Noncanonical regulation of insulin-mediated ERK activation by phosphoinositide 3-kinase γ

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ABSTRACT  Classically Class IB phosphoinositide 3-kinase (PI3Kγ) plays a role in extracellular signal–regulated kinase (ERK) activation following G-protein coupled receptor (GPCR) activation. Knock-down of PI3Kγ unexpectedly resulted in loss of ERK activation to receptor tyrosine kinase agonists such as epidermal growth factor or insulin. Mouse embryonic fibroblasts (MEFs) or primary adult cardiac fibroblasts isolated from PI3Kγ knockout mice (PI3KγKO) showed decreased insulin-stimulated ERK activation. However, expression of kinase-dead PI3Kγ resulted in rescue of insulin-stimulated ERK activation. Mechanistically, PI3Kγ sequesters protein phosphatase 2A (PP2A), disrupting ERK–PP2A interaction, as evidenced by increased ERK–PP2A interaction and associated PP2A activity in PI3KγKO MEFs, resulting in decreased ERK activation. Furthermore, β-blocker carvedilol-mediated β-arrestin-dependent ERK activation is significantly reduced in PI3KγKO MEF, suggesting accelerated dephosphorylation. Thus, instead of classically mediating the kinase arm, PI3Kγ inhibits PP2A by scaffolding and sequestering, playing a key parallel synergistic step in sustaining the function of ERK, a nodal enzyme in multiple cellular processes.

INTRODUCTION

Activation of the mitogen-activated protein kinase (MAPK) cascade plays a key role in transducing various extracellular signals following activation of either G-protein coupled receptors (GPCRs), such as β-adrenergic receptor (βAR) or receptor tyrosine kinases (RTKs), such as epidermal growth factor (EGF) receptor (EGFR) or insulin growth factor receptor (IGFIR) (Boulton et al., 1990, 1991; Cobb et al., 1991; Rozengurt, 2007; Chakraborty et al., 2014). The MAPK cascade Ras-Raf-MEK-ERK pathway is at the heart of signaling networks that govern cellular proliferation, cellular differentiation, and cell survival (Kolch, 2000). Phosphorylation of extracellular signal–regulated kinase (ERK) mediated by specific upstream protein kinases is the most common mechanism of ERK activation (Pearson et al., 2001). Activated ERK can either be translocated to the nucleus to initiate transcriptional responses or phosphorylate proteins in the cytosol to mediate anti-apoptotic or survival pathways (Pearson et al., 2001; Yang et al., 2013). The kinase cascade culminating in phosphorylation and activation of ERK by MAPK kinase (MEK) is well established and studied in depth (Pearson et al., 2001). However, less is understood about dephosphorylation of ERK to terminate its activation and restore its baseline inactive state. Dephosphorylation of ERK by protein phosphatases is a fundamental regulatory mechanism checking the activity of signal transduction but is generally considered to be a passive process. Therefore it is not known whether acute regulation of protein phosphatases could alter the state of ERK phosphorylation independent of the upstream kinase cascade regulating ERK function.

Protein phosphatase 2A (PP2A; Sontag, 2001) is an important negative regulator of ERK (Chen et al., 2001; Johnson and Lapadat, 2002; Ugi et al., 2002) and accounts for the majority of the serine/threonine protein phosphatase activity in most cells. In addition to PP2A-mediated dephosphorylation, ERK dephosphorylation can occur in a spatiotemporal manner; for example, dual-specificity phosphatase (DUSP) dephosphorylates and anchors ERK in the nucleus (Caunt et al., 2008). Additionally, ERK can be dephosphorylated in a cell-specific manner, as observed in the basal ganglionic nuclei of the brain striatum, wherein striatal enriched phosphatase...
PI3K belongs to the Class IB family of lipid kinases, which are activated by stimulation of GPCRs (Stoyanov et al., 1995; Vanhaesebroeck et al., 2010). Traditionally, the lipid kinase activity of PI3K activates Akt, which mediates important cellular functions (Foster et al., 2003; Rucke et al., 2006; Rommel et al., 2007). In addition, PI3K exhibits protein kinase activity (Dhand et al., 1994) that regulates PI3K autophosphorylation (Stoyanova et al., 1997; Czupalla et al., 2003) and βAR function (Naga Prasad et al., 2008; Perino et al., 2010; Martini et al., 2014). The scaffolding function of PI3K in regulation of signaling pathways attains prominence given that expression of the PI3K isoform is low in many organ systems (Vanhaesebroeck et al., 2010; Martini et al., 2014; Ghigo and Li, 2015) and is up-regulated in cardiac pathologies (Fougerat et al., 2008; Perino et al., 2011) and cancer (Edling et al., 2010; Xie et al., 2013). Although it is known that PI3K regulates ERK activation following GPCR stimulation through its protein kinase activity (Bondeva et al., 1998), the underlying mechanisms are not understood very well. Given that PI3K is known to regulate PP2A, we assessed whether regulation of PP2A by PI3K could alter the level and strength of ERK activation following GPCR or receptor tyrosine kinase activation. Here we show that PI3K promotes and sustains ERK phosphorylation by inhibiting PP2A activity downstream of the insulin-mediated signaling pathway. Furthermore, PI3K suppressed PP2A activity by decreasing the recruitment of PP2A catalytic subunits to the ERK complex through a kinase-independent mechanism. These studies put forward the important concept that inhibition of PP2A during a MAPK cascade following activation of receptors could be as critical to cellular signal transduction as phosphorylation mediated by kinases.

**RESULTS**

**PI3Kγ regulates ERK activation following G-protein coupled receptor or growth factor receptor agonist**

PI3Ks are known to be integral in activation of the Ras-Raf-MEK pathway (Vanhaesebroeck et al., 2010), and previous studies have shown that activation of GPCRs such as muscarinic receptors or lysophosphatidic acid receptors leads to ERK activation via PI3Kγ (Lopez-Ilasaca et al., 1997; Bondeva et al., 1998; Takeda et al., 1999). To test whether PI3Kγ plays a role in ERK activation following stimulation of beta-adrenergic receptor (βAR, a key regulator of cardiac function), HEK 293 cells were stimulated with the βAR agonist isoproterenol (Iso) in the presence or absence of the PI3K inhibitor wortmannin (Wort). Following Iso stimulation, ERK activation was assessed by phospho-ERK (p-ERK) immunoblotting. Consistent with previous studies (Zhang and Steinberg, 2013; Copik et al., 2015), Iso stimulation resulted in a significant increase in ERK activation. However, pretreatment of cells with Wort resulted in a significant loss of ERK activation (Figure 1A, top panel, bottom panel summary data, \( n = 5 \)). Total ERK1/2 was used as loading control. To directly test whether PI3Kγ plays a role in Iso-mediated ERK activation, HEK 293 cells with stable knockdown of PI3Kγ (PI3Kγ KD) or PI3Kγ (PI3Kγ KD) were generated using short hairpin RNA (shRNA). The efficiency of KD was checked by immunoblotting for PI3Kγ (Figure 1B), showing that these shRNA were specific for reducing the expression of PI3Kγ or γ. Actin immunoblotting was used as loading control. To arrive at the shRNA constructs that specifically depleted either PI3Kγ or γ, an initial screen of three independent small interfering RNAs (siRNAs) was used to reduce the expression of PI3Kγ or γ, respectively. The siRNA that mediated most significant knockdown of PI3Kγ or γ was used for generating shRNA constructs targeting either PI3Kγ or γ (for details see Materials and Methods).

PI3Kγ or γ KD cells or control vector cells were stimulated with Iso, and ERK activation was assessed. Iso-mediated ERK phosphorylation was significantly reduced in PI3Kγ KD cells in comparison with either vector or PI3Kγ KD cells (Figure 1C, top panel, bottom panel summary data, \( n = 4 \)), showing that PI3Kγ plays a key role in regulation of GPCR-mediated ERK activation. Even though baseline ERK phosphorylation was significantly reduced in both PI3Kγ and PI3Kγ KD cells, we made comparisons of ERK phosphorylation only after stimulation in all the experiments. Because PI3Kγ regulates ERK phosphorylation after activation of GPCR, we tested whether KD of PI3Kγ alters ERK activation following stimulation with epidermal growth factor (EGF) or 10% fetal bovine serum (FBS, which contains many growth factor components). Consistent with the role of PI3Kγ in EGF receptor (EGFR) signaling, there was a significant decrease in ERK activation after EGFR activation in PI3Kγ KD cells (Figure 1D, top panel, bottom panel summary data, \( n = 4 \)). Surprisingly, there was also a significant decrease in ERK activation after EGFR stimulation in PI3Kγ KD cells (Figure 1D, top panel, bottom panel summary data, \( n = 4 \)). Interestingly, KD of PI3Kγ resulted in significant decrease in ERK activation following FBS treatment of serum-starved cells, while absence of PI3Kγ did not alter ERK response (Figure 1D, top panel, bottom panel summary data, \( n = 4 \)). These results indicate that PI3Kγ plays a key role in ERK activation downstream of growth factor receptor stimulation. These observations reveal the presence of a hitherto unknown role for PI3Kγ in ERK phosphorylation following growth factor-mediated receptor tyrosine kinase activation.

To further dissect the underlying mechanisms of ERK activation, we isolated primary mouse embryonic fibroblasts (MEFs) from PI3Kγ knockout (KO) mice and wild-type (WT) mice. The primary aim of isolating and using MEFs in our study was to develop a cleaner cellular system to determine pathways underlying this unexpected observation. As a first step in validating the role of PI3Kγ in ERK activation following GPCR stimulation, WT and KO MEFs were serum-starved and stimulated with Iso. Robust ERK activation was observed in WT MEFs, which was significantly reduced in KO MEFs (Figure 1E, left panel, middle panel cumulative data, \( n = 3 \)), confirming the key role of PI3Kγ in GPCR-mediated ERK signaling. Total ERK 1/2 was used as loading control, and expression of PI3Kγ in the WT MEFs was confirmed by PI3Kγ immunoblotting (Figure 1E, right panel).

To determine whether PI3Kγ plays a role in growth factor–mediated ERK phosphorylation, MEFs were stimulated with insulin (Ins)
over a time course of 0–60 min and ERK activation was assessed. Significant ERK phosphorylation was observed following Ins stimulation in the WT MEFs for 5 min, which was slowly reduced over a period of 60 min (Figure 2A, left panel, right panel summary data, n = 4). In contrast, ERK phosphorylation was significantly blunted and followed by rapid dephosphorylation in KO MEFs over a period of 10 min (Figure 2A, left panel, right panel summary data, n = 4). Total ERK 1/2 was used as control. This observation shows that PI3Kγ plays a critical role in ERK activation following stimulation with growth factors such as Ins. To further confirm these findings, WT and KO MEFs were stimulated with Ins for 10 min and assessed for ERK activation by immunostaining for p-ERK using confocal microscopy. Loss of basal ERK phosphorylation was evident in KO MEFs (Figure 2B, panels 7 and 9) in comparison with WT MEFs (Figure 2B, panels 1 and 3). Consistently, WT MEFs showed robust ERK phosphorylation (Figure 2B, panels 4 and 6) following Ins stimulation. In contrast, KO MEFs showed significant loss of ERK phosphorylation despite Ins stimulation (Figure 2B, panels 10 and 12). The nucleus was stained using 4', 6-diamino-2-phenylindole, dihydrochloride (DAPI) (Figure 2B, panels 2, 3, 5, 6, 8, 9, 11, and 12). The quantification of fluorescence intensity is presented in Figure 2B (bottom panel). These results show that PI3Kγ is a prerequisite for eliciting ERK activation following Ins stimulation, suggesting an unexpected role for PI3Kγ in insulin receptor-mediated ERK activation/signaling.

PI3Kγ regulates ERK phosphorylation in a kinase-independent manner

Although the kinase arm of PI3Kγ is classically considered to play a role in ERK activation, increasing evidence has identified key roles for a kinase-independent function of PI3Kγ in regulating downstream signaling (Damiano et al., 2010; Mohan et al., 2013; Schmidt et al., 2013; Frister et al., 2014). Given that KO MEFs do not express PI3Kγ, we tested whether Ins-mediated regulation of ERK phosphorylation is kinase-dependent or -independent. KO MEFs were transfected with WT and inactive PI3Kγ (PI3Kγ\textsubscript{inactive}) – PI3Kγ with a deletion in the ATP binding site as a result of which the expressed protein lacks both lipid and protein kinase activities, ΔPI3Kγ\textsubscript{γ} and stimulated with Ins to assess ERK phosphorylation. Overexpression of WT PI3Kγ in KO MEFs restored ERK activation in response to Ins (Figure 3, top panel, bottom panel summary data, n = 4). Intriguingly, overexpression of PI3Kγ\textsubscript{inactive} in the KO MEFs also restored ERK activation in KO MEFs similar to WT expression (Figure 3, bottom panel, bottom panel summary data, n = 4). Molecular Biology of the Cell
cells were stimulated with Ins for 10 min. ERK phosphorylation was visualized by green fluorescence (Figure 4, panels 1, 4, 5, 8, 9, 12, 13, and 16), while expression of WT PI3Kγ (Figure 4, panels 2 and 10) or PI3K\(_\gamma\)\(_{\text{inact}}\) (Figure 4, panels 6 and 14) was assessed by red fluorescence using anti-HA antibody. Nuclear staining was performed with DAPI. Expression of either HA-WT PI3K\(_\gamma\) or HA-PI3K\(_\gamma\)\(_{\text{inact}}\) in KO MEFs resulted in restoration of Ins-mediated ERK phosphorylation (Figure 4, panels 9, 12, 13, and 16) in contrast to the loss in Ins-mediated ERK phosphorylation in KO MEFs (Figure 4B, panels 10 and 12). A comparison of green fluorescence intensity quantification for cells without and with HA expression is presented (right column). These observations show that the kinase-independent function of PI3K\(_\gamma\) may play a critical role in ERK activation/sustaining ERK activation following stimulation with Ins.

### PI3K\(_\gamma\) inhibits PP2A in regulation of ERK signaling

We have previously reported that PI3K\(_\gamma\) inhibits protein phosphatase 2A (PP2A) activity (Vasudevan et al., 2011; Mohan et al., 2013), and as ERK dephosphorylation is, in part, mediated by PP2A (Silverstein et al., 2002; Ugi et al., 2002; Letourneux et al., 2006), we tested whether ERK-associated phosphatase activity is altered in the absence of PI3K\(_\gamma\) in the KO MEFs. ERK was immunoprecipitated from lysates of control and Ins-stimulated MEFs, and the immunoprecipitates were subjected to protein phosphatase assay. ERK-associated phosphatase activity (pmol phosphate/min) was significantly lower in KO MEFs at baseline in comparison with WT MEFs (Figure 5A, \(n = 3\)). However, the levels of ERK-associated phosphatase activity post-Ins were similar and comparable between KO MEFs and WT MEFs (Figure 5A, \(n = 3\)). Interestingly, ERK-associated phosphatase activity post-Ins in WT MEFs was significantly decreased in comparison with that in untreated controls (Figure 5A, \(n = 3\)). In contrast to WT MEFs, there was a significant increase in ERK-associated phosphatase activity in KO MEFs following Ins stimulation in comparison with its baseline (Figure 5A, \(n = 3\)). Because we observed opposing changes in ERK-associated phosphatase activity in WT and KO MEFs following Ins stimulation, a comparison of fold over untreated is presented in Supplemental Figure 1A. Also, the specificity of the ERK antibody used for pull-down assay was tested using anti-rabbit immunoglobulin G (IgG) as control and blotting for ERK and coimmunoprecipitating PP2Ac (Supplemental Figure 2).

Because we observed an increase in phosphatase activity associated with ERK in KO MEFs following Ins stimulation, we tested whether ERK phosphorylation could be recovered in KO MEFs after Ins-stimulation by inhibiting PP2A with a PP2A-specific inhibitor, Fosfocin (Fos). WT and KO MEFs were stimulated with Ins following pretreatment with Fos. Consistently, Fos pretreatment significantly increased the level of ERK phosphorylation in the Ins-stimulated WT MEFs compared with vehicle (Veh)-treated Ins-stimulated WT MEFs (Figure 5B). Importantly, there was complete recovery of ERK phosphorylation in Ins-stimulated KO MEFs pretreated with Fos, in contrast to Veh-treated KO MEFs (Figure 5B). Total ERK was used as control, and summary data (\(n = 4\)) are presented in the bottom panel (Figure 5B). These observations show that in the absence of PI3K\(_\gamma\), there is a significant increase in ERK-associated PP2A activity in the KO MEFs, which indeed determines the strength and sustainability of ERK activation following Ins stimulation.

As our data in Figure 3 show that PI3K\(_\gamma\) can restore ERK phosphorylation following Ins stimulation in KO MEFs in a kinase-independent manner, we tested whether PI3K\(_\gamma\) kinase-independent function alters ERK-associated phosphatase activity. KO MEFs were transfected with vector control (Vec), WT PI3K\(_\gamma\), or PI3K\(_\gamma\)\(_{\text{inact}}\), and ERK was immunoprecipitated following Ins stimulation to measure ERK-associated phosphatase activity. Consistent with our observation in Figure 5A, Ins stimulation in Vec-transfected WT MEFs showed a significant loss of ERK-associated phosphatase activity, while a significant increase in ERK-associated phosphatase activity was observed in Vec-transfected KO MEFs (Figure 5C, \(n = 3\)). Critically, expression of either WT PI3K\(_\gamma\) or PI3K\(_\gamma\)\(_{\text{inact}}\) in KO MEFs significantly reduced ERK-associated phosphatase activity (Figure 5C, \(n = 3\)), indicating inhibition of ERK-associated phosphatase by PI3K\(_\gamma\) through kinase-independent mechanisms. A comparison of fold over untreated is presented in Supplemental Figure 1B. To determine whether the scaffolding function of PI3K\(_\gamma\) underlies the kinase-independent regulation of ERK activation, WT and KO MEFs were stimulated with Ins. Following Ins treatment, a comparison of fold over untreated is presented in Supplemental Figure 1B. To determine whether the scaffolding function of PI3K\(_\gamma\) underlies the kinase-independent regulation of ERK activation, WT and KO MEFs were stimulated with Ins.
stimulation, ERK was immunoprecipitated and immunoblotted for common immunoprecipitating PP2A. Even though baseline interaction of PP2A with ERK was not altered in KO MEFs in comparison with WT MEFs, there was a marked decrease in PP2A interaction with ERK in WT MEFs following Ins stimulation (Figure 5D, right panel, summary data, n = 3). Interestingly, there was a significant increase in PP2A interaction with ERK following Ins stimulation in KO MEFs (Figure 5D, right panel, summary data, n = 3).

To further confirm the kinase-independent function of PI3Kγ in regulation of ERK–PP2A interaction, KO MEFs were transfected with inactive PI3Kδ (Δ PI3Kγ) and serum-starved before stimulation with Ins. ERK was immunoprecipitated and blotted for immunoprecipitating PP2A. There was a significant decrease in ERK–PP2A interaction with the overexpression of inactive PI3Kγ (Δ PI3Kγ) (Figure 6A, right panel, summary data, n = 3), even at baseline. Importantly, Ins stimulation resulted in a significant increase in interaction between ERK and PP2A in the control vector-transfected KO MEFs (Figure 6A, n = 3). Interestingly, overexpression of Δ PI3Kγ prevented recruitment of PP2A to ERK following Ins treatment (Figure 6A, right panel, summary data, n = 3). To test whether PI3Kγ sequesters PP2A during insulin-mediated signaling, WT MEFs were treated with Ins and PI3Kγ was immunoprecipitated and blotted for immunoprecipitating PP2A. Following Ins stimulation, there was a significant increase in interaction between PP2A and PI3Kγ (Figure 6B, right panel, summary data, n = 3), suggesting that PI3Kγ is involved in sequestration of PP2A. Together these data show that PI3Kγ regulates ERK activation post-Ins stimulation by the kinase-independent scaffolding function, during which expression of PI3Kγ may sequester PP2A from ERK, leading to sustained phosphorylation of ERK.

PI3Kγ regulates ERK signaling in primary adult mouse cardiac fibroblasts

We have previously shown that ERK activation in response to Ins in total heart lysates from WT and PI3Kγ knockout mice is similar (Mohan et al., 2013), suggesting that kinase-independent regulation of ERK activation by PI3Kγ may be a cell-specific response. Because cardiomyocytes make up the major share of cells in a normal heart, we tested whether the kinase-independent mechanism of PI3Kγ regulates ERK activation in isolated primary adult cardiac fibroblasts. To test whether PI3Kγ-mediated ERK signaling is preserved in adult fibroblasts, adult cardiac fibroblasts (CF) were isolated from the hearts of 3-mo-old WT and PI3Kγ KO mice. Adult CFs were stimulated with Ins and ERK activation was measured. In contrast to WT CFs, CFs from PI3Kγ KO mice showed minimal ERK activation in response to Ins (Figure 7A, n = 3; summary data from three independent hearts excised from WT and PI3Kγ KO mice). These data show that ERK signaling in adult CFs could be regulated in part by a kinase-independent mechanism of PI3Kγ in the adult heart. The expression of PI3Kγ in CFs was confirmed by immunoblotting and is presented in Supplemental Figure 3.

β-Arrestin–biased agonist–mediated ERK activation is regulated by PI3Kγ expression

It is known that the βAR blocker (β-blocker) carvedilol mediates G-protein–independent β-arrestin–dependent ERK signaling (Widler et al., 2007; Kim et al., 2008), and β-arrestin–mediated signaling is thought to mediate beneficial effects (Noma et al., 2007). Because PI3Kγ is upregulated under cardiac stress (Patrucco et al., 2004; Perino et al., 2011), we determined whether PI3Kγ plays a role in ERK activation by the β-blocker carvedilol. WT or PI3Kγ KO MEFs were stimulated with carvedilol for 0–60 min and ERK activation was assessed. Significant and sustained ERK activation was observed following carvedilol treatment in WT MEFs (Figure 7B, top panel, bottom panel summary data, n = 3), which was abolished in KO MEFs (Figure 7B, top panel, bottom panel summary data, n = 3). These results demonstrate that expression of PI3Kγ plays a critical role in sustaining ERK activation following extracellular stimuli such as agonists or antagonists. This is a key observation, as expression of PI3Kγ is low in many cell types in normal physiology, but its expression is significantly upregulated in pathology (Fougerat et al., 2008; Perino et al., 2011). Such upregulation suggests that kinase-independent functioning of PI3Kγ may regulate critical cellular responses, in addition to its classical role of kinase-dependent signaling. Representative full scans of all the Western blots in this article are presented in Supplemental Figure 4.

DISCUSSION

Here we report identification of a unique regulation of ERK signaling by a kinase-independent function of PI3Kγ that regulates PP2A function. We show that inhibition of PI3Kγ with the pan PI3K inhibitor Wort, knockdown of PI3Kγ, or absence of PI3Kγ results in loss of ERK phosphorylation. The decrease in ERK activation occurs in response not only to classical GPCR-mediated PI3Kγ-dependent pathway but also to growth factor (Ins)–driven signaling, in which PI3Kγ is not known to play a role in regulating ERK. The severely compromised Ins-dependent ERK activation in the absence of PI3Kγ is restored by expression of inactive PI3Kγ (PI3KγΔ) and Δ PI3Kγ, showing a key role for kinase-independent function, in contrast to the classical kinase-dependent role of PI3Kγ. Furthermore, our study shows that PI3Kγ is critical for ERK signaling mediated by the β-blocker carvedilol. Interestingly, our data show that the kinase-independent function of
PI3Kγ regulates PP2A activity by sequestering PP2A from ERK, aiding in sustained ERK activation. Finally, the PI3Kγ-PP2A-ERK axis is preserved in primary adult cardiac fibroblasts. We believe that this unique role of PI3Kγ in signaling may help explain the progression of diseases in which the abundance of PI3Kγ protein is increased in pathology. Our data thus provide evidence of a hitherto unappreciated role of PI3Kγ in sequestering PP2A by the scaffolding function, thereby regulating ERK dephosphorylation by PP2A (Figure 8).

The role of PI3Kγ in regulating downstream signaling following stimulation of GPCRs such as muscarinic (m2) or formyl-MET-LEU-PHE (fMLP) receptors is well known (Lopez-Ilasaca et al., 1997; Burelout et al., 2004). Studies using inactive PI3Kγ mutants have shown that PI3Kγ can regulate the MAPK pathway (Lopez-Ilasaca et al., 1997; Bondeva et al., 1998) in response to agonist. Furthermore, studies using chimeric PI3K mutants containing the PI3Kγ catalytic core replaced with Class II and III sequences that retained protein kinase activity showed marked Wort sensitivity toward ERK activation (Bondeva et al., 1998). This observation suggested that protein kinase activity of PI3Kγ could play a role in MAPK signaling following GPCR activation. Consistently, our studies show that inhibition of PI3K by Wort inhibits ERK activation in response to βAR stimulation. Critically, ERK activation following βAR stimulation by the agonist isoproterenol (Iso) is mediated by PI3Kγ, as selective knockdown of PI3Kγ or MEFs from the PI3Kγ KO mice shows loss of ERK activation following Iso. These observations together suggest that PI3Kγ is a key mediator of βAR-mediated ERK activation.

In addition to the preserved role of PI3Kγ in GPCR-mediated ERK activation, we observed that the absence of PI3Kγ (KO MEFs) or the knockdown of PI3Kγ in HEK 293 cells resulted in reduction of ERK activation following growth factor stimulation (EGF or Ins). Importantly, knockdown of both PI3Kα and PI3Kγ has similar effects on ERK activation following EGF stimulation, suggesting a unique role for PI3Kγ downstream of growth factor receptors in regulating ERK activation. This observation was surprising, given that our previous studies of the hearts from PI3Kγ KO mice showed no differences in ERK activation in response to GPCR agonist Iso or growth factor Ins administration (Mohan et al., 2013). These data provide the insight that PI3Kγ-mediated regulation of ERK could be a cell-specific temporal regulation that may depend upon the cellular functioning, including proliferation, given that ERK is a key regulator of cell division (Crews et al., 1992a; Chang et al., 2003). Because cardiac myocytes account for a majority of the cells by volume in the heart and as they are quiescent and do not divide, this unique ERK regulation may be absent in adult cardiomyocytes. In contrast, cardiac fibroblasts...
The presence of PI3Kγ in MEFs was utilized to measure the PI3Kγ activity using a malachite green phosphatase assay kit. The assays were performed in triplicate with the following rundown. WT MEFs and KO MEFs were serum-starved and stimulated with 10 μM Insulin for 10 min. ERK was immunoprecipitated using 500 μg of cell lysates, and immunoprecipitates were utilized to measure serine/threonine phosphatase activity. The phosphatase activities are presented as pmol phosphate released per minute. Amalgamated data are presented as bar graphs (n = 3). *p ≤ 0.01, Ins vs. Ctrl. #p ≤ 0.001, WT MEF-Ctrl/KO MEF-Ctrl. (A) WT MEFs and KO MEFs were serum-starved, treated with 1 μM Fostreicin (Fos), and stimulated with Ins. The cells were lysed and immunoblotted with α-ERK antibody. The blots were stripped and immunoblotted with α-ERK antibody as a loading control. Cumulative data are presented as bar graphs (n = 4). #p ≤ 0.001, WT MEF-Fos-Ctrl vs. MEF-Veh-Ctrl. (B) WT MEFs and KO MEFs were serum-starved, treated with 10 μM Insulin for 10 min. The cells were lysed, ERK was immunoprecipitated using 500 μg of cell lysates, and immunoprecipitates were utilized to measure serine/threonine phosphatase activity. The phosphatase activities are presented as pmol phosphate released pmol phosphate released per minute. Amalgamated data are presented as bar graphs (n = 3). *p ≤ 0.01, Ins vs. Ctrl. #p ≤ 0.001, WT MEF-Ins/KO MEF-Ins. **p ≤ 0.001, KO MEF-Ins with overexpression of WT or PI3Kγ inact vs. KO MEF-Ctrl. (C) WT MEFs and KO MEFs were serum-starved, treated with 1 μM Fostreicin (Fos), and stimulated with Ins. The cells were lysed, ERK was immunoprecipitated using 500 μg of cell lysates, and immunoprecipitates were immunoblotted for coimmunoprecipitating PP2Ac. The blots were stripped and immunoblotted with α-ERK antibody as a loading control. Amalgamated data of coimmunoprecipitating PP2Ac normalized to immunoprecipitated ERK presented as bar graphs (n = 3). *p ≤ 0.01, Ins vs. Ctrl. #p ≤ 0.001, WT MEF-Ins/KO MEF-Ins. 

Regulation of ERK by PI3Kγ in response to insulin suggests a hitherto unknown mechanism of ERK regulation. As previous studies have elegantly shown that activation of ERK in response to GPCR stimulation is dependent on the kinase function of PI3Kγ (Lopez-Ila-sama et al., 1997; Bondeva et al., 1998), we overexpressed inactive PI3Kγ (PI3Kγ inact – deletion in ATP binding site, Δ PI3Kγ) in the KO MEFs. A recovery in ERK response to Ins was observed even in the presence of PI3Kγ inact, showing that regulation of ERK phosphorylation to insulin could be a kinase-independent mechanism. There has been increasing recognition of the kinase-independent function of PI3Kγ in regulating signal pathways, such as modulating cAMP levels by scaffolding phosphodiesterase (PDE) (Patrucco et al., 2004; Perino et al., 2011). Furthermore, we have shown that the kinase-independent scaffolding function of PI3Kγ regulates the interaction between PP2A and its activator PP2A methyltransferase, thereby regulating the function of glycogen synthase kinase 3 (GSK3) in vivo in the hearts (Mohan et al., 2013). Correspondingly, we observed significantly increased direct interaction between PP2A and ERK in the KO MEFs in comparison with WT, suggesting that PI3Kγ may directly interact with and scaffold PP2A from ERK. In contrast to our heart studies on GSK3, there were no differences in the interaction...
between methylated PP2A with ERK (unpublished data). This suggests that regulation of direct interaction between PP2A and ERK by PI3Kγ may be a temporal cell-specific mechanism that could be occurring in a subset of actively dividing cells. This idea is supported by observations of primary adult cardiac fibroblasts.

Our studies suggest the presence of a powerful regulation of ERK dephosphorylation by PI3Kγ that cooperatively promotes ERK signaling. Decreased phosphorylation of ERK, downstream of both the Isot–GPCR and Ins–RTK pathway in PI3Kγ KO MEFs, indicates a critical role for PI3Kγ in enhancing ERK function. Although the majority of studies have been confined to understanding the kinase-dependent phosphorylation of ERK by upstream regulators (Crews et al., 1992b; Zheng and Guan, 1993a,b; Pearson et al., 2001), less is known about active regulation of ERK by PP2A-mediated dephosphorylation. In this context, our studies show that PI3Kγ-mediated suppression of PP2A is an active and potentially an equal component necessary for sustaining and initiating the activation of ERK. Correspondingly, our data show drastically reduced phosphorylation of ERK in the MEFs from PI3Kγ KO mice irrespective of the stimulant (Iso, Ins, EGF, FBS, or carvedilol). This implies that PI3Kγ-mediated ERK activation via the PP2A is not limited to a single signal transduction pathway but could synergize and underlie the multiple upstream signaling pathways.

FIGURE 6: PI3Kγ sequesters PP2A from ERK. (A) KO MEFs transfected with Vec and PI3Kγinact were serum-starved and stimulated with Ins for 10 min. The cells were lysed, ERK was immunoprecipitated using 500 μg of cell lysates, and immunoprecipitates were immunoblotted for coimmunoprecipitating PP2Ac. The blots were stripped and immunoblotted with α-ERK antibody as loading control. Amalgamated data of coimmunoprecipitating PP2Ac normalized to immunoprecipitated ERK antibody as loading control. Cumulative data of coimmunoprecipitates were immunoblotted for coimmunoprecipitating PP2Ac. The blots were stripped and immunoblotted with α-PI3Kγ antibody as loading control. Cumulative data of coimmunoprecipitating PP2Ac normalized to immunoprecipitated PI3Kγ presented as bar graphs (n = 3). *p ≤ 0.01, Ins vs. Ctrl.

FIGURE 7: PI3Kγ regulates ERK phosphorylation in adult cardiac fibroblasts and modulates β-arrestin–mediated ERK phosphorylation. (A) PI3Kγ modulates Ins-mediated ERK phosphorylation in adult cardiac fibroblasts. Adult cardiac fibroblasts isolated from WT and PI3Kγ KO mice were serum-starved and stimulated with Ins for 10 min. The cells were lysed and immunoblotted with α-pERK antibody. The blots were stripped and immunoblotted with α-ERK antibody as loading control. Amalgamated densitometric data are presented as bar graphs (n = 3). *p ≤ 0.001, WT CF vs. KO CF. (B) WT MEFs and KO MEFs were serum-starved and stimulated with 10 μM of the β-blocker carvedilol (Carv) for 5–60 min. The cells were lysed and immunoblotted with α-pERK antibody. The blots were stripped and immunoblotted with α-ERK antibody as loading control. Amalgamated densitometric data are presented as bar graphs (n = 3). *p ≤ 0.001, WT MEF-Carv vs. KO MEF-Carv.

Understanding the regulation of ERK activation through phosphatase regulation is critical, as it is a parallel regulatory pathway that has significant consequences for downstream effects. This concept is supported by significant loss of carvedilol (a β-blocker treatment)–mediated ERK activation, in which the activated kinase is not able to overcome the accelerated dephosphorylation by the phosphatase overwhelming the kinase arm. This observation suggests the existence of tight phosphatase regulation by PI3Kγ, although through a kinase-independent mechanism, as there is a restoration of ERK activation following expression of inactive PI3Kγ in the PI3Kγ KO MEFs. A corollary to these observations is our data showing decrease in ERK activation in primary adult cardiac fibroblasts. These data support the idea that regulation of ERK-associated phosphatase activity by a noncanonical kinase-independent mechanism of PI3Kγ could be a key determining factor in mediating downstream pathways. In this regard, it is important to note that had we used isofrom specific kinase inhibitors, we would have missed this unique noncanonical regulation of the ERK-associated phosphatase activity by PI3Kγ. The kinase-independent regulation by PI3Kγ is significant, given that its expression is not widespread but mainly confined to hematopoietic systems, pancreas, skeletal muscle, and to a certain extent the heart and lungs. However, the level of PI3Kγ increases markedly in heart failure pathology, and therefore in addition to its role as a kinase, increased PI3Kγ protein levels could also
effectively regulate kinase-independent functions such as sustaining ERK activation, which may be a key to β-blocker response.

Mechanistically, decreased phosphorylation of ERK in PI3Kγ KO MEFs was associated with increased PP2A activity. Furthermore, inhibition of PP2A with a selective inhibitor, Fos, was able to restore the pERK response to Ins in KO MEFs, suggesting that PP2A activity underlies ERK regulation by PI3Kγ. Although we have observed nearly complete recovery in ERK phosphorylation following inhibition of PP2A, it is important to note that this may not be the only mechanism for dephosphorylation of ERK. Studies have identified DUSPs that bind to ERK and inactivate it in the nucleus in response to EGF stimulations (Caunt et al., 2008). In this regard, our studies indicate that the kinase-independent scaffolding function of PI3Kγ may regulate ERK function in the cytosol. Importantly, studies have shown that β-arrestin–dependent G-protein–independent ERK signaling is primarily cytosolic, in contrast to G-protein–dependent nuclear signals (Tohgo et al., 2002). In addition to PP2A and DUSP mechanisms of ERK dephosphorylation, ERK can also be dephosphorylated by unique phosphatases in a cell-specific manner, like STEP in striatal ganglion (Shiflett and Balleine, 2011). These observations suggest that dephosphorylation of ERK can be regulated in a spatiotemporal manner as well as in a cell-specific manner, suggesting redundant regulation of this key step.

The recovery in pERK levels following inhibition of PP2A lays a foundation for the concept that kinase-mediated phosphorylation of ERK is as effective as the ability of the cell to inhibit PP2A activity by PI3Kγ during the signal transduction event. Consistently, we observed significant recruitment of PP2A to ERK in PI3Kγ KO MEFs, associated with increased phosphatase activity that could account for increased dephosphorylation of ERK. Conversely, the PP2A–ERK interaction was reversed, with overexpression of PI3Kγ inactive and PI3Kγ interaction with PP2A increased with Ins stimulation. Thus scaffolding of PP2A by PI3Kγ (active or inactive) sequesters PP2A, leading to a decrease in recruitment of PP2A to ERK, thus sustaining ERK phosphorylation following stimulation. These multiple lines of evidence support the idea that the expression level of PI3Kγ could be a critical factor in resetting PP2A activity and thereby ERK activation, which is key to many cellular responses, including proliferation, differentiation, and survival (Crews et al., 1992a; Chen et al., 2001; Pearson et al., 2001; Chang et al., 2003). More importantly, the reset initiated by PI3Kγ could play a role in sustained signaling, as increasing levels of PI3Kγ expression could prolong ERK activation.

The appreciation that the intrinsic magnitude of protein phosphorylation in response to external stimuli is not just based on kinase activity of the upstream molecules but also on the simultaneous inhibition of dephosphorylating phosphatase, brings to the fore the idea that signal transduction occurring in the cells is much more dynamic, involving inhibition of the phosphatase to sustain a signal, with the primary goal of executing a response. We have identified in our studies a signaling profile that indicates that phosphatase inhibition rather than kinase activity may be responsible for efficiency of signal transduction. In our study, the absence of PI3Kγ leads to weak ERK phosphorylation due to rapid dephosphorylation mediated by overactive PP2A, which overwheels the kinase-driven mechanism. Even though PI3Kγ plays a major role as a kinase regulating key signaling events (Naga Prasad et al., 2002, 2005; Nienaber et al., 2013), the mechanism of sustaining ERK activation depends on the kinase-independent scaffolding function of sequestering PP2A from interacting with ERK. These observations have significant implications, as an increase in PI3Kγ expression could alter the magnitude of a signal through both kinase-dependent and kinase-independent mechanisms.

MATERIALS AND METHODS

Pharmacological compounds

Isoproterenol hydrochloride, insulin, carvedilol, and wortmannin were all obtained from Sigma-Aldrich, St. Louis, MO. Fostreicin was from Clayman Chemical, MI. Human EGF was from STEMCELL Technologies, Vancouver, BC, Canada. FBS was from Atlanta Biologicals.

siRNAs and shRNA constructs

Sequences and details of the siRNAs initially used and development of stable shRNA knockdown cells have been described previously (Mohan et al., 2013).

Cell cultures and treatments

Cell lines used. HEK293 cells; stable knockdown of PI3Kα and PI3Kγ in HEK293 cells; MEFs isolated from PI3Kγ-knockout mice (KO MEF) as well as from wild-type mice (WT MEF).

HEK293 cell lines were grown and maintained in DMEM (Life Technologies) with 10% FBS (Life Technologies) and penicillin/streptomycin (Life Technologies). Stable knockdown of PI3Kα and PI3Kγ in HEK293 cells was also maintained in DMEM with 10% FBS supplemented with hygromycin (400 mg/ml). Wild-type MEFs were maintained in DMEM with 10% FBS and penicillin/streptomycin. Cells were maintained in a humidified incubator at 37°C under 5% CO₂.

Isolation and maintenance of MEFs and cardiac fibroblasts from wild-type and PI3Kγ-knockout mice. Isolation and maintenance of MEFs from wild-type and knockout mice have been described earlier (Naga Prasad et al., 2005).

Isolation of cardiac fibroblasts (CFs). Hearts were washed several times with cold phosphate-buffered saline (PBS) and minced into 1–2 mm pieces. The tissue was then subjected to digestion at 35°C by a mixture of 0.1% trypsin and 200 U/ml collagenase (type IV; Sigma-Aldrich) for 10 min by constant stirring. At the end of digestion, the mixture was aspirated without taking any undigested tissue. The digestion process was repeated three to four times for more yield. After the first two digestion mixtures were discarded (as they contained blood cells and cell debris), the next phases of digestion mixtures were collected in a fresh tube. Then the mixture was
pelleted by centrifuging at 1000 rpm for 5 min and the pellet was resuspended in DMEM containing 10% FBS, plated on a 100-mm culture dish, and incubated for 2 h at 37°C in a humidified incubator under 10% CO2. At the end of this period, unattached cells were discarded and attached cells were grown in DMEM with sodium pyruvate and d-glucose (4.5 g/l) containing 10% FBS and 2.2% antibiotic/antimycotic (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B; Life Technologies) at 37°C. Cells were maintained in a humidified incubator at 37°C and under 5% CO2. The primary cultures of cardiac fibroblasts were maintained in the above-mentioned medium and the medium was changed every 2 d.

**Treatment.** Cells were separately treated with ligands such as Ins (3 μg/ml), Iso hydrochloride (100 μM), carvedilol (10 μM), and EGF (10 ng/ml) either for 10 min or until different time points to capture the phosphorylation of ERK. Cells were pretreated with wortmannin (100 nM) or Fostreicin (1 μM), separately or in combination, followed by treatment with specific ligands. Controls in the experiments were maintained under conditions similar to those for treated cells, but without ligands.

**Transfection.** WT and KO MEFs were transfected with 6 μg WT and PI3K\textsubscript{fynct} plasmid DNA [pCDNA3.1+] using FuGENE 6 (Promega) following the manufacturer's protocol. Cells were under transfection for 48 h, followed by treatment of either ligand alone or with a specific inhibitor.

**Western blot**

Protein extracts was prepared from cells using either NP-40 lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol, 10 mM NaF, 1% NP-40, sodium orthovanadate, leupeptin, aprotinin, phosphatase inhibitor cocktail [no phosphatase inhibitor cocktail if used for phosphatase assay]) or Triton X-100 lysis buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 1 mM PMSF, 20% glycerol, 0.8% Triton X-100, leupeptin, aprotinin, phosphatase inhibitor cocktail [no phosphatase inhibitor cocktail if used for phosphatase assay]) for the immunoprecipitation experiment. Samples of 50 μg of cell lysates were subjected to immunoblotting using primary antibody specific for pERK, ERK (Cell Signaling), PI3K\textsubscript{α} (Santa Cruz Biotechnology), PI3K\textsubscript{β} (Santa Cruz Biotechnology), PP2Ac (Santa Cruz Biotechnology), and horseradish peroxidase–conjugated secondary antibodies (GE Healthcare or Thermo Scientific). Immune complexes were detected using chemiluminescence reagents and the images were captured on x-ray films. Image analysis was performed with National Institutes of Health (NIH) ImageJ software. Alternatively, primary antibody incubation was followed by incubation with infrared dye (IRDye)–conjugated secondary antibodies (either 926-68072 IRDye 680RD donkey anti-mouse IgG [H + L], 926-32213 IRDye 800CW donkey anti-rabbit IgG [H + L], or 926-32214 IRDye 800CW donkey anti-goat IgG [H + L]; LI-COR), and immunoreactive bands were visualized under an Odyssey scanner (Odyssey CLx; LI-COR). The bands were quantitated using Image Studio Version 3.1 (LI-COR) and normalized by ERK.

**Phosphatase assay**

A phosphatase activity was performed using a serine–threonine phosphatase kit (Cat#20-105; Upstate Biotechnology). Samples of 500 μg total protein from cell lysates were used to immunoprecipitate ERK; samples were resuspended in the phosphate-free assay buffer and incubated in the presence or absence of serine–threonine specific phosphopeptide substrate for 10 min. The reaction mix was incubated with acidic malachite green solution, and its absorbance at 630 nm was measured in a plate reader.

**Confocal microscopy**

MEFs were transfected with vector or HA-tagged PI3K\textsubscript{γ} and plated onto coverslips treated with poly-l-lysine. Cells were serum-starved for 4 h, stimulated, fixed (4% para-formaldehyde), permeabilized with ice cold methanol for 10 min at ~20°C, and blocked with 5% normal goat serum (NGS) in PBS. Anti-phospho ERK (1:1000; Cell Signaling) and/or anti-HA (1:100, Roche) were used as primary antibodies, while goat anti-rabbit AlexaFlour 488 (1:200; Molecular Probes) and anti-mouse AlexaFlour 568 (1:200; Molecular Probes) were used as secondary antibodies. Samples were visualized using sequential line excitation at 488 and 568 nm for green and red, respectively. Quantitation of the fluorescence intensity of the cells was performed using the IMAGE PRO PLUS 7 program (Media Cybernetics).

**Statistical analysis**

All data expressed as mean ± SEM (n ≥ 3 experiments performed under identical conditions). Analysis of variance was used for multiple comparisons of the data. Statistical analyses were performed using GraphPad Prism, and the significance between the treatments was determined by Student's t test. A p value less than 0.05 was considered statistically significant.

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