated, which is addressed in Chapter 3, will be critical in designing future mass spectrometry experiments to more effectively identify relevant phosphorylation sites.

Studies with PREX1 have shown that the region of the IP4P domain that is homologous to the region surrounding S1107 on PREX2 is important for the regulation of PREX1 by phosphorylation. The residue on PREX1 (S1169) that is homologous to S1107 on PREX2, was identified as one of the residues with elevated phosphorylation following growth factor treatment, including IGF (Montero et al., 2011; Montero et al., 2013). In addition, many of the sites on PREX1 that are de-phosphorylated by PP1α are in this region, including the prominent site, S1165 (Barber et al., 2012). Importantly, the

kinase that phosphorylates PREX1 after treatment with growth factors and the kinase or kinases that phosphorylate the PP1α sensitive sites on PREX1 have not been identified. In Chapter 3, we attempt to identify the PREX2 kinase, and given the similarities between PREX1 and PREX2 phosphorylation, these studies have implications for PREX1 as well.

We also found that phosphorylation of PREX2, including at S1107, was sensitive to PP1α and PP2A dephosphorylation. This is consistent with the presence of a PP1α binding site near S1107 and the previous reports showing that a fragment of PREX2 containing this binding site can interact with PP1α (Barber et al., 2012; Hendrickx et al., 2009). Our data showed that PP1α can completely ablate insulin and Gβγ-induced phosphorylation of PREX2; however, it is still unclear how these dephosphorylation events are regulated downstream of insulin receptor or GPCR activation. PP1α dephosphorylation activates PREX1, and if this is also the case for PREX2, then it is possible that PP1α and PP2A are activated downstream of insulin receptor or GPCR activation to stimulate PREX2 (Barber et al., 2012). In this model, PREX2 would be held in an inactive conformation by a kinase that is constitutively active in a basal cellular state. However, our data would suggest that this model is unlikely given that PREX2 appears to be dephosphorylated when cells are starved and then phosphorylated under conditions where PREX2 is activated. A more likely model from our data would be that phosphatases are active in starved cells, keeping PREX2 dephosphorylated and primed for activation by PI(3,4,5)P3 or Gβγ. In this case, the function of phosphorylation is less clear, and could potentially be a mechanism to turn off PREX2 GEF activity after it has been activated by a signal. This is a possibility that will be explored in Chapter 3.

Acknowledgements: The mass spectrometry experiments were performed by Min Yuan and John Asara at the Mass Spectrometry Core at Beth Israel Deaconess Medical Center. The S1107 antibody was made in collaboration with Anthony Couvillon at Cell Signaling Technologies. The PREX2 PH domain model was created by Antonina Silkov from the Barry Honig laboratory at Columbia University Medical Center.

CHAPTER 3: PAK kinases phosphorylate PREX2 to initiate feedback inhibition of RAC1

BACKGROUND AND SIGNIFICANCE

In Chapter 2, we showed that PREX2 is phosphorylated after insulin receptor activation, and that these phosphorylation events require PI3K and binding of PREX2 to PI(3,4,5)P3. We also found that PREX2 was phosphorylated downstream of Gβγ. Given that phosphorylation commonly regulates the function of other GEFs, including PREX1, we hypothesized that it was important for PREX2 function as well. Further, PREX2 has a functional role in insulin signaling in the mouse, suggesting that regulation of PREX2 function by phosphorylation downstream of insulin stimulation could have important cellular consequences for glucose metabolism. In this chapter, we analyze the effect of phosphorylation on PREX2 function, and use clues from our functional studies to identify the PREX2 kinase.

RESULTS

Phosphorylation of PREX2 prevents membrane binding

RAC1, the target of PREX2 GEF activity, is activated at the membrane, and further, membrane bound PREX1 is more active than cytosolic PREX1 (Barber et al., 2007). This suggests that alterations in the ability of PREX2 to bind to the membrane could have important implications for its function. To determine whether phosphorylation of PREX2 alters membrane binding, we performed cellular fractionation on V5 PREX2 expressing cells. After insulin or Gβγ stimulation, both the phosphorylated and dephosphorylated forms of PREX2 were present in the cytoplasmic fraction (Figure 3.1A). Importantly, only the dephosphorylated form of PREX2 was present in the membrane fraction, indicating that the phosphorylated form of PREX2 is unable to bind to the membrane. To observe whether the same effect occurs with endogenous PREX2,

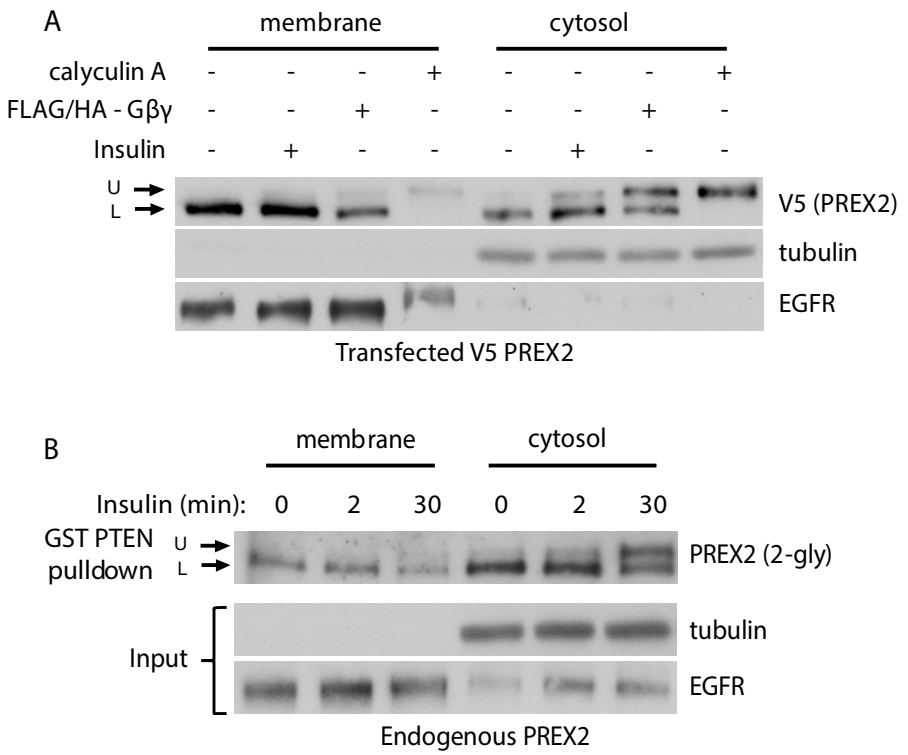


Figure 3.1 – Phosphorylation of PREX2 prevents membrane localization A) Western blot analysis of cytosolic and membrane fractions from starved or insulin treated HEK293 cells expressing V5 PREX2 with or without co-expression of FLAG/HA Gβγ. B) Western blot analysis of GST PTEN pull downs of endogenous PREX2 from cytosolic and membrane fractions of HEK293 cells. Cells were starved and then treated with insulin for indicated times. U - upper band, L – lower band.

we performed GST PTEN pull downs from the membrane and cytosolic fractions of HEK293 cells in order to isolate PREX2 and obtain a clear Western blot signal. As seen with V5 PREX2, phosphorylated endogenous PREX2 was not at the membrane, despite being present in the cytoplasm (Figure 3.1B). These data show that phosphorylation prevents PREX2 from binding to the membrane where it can stimulate RAC1, suggesting that phosphorylation may negatively regulate PREX2 function.

Phosphorylation of PREX2 blocks binding to PI(3,4,5)P3 and Gβγ

Both PI(3,4,5)P3 and Gβγ are membrane-associated, so we hypothesized that phosphorylated PREX2 may have reduced affinity for PI(3,4,5)P3 and Gβγ, resulting in the localization patterns we observed. PI(3,4,5)P3 pull downs of V5 PREX2 were performed from insulin stimulated or Gβγ expressing cells, and we found that the phosphorylated form of PREX2 was completely absent from the pull down, suggesting that phosphorylation was preventing PREX2 from binding to PI(3,4,5)P3 (Figure 3.2A). In Chapter 1, we established that endogenous PREX2 is stoichiometrically phosphorylated by an insulin dependent signal in HEK293 cells. Given that finding, it was not surprising that the effect of insulin stimulation on PI(3,4,5)P3 binding was even more striking with endogenous PREX2, as insulin significantly reduced the ability of PREX2 to bind to PI(3,4,5)P3, an effect that was reversed with PI3K inhibition (Figure 3.2B). In addition, PI(3,4,5)P3 pull-downs were performed with V5 PREX2 from cells where PP1α was co-expressed in order to generate the least phosphorylated form of PREX2, or from cells treated with calyculin A to generate a maximally phosphorylated form of PREX2 (Figure 3.2C). The result was clear as PP1α co-expression increased the ability of PREX2 to bind PI(3,4,5)P3, and calyculin A treatment almost completely ablated PI(3,4,5)P3 binding. Further, in the calyculin A treated sample, while almost all of the PREX2 in the input was enriched in the slower migrating, phosphorylated form of PREX2, the small amount that did bind to PI(3,4,5)P3 was the faster migrating, dephosphorylated form. This result also showed that phosphatases can regulate PREX2 binding to PI(3,4,5)P3. One alternative explanation for our results could be that the phosphorylated form of PREX2 actually binds better to PI(3,4,5)P3 than the

dephosphorylated form, and we only see binding of dephosphorylated PREX2 to PI(3,4,5)P3 *in vitro* because endogenous PI(3,4,5)P3 is binding all of the phosphorylated protein. However, considering calyculin A-treated PREX2 could not bind to PI(3,4,5)P3 in the presence of PI3K inhibitor (which will make PI(3,4,5)P3 levels in the cell very

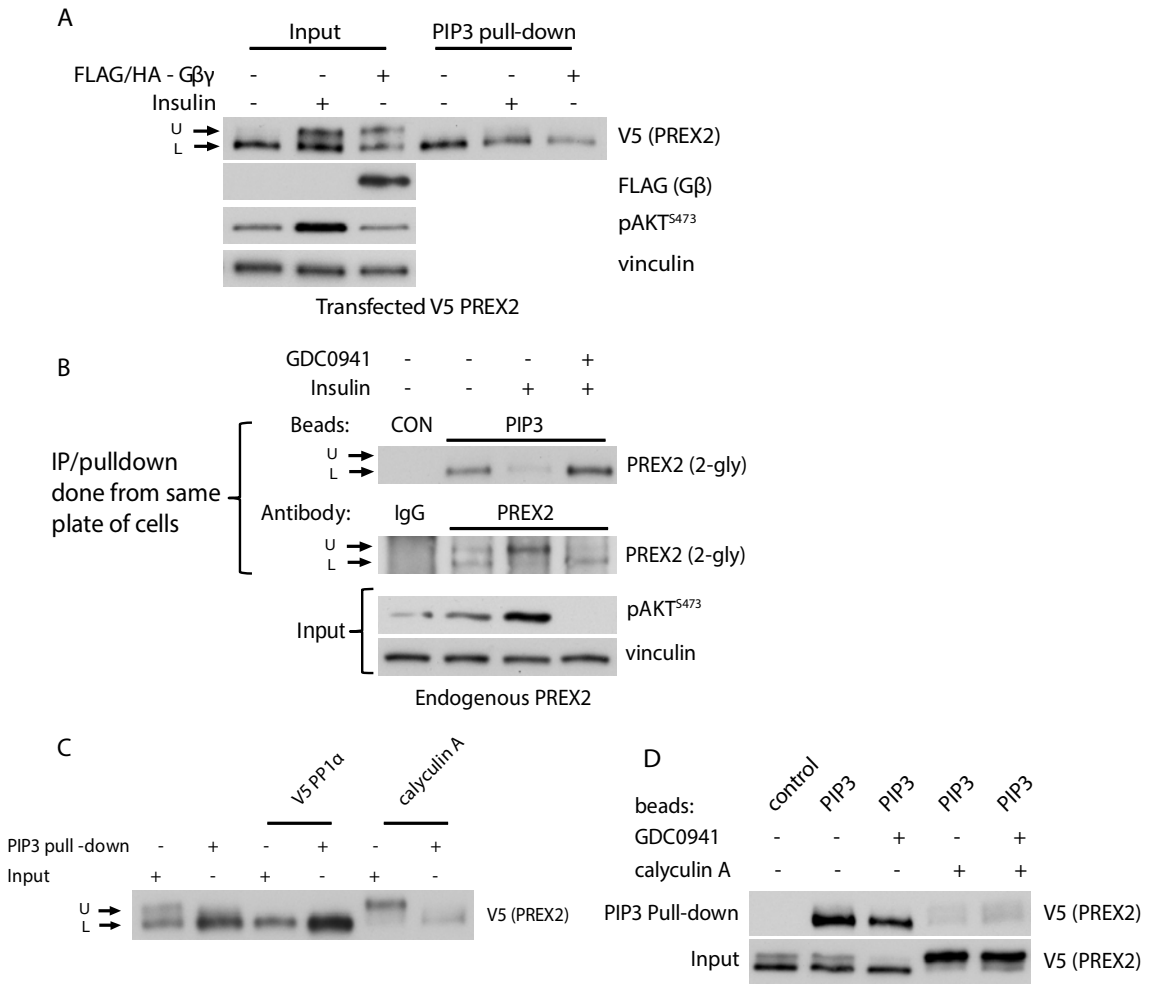


Figure 3.2 – Phosphorylation of PREX2 blocks binding to PI(3,4,5)P3 A) Western blot analysis of PI(3,4,5)P3 pull downs from starved or insulin treated HEK293 cells expressing V5 PREX2 with or without FLAG/HA Gβγ. B) Western blot analysis of endogenous PREX2 isolated from HEK293 cells with either PI(3,4,5)P3 beads (top) or PREX2 antibody (bottom). C) Western blot analysis of PI(3,4,5)P3 pull downs from V5 PREX2 expressing cells with either V5 PP1α co-overexpression or treatment with 100nM calyculin A. D) Western blot analysis of PI(3,4,5)P3 pull downs from V5 PREX2 expressing cells that were starved and treated with either 500nM GDC0941 or 100nM calyculin A. U - upper band, L – lower band.

low), it is more likely that phosphorylated PREX2 has a lower affinity for PI(3,4,5)P3 (Figure 3.2D).

Next, we tested whether phosphorylation of PREX2 affected its ability to bind to Gβγ. Co-immunoprecipitation of V5 PREX2 and FLAG-Gβγ with a FLAG antibody showed that the dephosphorylated and faster migrating species of PREX2 preferentially bound to Gβγ (Figure 3.3A). Phosphorylating PREX2 even further with calyculin A

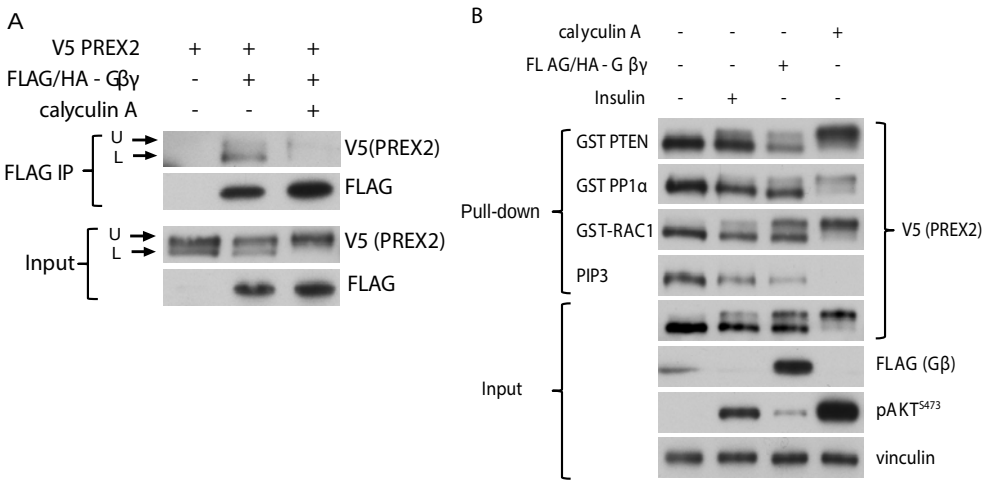


Figure 3.3 – Phosphorylation of PREX2 blocks binding to Gβγ, but not other known PREX2 interacting proteins A) Western blot analysis of FLAG immunoprecipitations from HEK293 cells co-expressing FLAG/HA Gβγ and V5 PREX2 with and without 100nM calyculin A treatment. B) Western blot analysis of pull downs with GST PTEN, GST PP1α, GST RAC1, or PI(3,4,5)P3 beads from HEK293 cells expressing V5 PREX2 with or without FLAG/HA Gβγ. The cells were starved and treated with insulin or 100nM calyculin A. U - upper band, L – lower band.

treatment reduced overall binding of PREX2 and Gβγ. These data show that phosphorylation of PREX2 reduces binding to Gβγ. To determine whether phosphorylation prevented binding of PREX2 to other known interacting partners, we performed pull downs with GST tagged PTEN, PP1α, and RAC1. While PI(3,4,5)P3 only bound the dephosphorylated form of PREX2, RAC1 and PTEN did not discriminate

between any forms of PREX2 (Figure 3.3B). The dephosphorylated form of PREX2 was somewhat enriched in the PP1α pull downs (though not as much as in the PI(3,4,5)P3 pull downs), but this was likely because PP1α was dephosphorylating PREX2 during the reaction. These findings suggest that phosphorylation selectively blocks binding of PREX2 to PI(3,4,5)P3 and Gβγ.

Phosphorylation of PREX2 reduces PI(3,4,5)P3 and Gβγ activation of GEF activity

Given that phosphorylation blocked PREX2 binding to PI(3,4,5)P3 and Gβγ, it was then important to determine the effect of phosphorylation on PI(3,4,5)P3 and Gβγ stimulation of GEF activity. V5 PREX2 was purified from HEK293 cells after being treated with either GDC0941, insulin in combination with Gβγ co-expression, or calyculin A, to achieve relatively dephosphorylated, partially phosphorylated, and maximally phosphorylated states of PREX2, respectively (Figure 3.4A). When compared to dephosphorylated PREX2, partially phosphorylated PREX2 showed decreased activation of GEF activity at multiple PI(3,4,5)P3 concentrations, while activation of the maximally phosphorylated form of PREX2 was reduced even further (Figure 3.4B). In addition, Gβγ dependent stimulation of PREX2 GEF activity was similarly affected by phosphorylation across many Gβγ concentrations (Figure 3.4C). We next sought to determine whether this negative regulation by phosphorylation was important in cells, and to probe this, we used a mutant containing a phospho-mimicking glutamate mutation at S1107 (S1107E) on PREX2. Previous studies from our lab demonstrated that PREX2 expression in BT549 breast cancer cells stimulates invasion through matrigel. Importantly, the S1107E mutation reduced the ability of PREX2 to stimulate invasion in these cells, which is consistent with the *in vitro* data showing that phosphorylation

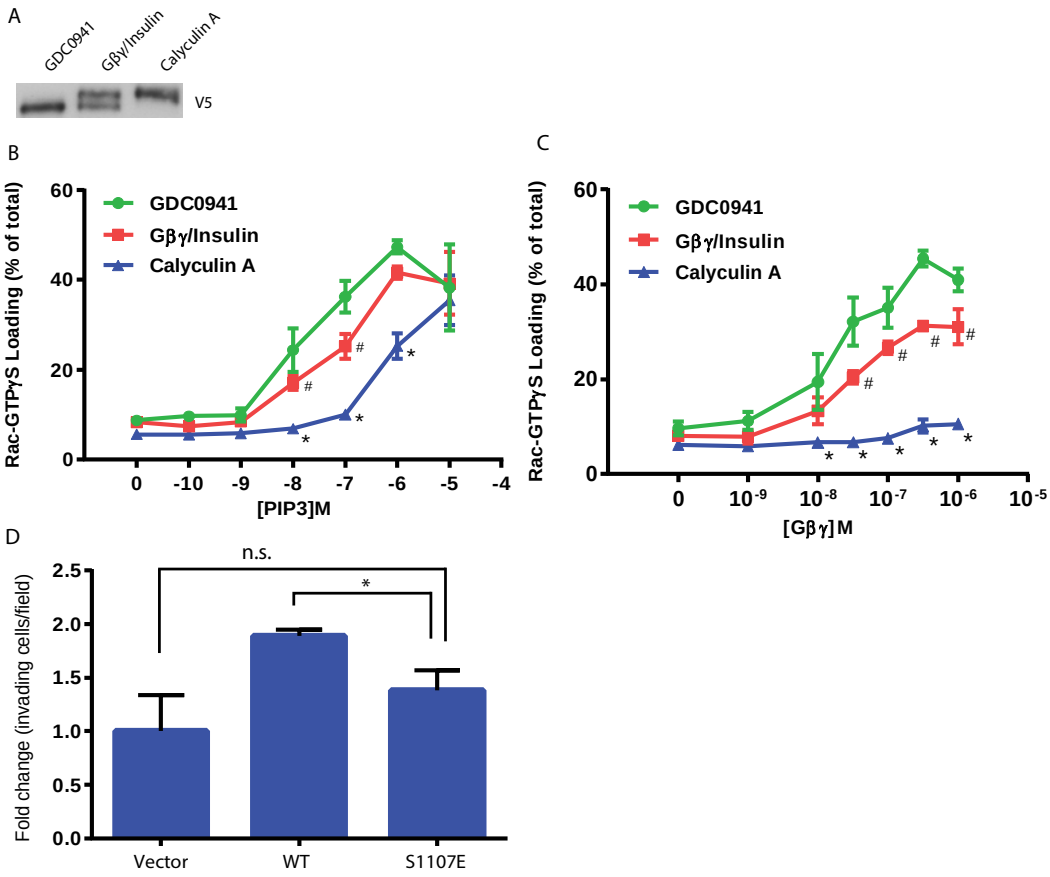


Figure 3.4 – Phosphorylation of PREX2 reduces PREX2 GEF activity A) V5 PREX2 was expressed in HEK293 cells with or without co-expression of FLAG/HA Gβγ and then was purified after being treated with either 500nM GDC0941, insulin (with co-expression of FLAG/HA Gβγ), or 100nM calyculin A. B and C) The ability of increasing doses of PI(3,4,5)P3 in (B) or Gβγ in (C) to stimulate PREX2 GEF activity towards GST RAC1 was assessed in an in vitro GEF assay. Data at each PI(3,4,5)P3 and Gβγ concentration are mean ± SD for 3 experiments and # represents $p < 0.05$ for GDC0941 vs. Gβγ/Insulin, and * represents $p < 0.005$ for GDC0941 vs. calyculin A by t-test. D) V5 PREX2 WT or S1107E were expressed in BT549 cells and invasion assays were performed. Representative shown of two experiments, data are mean ± SD, and * represents $p < 0.05$ by t-test.

reduces GEF activity (Figure 3.4D). Analysis of the time course of insulin-induced phosphorylation of PREX2 revealed that phosphorylation of both endogenous PREX2 and transfected V5 PREX2 did not peak until the 30 minute time point (Figure 3.5A, B).

This is in contrast to phosphorylation of the PI(3,4,5)P3 dependent kinase AKT, which is highly phosphorylated within the first 30 seconds after insulin treatment, and peaks between 1-5 minutes. This delayed time course of PREX2 phosphorylation, in combination with our data showing that phosphorylation reduces GEF activity and PREX2 stimulated invasion, supports a model where phosphorylation occurs after PREX2 activation by PI(3,4,5)P3 and Gβγ to attenuate its function and the RAC1 signal.

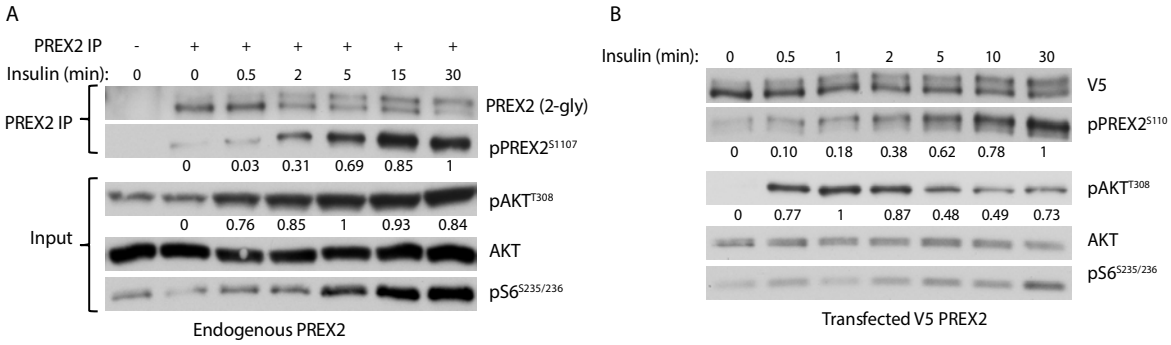


Figure 3.5 – Time course of PREX2 phosphorylation A) Western blot analysis of endogenous PREX2 that was immunoprecipitated from HEK293 cells that were starved and treated with insulin for the indicated times. B) Western blot analysis of V5 PREX2 expressing HEK293 cells that were starved and then treated with insulin for the indicated times. For quantification of phosphorylation, intensity of band was normalized to total protein of that sample. The highest value was set to 1, and the remaining samples were normalized to this value.

RAC1 promotes PAK phosphorylation of PREX2

Our data suggest that PREX2 phosphorylation is part of negative feedback following RAC1 activation to decrease PREX2 GEF activity and allow for tight control of the RAC1 signal. If this were true, then higher RAC1 activity within the cell should increase phosphorylation of PREX2. Indeed, expression of RAC1 in HEK293 cells induced phosphorylation of PREX2 even in the absence of insulin (Figure 3.6A). The dominant negative mutant T17N RAC1 mutant did not increase PREX2 phosphorylation,

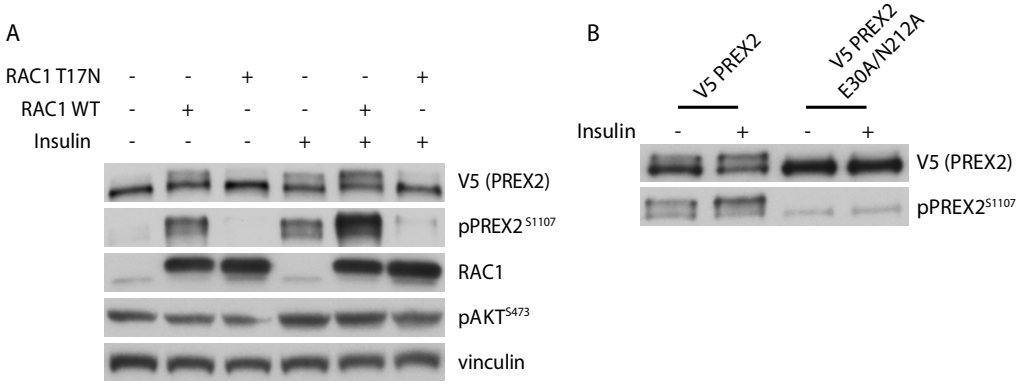


Figure 3.6 – RAC1 GTP loading and PREX2 GEF activity are required for phosphorylation of PREX2 A) Western blot analysis of starved or insulin treated HEK293 cells expressing V5 PREX2 with or without MYC RAC1 WT or the dominant negative mutant T17N. B) Western blot analysis of starved or insulin treated HEK293 cells expressing V5 PREX2 WT or the GEF-dead E30A/N212A mutant.

and further, the T17N mutant blocked the insulin stimulated phosphorylation, indicating that RAC1 is downstream of PI(3,4,5)P3 generation. In addition, a GEF dead PREX2 mutant (E30A/N212A) was not phosphorylated following insulin treatment, confirming that PREX2 must be able to stimulate RAC1 in order to become phosphorylated (Figure 3.6B) (Mense et al., 2015). These results suggested that the kinase or kinases that are phosphorylating PREX2 are downstream of RAC1 activation.

The PAK family of kinases, consisting of group I PAKs (PAK1-3) and group II PAKs (PAK4-6), are important mediators of signaling downstream of RHO family GTPases. The regulation of PAK1 is best understood, and its kinase activity is activated downstream of insulin and upon binding to GTP bound RAC1, making it a candidate kinase for PREX2 (Manser et al., 1994; Tsakiridis et al., 1996). Inhibition of both groups

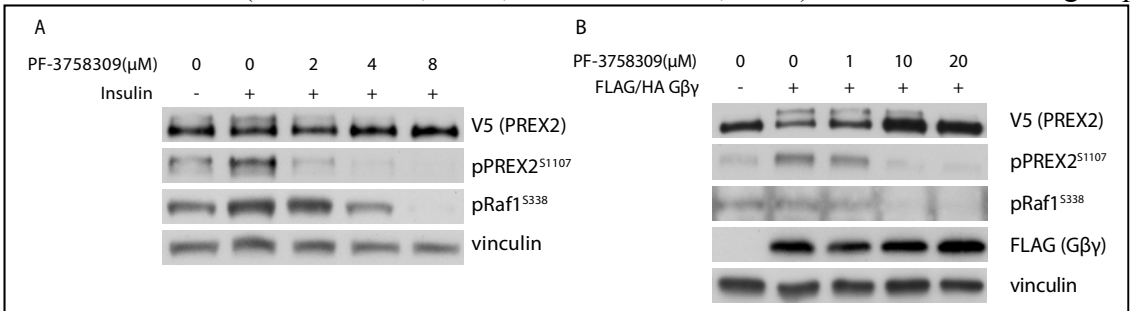


Figure 3.7 – Inhibition of PAK kinases blocks the phosphorylation of PREX2 A) Western blot analysis of V5 PREX2 expressing HEK293 cells that were starved and then treated with DMSO or varying concentrations of the pan-PAK inhibitor PF-3758309 followed by insulin treatment. B) Western blot analysis of HEK293 cells expressing V5 PREX2 with or without Gβγ. Cells were starved and treated with DMSO or varying concentrations of PF-3758309.

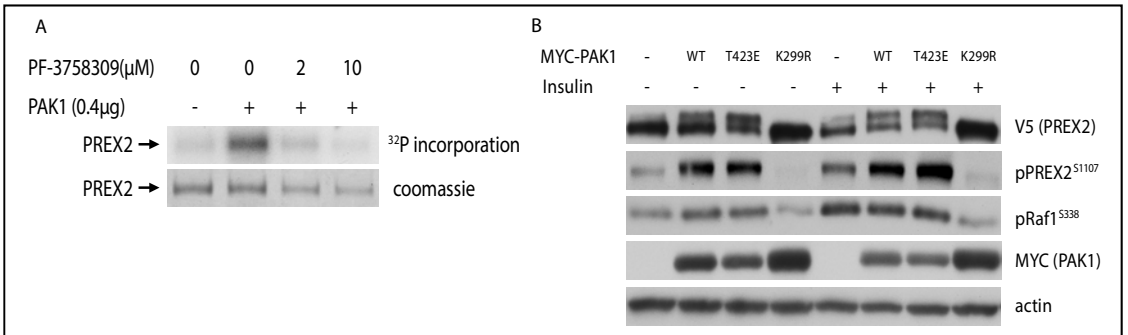


Figure 3.8 – PREX2 is a direct substrate of PAK1 *in vitro* and PAK1 increases phosphorylation in cells A) *In vitro* kinase assay with purified PAK1 and V5 PREX2 isolated on V5 agarose beads, performed with or without PF-3758309. Incorporation of [γ-^{32}P]-ATP was analyzed. B) A) Western blot analysis of starved or insulin treated HEK293 cells expressing V5 PREX2 with or without MYC PAK1 WT, the constitutively active mutant T423E, or the kinase dead mutant K299R.

of PAKs with the inhibitor PF-3758309 blocked the insulin and Gβγ-induced phosphorylation of PREX2 (Figure 3.7A, B). As is the case for many small molecule inhibitors of kinases, they are likely not entirely specific for their proposed target kinase. So we then tested whether specific isoforms of PAK kinases could regulate PREX2 phosphorylation with *in vitro* kinase assays and overexpression of PAK isoforms in cells. We first asked whether PREX2 was a direct substrate of PAK1 and found that PREX2 was indeed phosphorylated by PAK1 *in vitro* (Figure 3.8A). To determine whether PAK1 can affect PREX2 phosphorylation in cells, we overexpressed PAK1 in HEK293 cells and analyzed the effect on PREX2 phosphorylation. Overexpression of PAK1 induced the phosphorylation of PREX2, and the induction of the PREX2 mobility shift was even more striking with the constitutively active PAK1 mutant T423E (Figure 3.8B). The kinase dead and dominant negative PAK1 mutant K299R completely eliminated any phosphorylation of PREX2 in either the starved or insulin treated contexts. Interestingly, purified PAK4 was also able to phosphorylate PREX2 *in vitro* and increase phosphorylation of PREX2 in cells (Figure 3.9A, B). Further, as we would expect from

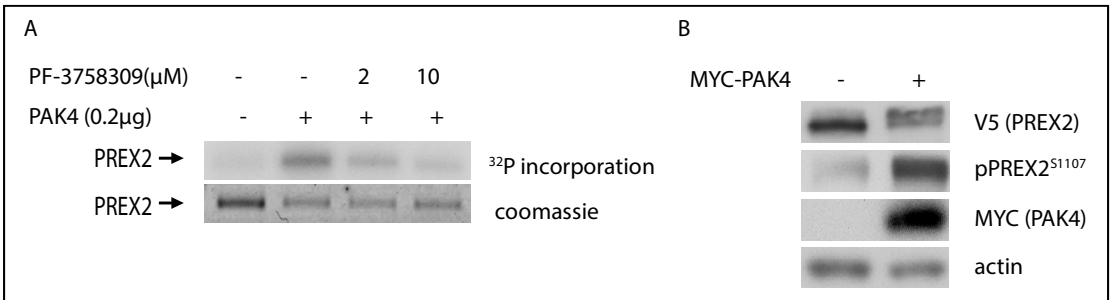


Figure 3.9 – PREX2 is a direct substrate of PAK4 *in vitro* and PAK4 increases phosphorylation in cells A) *In vitro* kinase assay with purified PAK4 and V5 PREX2 isolated on V5 agarose beads, performed with or without PF-3758309. Incorporation of [γ-^{32}P]-ATP was analyzed. B) Western blot analysis of starved HEK293 cells expressing V5 PREX2 with or without MYC PAK4.

the functional data in Figure 3.4, PAK1-induced phosphorylation also reduced PREX2 GEF activity towards RAC1 (Figure 3.10). PREX2 that was purified from cells co-expressing PAK1 had lower GEF activity towards RAC1 over multiple PI(3,4,5)P3 concentrations compared to PREX2 purified from cells co-expressing dominant negative PAK1. Taken together, these data show that RAC1 activates PAK kinases to initiate this negative regulation of PREX2 GEF activity following insulin and Gβγ stimulation.

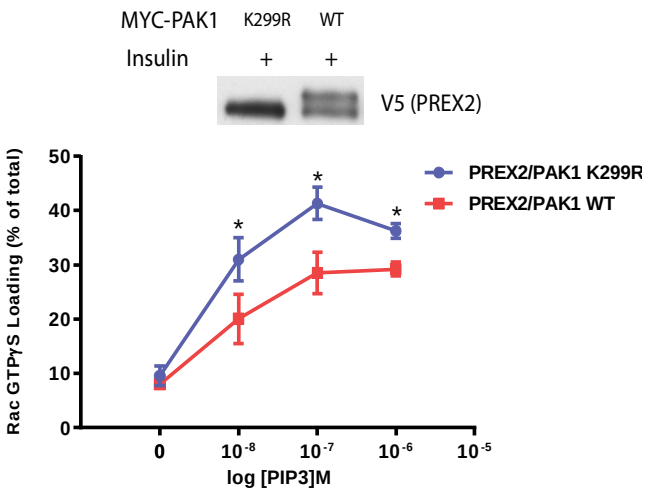


Figure 3.10 – PAK1 phosphorylation reduces PREX2 GEF activity PREX2 was purified from HEK293 cells co-expressing either MYC PAK1 WT or K299R. The ability of increasing doses of PI(3,4,5)P3 to stimulate GEF activity towards GST RAC1 was assessed in an *in vitro* GEF assay. Data are mean ± SD for two experiments with samples done in triplicate at each PI(3,4,5)P3 concentration and * represents $p < 0.005$ by t-test.

In summary, the data from chapters 2 and 3 outline a novel signaling pathway downstream of insulin receptor and Gβγ dependent activation of PREX2 where PAK kinases, which are activated by RAC1, phosphorylate PREX2 to reduce its GEF activity. Altogether, we propose that PREX2 is dephosphorylated in an unstimulated cell, and then upon binding of insulin or a GPCR agonist to its receptor, PI(3,4,5)P3 or Gβγ levels increase and dephosphorylated PREX2 can bind these molecules on the membrane and activate RAC1 (Figure 3.11). Higher levels of GTP bound RAC1 activate PAK kinases, which phosphorylate PREX2 to

release it from the membrane, decreasing RAC1 activation. Additionally, either PP1α or PP2A can complete the circuit by dephosphorylating PREX2, priming it for future activation by PI(3,4,5)P3 or Gβγ. This model allows for tight regulation of the length and amplitude of RAC1 activation, resulting in a transient RAC1 signal following ligand binding.

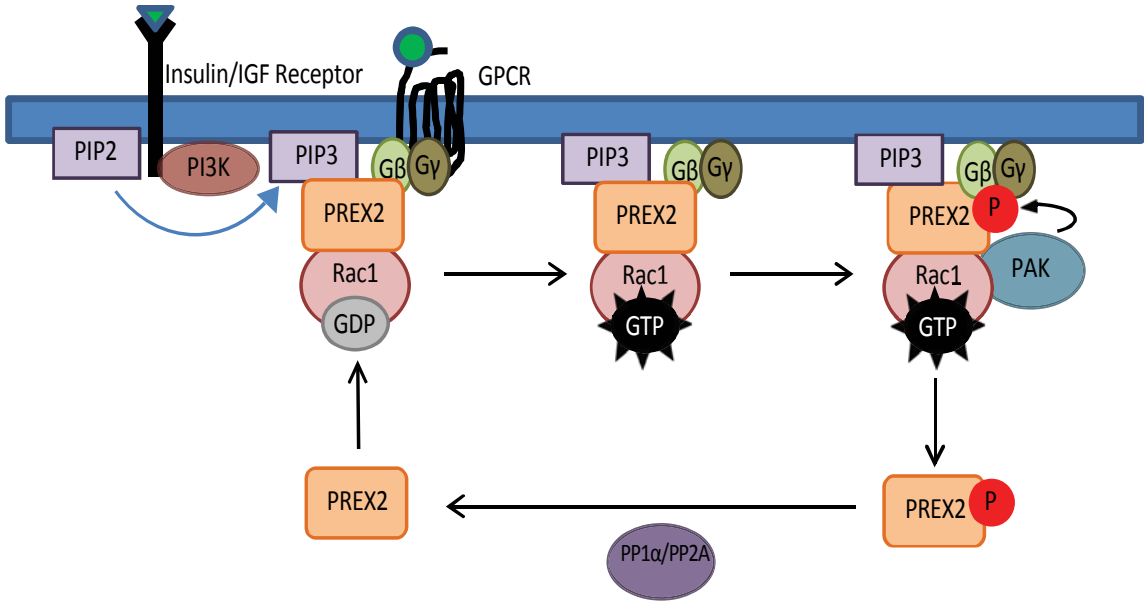


Figure 3.11 – Proposed model for PAK-mediated regulation of PREX2 GEF activity and localization

DISCUSSION

It has previously been reported that PI(3,4,5)P3 and Gβγ can activate PREX2, and as we described in Chapter 2, these two molecules also stimulate PREX2 phosphorylation. Here we uncovered the functional consequences of these phosphorylation events. We first found that PREX2 phosphorylation blocked the translocation of PREX2 to the cell membrane, and prevented binding to PI(3,4,5)P3 and

Gβγ. There are important parallels between our findings and PREX1 studies that analyzed the relationship between phosphorylation and membrane binding, which could inform future experiments involving PREX2. As mentioned in Chapter 1, *in vitro* binding of PREX1 to Gβγ is reduced by PKA phosphorylation, and agents that increase cAMP, and therefore activate PKA, decrease PREX1 levels at the membrane (Urano et al., 2008; Zhao et al., 2007). Further, it was reported that PREX1 requires certain intramolecular interactions to bind to Gβγ, and these intramolecular interactions are disrupted by PKA phosphorylation, blocking PREX1 binding to Gβγ (Urano et al., 2008). The phosphorylation events identified here on PREX2 do not appear to be mediated by PKA; however, it is possible that PAK1 phosphorylation of PREX2 could be reducing PI(3,4,5)P3 and Gβγ binding through a similar mechanism, by preventing intramolecular interactions. Intramolecular interactions have also been shown to regulate GEF activity of other RHO family GEFS, such as DBL and VAV1 (Aghazadeh et al., 2000; Bi et al., 2001).

We also found that phosphorylation of PREX2 not only blocked binding to PI(3,4,5)P3 and Gβγ, but it prevented activation by these second messengers. Further, a phospho-mimicking mutation at S1107 reduced PREX2 stimulated invasion. These data suggest that phosphorylation is part of a negative feedback circuit initiated by PREX2 activation of RAC1. The time course of PREX2 phosphorylation was delayed compared to AKT, which is also consistent with phosphorylation functioning to attenuate PREX2 function after it has been activated. The mechanisms of RAC1 inactivation are generally not as well characterized as the mechanisms of RAC1 activation, especially after insulin stimulation, and negative feedback circuits like the one identified here could be a

significant and more commonly utilized mechanism within the cell to control the time course of RAC1 activation (Wertheimer et al., 2012).

Furthermore, we discovered that PREX2 was a substrate of the RAC1 dependent PAK family of kinases. These kinases are well known for their role in propagating RAC1 dependent outputs. Given this role, it is unexpected for PAK kinases to mediate negative feedback to turn off RAC1 activity. However, there is precedent for this in lower eukaryotes. In the yeast *Saccharomyces cerevisiae*, the Pak family member Cla4 phosphorylates Cdc24, a GEF for the Cdc42 GTPase, and phosphorylation reduces GEF activity in vitro and reduces levels of Cdc42 at the front of a cell during polarization (Bose et al., 2001; Gulli et al., 2000; Kuo et al., 2014). Further, in the fungus *Ustilago maydis*, where Cdc24 is also a Rac1 GEF, Cla4 phosphorylates Cdc24, causing its degradation and a reduction in Rac1 activation (Frieser et al., 2011). Here, we show that this type of PAK mediated negative regulation of GEFs occurs in higher eukaryotes as well.

The mode of regulation identified in this chapter, where a pathway evolves a mechanism to turn a signal off after it has been activated, has been reported previously in mammalian cells, including within the insulin signaling pathway. For example, mTORC1, an important effector of insulin signaling, also initiates negative feedback to reduce activation of the pathway. Insulin activation of mTORC1 leads to the stimulation of various downstream kinases to regulate protein synthesis. However, mTORC1 activation also results in serine phosphorylation of IRS proteins, reducing their stability and decreasing the insulin signal. IRS protein levels can then be restored upon pathway inactivation caused by PTEN expression or PI3K inhibition (Harrington et al., 2004;

Haruta et al., 2000; Simpson et al., 2001; Takano et al., 2001). Importantly, mTORC1 dependent regulation of IRS stability appears to be a critical consideration for therapeutics given that inhibition of mTORC1 leads to increased AKT phosphorylation in cancer cell lines and human patients, which may partially explain the modest effects of these inhibitors in cancer patients (O'Reilly et al., 2006).

The role of mTORC1 in insulin signaling is very similar to the model we propose for PAK kinases, where PAK can both propagate the insulin dependent RAC1 signal and negatively regulate it through PREX2. Furthermore, similar to mTORC1 dependent negative feedback, PAK phosphorylation of PREX2 could have important therapeutic implications. PAK inhibitors are currently being evaluated as cancer therapeutics, and if PAK negative regulation of PREX2 is playing a significant role in insulin signaling, or more generally in RAC1 activation, then it may be important to consider in the context of certain cancers (Baker et al., 2014). For example, a tumor with PREX2 overexpression may not respond well to PAK inhibitors due to an increase in PREX2 GEF activity towards RAC1. Further, given the role of PREX2 in cancer, it will be important to study PAK mediated inactivation of PREX2 not only in the context of resistance to therapeutics, but in tumorigenesis. Many PREX2 mutations have been identified in different types of cancer, and it is possible that some of these could disrupt the PAK1 dependent negative feedback and activate RAC1 signaling, a possibility we will explore in Chapter 4.

PREX2 has previously been identified as a critical mediator of insulin signaling, and our data showing that PREX2 function is tightly regulated by insulin-induced phosphorylation further supports this idea (Hodakoski et al., 2014). Future experiments

should focus on the effect of this feedback circuit on both PREX2 and RAC1 dependent physiological outputs downstream of insulin receptor activation, including glucose metabolism. It will also be of interest to study this signaling pathway in the context of insulin related disease, such as diabetes. PREX2 protein expression appears to be lower in adipose tissue of insulin resistant human patients, suggesting that reducing PREX2 activity to prevent glucose uptake is a possible mechanism for insulin resistance (Hodakoski et al., 2014). In addition, the importance of RAC1 in insulin resistance is highlighted by the fact that RAC1 activity can be decreased in muscle tissue of insulin resistant mice and humans (Sylow et al., 2013; Sylow et al., 2014). Our data present the possibility that a reduction in PREX2 GEF activity and RAC activation could also be achieved through PAK mediated phosphorylation, meaning phosphorylation of PREX2 could be altered in diabetes and could potentially be a cause of insulin resistance. High or chronic levels of insulin (in addition to GPCR-activating fatty acids) could lead to activation of PAK phosphorylation of PREX2, keeping PREX2 inactive and potentially disrupting insulin signaling. Interestingly, the activation of feedback mechanisms in insulin resistance has been identified previously, including mTORC1 dependent ribosomal S6K phosphorylation of IRS1 (Pende et al., 2000; Um et al., 2004).

Our findings in this chapter could also have important implications for PREX1. In this study, we identified S1107 as an insulin, Gβγ, and PAK sensitive phosphorylation site. Interestingly, the residue on PREX1 (S1169) that is phosphorylated following treatment with neuregulin and IGF is the homologous residue to S1107 (Montero et al., 2011; Montero et al., 2013). In addition, PP1α dephosphorylation of PREX1 activates its GEF activity, which is consistent with our model for PREX2 (Barber et al., 2012). Lastly,

PI3K and Gβγ co-expression in sf9 insect cells leads to an electrophoretic mobility shift of PREX1, and the slower migrating, presumably phosphorylated species of PREX1 is mostly excluded from the membrane fraction (Barber et al., 2007). The similarities between phosphorylation events on PREX1 and PREX2 suggest PREX1 may also be regulated by PAK phosphorylation.

Collectively, our data represent a novel paradigm for maintaining a tightly regulated RAC1 signal by blocking the stimulation of the specific GEF that was activated, in this case PREX2. This type of regulation adds a layer of complexity to the current model where RAC1 is activated by GEFs and inactivated by GAPs and GDIs, and if PAK kinases regulate other GEFs the same way, then this signaling pathway could have implications in many aspects of cellular physiology.

Acknowledgements: The purification of Gβγ for the *in vitro* RAC1 GEF assays was done by Aliaksei Shymanets and Bernd Nürnberg at the University of Tübingen.

CHAPTER 4: PREX2 tumor mutants have altered levels of phosphorylation

BACKGROUND AND SIGNIFICANCE

The important role of PREX2 in cancer is evidenced by the fact that PREX2 expression is increased in many different tumor types and that it is one of the most commonly mutated GEFs in cancer. PREX2 tumor mutations are spread throughout the entire length of the protein and do not cluster in any particular region, making it difficult to hypothesize the potential functional consequences of these mutations (Cerami et al., 2012; Gao et al., 2013). Two studies have analyzed the effect of PREX2 tumor mutations on its function and oncogenicity. In a study by Berger et al., a number of PREX2 mutations found in melanoma were analyzed, and they showed that overexpression of K278*, E824*, Q1430*, or G844D PREX2 cooperate with NRAS to accelerate tumorigenesis in a melanocyte xenograft mouse model (Berger et al., 2012). Another study from our lab has shown that the G844D mutant, along with P948S from melanoma, and V432M from pancreatic cancer, are able to avoid inhibition of *in vitro* GEF activity by PTEN. Further, PTEN is unable to inhibit invasion stimulated by these three PREX2 tumor mutants in BT549 breast cancer cells (Mense et al., 2015). These studies show that PREX2 tumor mutations are likely selected in cancer to give cells a tumorigenic advantage.

However, given the large number of PREX2 mutations that have been reported, we still know relatively little about why the vast majority of these mutations are selected in cancer. In Chapters 2 and 3, we identified PAK mediated phosphorylation of PREX2 downstream of RAC1 activation, and we showed that these phosphorylation events reduced PREX2 GEF activity. Here, we investigate the possibility that PAK dependent negative feedback is important for the role of PREX2 as an oncogene by testing whether

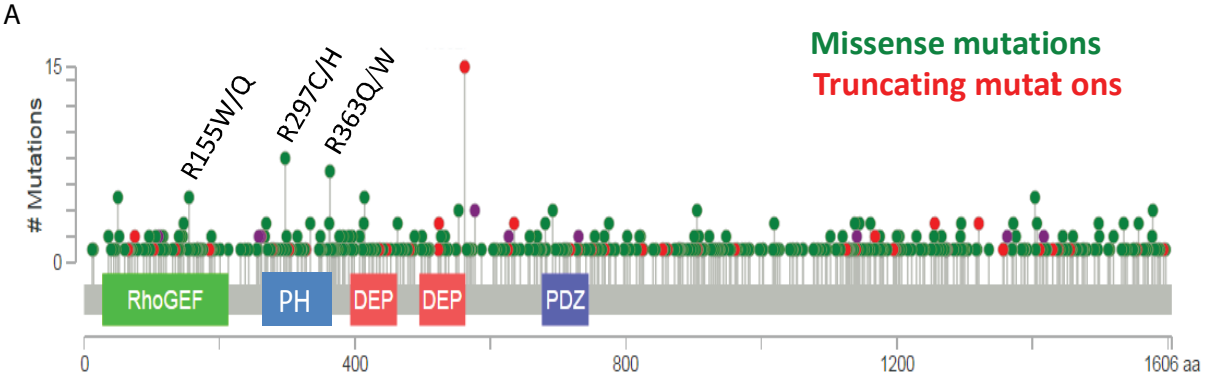
common PREX2 tumor mutations have altered PAK phosphorylation and RAC1 GEF activity.

RESULTS

PREX2 hotspot tumor mutations reduce insulin and PAK1 mediated phosphorylation

The published experiments that analyzed the functional consequences of PREX2 tumor mutations occurred early on in the generation of The Cancer Genome Atlas and the cBio web portal, which made publically available a wealth of whole genome sequencing data from many different tumor types. Because of this, these studies on specific PREX2

A



B

Mutation	# of Occurrences	Tumor Types
R155W/Q	5	Breast, Uterine, Leukemia, Colorectal, Stomach
R297C/H	6	Melanoma (4), Pancreas, Colorectal
R299Q	2	Prostate, Stomach
R363Q/W	7	Breast (3), Lung, Uterine, Colorectal, Sarcoma

Figure 4.1 - Landscape of PREX2 tumor mutations (figure adapted from cBio web portal) A) Histogram of PREX2 showing current landscape of tumor mutations. B) Table showing the total number of reported cases for each PREX2 tumor mutant that was selected for further study and the types of tumors in which these mutations were found.

tumor mutants were forced to use the limited data sets that were available at the time in choosing their mutations to study. Currently, over 700 mutations on PREX2 in many different types of cancer have been reported. This allowed us to identify recurrent, or "hot spot" mutations, that are more likely to have important functional alterations that are selected for by the tumor. Using the cBio portal to analyze the reported PREX2 mutations, we selected three of the most common somatic tumor missense mutations to study: R155W, R297C, and R363Q (Figure 4.1A) (Cerami et al., 2012; Gao et al., 2013). We also studied R299Q because it was another arginine that was just two amino acids away from a common missense mutation, R297C, and was also mutated in two separate tumor samples. R155W is in the catalytic DH domain, while R297C, R299Q, and R363Q are in the PH domain. These mutations are found in a variety of human cancers (Figure 4.1B).

First, the effect of insulin stimulation on the phosphorylation of these mutants was determined in HEK293 cells. All four mutants, R155W, R297C, R299Q, and R363Q had reduced phosphorylation compared to wild type PREX2. Phosphorylation of R297C and R299Q was almost completely ablated, while the reduction of phosphorylation on R155W and R363Q was more subtle (Figure 4.2A). In response to PAK1 overexpression, we observed a very similar pattern of reduced phosphorylation of these four tumor mutants (Figure 4.2B). Taken together, these data show that insulin and PAK1 dependent phosphorylation of PREX2 hotspot tumor mutations was reduced, particularly in the case of R297C and R299Q.

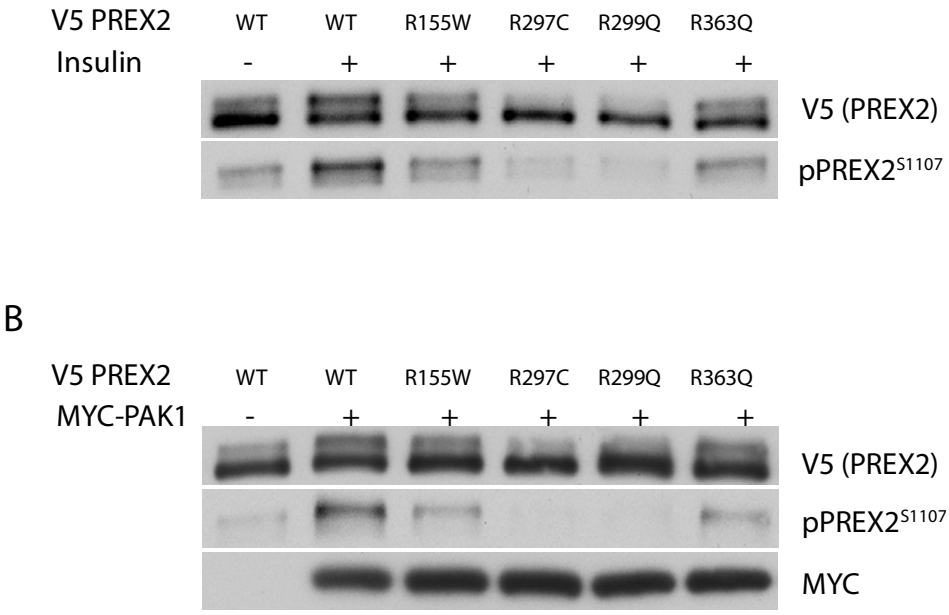


Figure 4.2 – PREX2 hotspot mutations have reduced levels of phosphorylation A) Western blot analysis of starved or insulin treated HEK293 cells expressing V5 PREX2 WT, R155W, R297C, R299Q, or R363Q. B) Western blot analysis of starved HEK293 cells expressing V5 PREX2 WT, R155W, R297C, R299Q, with or without co-expression of MYC PAK1 R363Q.

Phosphorylation of PREX2 R297C induced by activating PI3K tumor mutants is reduced

PI3K is frequently mutated in cancer, particularly in breast cancer, and two hot spot mutations, E545K in the helical domain and H1047R in the kinase domain, have been shown to activate PI3K kinase activity, increase phosphorylation of downstream kinases such as AKT, promote anchorage independent cell growth, and cause tumor formation in mice (Cerami et al., 2012; Gao et al., 2013; Ikenoue et al., 2005; Isakoff et al., 2005; Kang et al., 2005; Koren and Bentires-Alj, 2013; Zhao et al., 2005). Given that PREX2 can cooperate with the E545K activating mutant of PI3K to promote colony formation in soft agar (Fine et al., 2009), we tested whether these PREX2 tumor mutants

had decreased phosphorylation when co-expressed with these PI3K mutants. Interestingly, in U87MG cells, only the R297C mutant showed significantly reduced phosphorylation (as shown by the extent of the PREX2 electrophoretic mobility shift) when co-expressed with either the H1047R or E545K PI3K mutants (Figure 4.3). The

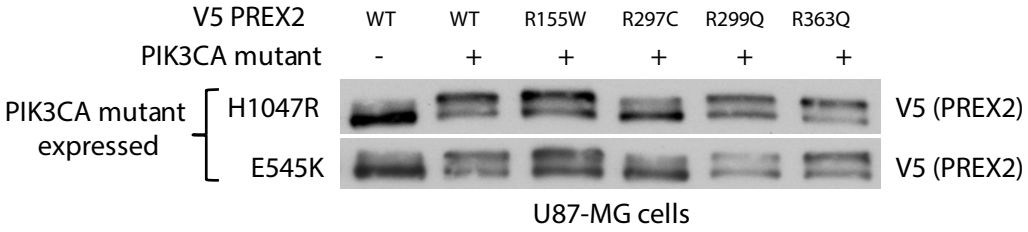


Figure 4.3 – R297C mutant has reduced phosphorylation after expression of PI3K activating mutations Western blot analysis of starved U87MG cells expressing V5 PREX2 WT, R155W, R297C, R299Q, or R363Q with or without co-expression of p110α H1047R or E545K.

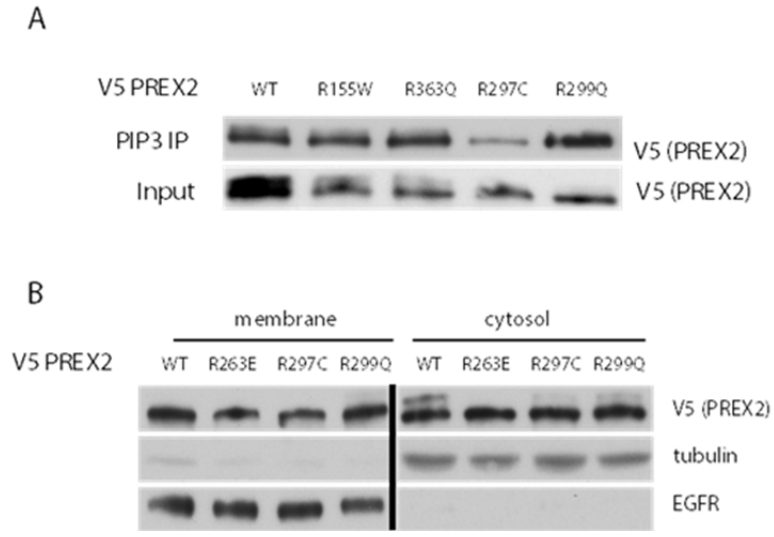


Figure 4.4 – R297C mutant does not efficiently bind PI(3,4,5)P3, but retains ability to bind cellular membrane A) Western blot analysis of PI(3,4,5)P3 pull downs from HEK293 cells expressing V5 PREX2 WT, R155W, R297C, R299Q, or R363Q. B) Western blot analysis of cytosolic and membrane fractions from HEK293 cells expressing V5 PREX2 WT, R263E, R297C, or R299Q.

R299Q mutant, which had comparable reductions in phosphorylation to R297C in response to insulin and PAK1 overexpression, had only a minor decrease in PI3K dependent phosphorylation. Further, a PI(3,4,5)P3 bead pull down showed that the R297C mutation significantly reduced PI(3,4,5)P3 binding, while PI(3,4,5)P3 binding to the other three PREX2 tumor mutants was unaffected (Figure 4.4A). This result could potentially explain why the R297C mutant is particularly susceptible to phosphorylation defects downstream of PI3K activation. When HEK293 cells expressing R297C were subjected to cell fractionation, this mutant was still present on the membrane (Figure 4.4B). Further, the mutant that cannot bind PI(3,4,5)P3, R263E, was also present on the membrane, suggesting that PREX2 can bind to the membrane through PI(3,4,5)P3 independent mechanisms. This is important because it suggests that R297C may still be at the membrane to activate RAC1, or inhibit PTEN, even though it cannot bind to PI(3,4,5)P3.

T158 on PREX2 is a potential PAK1 site that is altered in cancer

Previous studies have identified PAK consensus phosphorylation motifs for the different isoforms of the kinase, and all of these motifs contain arginine residues at positions directly upstream of the phosphorylation site (Figure 4.5) (Rennefahrt et al., 2007). Given that all of the recurrent tumor mutants we studied are on arginine residues, we hypothesized that mutating them in cancer is a mechanism to disrupt nearby PAK phosphorylation events, resulting in a more prolonged PREX2 GEF activation. Interestingly, R155W is directly upstream of T158, while R297C and R299Q are upstream of T302, supporting the possibility that these tumor mutants are reducing PAK phosphorylation by affecting PAK phosphorylation at these residues (Figure 4.6A). Mutation of T158 to alanine reduced the PAK1 mediated phosphorylation of PREX2 to a

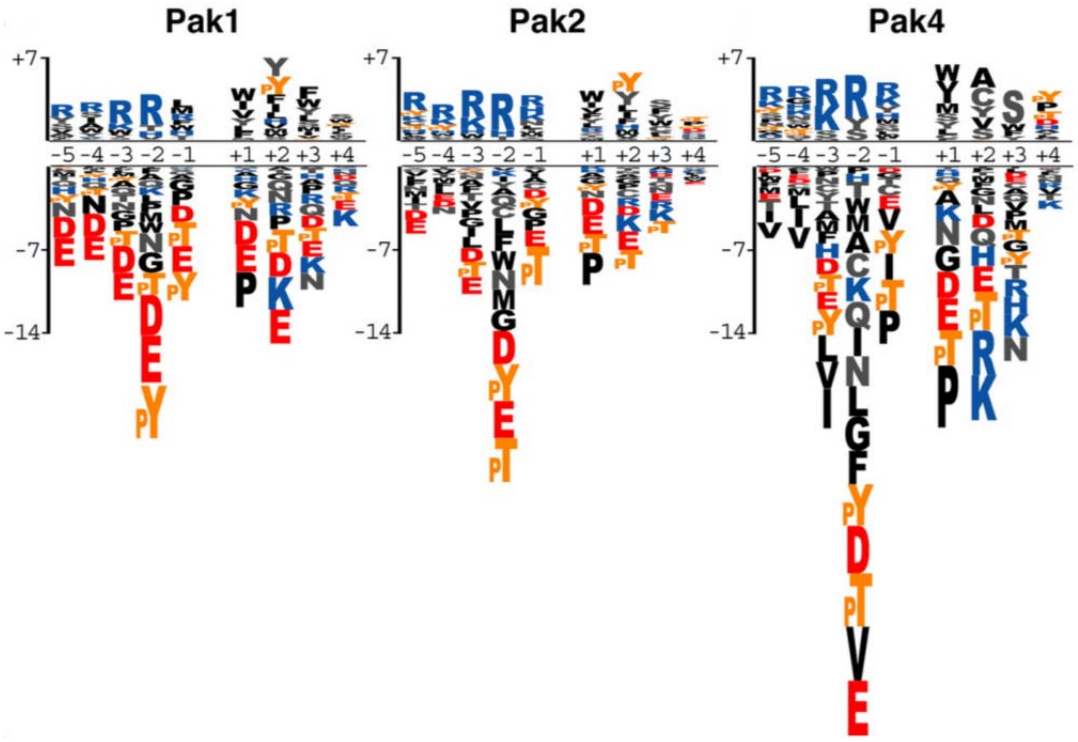


Figure 4.5 – Consensus phosphorylation motif for PAK kinases figure adapted from (Rennefahrt et al., 2007).

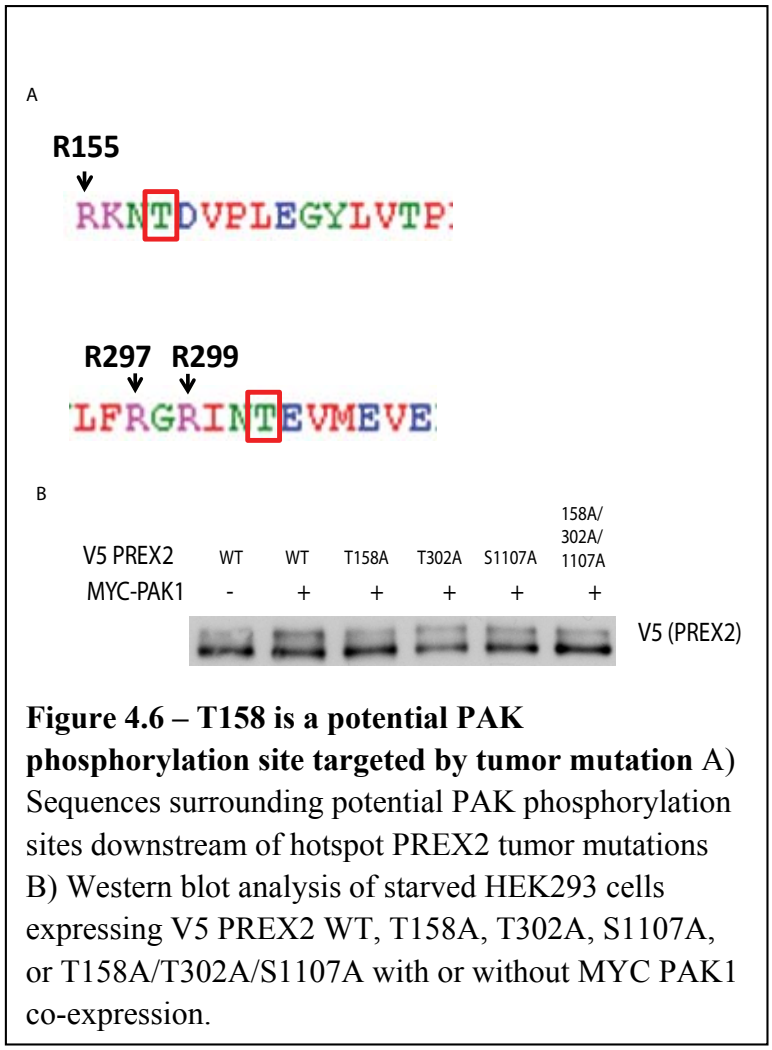


Figure 4.6 – T158 is a potential PAK phosphorylation site targeted by tumor mutation A) Sequences surrounding potential PAK phosphorylation sites downstream of hotspot PREX2 tumor mutations B) Western blot analysis of starved HEK293 cells expressing V5 PREX2 WT, T158A, T302A, S1107A, or T158A/T302A/S1107A with or without MYC PAK1 co-expression.

similar extent as the corresponding tumor mutant, R155W, while an alanine mutation at T302 had a minimal effect on phosphorylation, which is quite different from the clear reduction of R297C and R299Q phosphorylation (Figure 4.6B). From this data, it is possible that T158 could be an additional PAK1 site; however, R297C and R299Q are likely reducing phosphorylation through some alterative mechanism.

PREX2 tumor mutants have altered GEF activity towards RAC1

We then analyzed the functional consequences of these tumor mutations by determining their ability to be stimulated by either PI(3,4,5)P3 or Gβγ. PI(3,4,5)P3 was able to activate the GEF activity of the tumor mutants; however, the level of stimulation was reduced with all four mutants when compared to wild type PREX2 (Figure 4.7A). Interestingly, R297C and R299Q stimulation by Gβγ was unaffected compared to wild

type PREX2, while R155W and R363Q stimulation by Gβγ was reduced (Figure 4.7B). We then tested the ability of R297C to stimulate invasion in BT549 breast cancer cells. We found the R297C was still able to stimulate invasion towards an FBS gradient, suggesting that it is still a functional GEF in cells (Figure 4.7C). Taken together, the PI(3,4,5)P3 and Gβγ stimulated RAC1 GEF activity of these particular recurrent PREX2

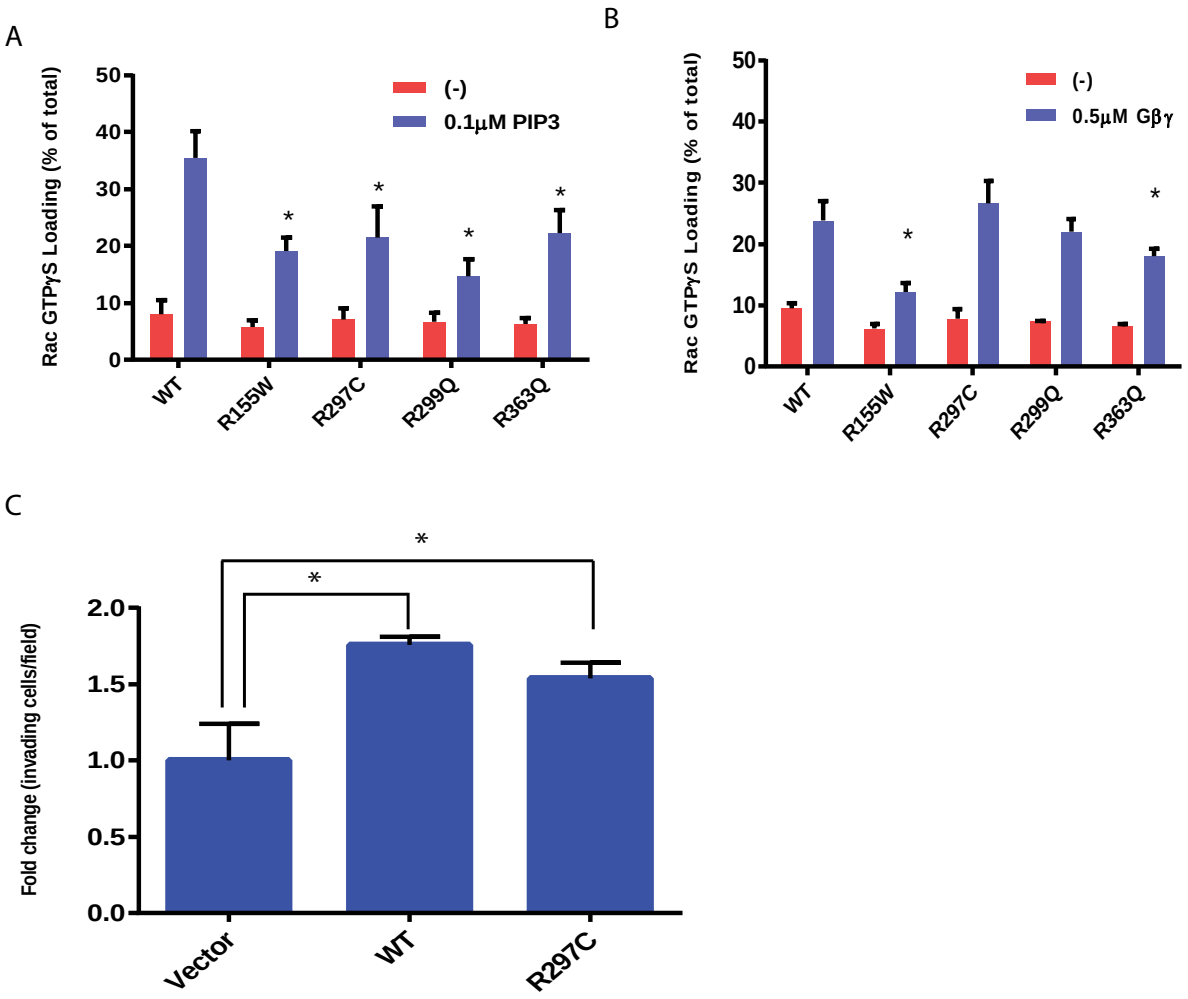


Figure 4.7 – Tumor mutants have altered PI(3,4,5)P3 and Gβγ stimulated GEF activity *in vitro* A and B) WT PREX2 and the tumor mutants were purified and the ability of 0.1μM PI(3,4,5)P3 (A) or 0.5μM Gβγ (B) to stimulate PREX2 GEF activity towards RAC1 was measured. Data are mean ± SD for two experiments with samples done in duplicate at each PI(3,4,5)P3 concentration and * represents $p < 0.001$ by t-test. C) V5 PREX2 WT or S1107E were expressed in BT549 cells and invasion assays were performed. Representative shown of two experiments, data are mean ± SD, and * represents $p < 0.05$ by t-test.

tumor mutants is altered, and surprisingly was often lower than that of the wild type protein.

DISCUSSION

The data from this chapter show that phosphorylation of PREX2 is altered in the four recurrent tumor mutants that we analyzed: R155W, R297C, R299Q, and R363Q. Phosphorylation was reduced for all mutants in response to both insulin stimulation and PAK1 overexpression. Two of the mutants that are located in the PH domain, R297C and R299Q, had almost no detectable phosphorylation in both of these contexts, while phosphorylation of R155W or R363Q was only slightly reduced. These results are exciting when put in the context of our data presented in Chapters 2 and 3 where we identified PAK phosphorylation as a negative regulatory event to reduce PREX2 GEF activity towards RAC1. The reduced phosphorylation of these tumor mutants presents the possibility that cancer cells are escaping PAK mediated negative regulation of PREX2 through mutations. This would be advantageous for a tumor cell, potentially prolonging RAC1 activation following insulin or GPCR stimulation, leading to a more invasive cell.

We also found that R297C had reduced phosphorylation when co-expressed with the constitutively activate PI3K mutations, E545K and H1047R. Given the prevalence and potency of these PI3K mutations in cancer, a PREX2 mutant that can avoid inactivation by PAK following PI3K-mediated PI(3,4,5)P3 generation could be especially advantageous for a tumor cell. Further, since PREX2 cooperates with activating PI3K mutations to form colonies in soft agar, it would not be surprising for a tumor cell to evolve a mechanism where it enhanced this cooperation through mutations in PREX2 that help avoid negative regulatory mechanisms (Fine et al., 2009). This hypothesis can be

tested by repeating these colony formation experiments to compare R297C to wild type PREX2, and then observing whether R297C increases the number of colonies formed when co-expressed with PI3K mutants. We also found that R297C showed significantly reduced PI(3,4,5)P3 binding compared to wild type PREX2, which could explain why this mutant was particularly insensitive to PI3K dependent phosphorylation. Importantly, R297C was still present on the membrane, suggesting that it does not require strong PI(3,4,5)P3 binding to translocate to the membrane. It is possible that R297C binding to Gβγ on the membrane is sufficient for membrane localization. It may also be the case that PREX2 has interfaces with the membrane that do not rely on binding to signal dependent second messengers.

In our analysis of the effect of these tumor mutations on PREX2 GEF activity, we were surprised to find that the mutants generally had reduced GEF activity. While PI(3,4,5)P3 stimulated the GEF activity of all four mutants, the level of PI(3,4,5)P3 stimulation was reduced compared to wild type PREX2. Interestingly, while R155W and R363Q both had reduced Gβγ stimulated GEF activity compared to wild type PREX2, activation of both R297C and R299Q by Gβγ was unaffected. It is unclear why these tumor mutants generally have lower PI(3,4,5)P3 and/or Gβγ dependent activation. It is possible that these mutants, particularly R297C and R299Q, are able to avoid PAK mediated negative regulation to create a more prolonged RAC1 activation, but in doing so they sacrifice a small amount of GEF activity. In this model, having a longer lasting RAC1 activation would be more beneficial to the tumor cell than a higher spike of RAC1 activity immediately following insulin stimulation that is subsequently turned off by PAK. Further work needs to be done to confirm whether the ability of these tumor

mutants to avoid PAK phosphorylation is important to create a more oncogenic form of PREX2. It is likely that these mutants have diverse mechanisms of promoting tumorigenesis, and given the role of PREX2 as an inhibitor of PTEN, some of these tumor mutants may be affecting tumorigenesis by altering PTEN function in tumors, a possibility discussed in detail in Chapter 6.

CHAPTER 5: Characterization of PREX2 binding to PTEN and other insulin related proteins

BACKGROUND AND SIGNIFICANCE

PREX2 is a PTEN interacting protein, and this interaction results in the inhibition of PTEN phosphatase activity (Fine et al., 2009). PREX2 inhibition of PTEN appears to be particularly important in insulin signaling (Hodakoski et al., 2014). Further characterization of PTEN inhibition by PREX2 revealed a complex mechanism. High affinity binding between PREX2 and PTEN is mediated by interactions between the IP4P domain of PREX2 and the PDZ binding domain on the C-terminus of PTEN; however, this interaction does not mediate PTEN inhibition. There is also low affinity binding that occurs between the PH domain of PREX2 and the phosphatase and C2 domains of PTEN, and it is this interaction that is required for inhibition of PTEN. Additionally, inhibition of PTEN by the PREX2 DHPH domain, but not the PH domain alone, requires the PTEN C-terminal tail to be phosphorylated. Taken together, the current model for PTEN inhibition is that the high affinity interaction mediated by the PREX2 IP4P domain initiates binding, which allows the PREX2 DHPH domain to sense the phosphorylation state of PTEN. Phosphorylated PTEN then causes conformational changes in the PREX2 DHPH domain, allowing the PH domain to inhibit PTEN (Figure 1.2) (Hodakoski et al., 2014). This is the first time that a PH domain has been shown to regulate PTEN, and in this chapter we sought to further characterize the binding region within the PREX2 PH domain. Further, we hypothesized that PTEN and RAC1 are not the only insulin related proteins that interact with PREX2. This hypothesis is supported by a previous study showing that PREX2 can interact with mTOR (Hernandez-Negrete et al., 2007). Herein, we identified additional interactors that may be important for the role of PREX2 within the insulin signaling pathway.

RESULTS

The $\beta_3\beta_4$ loop region of the PREX2 PH domain is responsible for PTEN binding

The structure of multiple PH domains has been solved, and these studies have revealed that PH domains share a high level of structural homology despite having low levels of homology among their sequences. PH domains consist of seven β strands that form two beta sheets that closely pack on top of one another, forming a sandwich like structure. The C-terminal portion of the PH domain contains an α-helix that closes one end of the sandwich. The variable loops that connect the $\beta_1\beta_2$, $\beta_3\beta_4$, and $\beta_6\beta_7$ strands protrude from the end of the PH domain opposite the helix, and the sequences and length of these loops can differ greatly between PH domains (Rebecchi and Scarlata, 1998). These loops are also often the site of lipid or ligand binding. In Chapter 2 we characterized PREX2 binding to PI(3,4,5)P3 and found that residues surrounding the $\beta_1\beta_2$ loop were critical for this interaction. Further, the $\beta_3\beta_4$ loop of the PREX2 PH domain was identified as the region determines PREX2 specificity for RAC1 (Joseph and Norris, 2005).

These conserved structures make it possible to predict secondary structures of PH domains when a crystal structure is not available. As shown in Figure 2.10, we made a model of the PREX2 PH domain, and the potential residues that comprise the $\beta_1\beta_2$ loop and $\beta_3\beta_4$ loop were identified (Figure 5.1A). To determine if these variable loops were important for PREX2 binding to PTEN, two fragments of the PH domain were generated. The first fragment was comprised of residues 245-300, which includes the $\beta_1\beta_2$ and $\beta_3\beta_4$

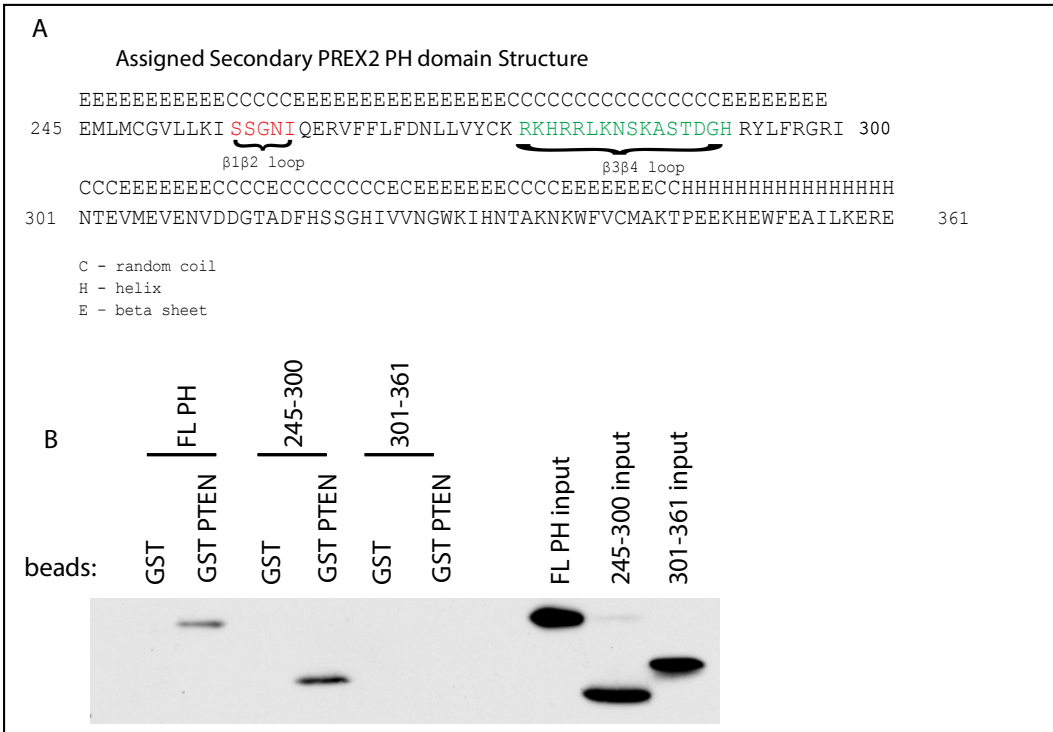


Figure 5.1 – The N-terminal half of the PREX2 PH domain confers binding to PTEN A) Sequence of PREX2 PH domain with potential secondary structures indicated from model. B) Western blot analysis of GST PTEN pull downs of *in vitro* translated PREX2 PH domain fragments.

loops, while the second fragment was comprised of residues 301-361. Using a rabbit reticulocyte lysate *in vitro* translation system, these PH domain fragments were made and their ability to bind to GST PTEN was assessed (Figure 5.1B). The 245-300 fragment bound to GST PTEN at a comparable level to that of the full length PH domain, while the 301-361 fragment did not bind at all. This suggests that the $\beta_1\beta_2$ and $\beta_3\beta_4$ loops are

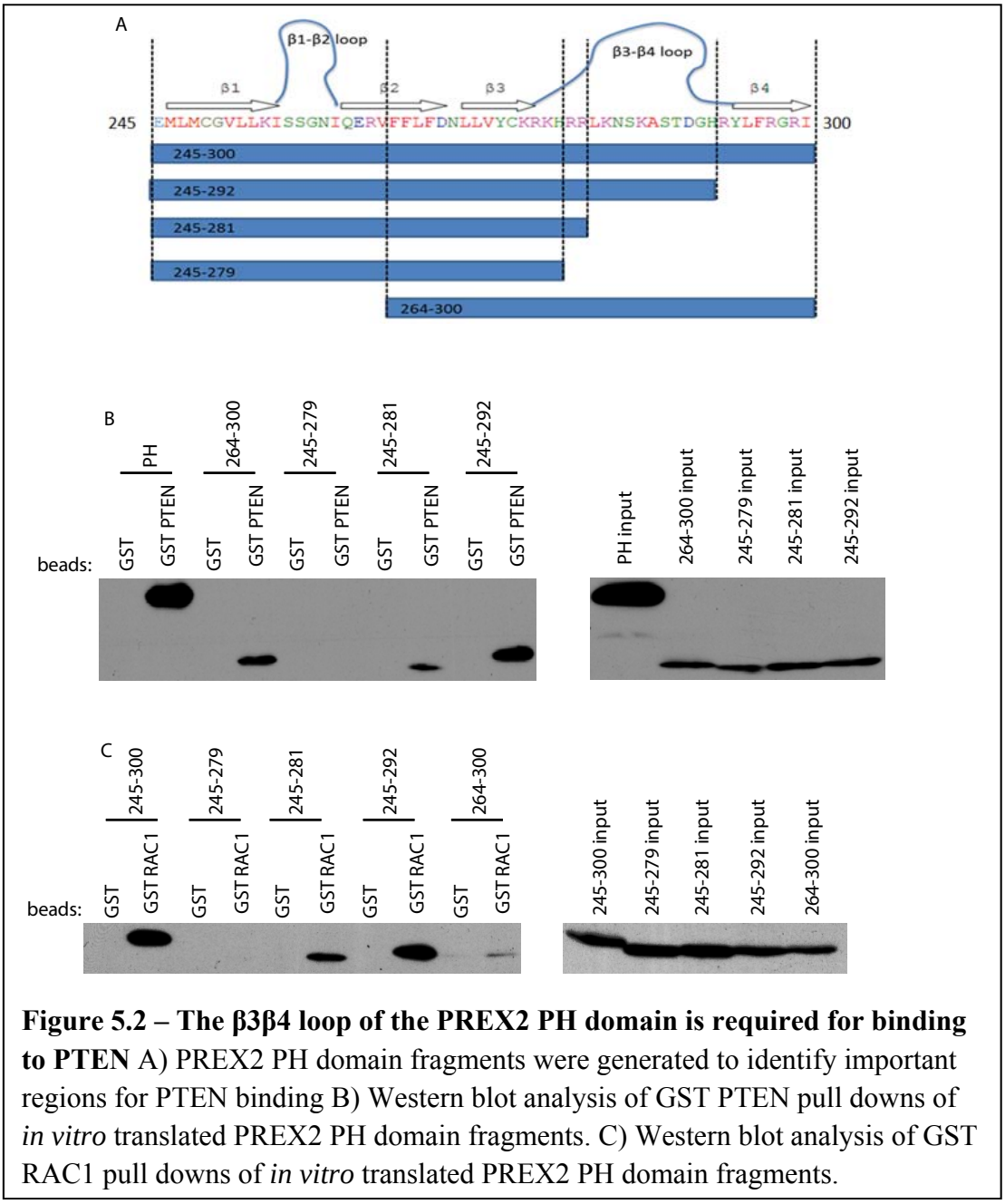


Figure 5.2 – The β3β4 loop of the PREX2 PH domain is required for binding to PTEN A) PREX2 PH domain fragments were generated to identify important regions for PTEN binding B) Western blot analysis of GST PTEN pull downs of *in vitro* translated PREX2 PH domain fragments. C) Western blot analysis of GST RAC1 pull downs of *in vitro* translated PREX2 PH domain fragments.

involved in PTEN binding. The 245-300 fragment was then broken down to separate the two putative variable loop structures that it contained (Figure 5.2A). The 264-300 fragment that eliminated the putative $\beta_1\beta_2$ loop retained PTEN binding ability, while the 245-279 fragment that removed the $\beta_3\beta_4$ loop did not bind (Figure 5.2B). Further, the 245-281 fragment, which added just two arginine residues to the non-binding fragment, recovered a small amount of PTEN binding, while the 245-292 fragment, which adds back most of the $\beta_3\beta_4$ loop, bound even better (Figure 5.2B). This suggests that the $\beta_3\beta_4$ loop is the region that is important for PTEN binding. As previously mentioned, the $\beta_3\beta_4$ loop was also reported to be the region of the PH domain that is required for PREX2 targeting of RAC1 (Joseph and Norris, 2005). We tested binding of these same PREX2 fragments in Figure 5.2A to GST RAC1, and observed a very similar binding pattern as we did for GST PTEN (Figure 5.2C). This is consistent with the other study by Joseph et al. and suggests that both PTEN and RAC1 are binding to the $\beta_3\beta_4$ loop of the PREX2 PH

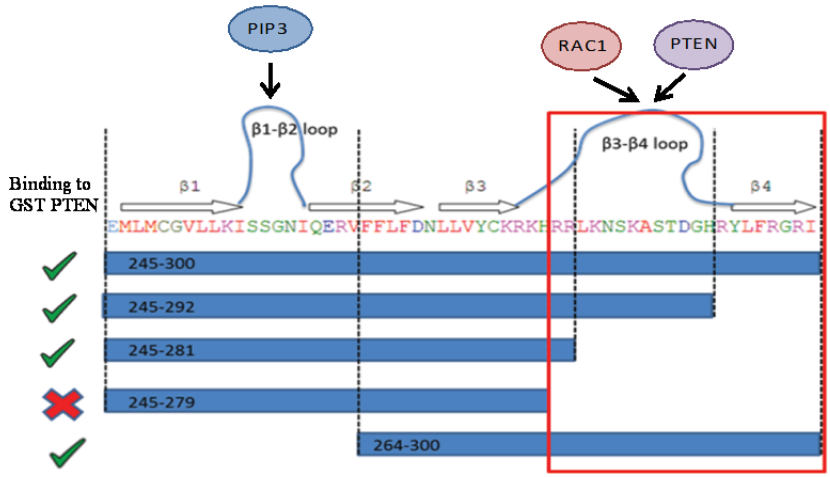


Figure 5.3 – Summary of PTEN, RAC1, and PI(3,4,5)P3 binding to the variable loops of PREX2 PH domain The PREX2 PH domain fragments tested are shown and their ability to bind to GST PTEN is indicated at left by either a green check mark to indicate it can bind to PTEN, or a red cross to indicate that it cannot bind to PTEN. The red box surrounds the minimal binding region identified by these fragments. Regions that interact with PI(3,4,5)P3 and RAC1 are also indicated.

domain. The binding pattern of these PREX2 PH domain fragments to GST PTEN are summarized in Figure 5.3 and the putative regions of PTEN, RAC1, and PI(3,4,5)P3 binding are indicated.

PREX2 co-immunoprecipitates with critical proteins in the insulin signaling pathway

In addition to characterizing the binding of PREX2 to PTEN, RAC1, and PI(3,4,5)P3, we investigated the binding of PREX2 to other proteins involved in the insulin signaling pathway. Interestingly, we consistently noticed that a 150kDa protein that was tyrosine phosphorylated after insulin treatment co-imunoprecipitated with V5

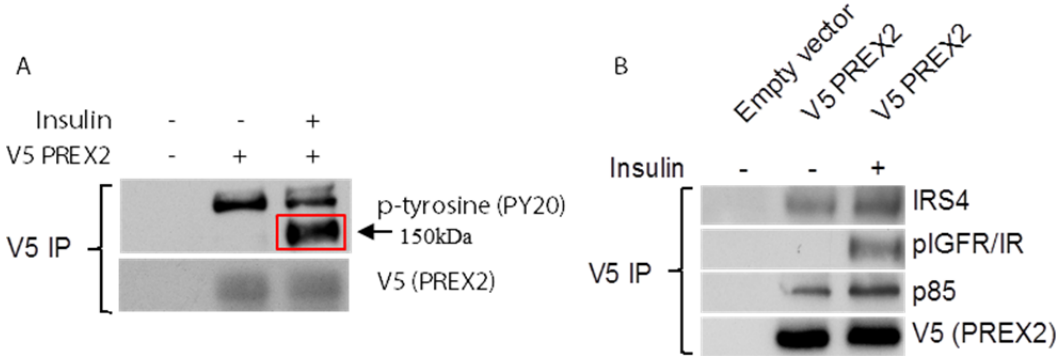


Figure 5.4 – PREX2 binds critical proteins in insulin signaling pathway A and B) Western blot analysis of V5 PREX2 immunoprecipitations.

PREX2 (Figure 5.4A). This is close in size to the IRS family of proteins, so the possibility of a PREX2-IRS interaction was investigated. In HEK293 cells, IRS4 is the primary isoform, so we probed for this protein in PREX2 immunoprecipitations (Fantin et al., 1998; Lavan et al., 1997). Using an antibody specific to IRS4, we found that IRS4 co-immunoprecipitated with V5 PREX2. In addition, the regulatory subunit of PI3K, p85, and the phosphorylated version of insulin receptor, was also present in V5 PREX2 immunoprecipitation (Figure 5.4B). The p85 and IRS4 interactions with PREX2 were not

affected by insulin stimulation. Further, p85 bound to the PREX2 DHPH domain but was unable to bind to a fragment of PREX2 containing everything except the DHPH domain, called ΔDHPH (Figure 5.5). Phosphorylated insulin receptor bound both DHPH and ΔDHPH, however it showed a significant preference for DHPH. The fact that these interactions with critical insulin related proteins occurs through the DHPH domain of PREX2 is an interesting result given that PTEN, RAC1, and PI(3,4,5)P3 all have binding sites in this region as well.

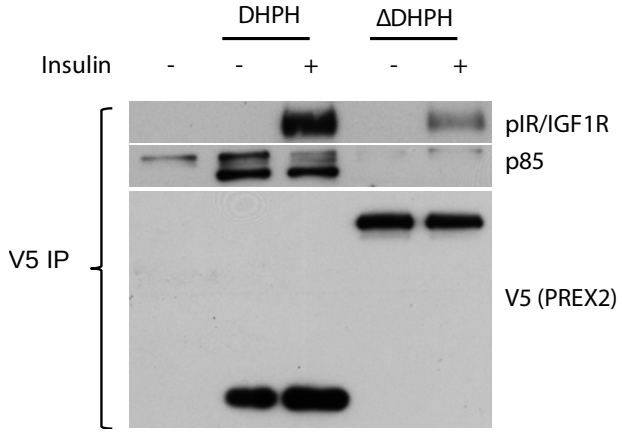


Figure 5.5 – Interactions with insulin receptor and p85 occur primarily through DHPH domain of PREX2 Western blot analysis of V5 immunoprecipitations from HEK293 cells expressing either V5 PREX2 DHPH or ΔDHPH.

DISCUSSION

As discussed in the introduction, PREX2 has a significant role in insulin signaling, both in cancer cell lines and in mice. Here, we analyzed PREX2 interactions with other proteins in the insulin-signaling pathway. We first focused on PREX2 binding to the PI(3,4,5)P3 lipid phosphatase PTEN, particularly the binding mediated by the PREX2 PH domain. While it is not the primary region responsible for PTEN binding, the PH domain is responsible for PTEN inhibition (Hodakoski et al., 2014). By analyzing a series of truncation mutants of the PREX2 PH domain, we discovered that the $\beta_3\beta_4$ loop

was required for PTEN binding. Given that the PH domain alone can inhibit PTEN both *in vitro* and in cells, it will be important to establish whether the $\beta_3\beta_4$ loop is also the minimal region required for inhibition. These small fragments of the PH domain are difficult to express in cells, so this type of analysis may require deletion mutants in the full length PH domain or in the full length protein. Additionally, it may be useful to perform mutational analysis on residues within the $\beta_3\beta_4$ loop to identify the specific residues that are required for PTEN binding or inhibition. This type of analysis could yield a full length PREX2 mutant that is unable to inhibit PTEN, but retains other PREX2 functions. Ideally this mutant would still be able to act as a RAC GEF, which would allow us to separate the roles of PTEN inhibition from RAC activation in different PREX2 outputs, such as glucose uptake. However, this may be difficult given that this region of the PH domain is also important for binding to RAC, and mutations that block PTEN inhibition may also affect GEF activity. If it is possible to make this type of mutant that is specifically deficient in PTEN inhibition, CRISPR technology could then be used to knock this mutant into cell lines and study the importance of PTEN inhibition. Further, this mutant could be knocked into a mouse to study the importance of PTEN inhibition *in vivo*.

Further characterization of the specific residues responsible for PREX2 PH domain mediated inhibition could also help in designing small molecules that modulate PTEN function. By knowing the specific interactions that are required for inhibition, it may be easier to rationally select types of molecules that could prevent PREX2 inhibition of PTEN and activate PTEN. Given the role of PTEN as a tumor suppressor, activating

PTEN phosphatase activity with a drug would have great potential as a cancer therapeutic.

PREX2 PH domain mediated inhibition of PTEN is also exciting given the fact that many proteins in the human proteome contain PH domains, and it presents the possibility that PH domain binding and inhibition may be a more universal mechanism of PTEN regulation. Given that PI(3,4,5)P3 is the primary substrate for PTEN, and that PI(3,4,5)P3 binds to PH domains, PTEN will often be in close proximity to PH domain containing proteins within the cell, making this a feasible possibility. If PTEN regulation by PH domains is common to PH domains other than PREX2, this would have an enormous impact on cancer biology given the role of PTEN as a tumor suppressor, and would more generally impact cellular biology given the wide range of important cellular functions that PTEN can influence. Our model of the PREX2 PH domain shows that the $\beta_3\beta_4$ loop is very long and flexible compared to $\beta_3\beta_4$ loops of other PH domains. In order to find other candidate PH domains that could also bind and inhibit PTEN, it may be important to identify those that have long and flexible loops like PREX2. Interestingly, we also generated a model of the PREX1 PH domain, which suggested that PREX1 also has a long and flexible $\beta_3\beta_4$ loop; however, full length PREX1 does not bind to PTEN (Hodakoski et al., 2014). This suggests that PH domain mediated inhibition of PTEN could be specific for PREX2. This may be especially more likely given the complexity of PREX2 mediated inhibition of PTEN. Even though the PH domain is required to inhibit PTEN, the PREX2-PTEN interaction is mediated primarily through the IP4P domain of PREX2. Given this model, it may be important to identify the precise region within the

IP4P domain of PREX2 that binds PTEN, and then look for candidates that have homology both to this region, and to the $\beta_3\beta_4$ loop region of the PREX2 PH domain.

In this chapter, we also established that PREX2 interacts with other insulin related proteins in addition to PTEN and RAC1. It has already been reported that PREX2 can bind to the phosphorylated insulin receptor, but not the EGF receptor (Hodakoski et al., 2014). Here, we show that p85 and IRS4 also co-immunoprecipitate with V5 PREX2. The functional consequences of these interactions are currently unclear; however it is possible that these interactions could play a role in the observed effect of PREX2 loss on insulin signaling in the mouse. PREX2 could serve as a scaffold near the insulin receptor, helping to stabilize complexes involving the receptor and these important signaling molecules that interact with PREX2, such as IRS proteins, p85, PTEN, and RAC1. From our data, it is not possible to determine whether these interactions are direct or whether PREX2 is indirectly interacting with these proteins as part of large signaling complex. Further experiments testing *in vitro* binding of purified proteins could be useful in determining whether these proteins are direct binding partners of PREX2. Interestingly, a complex including both RAC1 and p85 has been previously identified. This complex involves the GEF SOS1, and appears important for RAC1 function in MEFs (Innocenti et al., 2003). This suggests that the PREX2 interactions we have identified here may also occur through RAC1, and could be important for insulin related RAC1 outputs.

CHAPTER 6: Summary and Future Directions

The studies outlined in this thesis have characterized novel PREX2 phosphorylation events and protein interactions. PREX2 is a protein with established roles in important signaling pathways, such is insulin signaling, and its functions as a RAC1 GEF and PTEN inhibitor give PREX2 the potential to affect many cellular processes. Further, PREX2 is commonly altered in cancer and PREX2 mutants have been shown to promote tumorigenesis. Thus, it is critical to understand how PREX2 is regulated to not only gain a greater understanding of intracellular signaling but to potentially modulate PREX2, RAC1, or PTEN function in disease states where they are altered.

In Chapter 2, we showed that PREX2 was phosphorylated during insulin stimulation. We also identified S1107 as an insulin-stimulated phosphorylation site on PREX2. We found that phosphorylation of PREX2 depended on PI3K and required PREX2 binding to PI(3,4,5)P3, but was not regulated by the well characterized PI(3,4,5)P3 dependent kinases AKT, PDK1, and mTORC1. Through these analyses, we also demonstrated that the $\beta_1\beta_2$ loop region of the PREX2 PH domain was essential for binding to PI(3,4,5)P3. Importantly, PREX2 was also phosphorylated after expression of Gβγ, suggesting that phosphorylation was associated with PREX2 activation. Further, the protein phosphatase PP1α dephosphorylated both the insulin and Gβγ dependent phosphorylation events on PREX2, while PP2A reduced insulin-induced phosphorylation of PREX2.

In Chapter 3, we pursued the consequences of phosphorylation for PREX2 function, and used this information to identify a PREX2 kinase. We found that phosphorylation reduced PI(3,4,5)P3 and Gβγ stimulation of PREX2 GEF activity

towards RAC1, and that this is likely due to phosphorylated PREX2 having a much lower affinity for the membrane, PI(3,4,5)P3, and Gβγ. This was an interesting finding given the data in Chapter 2 showing that PREX2 phosphorylation depended on PI(3,4,5)P3 and Gβγ, the two second messengers known to activate its GEF activity. Taken together, these two findings suggested that phosphorylation was part of a negative feedback circuit to reduce PREX2 GEF activity after it had been stimulated by a signal. Consistent with negative feedback, RAC1 also induced PREX2 phosphorylation. This led to the discovery that PREX2 was phosphorylated and negatively regulated by the RAC1 dependent PAK family of kinases. Phosphorylation at S1107 was also regulated by PAK, suggesting that it may be a PAK phosphorylation site. Altogether, PAK phosphorylation of PREX2 allows for tight regulation of the length and amplitude of RAC1 activation, resulting in a transient RAC1 signal following ligand binding.

Our data showing that PREX2 function is tightly regulated by insulin-induced phosphorylation further supports a critical role for PREX2 in the insulin signaling pathway. Future experiments should focus on the effect of this feedback circuit on downstream insulin signaling. Given the major deficiencies in glucose uptake in PREX2$^{-/-}$ mice, and the importance of RAC1 in glucose uptake and GLUT4 trafficking, glucose uptake is a particular insulin dependent physiological process where this feedback loop should be studied. Glucose uptake occurs primarily in the muscle tissue, and also occurs to a lesser extent in the adipose tissue (Leto and Saltiel, 2012). Given this fact, it is surprising that the two published reports characterizing PREX2$^{-/-}$ mice show very low levels of PREX2 in the muscle (Donald et al., 2008; Hodakoski et al., 2014). Interestingly, mRNA levels of PREX2 are highest in the skeletal muscle, and PREX2 was

originally cloned from muscle tissue (Donald et al., 2004; Rosenfeldt et al., 2004). It is possible that there is an issue with detection of PREX2 in the muscle, potentially because of difficulties extracting it from this tissue or because of post-translational modifications that prevent detection. If PREX2 levels are actually very low in the muscle, then a small amount of protein may be enough to elicit an effect, or the knockout of PREX2 from adipose tissue is sufficient for the phenotypes that are seen. The presence of PREX2 in the muscle should be investigated in more detail before further studies are done to probe the role of PREX2 in the skeletal muscle, and the role of PAK1 mediated negative feedback. Once this is established, model systems of muscle and adipocytes that are set up to robustly measure glucose uptake and GLUT4 membrane translocation can be studied. For example, differentiated L6 rat myotubes or differentiated mouse 3T3-L1 adipocytes that stably express a tagged version of GLUT4 are often used as models to study glucose metabolism in culture. The effect of PREX2 on these processes can be studied through overexpression or knockdown of PREX2 in these systems. Further, by using the S1107E mutant, we can potentially determine whether PREX2 phosphorylation is important for these processes. Additionally, CRISPR technology could be used to knock-in phospho-mimicking or non-phosphorylatable mutants in these cell lines to study the effect of phosphorylation in the more physiologically relevant endogenous setting.

While we were able to show that these PAK dependent phosphorylation events were induced by Gβγ overexpression, future studies should try to identify specific GPCR mediated signals that activate this signaling pathway. Sphingosine-1-phosphate and fMLP are two potential candidates given that PREX2b and PREX1, respectively, have reported roles downstream of these GPCR ligands. Fatty acids also bind and activate

GPCRs. Two of these fatty acid-activated GPCRs, GPR41 and GPR120, increase glucose uptake in mouse adipose and muscle cells. This is particularly interesting given the role of PREX2 in glucose uptake. The short chain fatty acids propionate and valerate both increased glucose uptake through GPR41, while the long chain fatty acid docosahexaenoic acid and the agonist compound A (cpdA) increased glucose uptake through GPR120 (Han et al., 2014; Oh da et al., 2014; Oh et al., 2010). It is possible that these molecules, or any other fatty acids, activate PREX2 GEF activity, and therefore increase PAK phosphorylation of PREX2. In this case, chronically high levels of fatty acids could lead to hyperactivation of PAK1 mediated negative regulation of PREX2. This would presumably lead to inefficient insulin signaling and promote insulin resistance.

In order to most effectively study the effect of PAK phosphorylation *in vivo*, including the effect on glucose metabolism, it will likely be important to identify any additional PAK phosphorylation sites on PREX2 that may exist. Our data suggests that S1107 is not the only site regulated by insulin or PAK, and the identification of more PAK dependent phosphorylation sites may allow for the generation of a non-phosphorylatable mutant or a more physiologically potent phospho-mimicking mutant that has significant signaling and functional phenotypes. Our mass spectrometry revealed that in addition to S1107, the region around this residue was heavily phosphorylated, providing more candidate residues for future study.

Furthermore, now that we have a greater understanding of PREX2 phosphorylation, it will likely be easier to identify more relevant phosphorylation sites using mass spectrometry. In our mass spectrometry analysis, we compared PREX2 that

was purified from HEK293 cells that were either starved and treated with a PI3K inhibitor or treated with insulin. We also included a condition where insulin was combined with calyculin A treatment, which produces a very highly phosphorylated species of PREX2. We did indeed find many more residues that were phosphorylated after calyculin A treatment; however, calyculin A may increase phosphorylation at sites that are not specific to PAK kinases. Gβγ, RAC1, and PAK1 overexpression all strongly induce phosphorylation and would potentially be much more useful contexts to discover PAK phosphorylation sites. In addition, co-overexpression of the dominant negative forms of RAC1 and PAK1 result in completely dephosphorylated PREX2. Thus, mass spectrometry analysis comparing PREX2 that is dephosphorylated by co-overexpression of these dominant negative mutants to PREX2 that is phosphorylated by RAC1 or PAK1 co-overexpression would be more likely to reveal new sites that are phosphorylated by PAK kinases.

It will also be important to determine whether there is an effect of PAK phosphorylation on PREX2 inhibition of PTEN. We know from our data in Figure 3.3B that PAK phosphorylation of PREX2 has no effect on binding to GST PTEN. However, binding to PTEN does not necessarily correlate with inhibition, especially given the current model of inhibition where the high affinity binding between PREX2 and PTEN through the C-terminus is not the same interaction that regulates inhibition (Hodakoski et al., 2014). Phosphorylation of PREX2 may not affect the C-terminal binding, but could result in conformational changes that prevent or enhance the ability of the PH domain to access and inhibit the phosphatase domain of PTEN. *In vitro* inhibition assays with phosphorylated or dephosphorylated purified PREX2 and purified PTEN will be critical

to answer this question. However, given the data from the PREX2$^{-/-}$ mice and our data from this study, we would hypothesize that it is unlikely that PAK phosphorylation of PREX2 is affecting PTEN inhibition. In adipose tissue of PREX2$^{-/-}$ mice, there is reduced AKT phosphorylation compared to wild type mice as soon as two minutes after insulin stimulation, and in PREX2$^{-/-}$ MEFs, the reduction in AKT phosphorylation persists until at least the 60 minute time point (Hodakoski et al., 2014). This suggests that PREX2 is inhibiting PTEN at both early and later time points after insulin stimulation. Given that PAK phosphorylation of PREX2 changes significantly during this time course, and PTEN inhibition does not appear to be changing, PAK phosphorylation of PREX2 is likely not affecting PTEN inhibition. However, even if PAK has no effect on PTEN inhibition, it is certainly still possible that another kinase is mediating additional phosphorylation events on PREX2 to regulate inhibition of PTEN. This possibility will be discussed later in this chapter in the context of results obtained in Chapter 5.

In Chapter 4, we began to investigate whether PREX2 phosphorylation is altered in cancer. We identified four recurrent PREX2 tumor mutations: R155W, R297C, R299Q, and R363Q. We found that all of these mutants had lower levels of insulin and PAK1-induced phosphorylation compared to wild type PREX2. The phosphorylation of R297C and R299Q was almost completely eliminated. We also characterized the GEF activity of these mutants and showed that all four had reduced PI(3,4,5)P3 stimulated GEF activity, while only R155W and R363Q had reduced Gβγ stimulated GEF activity. In addition, the PREX2 R297C mutant had reduced phosphorylation when co-expressed with PI3K activating tumor mutations. This effect might be explained by the ineffective binding of R297C to PI(3,4,5)P3. However, despite having reduced PI(3,4,5)P3 binding,

R297C could still localize to the membrane and stimulate invasion of BT549 breast cancer cells.

Lastly, in Chapter 5 we looked at PREX2 interactions with other proteins within the insulin signaling pathway, with a focus on characterizing the interaction between the PREX2 PH domain and PTEN. Using fragments of the PREX2 PH domain, we showed that the $\beta_3\beta_4$ loop of the PREX2 PH domain was required for binding of the PH domain to PTEN. We also found that PREX2 could bind to p85, the insulin receptor, and IRS4, primarily through the DHPH domain; however, it is unclear whether these interactions with PREX2 are direct or what effect they have on insulin signaling or PREX2 function. It is possible that there is a complex at the membrane that includes all of these proteins and is necessary to propagate the insulin signal. This is a hypothesis that warrants further study.

The discovery that PTEN binds to the $\beta_3\beta_4$ loop of the PREX2 PH domain is interesting given the number of signaling molecules that appear to bind to these variable loops. Our data in Chapter 2 established that the nearby $\beta_1\beta_2$ loop region mediated PREX2 binding to PI(3,4,5)P3. Also, as previously mentioned and confirmed by our data, the $\beta_3\beta_4$ loop can bind to RAC1 (Joseph & Norris, 2005). Furthermore, it has also been reported that the PH domain is necessary for binding of PREX2b to $G\beta\gamma$ (Li et al., 2005b). This sets up a model where these variable loops within the PREX2 PH domain are potentially coordinating interactions with at least four molecules that are critical for its function within the cell: PTEN, RAC1, $G\beta\gamma$, and PI(3,4,5)P3. Future studies should focus on how these interactions are coordinated. For example, can PREX2 and RAC1 bind to the $\beta_3\beta_4$ loop at the same time? If not, it will be very interesting to explore the

possibility that competitive binding for the same location could be responsible controlling whether PREX2 is acting as a GEF for RAC1, or an inhibitor of PTEN. It could also be true that PTEN and RAC1 are able to promote binding of the other to the PREX2 PH domain, meaning that PREX2 would be a better inhibitor of PTEN in the same context where it is an active GEF. The effect of PI(3,4,5)P3 binding to the nearby $\beta_1\beta_2$ loop or Gβγ binding to the PH domain could also be influencing the binding of PTEN or RAC1 to the $\beta_3\beta_4$ loop, which is another hypothesis that should be addressed in the future.

The findings in Chapter 5 are also potentially important when combined with the mass spectrometry analysis in Chapter 2. Two phosphorylation events in the PTEN-binding $\beta_3\beta_4$ loop of the PREX2 PH domain were identified, S288 and T289. If these are legitimate phosphorylation events on PREX2, given their location in this critical region of the PH domain, it will be very interesting to see if they regulate PREX2 mediated inhibition of PTEN. The homologous site to S288 on PREX1, S319, was reported to have higher levels of phosphorylation in starved cells, and reduced levels of phosphorylation after neuregulin treatment (Montero et al., 2011). In our study, S288 of PREX2 was only phosphorylated in the starved and GDC0941 treated sample, which is consistent with the PREX1 data (Figure 2.4B). Interestingly, T289 had the opposite pattern and was only phosphorylated in the insulin and calyculin A treated sample of our mass spectrometry analysis. One potential hypothesis is that in starved cells, S288 is phosphorylated and T289 is dephosphorylated, and this specific combination prevents PTEN binding to the PH domain of PREX2, activating PTEN phosphatase activity to keep PI(3,4,5)P3 levels low (Figure 6.1). In this model, immediately upon growth factor stimulation a kinase (likely not PAK) phosphorylates PREX2 at T289, and a phosphatase dephosphorylates

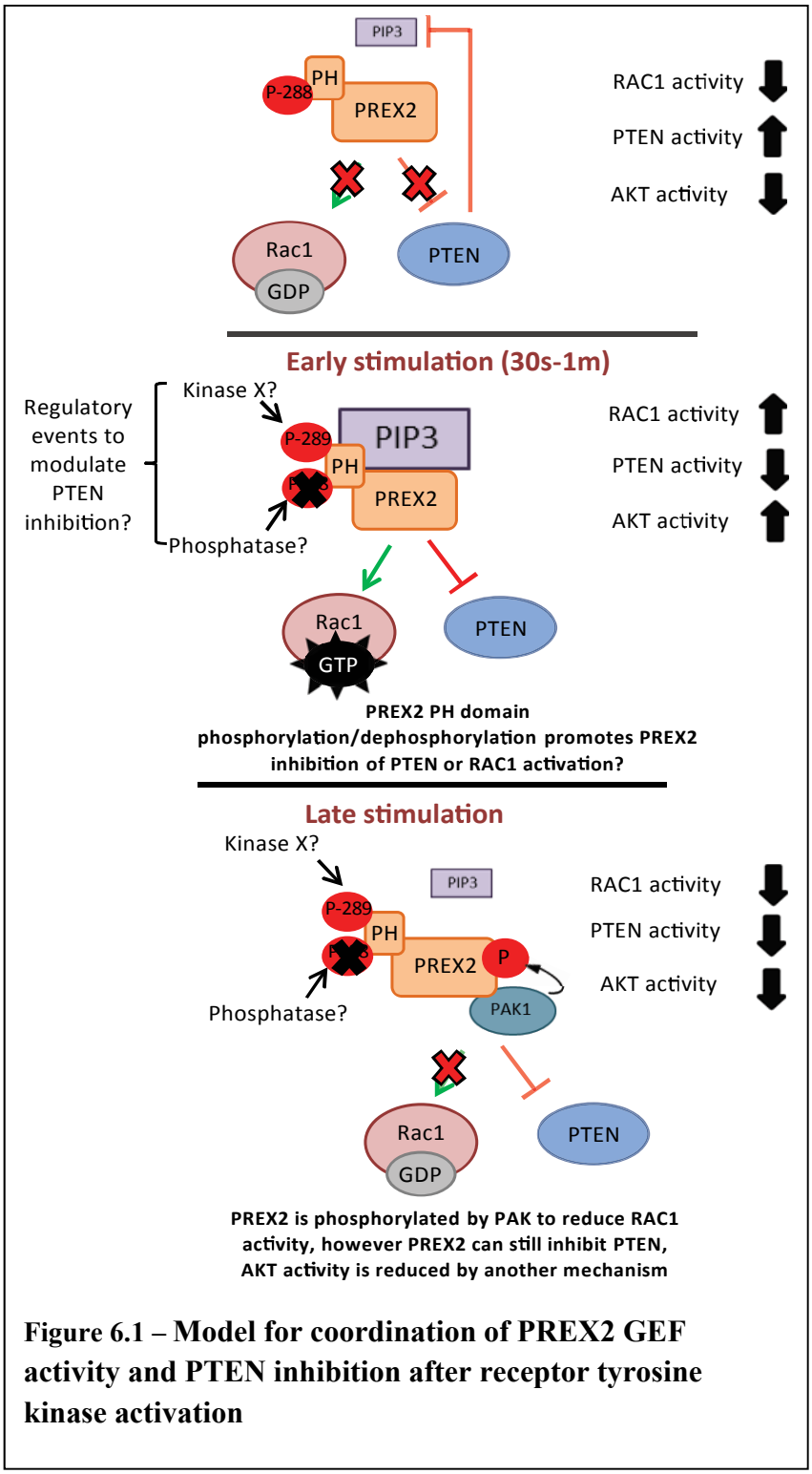


Figure 6.1 – Model for coordination of PREX2 GEF activity and PTEN inhibition after receptor tyrosine kinase activation

PREX2 at S288, resulting in a $\beta_3\beta_4$ loop that can bind and inhibit PTEN. This would promote the high PI(3,4,5)P3 levels very soon after insulin stimulation. Since the $\beta_3\beta_4$ loop also is important for RAC1 recognition, this hypothesis could potentially be extended to RAC1, where the regulation of phosphorylation in the $\beta_3\beta_4$ loop upon growth factor

stimulation would allow PREX2 to bind and activate RAC1. At later time points, PAK phosphorylates PREX2 to reduce RAC1 activation, and as the data from the PREX2$^{-/-}$ mice would suggest, PREX2 is likely still inhibiting PTEN.

Taken together, the findings from Chapters 4 and 5 have interesting implications for the role of PREX2 in cancer. One of most commonly mutated residues on PREX2 was at R297C, which also had one of the most striking biochemical phenotypes. We also characterized a recurrent tumor mutation, R299Q, which is in the same area of the PH domain as R297C. Both of these mutations are very close to the $\beta_3\beta_4$ loop of our model for the PREX2 PH domain, suggesting that they could potentially alter the ability of PREX2 to inhibit PTEN. Both of these residues are positively charged arginine residues, and mutating them would change the ionic properties of the area surrounding this variable loop, potentially altering its structure. If these mutations in this critical area led to an enhancement in PTEN inhibition, it could potentially explain why they were selected in cancer. Given that we did not see increases in GEF activity for these mutants, it would not be surprising if these mutants were affecting PTEN inhibition. This can be tested with purified protein in *in vitro* PTEN inhibition assays or in cells studying phosphorylation of AKT as a readout. If this hypothesis were true, one would expect these mutants to only be tumorigenic when PTEN is present. This could be tested by performing colony formation assays in cancer cell lines with or without PTEN. Further, in order to analyze these mutants in the endogenous setting and not in the context of overexpression, CRISPR can be used to knock-in these mutations into certain cell lines. These knock-in cell lines can then be used to look for differences in tumorigenicity, RAC1 activity, cell migration, or PTEN activity.

METHODS AND MATERIALS:

Plasmids and constructs: Full length PREX2 was cloned into the pcDNA3.1 V5/His vector (Life Technologies) and GST PTEN was cloned into the pGEX4T-1 vector (Amersham Bioscience) as previously described (Fine et al., 2009). PTEN G129E was made by mutating PCEP4-PTEN, and PREX2 point mutations were made by mutating the full length V5/His PREX2 pcDNA3.1 backbone using the QuikChange II XL site directed mutagenesis kit (Agilent). PH domain fragments of PREX2 were PCR amplified from the V5/His PREX2 pcDNA3.1 backbone, and then they were cloned into the pcDNA3.1 V5/His vector using the TOPO-TA cloning kit (Life Technologies). PP1α was amplified from cDNA of HEK293 cells, and cloned into the pcDNA3.1 V5/His vector using the TOPO-TA cloning kit. p110α mutants were cloned into the pcDNA3.1 vector. MYC-pCMV6M- PAK1 WT, PAK1 K299R, and PAK1 T423E were gifts from Jonathan Chernoff (Addgene plasmid #12210) (Sells et al., 1997). The HA PAK4 vector was a gift of Audrey Minden. The catalytic subunit of PP2A (pcDNA-PP2A/C-HA) was a gift of Gen Sheng Wu (Xu et al., 2013). PGEX encoding GST-tagged RAC1 was a gift of Katrin Rittinger. The FLAG-Gβ1/HA-Gγ2 vector in the pVITRO2-mcs(Hygro) backbone was a gift of Jonathan Backer. MYC RAC1-WT and MYC RAC1-T17N were gifts from Gary Bokoch (Addgene plasmids #12985, 12984).

Antibodies: Mouse V5 primary antibody (R960-25) was purchased from Life Technologies. Mouse M2 FLAG (F1804), vinculin (V9131), and actin (A5316) antibodies, in addition to mouse anti-V5 agarose affinity (A7345) and anti-M2-FLAG agarose affinity (A2220) gels were purchased from Sigma Aldrich. Mouse tubulin (MMS-410P) was purchased from Covance. Rabbit PTEN clone 138G6 (9559), total

AKT (9272), p308AKT (4056), p473AKT (9271), total ribosomal S6 (2271), p235/236 S6 (2211), ERK1/2 (9102), p202/204 ERK1/2 (4376), total EGFR (4267), and phospho-IGF1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151) (3024) primary antibodies were purchased from Cell Signaling Technologies. The rabbit polyclonal pS1107-PREX2 antibody to amino acids 1098-1116 (GHDTISNRDpSYSDCNSNRN) was made in collaboration with Cell Signaling Technologies. Mouse RAC1 (05-389), p338 RAF1 (05-538), p85 N-SH2 clone UB93-3 (05-217), p-tyrosine clone 4G10 (05-321) and rabbit IRS4 (06-771) primary antibodies were purchased from Millipore. Mouse MYC clone 9E10 (sc-40) primary antibody was purchased from Santa Cruz Biotechnology. The PREX2 rabbit polyclonal antibody was made by Zymed Laboratories. Secondary antibodies directed against rabbit, mouse, and rat IgG conjugated to HRP were purchased from Pierce.

Cell lines and Transfection: HEK293 and U87MG cells were cultured in DMEM (Cellgro) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 IU penicillin, and 100 μg/mL streptomycin (Cellgro). DBTRG-05MG and BT549 cells were cultured in RPMI supplemented with 10% (vol/vol) FBS, 100 IU penicillin, and 100 μg/mL streptomycin. When indicated, cells were starved for 16 hours in the appropriate media without FBS before treatment. Transfections were performed with Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Unless otherwise indicated, insulin treatments were for 30 minutes at 5 μg/mL. For treatment with a small molecule inhibitor, the indicated concentrations were incubated with the cells for 15 minutes prior to addition of insulin, or for 30 minutes prior to harvesting if no insulin was added.

GST fusion protein purification: Plasmids were transformed in to BL21(DE3) pLysE chemically competent cells (Invitrogen). Single colonies were picked and grown in a

small culture overnight, and this was used to inoculate a 500mL culture of LB broth until the OD600 was 0.3-0.5 when protein expression was induced by the addition of 0.1mM Isopropyl β-D-1-thiogalactopyranoside (Sigma) for 16 hours at 23°C. The bacterial pellet was resuspended in 10mL of lysis buffer (50 mM TRIS pH 7.2, 400 mM NaCl, 1% Triton X-100, 1 mM EDTA). The lysate was sonicated, and then centrifuged for 1 hour at 40,000 x g. The supernatant was incubated overnight with glutathione sepharose beads rotating at 4°C (GE Healthcare Life Sciences). Beads were then washed 3 times in lysis buffer followed by 3 times in a buffer containing 25 mM TRIS pH 7.5 and 150 mM NaCl. The beads were then stored and frozen in this same buffer. To elute, the beads were incubated with the bead bed volume of elution buffer (25 mM TRIS pH 8.0, 150 mM NaCl, 50 mM glutathione) for 30 minutes shaking at room temperature. The beads were removed, and eluted proteins were aliquoted and snap frozen. To quantify both eluted and bead bound proteins, samples were diluted in 2X Laemmli sample buffer (125 mM TRIS pH 6.8, 4% SDS, 20% glycerol, 10% beta-mercaptoethanol, 0.05% bromophenol-blue) and then were run on a gel with BSA standards and quantified using Gel Code Blue (Pierce).

In vitro Lambda phosphatase assay: For endogenous PREX2, HEK293 cells were harvested in lysis buffer (20 mM HEPES pH 7.4, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM NaOV3, 1 mM NaF, 100 nM calyculin A, 1x eukaryotic protease inhibitor cocktail (Sigma)). The lysates were vortexed, sonicated, centrifuged at 4°C for 30 minutes, and then combined with 5 μL PREX2 antibody and 20 μL of Protein A/G Plus agarose beads (Santa Cruz), rotating at 4°C for 4 hours. The beads were then washed 5 times in lysis buffer. For analysis of exogenous PREX2, HEK293 cells expressing V5

PREX2 were harvested in lysis buffer (25 mM TRIS pH 7.5, 0.2% Triton X-100, 200 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaOV3, 1 mM NaF, 100 nM calyculin A, 1x eukaryotic protease inhibitor cocktail). Lysates were vortexed, sonicated, centrifuged at 4°C for 30 minutes, and then incubated with V5 agarose beads (Sigma) for 4 hours rotating at 4°C. Beads were then washed 5 times in lysis buffer. For both V5 PREX2 and endogenous PREX2, the phosphatase reactions were set up in 100 µL using 1 µL of Lambda phosphatase from New England Biolabs along with the supplied 10x Lambda phosphatase buffer and 10x MnCl diluted in water. Reactions were incubated at 30°C for 20 minutes and were terminated with 2x Laemmli sample buffer.

Mass Spectrometry: V5 PREX2 was expressed in HEK293 cells. Cells were starved and then treated with either 500 nM GDC-0941 (referred to as the unstimulated state), 5 µg/ml insulin, or insulin and 100 nM calyculin A (both of these conditions are referred to as the stimulated state). Cells were lysed in buffer containing 25 mM TRIS pH 7.5, 0.2% Triton X-100, 200 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaOV3, 1 mM NaF, 100 nM calyculin A, and 1x eukaryotic protease inhibitor cocktail. The lysate was vortexed, sonicated, and centrifuged at 4°C for 30 minutes, and then pre-cleared with Protein A/G PLUS-agarose beads and mouse IgG (Santa Cruz). The beads were removed, and V5 PREX2 was isolated on V5 agarose beads by incubating them with the lysate for 4 hours rotating at 4°C. Beads were washed 5 times in lysis buffer, and resuspended in 2x Laemmli sample buffer. For each condition, two samples of approximately 3 micrograms of V5 PREX2 were run by SDS-PAGE and the bands were excised. Bands were digested overnight with sequencing grade trypsin and chymotrypsin (Promega). These digests were analyzed separately and phosphopeptides were enriched using titanium dioxide spin

tips (Protea) followed by microcapillary liquid chromatography / tandem mass spectrometry (LC-MS/MS). Enriched peptides were introduced to the Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) in positive ion CID mode using a data-dependent acquisition (DDA) with an EASY-NLC II nanoflow HPLC (Proxeon) (75 mm ID x 15 cm C18 column) at 300 nL/min. MS/MS spectra were searched against the reversed and concatenated SwissProt (Version 2012_3, Uniprot) protein database and the Prex2V5His custom database using Mascot server 2.4 and data were analyzed using Scaffold 4 software. Peptides were reported with a false discovery rate (FDR) threshold of 1%.

PREX2 siRNA: DBTRG cells in 6 well plates were transfected with 100 pmol of either control or PREX2 siRNA (5' CCCUGGACAGUGCAUUAUCAAUU, antisense – 5' UUGAUAAUGCACUGUCCAGGGUU)(Sigma). 48 hours later cells were treated as indicated, and harvested in 2X Laemmli sample buffer.

In vitro PI3K kinase assay (PREX2 as a substrate): Either V5 PREX2 or empty vector was expressed in HEK293 cells. Cells were starved and then lysed in buffer containing 25 mM TRIS pH 7.5, 0.2% Triton X-100, 200 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM $NaOV_3$, 1 mM NaF, 100 nM calyculin A, and 1x eukaryotic protease inhibitor cocktail. The lysate was vortexed, sonicated, and centrifuged at 4°C for 30 minutes, and then pre-cleared with Protein A/G PLUS-agarose beads and mouse IgG. The beads were removed, and V5 PREX2 was isolated on V5 agarose beads by incubating them with the lysate overnight rotating at 4°C. Beads were washed 2 times in lysis buffer, followed by 5 times in wash buffer containing 20 mM TRIS pH 7.4, 100 mM NaCl, 0.5 mM EDTA. After last wash, beads were resuspended in 70 µL of the wash buffer. 20 µL of 30 mM HEPES/1 mM EGTA solution was added to beads (this is buffer used for lipids

when the lipid is the substrate, but PREX2 is the substrate, so just added the buffer). 1 µL of 0.1 µg/ µL PIK3CA/PIK3R1 (Invitrogen) and 1 µL of 100 µM GDC0941 (1 µM final concentration) or 1 µL of DMSO was added to the reaction followed by 10 µL of the ATP mix. The ATP mix consisted of 5.5 µL 100 mM HEPES pH 7, 2 µL 500 mM $MgCl_2$, 0.5 µL 10 mM cold ATP, and 2 µL of 10 µCi/µL ATP (γ-^{32}P). The reactions were left at room temperature for 10 minutes, followed by 10 minutes at 37°C. Beads were then washed 2 times in wash buffer, and resuspended in 2x Laemmli sample buffer. Incorporation of [γ-^{32}P]-ATP was detected by gel electrophoresis.

In vitro PI3K kinase assay (lipid substrate): The silica gel thin layer chromatography (TLC) plate was activated by immersing in 1% potassium oxalate in 3:2 methanol:water solution for 5 minutes. Plate was then baked in 100°C oven for 1 hour. After removal from oven the plate was allowed to reach room temperature. Lipid mixture was then made by adding 4 µg phosphatidylinositol and 12 µg phosphotidylserine (both in 10 µg/µL chloroform solutions from Avanti) per sample to a tube. Lipid mixture was dried under gentle flow of nitrogen gas, and then lipids were dissolved in 20 µL/reaction of 30 mM HEPES/1 mM EGTA solution. Solution was vortexed, and then sonicated (periodically putting on ice if using a probe sonicator) until solution was no longer cloudy, and then left at room temperature. The reaction was then set up as follows: 70 µL of 20 mM TRIS pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 20 µL of lipid mix, 1 µL of 0.1 µg/µL PIK3CA/PIK3R1, 1 µL of 100 µM GDC0941 (1 µM final concentration) or 1 µL of DMSO, and then 10 µL of ATP mix (added last). ATP mix consisted of 5.5 µL 100 mM HEPES pH 7, 2µL 500mM $MgCl_2$, 0.5uL 10mM cold ATP, and 2µL of 10 µCi/µL ATP [γ-^{32}P]. Reaction was incubated for 10 minutes at room temperature and stopped by

adding 25 μL of 5N HCl. Then 160 μL of 1:1 chloroform:methanol was added to each reaction, solution was vortexed for 30 seconds, and then centrifuged for 2 minutes to separate aqueous and organic layers. 50 μL of the organic layer (bottom) was removed and added dropwise to the silica plate, and the spot was then dried. TLC chamber was set up by wetting a piece of filter paper within the chamber with mobile phase (65:35 n-propanol:acetic acid). Plate was put on the stand in the chamber and enough mobile phase was added to the chamber to cover the bottom of the plate, but remain lower than the spots of loaded sample. Mobile phase was allowed to run overnight, and the next morning the plate was exposed to the phosphorimager for 1 hour and scanned on a Typhoon Trio scanner (GE Healthcare Life Sciences).

Modeling of PREX2 PH domain: For modeling we used the software Modeller version 9v9 maintained by the Sali laboratory (Webb and Sali, 2014). The template for the PH domain of PREX2 was the crystal structure of the PH domain of CDC42 (PDB entry 2DFK, chain A). To explore the variations in query-template alignments, we used S4, a program that relies on information on secondary structure elements of the template (Kuziemko et al., 2011). The alternative alignments generated by S4 were evaluated with statistical potential-based pG score and structure verification program Verify3D. pG score is the posterior probability that the model has a correct three-dimensional conformation, given its normalized z-score (obtained using the program Prosa II, (Sippl, 1993)) and length. Verify3D assesses the compatibility of the segments of the sequence with their three-dimensional structures by plotting the average statistical preference scores in a window of 21 residues (Luthy et al., 1992). The best model among alternative

alignment had the pG score of 0.99. The minimum value of Verify3D profile window plot was 0.06.

V5 PREX2 Purification for RAC-GEF assay: V5 PREX2 was purified from HEK293 cells as previously described with small modifications (Fine et al., 2009). In short, 15 cm plates were transfected with V5 PREX2 and were harvested in 1.5mL of lysis buffer per plate (25 mM TRIS pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM NaOV3, 1 mM NaF, 100 nM calyculin A, 1x eukaryotic protease inhibitor cocktail). Cells were vortexed and sonicated, then centrifuged for 1 hour at 40,000 x g. The supernatant was incubated with V5 agarose (10 μL per plate) for 16 hours rotating at 4°C. The beads were then washed on a poly-prep column (Bio-Rad) 4 times with lysis buffer and 4 times with buffer containing 1X PBS, 10% glycerol, 1 mM EGTA, and 1 mM DTT. Then 1.5 bed volumes of elution buffer (1X PBS, 10% glycerol, 1 mM EGTA, 1 mM DTT, and 500 μg/mL V5 peptide (Sigma)) was added to the beads and the column was incubated at 37°C for 30 minutes. The column was then put into a 50 mL conical tube and centrifuged at 200 x g. This elution process was repeated a second time and elutions were combined. Protein samples were run on a gel with bovine serum albumin (BSA) standards and stained with Gel Code Blue (Thermo).

Expression and purification of recombinant Gβ1His-γ2: Recombinant purified Gβγ dimers were produced as described elsewhere (Shymanets et al., 2013). Essentially, Fall armyworm ovary cells (Sf9, from Gibco BRL, Eggenstein, Germany) were cultured in suspension with TNM-FH medium (Sigma, Deisenhofen, Germany) supplemented with 10 % (v/v) fetal calf serum (Gibco BRL), Lipid Medium Supplement (1:100; Sigma), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). For protein expression, Sf9 cells

(1.5 x 10^6 cells/mL) were infected with viruses encoding Gβ1 and N-terminally hexahistidine-tagged Gγ2. After 60 hours of infection, cells were collected by centrifugation at 1,000 x g for 5 min and washed twice with PBS. Recombinantly expressed isoprenylated Gβ1His-γ2 complexes were isolated from the membrane fraction of Sf9 cells as detailed earlier (Shymanets et al., 2012; Shymanets et al., 2009). Purified proteins were quantified by SDS-PAGE followed by Coomassie blue staining with BSA standards and stored at -80 °C.

In vitro RAC-GEF assay: Assay was performed as previously described with a few changes (Hill and Welch, 2006; Welch et al., 2002). Purification of GST RAC1 proteins was performed as described above. After elution with glutathione, a 500 µL elution was combined in a 10,000 MWCO Amicon filter with 15 mL of buffer containing 40 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1 mM DTT. Solution was concentrated to 1mL and this was repeated 3 more times. The solution was removed from the filter, GDP was added to 1 mM, and the solution was rotated at 4°C for 1 hour. $MgCl_2$ was then added to 15 mM to stop loading and the solution was added to a 10,000 MWCO Amicon filter with 15mL of 40 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 5 mM $MgCl_2$, and 10 µM GDP. Solution was concentrated to 1 mL and this was repeated 3 more times. Final protein was snap frozen and stored at -80°C. For the in vitro GEF assay, PI(3,4,5)P3 diC16 was purchased from Echelon Biosciences and was incorporated into liposomes. Liposomes were prepared with phosphatidylcholine, phosphatidylserine, and phosphatidylinositol at 1 mM, which is a 5X to the final assay concentration of 200 µM. In short, lipids were dried under argon gas, resuspended in lipid buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 2 mM EGTA)

with or without PI(3,4,5)P3 diC16 (Echelon), and then sonicated. Then, 2 μL of lipids were added to 1 μL of 1 μM GST RAC1 (diluted in lipid buffer) and 1 μL of 10x assay buffer (20 mM HEPES pH 7.4, 10 mM MgCl2, 100 mM NaCl, 1 mM EGTA, 100 mg/mL BSA, 10 mM DTT). The solution was then vortexed and left on ice for 10 minutes. Next, 4 μL purified V5 PREX2 from HEK293 cells was added, followed by 2 μL of the 5X GTPγS master mix. The 5X GTPγS master mix consisted of 25 μM cold GTPγS and 0.5 μCi/μL [^{35}S]GTPγS diluted in lipid buffer. The final concentrations of GST RAC1 and V5 PREX2 were 100 nM and 1 nM, respectively. In the final reaction, purified PREX2 and GST RAC1 were incubated in a reaction volume of 10 μL with PI(3,4,5)P3 or Gβγ, 5 μM cold GTPγS, and 1 μCi [^{35}S]GTPγS (Perkin Elmer) for 10 minutes at 30°C. Then GST RAC1 was pulled down by adding 400 μL of IP buffer (1X PBS, 1 mM EGTA, 10 mM $MgCl_2$, 1% Triton, 0.1 mM GTP) and glutathione sepharose beads (GE Healthcare Life Sciences), and rotating for 1 hour at 4°C. The beads were then washed 4 times in IP buffer and resuspended in 400 μL of scintillation fluid. This was then added to a scintillation vial containing 5 mL of scintillation fluid, and the [^{35}S]GTPγS-loading of GST RAC1 was detected by scintillation β counting.

Invasion assay: Matrigel (Trevigen) was diluted in water to a final concentration of 0.15 μg/μL in ice cold water and 40 μL of this dilution was put into the upper chamber of each Transwell (BD Biosciences) The Matrigel was dried at room temperature and 75 μL of empty media (no FBS) was added to the upper chamber. Then 600 μL of media with or without FBS was added to the lower chamber 30 minutes prior to starting the experiment. Empty vector or V5 PREX2 (WT or mutant) expressing BT549 cells were trypsinized and then allowed to recover in media with FBS. The cells were then washed twice in

empty media, counted, and either 50,000 cells (if using no chemoattractant), or 20,000 cells (when using FBS as a chemoattractant) were plated into the upper chamber of the Transwell. Cells were allowed to invade for 22 hours (no chemoattractant) or 3 hours (FBS as a chemoatractant) at 37°C. Three independent Transwell inserts were used per experiment. Cells were removed from the top of the insert, and invading cells were fixed in methanol for 15 minutes and then stained with 0.2% crystal violet in 2% ethanol for 15 minutes. Inserts were dried at room temperature, and pictures of at least 4 independent fields were taken per insert and invading cells were counted in each field. The average number of cells per field was calculated and data is presented as the fold-change increase over the average for the empty vector transfected cells.

GST and PI(3,4,5)P3 bead pull downs - For pull downs of V5 PREX2, HEK293 cells were transfected and then harvested in lysis buffer (20 mM HEPES pH 7.4, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM $NaOV_3$, 1 mM NaF, 100 nM calyculin A, 1x eukaryotic protease inhibitor cocktail). Lysate was then vortexed, sonicated, and centrifuged at 4°C for 30 minutes. For pull downs with GST fused proteins, lysates were pre-cleared with GST loaded glutathione sepharose beads for 1 hour rotating at 4°C. Supernatants were then incubated with 5 μg of GST PTEN, GST PP1α, or GST RAC1 loaded onto glutathione sepharose beads for 4 hours rotating at 4°C. For PI(3,4,5)P3 pull downs, 10 μL of PI(3,4,5)P3 bead slurry (Echelon) was incubated with lysates for 4 hours rotating at 4°C. Beads were washed 5 times in lysis buffer, and resuspended in 50 μL of 2X Laemmli sample buffer. For PI(3,4,5)P3 pull downs of endogenous PREX2, the same protocol was followed except that the lysates from 15 cm plates of HEK293 cells were

divided into two samples, one for the PI(3,4,5)P3 pull down and another for the immunoprecipitation of PREX2 with 5 μL of PREX2 antibody.

In vitro GST PP1α and PP2A dephosphorylation assay - HEK293 cells were transfected with V5 PREX2, and cells were lysed in buffer containing 20 mM HEPES pH 7.4, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM $NaOV_3$, 1 mM NaF, 100 nM calyculin A, and 1x eukaryotic protease inhibitor cocktail. The lysate was then vortexed, sonicated, and centrifuged at 4°C for 30 minutes. The supernatant was then incubated with V5 agarose beads for 4 hours rotating at 4°C. Next, the beads were washed 4 times in lysis buffer and once with either PP1α reaction buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM $MnCl_2$, 5 mM DTT) for PP1α assays, or protein phosphatase dilution buffer (Cat #: 20-169) from Millipore for PP2A assays. For PP1α dephosphorylation assays, 99 μL of PP1α reaction buffer was added to each tube followed by either 1 μL of GST elution buffer (25 mM TRIS pH 8.0, 100 mM NaCl, 50 mM glutathione) for the mock treatment, or 1 μL of GST PP1α (at a concentration of 1 μg/μL). For PP2A dephosphorylation assays, 45 μL of protein phosphatase dilution buffer was added to the V5 PREX2 beads, and then either 5 μL of protein phosphatase buffer or 0.5 units (5 μL) of purified PP2A (Millipore, Cat #:14-111) was added to each reaction. Reactions were incubated for 60 minutes at 30°C and stopped by removing the supernatant and resuspending the beads in 2X Laemmli sample buffer.

Gβγ-PREX2 co-immunoprecipitation - HEK293 cells were transfected with V5 PREX2 alone, or V5 PREX2 and FLAG/HA Gβγ together. Cells co-expressing PREX2 and Gβγ were either untreated, or treated with 100 nM calyculin A for 30 minutes. Cells were lysed in buffer containing 25 mM TRIS pH 7.5, 0.2% Triton X-100, 200 mM KCl, 10%

glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM $NaOV_3$, 1 mM NaF, 100 nM calyculin A, and 1x eukaryotic protease inhibitor cocktail. Lysates were rotated at 4°C for 15 minutes, vortexed, and then spun down at 4°C for 30 minutes. Protein concentrations of lysates were measured using the Biorad DC protein assay and protein concentrations were equalized for all samples. Samples were then pre-cleared with Protein A/G Plus agarose beads rotating at 4°C for 1 hour. Finally, samples were incubated with 10 µL of M2-FLAG agarose affinity gel for 4 hours rotating at 4°C. Beads were then washed 5 times in lysis buffer, and were resuspended in 2X Laemmli sample buffer.

Cell fractionation - Either untransfected (for endogenous PREX2 analysis) or V5 PREX2 transfected HEK293 cells were harvested in lysis buffer without detergents (50 mM TRIS pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaOV3, 1 mM NaF, 100 nM calyculin A, 1x eukaryotic protease inhibitor cocktail), and lysates were passed through a 25G needle 10 times. The nuclear fraction was removed by centrifuging the lysate at 720 x g for 5 minutes. The supernatant was centrifuged again at 17,000 x g for 20 minutes, followed by another spin at 17,000 x g for 10 minutes. The membrane fraction was separated by ultracenrifugation at 100,000 x g for 60 minutes. The supernatant (cytosolic fraction) was saved and the pellet (membrane fraction) was resuspended in 400 µL of lysis buffer without detergents and re-centrifuged at 100,000 x g for 45 minutes. For V5 PREX2, the pellet was resuspended in 100 µL of lysis buffer including 1% NP-40 and 0.25% sodium deoxycholate. For the endogenous fractionation, the membrane pellet was resuspended in 400 µL of lysis buffer plus detergents, and GST PTEN beads (5 µg GST PTEN per sample) were incubated with both the cytosolic and membrane fraction for 4 hours rotating at 4°C to concentrate the endogenous PREX2 to be visualized by Western blot.

In vitro PAK kinase assay - V5 PREX2 expressing HEK293 cells were starved and then treated with 500 nM GDC0941 for 30 minutes. One 15 cm plate was transfected for every two kinase reactions in a given experiment. Each plate was harvested in 1.5 mL of high salt lysis buffer containing 25 mM TRIS pH 7.5, 0.1% Triton X-100, 1 M NaCl, and 1x eukaryotic protease inhibitor cocktail, and the lysates were then vortexed, sonicated, and centrifuged for 30 minutes at 4°C. Lysates were combined and then incubated with V5 agarose beads for 4 hours rotating at 4°C. Beads were washed 3 times in lysis buffer followed by 3 washes with kinase buffer (25 mM MOPS, pH 7.2, 12.5 mM beta-glycerophosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT), and then were divided equally into the appropriate number of tubes. Purified active PAK1 or PAK4 (SignalChem) was diluted in kinase dilution buffer (kinase buffer diluted 1:4 with 50 ng/µl BSA). For the kinase reaction, 20 µL of volume, consisting of 10 µL PAK1 (containing 0.4 µg of protein) or PAK4 (containing 0.2 µg of protein), 4 µL water, 1 µL PF-3758309 (MedKoo), 50 µM cold ATP, and 10 µCi [γ-^{32}P]-ATP (both forms of ATP were diluted in kinase buffer), was incubated with the V5 PREX2 beads at 37°C for 30 minutes. The PAK1 or PAK4, water, and PF-3758309 were incubated on ice for 10 minutes before being added to the V5 PREX2 beads, and the cold and radioactive ATP was added to the reaction right before the incubation at 37°C. After the reaction, beads were then washed 2X in kinase buffer and resuspended in 2X Laemmli sample buffer. Incorporation of [γ-^{32}P]-ATP was detected by gel electrophoresis.

In vitro translation pull down assay using GST proteins: The TnT T7 Quick Coupled Transcription/Translation System from Promega was used to translate proteins *in vitro.* For the *in vitro* translation reactions, 20 µL of the TNT T7 master mix was combined

with 0.5μL of the supplied methionine stock, 1μg of DNA, and the appropriate amount of water to give a total reaction volume of 25 μL. The reactions were then incubated for 90 minutes at 30°C. Each reaction was divided into two pull downs, one with GST beads and the other with either GST PTEN or GST RAC1 beads. Meanwhile, 10 μg of either GST, GST PTEN, or GST RAC1 sepharose beads (for each translation reaction, one tube of GST beads and one tube either GST PTEN or GST RAC1 beads were required) were blocked for 1 hour at 4°C in 500 μL of buffer containing 25 mM TRIS pH 7.5, 0.2% Triton X-100, 200 mM KCl, 10% glycerol, 1 mM EDTA, and 1 mg/ml BSA. After the *in vitro* translation reaction, 18 μL of the reaction was added to 1mL of the GST bead blocking buffer used above. 20 μg of GST beads were added to this solution to remove anything in the reaction that binds to GST. This mixture was rotated for 1 hour at 4°C. The buffer was removed from the beads being blocked, and 500 μL of the pre-cleared lysate was added to GST beads, while the other 500 μL was added to the GST PTEN or GST RAC1 beads. These pull downs were the left rotating overnight at 4°C. Beads were then washed 5 times in the same buffer used for the pull downs, excluding the BSA, and then beads were resuspended in 2X Laemmli buffer.

REFERENCES:

Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C.G., and Segal, A.W. (1991). Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature *353*, 668-670.

Abo, A., Qu, J., Cammarano, M.S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998). PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia. The EMBO journal *17*, 6527-6540.

Aghazadeh, B., Lowry, W.E., Huang, X.Y., and Rosen, M.K. (2000). Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. Cell *102*, 625-633.

Al-Khouri, A.M., Ma, Y., Togo, S.H., Williams, S., and Mustelin, T. (2005). Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. The Journal of biological chemistry *280*, 35195-35202.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. The EMBO journal *15*, 6541-6551.

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., *et al.* (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Current biology : CB *7*, 776-789.

Alimonti, A., Carracedo, A., Clohessy, J.G., Trotman, L.C., Nardella, C., Egia, A., Salmena, L., Sampieri, K., Haveman, W.J., Brogi, E., *et al.* (2010). Subtle variations in Pten dose determine cancer susceptibility. Nature genetics *42*, 454-458.

Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., 3rd, Johnson, R.S., and Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature *372*, 186-190.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature *393*, 805-809.

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell *57*, 167-175.

Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W., and Osathanondh, R. (1982). Insulin-stimulated tyrosine phosphorylation of the insulin receptor in detergent extracts of human placental membranes. Comparison to epidermal growth factor-stimulated phosphorylation. The Journal of biological chemistry *257*, 15162-15166.

Backer, J.M., Myers, M.G., Jr., Shoelson, S.E., Chin, D.J., Sun, X.J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J., *et al.* (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. The EMBO journal *11*, 3469-3479.

Backman, S.A., Ghazarian, D., So, K., Sanchez, O., Wagner, K.U., Hennighausen, L., Suzuki, A., Tsao, M.S., Chapman, W.B., Stambolic, V., *et al.* (2004). Early onset of neoplasia in the prostate and skin of mice with tissue-specific deletion of Pten. Proceedings of the National Academy of Sciences of the United States of America *101*, 1725-1730.

Baker, N.M., Yee Chow, H., Chernoff, J., and Der, C.J. (2014). Molecular Pathways: Targeting RAC-p21-Activated Serine-Threonine Kinase Signaling in RAS-Driven Cancers. Clinical cancer research : an official journal of the American Association for Cancer Research *20*, 4740-4746.

Balamatsias, D., Kong, A.M., Waters, J.E., Sriratana, A., Gurung, R., Bailey, C.G., Rasko, J.E., Tiganis, T., Macaulay, S.L., and Mitchell, C.A. (2011). Identification of P-Rex1 as a novel Rac1-guanine nucleotide exchange factor (GEF) that promotes actin remodeling and GLUT4 protein trafficking in adipocytes. The Journal of biological chemistry *286*, 43229-43240.

Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R., and Fletcher, A.A. (1922). Pancreatic Extracts in the Treatment of Diabetes Mellitus. Canadian Medical Association journal *12*, 141-146.

Barber, M.A., Donald, S., Thelen, S., Anderson, K.E., Thelen, M., and Welch, H.C. (2007). Membrane translocation of P-Rex1 is mediated by G protein betagamma subunits and phosphoinositide 3-kinase. The Journal of biological chemistry *282*, 29967-29976.

Barber, M.A., Hendrickx, A., Beullens, M., Ceulemans, H., Oxley, D., Thelen, S., Thelen, M., Bollen, M., and Welch, H.C. (2012). The guanine-nucleotide-exchange factor P-Rex1 is activated by protein phosphatase 1alpha. The Biochemical journal *443*, 173-183.

Baskaran, Y., Ng, Y.W., Selamat, W., Ling, F.T., and Manser, E. (2012). Group I and II mammalian PAKs have different modes of activation by Cdc42. EMBO reports *13*, 653-659.

Bellacosa, A., Testa, J.R., Staal, S.P., and Tsichlis, P.N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science *254*, 274-277.

Berger, M.F., Hodis, E., Heffernan, T.P., Deribe, Y.L., Lawrence, M.S., Protopopov, A., Ivanova, E., Watson, I.R., Nickerson, E., Ghosh, P., *et al.* (2012). Melanoma genome sequencing reveals frequent PREX2 mutations. Nature *485*, 502-506.

Bi, F., Debreceni, B., Zhu, K., Salani, B., Eva, A., and Zheng, Y. (2001). Autoinhibition mechanism of proto-Dbl. Molecular and cellular biology *21*, 1463-1474.

Bose, I., Irazoqui, J.E., Moskow, J.J., Bardes, E.S., Zyla, T.R., and Lew, D.J. (2001). Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. The Journal of biological chemistry *276*, 7176-7186.

Brownawell, A.M., Kops, G.J., Macara, I.G., and Burgering, B.M. (2001). Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Molecular and cellular biology *21*, 3534-3546.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Campbell, A.D., Lawn, S., McGarry, L.C., Welch, H.C., Ozanne, B.W., and Norman, J.C. (2013). P-Rex1 cooperates with PDGFRbeta to drive cellular migration in 3D microenvironments. PloS one *8*, e53982.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J., and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. Nature *360*, 684-686.

Cancer Genome Atlas Research, N., Kandoth, C., Schultz, N., Cherniack, A.D., Akbani, R., Liu, Y., Shen, H., Robertson, A.G., Pashtan, I., Shen, R., *et al.* (2013). Integrated genomic characterization of endometrial carcinoma. Nature *497*, 67-73.

Cao, J., Wan, L., Hacker, E., Dai, X., Lenna, S., Jimenez-Cervantes, C., Wang, Y., Leslie, N.R., Xu, G.X., Widlund, H.R., *et al.* (2013). MC1R is a potent regulator of PTEN after UV exposure in melanocytes. Molecular cell *51*, 409-422.

Carpenter, C.L., Auger, K.R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L.C. (1993). Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. The Journal of biological chemistry *268*, 9478-9483.

Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., *et al.* (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery *2*, 401-404.

Chagpar, R.B., Links, P.H., Pastor, M.C., Furber, L.A., Hawrysh, A.D., Chamberlain, M.D., and Anderson, D.H. (2010). Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase. Proceedings of the National Academy of Sciences of the United States of America *107*, 5471-5476.

Chen, X., Pan, M., Han, L., Lu, H., Hao, X., and Dong, Q. (2013). miR-338-3p suppresses neuroblastoma proliferation, invasion and migration through targeting PREX2a. FEBS letters *587*, 3729-3737.

Chen, X.W., Leto, D., Chiang, S.H., Wang, Q., and Saltiel, A.R. (2007). Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Developmental cell *13*, 391-404.

Chen, X.W., Leto, D., Xiong, T., Yu, G., Cheng, A., Decker, S., and Saltiel, A.R. (2011). A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action. Molecular biology of the cell *22*, 141-152.

Chen, Z., Borek, D., Padrick, S.B., Gomez, T.S., Metlagel, Z., Ismail, A.M., Umetani, J., Billadeau, D.D., Otwinowski, Z., and Rosen, M.K. (2010). Structure and control of the actin regulatory WAVE complex. Nature *468*, 533-538.

Cherfils, J., and Zeghouf, M. (2013). Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiological reviews *93*, 269-309.

Chiu, T.T., Patel, N., Shaw, A.E., Bamburg, J.R., and Klip, A. (2010). Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells. Molecular biology of the cell *21*, 3529-3539.

Chiu, T.T., Sun, Y., Koshkina, A., and Klip, A. (2013). Rac-1 superactivation triggers insulin-independent glucose transporter 4 (GLUT4) translocation that bypasses signaling defects exerted by c-Jun N-terminal kinase (JNK)- and ceramide-induced insulin resistance. The Journal of biological chemistry *288*, 17520-17531.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B., 3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., and Birnbaum, M.J. (2001a). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728-1731.

Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F., and Birnbaum, M.J. (2001b). Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. The Journal of biological chemistry *276*, 38349-38352.

Cohen, P., Klumpp, S., and Schelling, D.L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. FEBS letters *250*, 596-600.

Cotteret, S., Jaffer, Z.M., Beeser, A., and Chernoff, J. (2003). p21-Activated kinase 5 (Pak5) localizes to mitochondria and inhibits apoptosis by phosphorylating BAD. Molecular and cellular biology *23*, 5526-5539.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Damoulakis, G., Gambardella, L., Rossman, K.L., Lawson, C.D., Anderson, K.E., Fukui, Y., Welch, H.C., Der, C.J., Stephens, L.R., and Hawkins, P.T. (2014). P-Rex1 directly activates RhoG to regulate GPCR-driven Rac signalling and actin polarity in neutrophils. Journal of cell science *127*, 2589-2600.

Dan, C., Kelly, A., Bernard, O., and Minden, A. (2001). Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. The Journal of biological chemistry *276*, 32115-32121.

Dan, C., Nath, N., Liberto, M., and Minden, A. (2002). PAK5, a new brain-specific kinase, promotes neurite outgrowth in N1E-115 cells. Molecular and cellular biology *22*, 567-577.

Das, B., Shu, X., Day, G.J., Han, J., Krishna, U.M., Falck, J.R., and Broek, D. (2000). Control of intramolecular interactions between the pleckstrin homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. The Journal of biological chemistry *275*, 15074-15081.

Davis, M.J., Ha, B.H., Holman, E.C., Halaban, R., Schlessinger, J., and Boggon, T.J. (2013). RAC1P29S is a spontaneously activating cancer-associated GTPase. Proceedings of the National Academy of Sciences of the United States of America *110*, 912-917.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P.P. (1998). Pten is essential for embryonic development and tumour suppression. Nature genetics *19*, 348-355.

Didsbury, J., Weber, R.F., Bokoch, G.M., Evans, T., and Snyderman, R. (1989). rac, a novel ras-related family of proteins that are botulinum toxin substrates. The Journal of biological chemistry *264*, 16378-16382.

Donald, S., Hill, K., Lecureuil, C., Barnouin, R., Krugmann, S., John Coadwell, W., Andrews, S.R., Walker, S.A., Hawkins, P.T., Stephens, L.R.*, et al.* (2004). P-Rex2, a new guanine-nucleotide exchange factor for Rac. FEBS letters *572*, 172-176.

Donald, S., Humby, T., Fyfe, I., Segonds-Pichon, A., Walker, S.A., Andrews, S.R., Coadwell, W.J., Emson, P., Wilkinson, L.S., and Welch, H.C. (2008). P-Rex2 regulates Purkinje cell dendrite morphology and motor coordination. Proceedings of the National Academy of Sciences of the United States of America *105*, 4483-4488.

Dong, X., Mo, Z., Bokoch, G., Guo, C., Li, Z., and Wu, D. (2005). P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. Current biology : CB *15*, 1874-1879.

Dresner, A., Laurent, D., Marcucci, M., Griffin, M.E., Dufour, S., Cline, G.W., Slezak, L.A., Andersen, D.K., Hundal, R.S., Rothman, D.L.*, et al.* (1999). Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. The Journal of clinical investigation *103*, 253-259.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.H., Masiarz, F., Kan, Y.W., Goldfine, I.D.*, et al.* (1985). The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. Cell *40*, 747-758.

Eden, S., Rohatgi, R., Podtelejnikov, A.V., Mann, M., and Kirschner, M.W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature *418*, 790-793.

Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nature cell biology *1*, 253-259.

Escobedo, J.A., Navankasattusas, S., Kavanaugh, W.M., Milfay, D., Fried, V.A., and Williams, L.T. (1991). cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. Cell *65*, 75-82.

Fantin, V.R., Sparling, J.D., Slot, J.W., Keller, S.R., Lienhard, G.E., and Lavan, B.E. (1998). Characterization of insulin receptor substrate 4 in human embryonic kidney 293 cells. The Journal of biological chemistry *273*, 10726-10732.

Ferguson, G.J., Milne, L., Kulkarni, S., Sasaki, T., Walker, S., Andrews, S., Crabbe, T., Finan, P., Jones, G., Jackson, S.*, et al.* (2007). PI(3)Kgamma has an important context-dependent role in neutrophil chemokinesis. Nature cell biology *9*, 86-91.

Fine, B., Hodakoski, C., Koujak, S., Su, T., Saal, L.H., Maurer, M., Hopkins, B., Keniry, M., Sulis, M.L., Mense, S.*, et al.* (2009). Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. Science *325*, 1261-1265.

Fleming, I.N., Elliott, C.M., Buchanan, F.G., Downes, C.P., and Exton, J.H. (1999). Ca2+/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. The Journal of biological chemistry *274*, 12753-12758.

Fleming, I.N., Elliott, C.M., Collard, J.G., and Exton, J.H. (1997). Lysophosphatidic acid induces threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts via activation of protein kinase C. The Journal of biological chemistry *272*, 33105-33110.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., and Schioth, H.B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Molecular pharmacology *63*, 1256-1272.

Freychet, P., Roth, J., and Neville, D.M., Jr. (1971). Insulin receptors in the liver: specific binding of (125 I)insulin to the plasma membrane and its relation to insulin bioactivity. Proceedings of the National Academy of Sciences of the United States of America *68*, 1833-1837.

Frieser, S.H., Hlubek, A., Sandrock, B., and Bolker, M. (2011). Cla4 kinase triggers destruction of the Rac1-GEF Cdc24 during polarized growth in Ustilago maydis. Molecular biology of the cell *22*, 3253-3262.

Fritsch, R., de Krijger, I., Fritsch, K., George, R., Reason, B., Kumar, M.S., Diefenbacher, M., Stamp, G., and Downward, J. (2013). RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. Cell *153*, 1050-1063.

Fritz, G., Just, I., and Kaina, B. (1999). Rho GTPases are over-expressed in human tumors. International journal of cancer Journal international du cancer *81*, 682-687.

Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. Cell *109*, 611-623.

Funamoto, S., Milan, K., Meili, R., and Firtel, R.A. (2001). Role of phosphatidylinositol 3' kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in dictyostelium. The Journal of cell biology *153*, 795-810.

Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E.*, et al.* (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science signaling *6*, pl1.

George, S., Rochford, J.J., Wolfrum, C., Gray, S.L., Schinner, S., Wilson, J.C., Soos, M.A., Murgatroyd, P.R., Williams, R.M., Acerini, C.L.*, et al.* (2004). A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science *304*, 1325-1328.

Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. Annual review of biochemistry *56*, 615-649.

Gnesutta, N., Qu, J., and Minden, A. (2001). The serine/threonine kinase PAK4 prevents caspase activation and protects cells from apoptosis. The Journal of biological chemistry *276*, 14414-14419.

Gross, D.N., van den Heuvel, A.P., and Birnbaum, M.J. (2008). The role of FoxO in the regulation of metabolism. Oncogene *27*, 2320-2336.

Grotendorst, G.R., Chang, T., Seppa, H.E., Kleinman, H.K., and Martin, G.R. (1982). Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. Journal of cellular physiology *113*, 261-266.

Gulli, M.P., Jaquenoud, M., Shimada, Y., Niederhauser, G., Wiget, P., and Peter, M. (2000). Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. Molecular cell *6*, 1155-1167.

Guo, B., Liu, L., Yao, J., Ma, R., Chang, D., Li, Z., Song, T., and Huang, C. (2014). miR-338-3p suppresses gastric cancer progression through a PTEN-AKT axis by targeting P-REX2a. Molecular cancer research : MCR *12*, 313-321.

Gupta, S., Ramjaun, A.R., Haiko, P., Wang, Y., Warne, P.H., Nicke, B., Nye, E., Stamp, G., Alitalo, K., and Downward, J. (2007). Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell *129*, 957-968.

Ha, B.H., Davis, M.J., Chen, C., Lou, H.J., Gao, J., Zhang, R., Krauthammer, M., Halaban, R., Schlessinger, J., Turk, B.E., *et al.* (2012). Type II p21-activated kinases (PAKs) are regulated by an autoinhibitory pseudosubstrate. Proceedings of the National Academy of Sciences of the United States of America *109*, 16107-16112.

Haataja, L., Groffen, J., and Heisterkamp, N. (1997). Characterization of RAC3, a novel member of the Rho family. The Journal of biological chemistry *272*, 20384-20388.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A., and Broek, D. (1998). Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science *279*, 558-560.

Han, J.H., Kim, I.S., Jung, S.H., Lee, S.G., Son, H.Y., and Myung, C.S. (2014). The effects of propionate and valerate on insulin responsiveness for glucose uptake in 3T3-L1 adipocytes and C2C12 myotubes via G protein-coupled receptor 41. PloS one *9*, e95268.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R., *et al.* (2004). The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. The Journal of cell biology *166*, 213-223.

Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P.M., Olefsky, J.M., and Kobayashi, M. (2000). A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Molecular endocrinology *14*, 783-794.

Haugh, J.M., Codazzi, F., Teruel, M., and Meyer, T. (2000). Spatial sensing in fibroblasts mediated by 3' phosphoinositides. The Journal of cell biology *151*, 1269-1280.

Hawkins, P.T., Jackson, T.R., and Stephens, L.R. (1992). Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. Nature *358*, 157-159.

He, L., Fan, C., Kapoor, A., Ingram, A.J., Rybak, A.P., Austin, R.C., Dickhout, J., Cutz, J.C., Scholey, J., and Tang, D. (2011). alpha-Mannosidase 2C1 attenuates PTEN function in prostate cancer cells. Nature communications *2*, 307.

Hendrickx, A., Beullens, M., Ceulemans, H., Den Abt, T., Van Eynde, A., Nicolaescu, E., Lesage, B., and Bollen, M. (2009). Docking motif-guided mapping of the interactome of protein phosphatase-1. Chemistry & biology *16*, 365-371.

Hernandez-Negrete, I., Carretero-Ortega, J., Rosenfeldt, H., Hernandez-Garcia, R., Calderon-Salinas, J.V., Reyes-Cruz, G., Gutkind, J.S., and Vazquez-Prado, J. (2007). P-Rex1 links mammalian target of rapamycin signaling to Rac activation and cell migration. The Journal of biological chemistry *282*, 23708-23715.

Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F., *et al.* (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. Cell *70*, 419-429.

Hill, K., and Welch, H.C. (2006). Purification of P-Rex1 from neutrophils and nucleotide exchange assay. Methods in enzymology *406*, 26-41.

Hirshberg, M., Stockley, R.W., Dodson, G., and Webb, M.R. (1997). The crystal structure of human rac1, a member of the rho-family complexed with a GTP analogue. Nature structural biology *4*, 147-152.

Hodakoski, C., Hopkins, B.D., Barrows, D., Mense, S.M., Keniry, M., Anderson, K.E., Kern, P.A., Hawkins, P.T., Stephens, L.R., and Parsons, R. (2014). Regulation of PTEN inhibition by the pleckstrin homology domain of P-REX2 during insulin signaling and glucose homeostasis. Proceedings of the National Academy of Sciences of the United States of America *111*, 155-160.

Hoeller, O., and Kay, R.R. (2007). Chemotaxis in the absence of PIP3 gradients. Current biology : CB *17*, 813-817.

Hopkins, B.D., Hodakoski, C., Barrows, D., Mense, S.M., and Parsons, R.E. (2014). PTEN function: the long and the short of it. Trends in biochemical sciences *39*, 183-190.

Hu, P., Mondino, A., Skolnik, E.Y., and Schlessinger, J. (1993). Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. Molecular and cellular biology *13*, 7677-7688.

Iijima, M., and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. Cell *109*, 599-610.

Ikeda, S.R. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. Nature *380*, 255-258.

Ikenoue, T., Kanai, F., Hikiba, Y., Obata, T., Tanaka, Y., Imamura, J., Ohta, M., Jazag, A., Guleng, B., Tateishi, K., *et al.* (2005). Functional analysis of PIK3CA gene mutations in human colorectal cancer. Cancer research *65*, 4562-4567.

Innocenti, M., Frittoli, E., Ponzanelli, I., Falck, J.R., Brachmann, S.M., Di Fiore, P.P., and Scita, G. (2003). Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. The Journal of cell biology *160*, 17-23.

Isakoff, S.J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K.M., Abagyan, R., Lemmon, M.A., Aronheim, A., and Skolnik, E.Y. (1998). Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. The EMBO journal *17*, 5374-5387.

Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearline, R.V., Cantley, L.C., and Brugge, J.S. (2005). Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer research *65*, 10992-11000.

Ismail, A.M., Padrick, S.B., Chen, B., Umetani, J., and Rosen, M.K. (2009). The WAVE regulatory complex is inhibited. Nature structural & molecular biology *16*, 561-563.

JeBailey, L., Rudich, A., Huang, X., Di Ciano-Oliveira, C., Kapus, A., and Klip, A. (2004). Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. Molecular endocrinology *18*, 359-372.

JeBailey, L., Wanono, O., Niu, W., Roessler, J., Rudich, A., and Klip, A. (2007). Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells. Diabetes *56*, 394-403.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., and Czech, M.P. (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proceedings of the National Academy of Sciences of the United States of America *100*, 7569-7574.

Jin, S., Zhuo, Y., Guo, W., and Field, J. (2005). p21-activated Kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. The Journal of biological chemistry *280*, 24698-24705.

Jones, P.F., Jakubowicz, T., and Hemmings, B.A. (1991). Molecular cloning of a second form of rac protein kinase. Cell regulation *2*, 1001-1009.

Jordan, P., Brazao, R., Boavida, M.G., Gespach, C., and Chastre, E. (1999). Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. Oncogene *18*, 6835-6839.

Joseph, R.E., and Norris, F.A. (2005). Substrate specificity and recognition is conferred by the pleckstrin homology domain of the Dbl family guanine nucleotide exchange factor P-Rex2. The Journal of biological chemistry *280*, 27508-27512.

Kane, S., Sano, H., Liu, S.C., Asara, J.M., Lane, W.S., Garner, C.C., and Lienhard, G.E. (2002). A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. The Journal of biological chemistry *277*, 22115-22118.

Kang, S., Bader, A.G., and Vogt, P.K. (2005). Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proceedings of the National Academy of Sciences of the United States of America *102*, 802-807.

Kaplan, D.R., Whitman, M., Schaffhausen, B., Raptis, L., Garcea, R.L., Pallas, D., Roberts, T.M., and Cantley, L. (1986). Phosphatidylinositol metabolism and polyoma-mediated transformation. Proceedings of the National Academy of Sciences of the United States of America *83*, 3624-3628.

Kasuga, M., Karlsson, F.A., and Kahn, C.R. (1982a). Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science *215*, 185-187.

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., and Kahn, C.R. (1982b). Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature *298*, 667-669.

Khan, S.M., Sleno, R., Gora, S., Zylbergold, P., Laverdure, J.P., Labbe, J.C., Miller, G.J., and Hebert, T.E. (2013). The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. Pharmacological reviews *65*, 545-577.

Khayat, Z.A., Tong, P., Yaworsky, K., Bloch, R.J., and Klip, A. (2000). Insulin-induced actin filament remodeling colocalizes actin with phosphatidylinositol 3-kinase and GLUT4 in L6 myotubes. Journal of cell science *113 Pt 2*, 279-290.

Kichina, J.V., Goc, A., Al-Husein, B., Somanath, P.R., and Kandel, E.S. (2010). PAK1 as a therapeutic target. Expert opinion on therapeutic targets *14*, 703-725.

King, A.J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M.S. (1998). The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature *396*, 180-183.

King, H., Nicholas, N.S., and Wells, C.M. (2014). Role of p-21-activated kinases in cancer progression. International review of cell and molecular biology *309*, 347-387.

Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T., and Bokoch, G.M. (1991). Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. Science *254*, 1512-1515.

Kobilka, B.K. (2007). G protein coupled receptor structure and activation. Biochimica et biophysica acta *1768*, 794-807.

Kohn, A.D., Kovacina, K.S., and Roth, R.A. (1995). Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. The EMBO journal *14*, 4288-4295.

Kohn, A.D., Summers, S.A., Birnbaum, M.J., and Roth, R.A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. The Journal of biological chemistry *271*, 31372-31378.

Komander, D., Fairservice, A., Deak, M., Kular, G.S., Prescott, A.R., Peter Downes, C., Safrany, S.T., Alessi, D.R., and van Aalten, D.M. (2004). Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. The EMBO journal *23*, 3918-3928.

Komazawa, N., Matsuda, M., Kondoh, G., Mizunoya, W., Iwaki, M., Takagi, T., Sumikawa, Y., Inoue, K., Suzuki, A., Mak, T.W., *et al.* (2004). Enhanced insulin sensitivity, energy expenditure and thermogenesis in adipose-specific Pten suppression in mice. Nature medicine *10*, 1208-1215.

Koren, S., and Bentires-Alj, M. (2013). Mouse models of PIK3CA mutations: one mutation initiates heterogeneous mammary tumors. The FEBS journal *280*, 2758-2765.

Kotelevets, L., van Hengel, J., Bruyneel, E., Mareel, M., van Roy, F., and Chastre, E. (2005). Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 115-117.

Krauthammer, M., Kong, Y., Ha, B.H., Evans, P., Bacchiocchi, A., McCusker, J.P., Cheng, E., Davis, M.J., Goh, G., Choi, M., *et al.* (2012). Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nature genetics *44*, 1006-1014.

Krook, A., Roth, R.A., Jiang, X.J., Zierath, J.R., and Wallberg-Henriksson, H. (1998). Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. Diabetes *47*, 1281-1286.

Kuo, C.C., Savage, N.S., Chen, H., Wu, C.F., Zyla, T.R., and Lew, D.J. (2014). Inhibitory GEF phosphorylation provides negative feedback in the yeast polarity circuit. Current biology : CB *24*, 753-759.

Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Ui, M., Hazeki, O., and Katada, T. (1997). Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. The Journal of biological chemistry *272*, 24252-24256.

Kushner, J.A., Simpson, L., Wartschow, L.M., Guo, S., Rankin, M.M., Parsons, R., and White, M.F. (2005). Phosphatase and tensin homolog regulation of islet growth and glucose homeostasis. The Journal of biological chemistry *280*, 39388-39393.

Kuziemko, A., Honig, B., and Petrey, D. (2011). Using structure to explore the sequence alignment space of remote homologs. PLoS computational biology *7*, e1002175.

Lavan, B.E., Fantin, V.R., Chang, E.T., Lane, W.S., Keller, S.R., and Lienhard, G.E. (1997). A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. The Journal of biological chemistry *272*, 21403-21407.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., and Pavletich, N.P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell *99*, 323-334.

Lei, M., Lu, W., Meng, W., Parrini, M.C., Eck, M.J., Mayer, B.J., and Harrison, S.C. (2000). Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell *102*, 387-397.

Lemmon, M.A. (2008). Membrane recognition by phospholipid-binding domains. Nature reviews Molecular cell biology *9*, 99-111.

Leto, D., and Saltiel, A.R. (2012). Regulation of glucose transport by insulin: traffic control of GLUT4. Nature reviews Molecular cell biology *13*, 383-396.

Levine, R., Goldstein, M.S., Huddlestun, B., and Klein, S.P. (1950). Action of insulin on the 'permeability' of cells to free hexoses, as studied by its effect on the distribution of galactose. The American journal of physiology *163*, 70-76.

Li, D.M., and Sun, H. (1998). PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proceedings of the National Academy of Sciences of the United States of America *95*, 15406-15411.

Li, G., Robinson, G.W., Lesche, R., Martinez-Diaz, H., Jiang, Z., Rozengurt, N., Wagner, K.U., Wu, D.C., Lane, T.F., Liu, X., *et al.* (2002). Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. Development *129*, 4159-4170.

Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M.P., Tonks, N., and Parsons, R. (1998). The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer research *58*, 5667-5672.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., *et al.* (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.

Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., *et al.* (2005a). Regulation of PTEN by Rho small GTPases. Nature cell biology *7*, 399-404.

Li, Z., Paik, J.H., Wang, Z., Hla, T., and Wu, D. (2005b). Role of guanine nucleotide exchange factor P-Rex-2b in sphingosine 1-phosphate-induced Rac1 activation and cell migration in endothelial cells. Prostaglandins & other lipid mediators *76*, 95-104.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., *et al.* (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature genetics *16*, 64-67.

Lima-Fernandes, E., Enslen, H., Camand, E., Kotelevets, L., Boularan, C., Achour, L., Benmerah, A., Gibson, L.C., Baillie, G.S., Pitcher, J.A., *et al.* (2011). Distinct functional outputs of PTEN signalling are controlled by dynamic association with beta-arrestins. The EMBO journal *30*, 2557-2568.

Lindsay, C.R., Lawn, S., Campbell, A.D., Faller, W.J., Rambow, F., Mort, R.L., Timpson, P., Li, A., Cammareri, P., Ridgway, R.A., *et al.* (2011). P-Rex1 is required for efficient melanoblast migration and melanoma metastasis. Nature communications *2*, 555.

Logothetis, D.E., Kurachi, Y., Galper, J., Neer, E.J., and Clapham, D.E. (1987). The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature *325*, 321-326.

Loovers, H.M., Postma, M., Keizer-Gunnink, I., Huang, Y.E., Devreotes, P.N., and van Haastert, P.J. (2006). Distinct roles of PI(3,4,5)P3 during chemoattractant signaling in Dictyostelium: a quantitative in vivo analysis by inhibition of PI3-kinase. Molecular biology of the cell *17*, 1503-1513.

Lopez-Lago, M., Lee, H., Cruz, C., Movilla, N., and Bustelo, X.R. (2000). Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav. Molecular and cellular biology *20*, 1678-1691.

Lu, Y., Yu, Q., Liu, J.H., Zhang, J., Wang, H., Koul, D., McMurray, J.S., Fang, X., Yung, W.K., Siminovitch, K.A., *et al.* (2003). Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. The Journal of biological chemistry *278*, 40057-40066.

Luthy, R., Bowie, J.U., and Eisenberg, D. (1992). Assessment of protein models with three-dimensional profiles. Nature *356*, 83-85.

Macara, I.G., Marinetti, G.V., and Balduzzi, P.C. (1984). Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *81*, 2728-2732.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. The Journal of biological chemistry *273*, 13375-13378.

Maira, S.M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., Brachmann, S., Chene, P., De Pover, A., Schoemaker, K., *et al.* (2008). Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Molecular cancer therapeutics *7*, 1851-1863.

Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S., and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature *367*, 40-46.

Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L., Zheng, Z., Liaw, D., Caron, S., Duboue, B., Lin, A.Y., *et al.* (1998). Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. Human molecular genetics *7*, 507-515.

Matos, P., Collard, J.G., and Jordan, P. (2003). Tumor-related alternatively spliced Rac1b is not regulated by Rho-GDP dissociation inhibitors and exhibits selective downstream signaling. The Journal of biological chemistry *278*, 50442-50448.

Mayeenuddin, L.H., and Garrison, J.C. (2006). Phosphorylation of P-Rex1 by the cyclic AMP-dependent protein kinase inhibits the phosphatidylinositiol (3,4,5)-trisphosphate and Gbetagamma-mediated regulation of its activity. The Journal of biological chemistry *281*, 1921-1928.

Meili, R., Ellsworth, C., Lee, S., Reddy, T.B., Ma, H., and Firtel, R.A. (1999). Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. The EMBO journal *18*, 2092-2105.

Mense, S.M., Barrows, D., Hodakoski, C., Steinbach, N., Schoenfeld, D., Su, W., Hopkins, B.D., Su, T., Fine, B., Hibshoosh, H.*, et al.* (2015). PTEN inhibits PREX2-catalyzed activation of RAC1 to restrain tumor cell invasion. Science signaling *8*, ra32.

Miller, S.J., Lou, D.Y., Seldin, D.C., Lane, W.S., and Neel, B.G. (2002). Direct identification of PTEN phosphorylation sites. FEBS letters *528*, 145-153.

Montero, J.C., Seoane, S., Ocana, A., and Pandiella, A. (2011). P-Rex1 participates in Neuregulin-ErbB signal transduction and its expression correlates with patient outcome in breast cancer. Oncogene *30*, 1059-1071.

Montero, J.C., Seoane, S., and Pandiella, A. (2013). Phosphorylation of P-Rex1 at serine 1169 participates in IGF-1R signaling in breast cancer cells. Cellular signalling *25*, 2281-2289.

Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P., and Tonks, N.K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proceedings of the National Academy of Sciences of the United States of America *95*, 13513-13518.

Nakae, J., Biggs, W.H., 3rd, Kitamura, T., Cavenee, W.K., Wright, C.V., Arden, K.C., and Accili, D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nature genetics *32*, 245-253.

Nakatani, K., Sakaue, H., Thompson, D.A., Weigel, R.J., and Roth, R.A. (1999). Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site. Biochemical and biophysical research communications *257*, 906-910.

Nie, B., Cheng, N., Dinauer, M.C., and Ye, R.D. (2010). Characterization of P-Rex1 for its role in fMet-Leu-Phe-induced superoxide production in reconstituted COS(phox) cells. Cellular signalling *22*, 770-782.

Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L., *et al.* (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer research *66*, 1500-1508.

Obenauer, J.C., Cantley, L.C., and Yaffe, M.B. (2003). Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic acids research *31*, 3635-3641.

Oh da, Y., Walenta, E., Akiyama, T.E., Lagakos, W.S., Lackey, D., Pessentheiner, A.R., Sasik, R., Hah, N., Chi, T.J., Cox, J.M., *et al.* (2014). A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nature medicine *20*, 942-947.

Oh, D.Y., Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M., and Olefsky, J.M. (2010). GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell *142*, 687-698.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., *et al.* (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. Cell *65*, 91-104.

Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H., Hawkins, P.T., Stephens, L., Eccleston, J.F., and Williams, R.L. (2000). Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. Cell *103*, 931-943.

Pal, A., Barber, T.M., Van de Bunt, M., Rudge, S.A., Zhang, Q., Lachlan, K.L., Cooper, N.S., Linden, H., Levy, J.C., Wakelam, M.J., *et al.* (2012). PTEN mutations as a cause of constitutive insulin sensitivity and obesity. The New England journal of medicine *367*, 1002-1011.

Panayotou, G., Bax, B., Gout, I., Federwisch, M., Wroblowski, B., Dhand, R., Fry, M.J., Blundell, T.L., Wollmer, A., and Waterfield, M.D. (1992). Interaction of the p85 subunit of PI 3-kinase and its N-terminal SH2 domain with a PDGF receptor phosphorylation site: structural features and analysis of conformational changes. The EMBO journal *11*, 4261-4272.

Park, W.S., Heo, W.D., Whalen, J.H., O'Rourke, N.A., Bryan, H.M., Meyer, T., and Teruel, M.N. (2008). Comprehensive identification of PIP3-regulated PH domains from

C. elegans to H. sapiens by model prediction and live imaging. Molecular cell *30*, 381-392.

Pende, M., Kozma, S.C., Jaquet, M., Oorschot, V., Burcelin, R., Le Marchand-Brustel, Y., Klumperman, J., Thorens, B., and Thomas, G. (2000). Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. Nature *408*, 994-997.

Petruzzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S., and Rosen, O.M. (1982). Insulin activates a tyrosine-specific protein kinase in extracts of 3T3-L1 adipocytes and human placenta. Proceedings of the National Academy of Sciences of the United States of America *79*, 6792-6796.

Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proceedings of the National Academy of Sciences of the United States of America *96*, 1563-1568.

Qin, J., Xie, Y., Wang, B., Hoshino, M., Wolff, D.W., Zhao, J., Scofield, M.A., Dowd, F.J., Lin, M.F., and Tu, Y. (2009). Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis. Oncogene *28*, 1853-1863.

Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S., and Hall, A. (2004). Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. Science *303*, 1179-1181.

Rahdar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F., and Devreotes, P.N. (2009). A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. Proceedings of the National Academy of Sciences of the United States of America *106*, 480-485.

Rebecchi, M.J., and Scarlata, S. (1998). Pleckstrin homology domains: a common fold with diverse functions. Annual review of biophysics and biomolecular structure *27*, 503-528.

Rennefahrt, U.E., Deacon, S.W., Parker, S.A., Devarajan, K., Beeser, A., Chernoff, J., Knapp, S., Turk, B.E., and Peterson, J.R. (2007). Specificity profiling of Pak kinases allows identification of novel phosphorylation sites. The Journal of biological chemistry *282*, 15667-15678.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell *70*, 401-410.

Roden, M., Price, T.B., Perseghin, G., Petersen, K.F., Rothman, D.L., Cline, G.W., and Shulman, G.I. (1996). Mechanism of free fatty acid-induced insulin resistance in humans. The Journal of clinical investigation *97*, 2859-2865.

Rodriguez-Viciana, P., Sabatier, C., and McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Molecular and cellular biology *24*, 4943-4954.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature *370*, 527-532.

Rodriguez-Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D., and Downward, J. (1996). Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. The EMBO journal *15*, 2442-2451.

Rosenfeldt, H., Vazquez-Prado, J., and Gutkind, J.S. (2004). P-REX2, a novel PI-3-kinase sensitive Rac exchange factor. FEBS letters *572*, 167-171.

Rossman, K.L., Der, C.J., and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nature reviews Molecular cell biology *6*, 167-180.

Ruderman, N.B., Kapeller, R., White, M.F., and Cantley, L.C. (1990). Activation of phosphatidylinositol 3-kinase by insulin. Proceedings of the National Academy of Sciences of the United States of America *87*, 1411-1415.

Samuel, V.T., and Shulman, G.I. (2012). Mechanisms for insulin resistance: common threads and missing links. Cell *148*, 852-871.

Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. Science *283*, 2083-2085.

Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W., and Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. The Journal of biological chemistry *278*, 14599-14602.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes & development *16*, 1587-1609.

Schnelzer, A., Prechtel, D., Knaus, U., Dehne, K., Gerhard, M., Graeff, H., Harbeck, N., Schmitt, M., and Lengyel, E. (2000). Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene *19*, 3013-3020.

Sells, M.A., Knaus, U.G., Bagrodia, S., Ambrose, D.M., Bokoch, G.M., and Chernoff, J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Current biology : CB *7*, 202-210.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science *287*, 1037-1040.

Servant, G., Weiner, O.D., Neptune, E.R., Sedat, J.W., and Bourne, H.R. (1999). Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. Molecular biology of the cell *10*, 1163-1178.

Shinohara, M., Terada, Y., Iwamatsu, A., Shinohara, A., Mochizuki, N., Higuchi, M., Gotoh, Y., Ihara, S., Nagata, S., Itoh, H., *et al.* (2002). SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. Nature *416*, 759-763.

Shymanets, A., Ahmadian, M.R., Kossmeier, K.T., Wetzker, R., Harteneck, C., and Nurnberg, B. (2012). The p101 subunit of PI3Kgamma restores activation by Gbeta mutants deficient in stimulating p110gamma. The Biochemical journal *441*, 851-858.

Shymanets, A., Ahmadian, M.R., and Nurnberg, B. (2009). Gbetagamma-copurified lipid kinase impurity from Sf9 cells. Protein and peptide letters *16*, 1053-1056.

Shymanets, A., Prajwal, Bucher, K., Beer-Hammer, S., Harteneck, C., and Nurnberg, B. (2013). p87 and p101 subunits are distinct regulators determining class IB phosphoinositide 3-kinase (PI3K) specificity. The Journal of biological chemistry *288*, 31059-31068.

Simpson, L., Li, J., Liaw, D., Hennessy, I., Oliner, J., Christians, F., and Parsons, R. (2001). PTEN expression causes feedback upregulation of insulin receptor substrate 2. Molecular and cellular biology *21*, 3947-3958.

Singh, B., Ittmann, M.M., and Krolewski, J.J. (1998). Sporadic breast cancers exhibit loss of heterozygosity on chromosome segment 10q23 close to the Cowden disease locus. Genes, chromosomes & cancer *21*, 166-171.

Sippl, M.J. (1993). Recognition of errors in three-dimensional structures of proteins. Proteins *17*, 355-362.

Skolnik, E.Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A., and Schlessinger, J. (1991). Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell *65*, 83-90.

Slack-Davis, J.K., Eblen, S.T., Zecevic, M., Boerner, S.A., Tarcsafalvi, A., Diaz, H.B., Marshall, M.S., Weber, M.J., Parsons, J.T., and Catling, A.D. (2003). PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. The Journal of cell biology *162*, 281-291.

Sosa, M.S., Lopez-Haber, C., Yang, C., Wang, H., Lemmon, M.A., Busillo, J.M., Luo, J., Benovic, J.L., Klein-Szanto, A., Yagi, H.*, et al.* (2010). Identification of the Rac-GEF P-Rex1 as an essential mediator of ErbB signaling in breast cancer. Molecular cell *40*, 877-892.

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Stambolic, V., Tsao, M.S., Macpherson, D., Suzuki, A., Chapman, W.B., and Mak, T.W. (2000). High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/- mice. Cancer research *60*, 3605-3611.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T.*, et al.* (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nature genetics *15*, 356-362.

Stephens, L., Smrcka, A., Cooke, F.T., Jackson, T.R., Sternweis, P.C., and Hawkins, P.T. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell *77*, 83-93.

Stephens, L.R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S., Thelen, M., Cadwallader, K., Tempst, P.*, et al.* (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell *89*, 105-114.

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

Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F., and Hawkins, P.T. (1997). Dual role of

phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science *277*, 567-570.

Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., *et al.* (1995). Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. Science *269*, 690-693.

Sugimoto, Y., Whitman, M., Cantley, L.C., and Erikson, R.L. (1984). Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. Proceedings of the National Academy of Sciences of the United States of America *81*, 2117-2121.

Suire, S., Coadwell, J., Ferguson, G.J., Davidson, K., Hawkins, P., and Stephens, L. (2005). p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. Current biology : CB *15*, 566-570.

Suire, S., Condliffe, A.M., Ferguson, G.J., Ellson, C.D., Guillou, H., Davidson, K., Welch, H., Coadwell, J., Turner, M., Chilvers, E.R., *et al.* (2006). Gbetagammas and the Ras binding domain of p110gamma are both important regulators of PI(3)Kgamma signalling in neutrophils. Nature cell biology *8*, 1303-1309.

Sumitomo, M., Iwase, A., Zheng, R., Navarro, D., Kaminetzky, D., Shen, R., Georgescu, M.M., and Nanus, D.M. (2004). Synergy in tumor suppression by direct interaction of neutral endopeptidase with PTEN. Cancer cell *5*, 67-78.

Sun, H., King, A.J., Diaz, H.B., and Marshall, M.S. (2000). Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. Current biology : CB *10*, 281-284.

Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., *et al.* (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Current biology : CB *8*, 1169-1178.

Sylow, L., Jensen, T.E., Kleinert, M., Hojlund, K., Kiens, B., Wojtaszewski, J., Prats, C., Schjerling, P., and Richter, E.A. (2013). Rac1 signaling is required for insulin-stimulated glucose uptake and is dysregulated in insulin-resistant murine and human skeletal muscle. Diabetes *62*, 1865-1875.

Sylow, L., Kleinert, M., Pehmoller, C., Prats, C., Chiu, T.T., Klip, A., Richter, E.A., and Jensen, T.E. (2014). Akt and Rac1 signaling are jointly required for insulin-stimulated glucose uptake in skeletal muscle and downregulated in insulin resistance. Cellular signalling *26*, 323-331.

Takahashi, Y., Morales, F.C., Kreimann, E.L., and Georgescu, M.M. (2006). PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. The EMBO journal *25*, 910-920.

Takano, A., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M., and Kobayashi, M. (2001). Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Molecular and cellular biology *21*, 5050-5062.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., *et al.* (1994). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature *372*, 182-186.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K.M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science *280*, 1614-1617.

Tang, W.J., and Gilman, A.G. (1991). Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science *254*, 1500-1503.

Tang, Y., Chen, Z., Ambrose, D., Liu, J., Gibbs, J.B., Chernoff, J., and Field, J. (1997). Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. Molecular and cellular biology *17*, 4454-4464.

Taniguchi, C.M., Emanuelli, B., and Kahn, C.R. (2006). Critical nodes in signalling pathways: insights into insulin action. Nature reviews Molecular cell biology *7*, 85-96.

Thomas, C.C., Deak, M., Alessi, D.R., and van Aalten, D.M. (2002). High-resolution structure of the pleckstrin homology domain of protein kinase b/akt bound to phosphatidylinositol (3,4,5)-trisphosphate. Current biology : CB *12*, 1256-1262.

Tibarewal, P., Zilidis, G., Spinelli, L., Schurch, N., Maccario, H., Gray, A., Perera, N.M., Davidson, L., Barton, G.J., and Leslie, N.R. (2012). PTEN protein phosphatase activity correlates with control of gene expression and invasion, a tumor-suppressing phenotype, but not with AKT activity. Science signaling *5*, ra18.

Tolkacheva, T., Boddapati, M., Sanfiz, A., Tsuchida, K., Kimmelman, A.C., and Chan, A.M. (2001). Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383. Cancer research *61*, 4985-4989.

Tong, P., Khayat, Z.A., Huang, C., Patel, N., Ueyama, A., and Klip, A. (2001). Insulin-induced cortical actin remodeling promotes GLUT4 insertion at muscle cell membrane ruffles. The Journal of clinical investigation *108*, 371-381.

Torres, J., and Pulido, R. (2001). The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. The Journal of biological chemistry *276*, 993-998.

Tran, N.H., and Frost, J.A. (2003). Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. The Journal of biological chemistry *278*, 11221-11226.

Traynor-Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G.M., and Sklar, L.A. (1989). Transient increase in phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. The Journal of biological chemistry *264*, 15668-15673.

Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., *et al.* (2003). Pten dose dictates cancer progression in the prostate. PLoS biology *1*, E59.

Tsakiridis, T., Taha, C., Grinstein, S., and Klip, A. (1996). Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. The Journal of biological chemistry *271*, 19664-19667.

Tsakiridis, T., Vranic, M., and Klip, A. (1994). Disassembly of the actin network inhibits insulin-dependent stimulation of glucose transport and prevents recruitment of glucose transporters to the plasma membrane. The Journal of biological chemistry *269*, 29934-29942.

Tunduguru, R., Chiu, T.T., Ramalingam, L., Elmendorf, J.S., Klip, A., and Thurmond, D.C. (2014). Signaling of the p21-activated kinase (PAK1) coordinates insulin-stimulated actin remodeling and glucose uptake in skeletal muscle cells. Biochemical pharmacology *92*, 380-388.

Ueda, S., Kataoka, T., and Satoh, T. (2008). Activation of the small GTPase Rac1 by a specific guanine-nucleotide-exchange factor suffices to induce glucose uptake into skeletal-muscle cells. Biology of the cell / under the auspices of the European Cell Biology Organization *100*, 645-657.

Ueda, S., Kitazawa, S., Ishida, K., Nishikawa, Y., Matsui, M., Matsumoto, H., Aoki, T., Nozaki, S., Takeda, T., Tamori, Y., *et al.* (2010). Crucial role of the small GTPase Rac1 in insulin-stimulated translocation of glucose transporter 4 to the mouse skeletal muscle sarcolemma. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *24*, 2254-2261.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.C., Tsubokawa, M.*, et al.* (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature *313*, 756-761.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J.*, et al.* (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature *431*, 200-205.

Urano, D., Nakata, A., Mizuno, N., Tago, K., and Itoh, H. (2008). Domain-domain interaction of P-Rex1 is essential for the activation and inhibition by G protein betagamma subunits and PKA. Cellular signalling *20*, 1545-1554.

Van Obberghen, E., and Kowalski, A. (1982). Phosphorylation of the hepatic insulin receptor: stimulating effect of insulin on intact cells and in a cell-free system. FEBS letters *143*, 179-182.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. Nature reviews Molecular cell biology *11*, 329-341.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J., and Waterfield, M.D. (1997). P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proceedings of the National Academy of Sciences of the United States of America *94*, 4330-4335.

Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W.R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. Molecular and cellular biology *20*, 5010-5018.

Vetter, I.R., and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. Science *294*, 1299-1304.

Voigt, P., Dorner, M.B., and Schaefer, M. (2006). Characterization of p87PIKAP, a novel regulatory subunit of phosphoinositide 3-kinase gamma that is highly expressed in heart and interacts with PDE3B. The Journal of biological chemistry *281*, 9977-9986.

Wang, J., Rao, Q., Wang, M., Wei, H., Xing, H., Liu, H., Wang, Y., Tang, K., Peng, L., Tian, Z.*, et al.* (2009). Overexpression of Rac1 in leukemia patients and its role in leukemia cell migration and growth. Biochemical and biophysical research communications *386*, 769-774.

Wang, Q., Bilan, P.J., Tsakiridis, T., Hinek, A., and Klip, A. (1998). Actin filaments participate in the relocalization of phosphatidylinositol3-kinase to glucose transporter-

containing compartments and in the stimulation of glucose uptake in 3T3-L1 adipocytes. The Biochemical journal *331 (Pt 3)*, 917-928.

Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., *et al.* (2003). Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer cell *4*, 209-221.

Wang, S.I., Puc, J., Li, J., Bruce, J.N., Cairns, P., Sidransky, D., and Parsons, R. (1997). Somatic mutations of PTEN in glioblastoma multiforme. Cancer research *57*, 4183-4186.

Wang, W., Lim, L., Baskaran, Y., Manser, E., and Song, J. (2013). NMR binding and crystal structure reveal that intrinsically-unstructured regulatory domain auto-inhibits PAK4 by a mechanism different from that of PAK1. Biochemical and biophysical research communications *438*, 169-174.

Wang, Z., Oh, E., Clapp, D.W., Chernoff, J., and Thurmond, D.C. (2011). Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. The Journal of biological chemistry *286*, 41359-41367.

Webb, B., and Sali, A. (2014). Comparative Protein Structure Modeling Using MODELLER. Current protocols in bioinformatics / editoral board, Andreas D Baxevanis [et al] *47*, 5 6 1-5 6 32.

Welch, H.C., Coadwell, W.J., Ellson, C.D., Ferguson, G.J., Andrews, S.R., Erdjument-Bromage, H., Tempst, P., Hawkins, P.T., and Stephens, L.R. (2002). P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell *108*, 809-821.

Welch, H.C., Condliffe, A.M., Milne, L.J., Ferguson, G.J., Hill, K., Webb, L.M., Okkenhaug, K., Coadwell, W.J., Andrews, S.R., Thelen, M., *et al.* (2005). P-Rex1 regulates neutrophil function. Current biology : CB *15*, 1867-1873.

Wertheimer, E., Gutierrez-Uzquiza, A., Rosemblit, C., Lopez-Haber, C., Sosa, M.S., and Kazanietz, M.G. (2012). Rac signaling in breast cancer: a tale of GEFs and GAPs. Cellular signalling *24*, 353-362.

White, M.F., Maron, R., and Kahn, C.R. (1985). Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. Nature *318*, 183-186.

Whitehead, J.P., Humphreys, P., Krook, A., Jackson, R., Hayward, A., Lewis, H., Siddle, K., and O'Rahilly, S. (1998). Molecular scanning of the insulin receptor substrate 1 gene in subjects with severe insulin resistance: detection and functional analysis of a naturally occurring mutation in a YMXM motif. Diabetes *47*, 837-839.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., and Cantley, L. (1988). Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature *332*, 644-646.

Whitman, M., Kaplan, D.R., Schaffhausen, B., Cantley, L., and Roberts, T.M. (1985). Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature *315*, 239-242.

Wijesekara, N., Konrad, D., Eweida, M., Jefferies, C., Liadis, N., Giacca, A., Crackower, M., Suzuki, A., Mak, T.W., Kahn, C.R.*, et al.* (2005). Muscle-specific Pten deletion protects against insulin resistance and diabetes. Molecular and cellular biology *25*, 1135-1145.

Withers, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I.*, et al.* (1998). Disruption of IRS-2 causes type 2 diabetes in mice. Nature *391*, 900-904.

Worthylake, D.K., Rossman, K.L., and Sondek, J. (2000). Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature *408*, 682-688.

Wu, X., Carr, H.S., Dan, I., Ruvolo, P.P., and Frost, J.A. (2008). p21 activated kinase 5 activates Raf-1 and targets it to mitochondria. Journal of cellular biochemistry *105*, 167-175.

Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M.B., Yuan, X.J., Wood, J., Ross, C., Sawyers, C.L., and Whang, Y.E. (2000a). Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. Proceedings of the National Academy of Sciences of the United States of America *97*, 4233-4238.

Wu, Y., Dowbenko, D., Spencer, S., Laura, R., Lee, J., Gu, Q., and Lasky, L.A. (2000b). Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. The Journal of biological chemistry *275*, 21477-21485.

Xiao, Z., Zhang, N., Murphy, D.B., and Devreotes, P.N. (1997). Dynamic distribution of chemoattractant receptors in living cells during chemotaxis and persistent stimulation. The Journal of cell biology *139*, 365-374.

Xie, X., Gong, Z., Mansuy-Aubert, V., Zhou, Q.L., Tatulian, S.A., Sehrt, D., Gnad, F., Brill, L.M., Motamedchaboki, K., Chen, Y.*, et al.* (2011). C2 domain-containing phosphoprotein CDP138 regulates GLUT4 insertion into the plasma membrane. Cell metabolism *14*, 378-389.

Xu, J., Xu, Z., Zhou, J.Y., Zhuang, Z., Wang, E., Boerner, J., and Wu, G.S. (2013). Regulation of the Src-PP2A interaction in tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. The Journal of biological chemistry *288*, 33263-33271.

Yang, F., Li, X., Sharma, M., Zarnegar, M., Lim, B., and Sun, Z. (2001). Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6. The Journal of biological chemistry *276*, 15345-15353.

Ye, D.Z., Jin, S., Zhuo, Y., and Field, J. (2011). p21-Activated kinase 1 (Pak1) phosphorylates BAD directly at serine 111 in vitro and indirectly through Raf-1 at serine 112. PloS one *6*, e27637.

Yim, E.K., Peng, G., Dai, H., Hu, R., Li, K., Lu, Y., Mills, G.B., Meric-Bernstam, F., Hennessy, B.T., Craven, R.J.*, et al.* (2009). Rak functions as a tumor suppressor by regulating PTEN protein stability and function. Cancer cell *15*, 304-314.

Yoshizawa, M., Kawauchi, T., Sone, M., Nishimura, Y.V., Terao, M., Chihama, K., Nabeshima, Y., and Hoshino, M. (2005). Involvement of a Rac activator,P-Rex1, in neurotrophin-derived signaling and neuronal migration. The Journal of neuroscience : the official journal of the Society for Neuroscience *25*, 4406-4419.

Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J.K., Cushman, S.W., Cooney, G.J.*, et al.* (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. The Journal of biological chemistry *277*, 50230-50236.

Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G.A., and Backer, J.M. (1998). Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Molecular and cellular biology *18*, 1379-1387.

Zhang, X.C., Piccini, A., Myers, M.P., Van Aelst, L., and Tonks, N.K. (2012). Functional analysis of the protein phosphatase activity of PTEN. The Biochemical journal *444*, 457-464.

Zhao, J.J., Liu, Z., Wang, L., Shin, E., Loda, M.F., and Roberts, T.M. (2005). The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells. Proceedings of the National Academy of Sciences of the United States of America *102*, 18443-18448.

Zhao, T., Nalbant, P., Hoshino, M., Dong, X., Wu, D., and Bokoch, G.M. (2007). Signaling requirements for translocation of P-Rex1, a key Rac2 exchange factor involved

in chemoattractant-stimulated human neutrophil function. Journal of leukocyte biology *81*, 1127-1136.

Zhou, C., Licciulli, S., Avila, J.L., Cho, M., Troutman, S., Jiang, P., Kossenkov, A.V., Showe, L.C., Liu, Q., Vachani, A.*, et al.* (2013). The Rac1 splice form Rac1b promotes K-ras-induced lung tumorigenesis. Oncogene *32*, 903-909.