RAS and RHO Families of GTPases Directly Regulate Distinct Phosphoinositide 3-Kinase Isoforms

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SUMMARY

RAS proteins are important direct activators of p110α, p110γ, and p110δ type I phosphoinositide 3-kinases (PI3Ks), interacting via an amino-terminal RAS-binding domain (RBD). Here, we investigate the regulation of the ubiquitous p110β isoform of PI3K, implicated in G-protein-coupled receptor (GPCR) signaling, PTEN-loss-driven cancers, and thrombocyte function. Unexpectedly, RAS is unable to interact with p110β, but instead Rac1 and CDC42 from the RHO subfamily of small GTPases bind and activate p110β via its RBD. In fibroblasts, GPCRs couple to PI3K through Dock180/Elmo1-mediated Rac activation and subsequent interaction with p110β. Cells from mice carrying mutations in the p110β RBD show reduced PI3K activity and defective chemotaxis, and these mice are resistant to experimental lung fibrosis. These findings revise our understanding of the regulation of type I PI3K by showing that both RAS and RHO family GTPases directly regulate distinct ubiquitous PI3K isoforms and that Rac activates p110β downstream of GPCRs.

INTRODUCTION

The type I phosphoinositide 3-kinases (PI3Ks) are critical signaling proteins involved in the regulation of cell growth, survival, motility, and metabolism. In mammals, there exist four isoforms of the type I PI3K catalytic p110 subunits: α, β, γ, and δ. Of these, α and β are ubiquitously expressed, whereas γ and δ have more limited distribution, most notably in hematopoietic cells (Vanhaesebroeck et al., 2010). The lipid kinase activity of p110α is regulated downstream of receptor tyrosine kinases by the binding of tyrosine-phosphorylated proteins to its regulatory p85 subunit, resulting in attenuation of its autoinhibitory activity. In addition, activated RAS proteins bind directly to an N-terminal RAS-binding domain (RBD) on p110α, acting synergistically with the input from tyrosine-phosphorylated proteins to optimally activate lipid kinase activity (Rodriguez-Viciana et al., 1994, 1996). Proof of the pathophysiological importance of the direct interaction of RAS with p110α came from the generation of mice bearing germline mutations in the RBD of p110α, which were found to be highly resistant to mutant-RAS-induced lung and skin cancer formation (Gupta et al., 2007).

The direct binding of RAS to p110γ has also been studied in detail. The 3D structure of RAS bound to p110γ has been determined, and RAS has been shown to activate the lipid kinase activity of p110γ cooperatively with input from Gα subunits via the regulatory p101 subunit (Pacold et al., 2000). Mice with mutations in the RBD of p110γ show neutrophil defects in the regulation of PI3K activity by some G-protein-coupled receptors (GPCRs) (Suire et al., 2006). RAS also has been reported to bind and activate p110δ in vitro (Vanhaesebroeck et al., 1997). In addition, RBD mutations have been used to demonstrate that input of RAS binding to the single Drosophila type I PI3K is critical in insulin-pathway-controlled developmental growth (Orme et al., 2006) and that RAS binding is required for PI3K activation by chemoattractants in Dictyostelium (Funamoto et al., 2002). p110δ has been much less thoroughly studied than p110α. It appears to be relatively insensitive to activation by growth factor receptor tyrosine kinase signaling but important downstream of certain GPCRs, including those for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), making p110δ the only GPCR-regulated type I PI3K isoform outside the hematopoietic system (Ciraolo et al., 2008; Guillermot-Guibert et al., 2008; Jia et al., 2008). p110δ may also play an important role in cancer because mouse models of breast and prostate cancer, as well as a number of human cancer cell lines, depend on p110δ, particularly in the setting of PTEN loss (Ciraolo et al., 2008; Jia et al., 2008). In platelets, p110δ is essential for integrin-dependent adhesion and clot formation (Jackson et al., 2005; Martin et al., 2008).
et al., 2010), leading to the intense effort to develop isoform-specific p110β inhibitors, some of which are now in clinical trials as antiplatelet and anticancer agents (NCT01458067, NCT00688714).

The molecular basis of how p110β can exert these distinct functions is poorly understood. p110β is overall structurally similar to other p110 catalytic subunits and engages the very same p85 type regulatory subunits as p110α, albeit in a somewhat different way (Zhang et al., 2011). Early reports have found p110β to associate with Gβγ subunits from heterotrimeric G proteins, which can directly stimulate its lipid kinase activity in vitro (Kurosu et al., 1997; Maier et al., 1999). It has, however, remained entirely unclear whether the p110β RBD contributes to p110β activation and function, and despite the apparently similar level of relatedness between the RBDs across the four isoforms, a systematic analysis of RAS effector proteins failed to detect any activation of p110β by RAS in cotransfected cells (Rodriguez-Viciana et al., 2004).

In this report, we explore the role of p110β regulation through its RBD for PI3K signaling and function. We present extensive in vitro work to show that p110β is the only type I PI3K isoform not regulated by RAS and to identify the RHO family GTPases RAC and CDC42 as direct isoform-specific RBD interactors and activators of p110β. We go on to show that GPCRs couple to PI3K via Dock180/Elim1-mediated RAC activation and subsequent interaction with p110β. Mouse embryonic fibroblasts (MEFs) from p110β RBD mutant mice show reduced PI3K activity and mice are resistant to bleomycin-induced lung fibrosis, a pathology that has been linked with LPA signaling. These findings explain longstanding inconsistencies and revise our understanding of type I PI3K regulation by small GTP-binding proteins, providing molecular insight into the regulation and function of the ubiquitous p110β isoform.

RESULTS

**PI3K p110β Is Not a RAS Target Protein**

To characterize the role of RAS in regulating p110β, we set out to characterize the biochemical interaction between the two in vitro. In glutathione S-transferase (GST) pull-down studies using recombinant, GTPγS-loaded HRAS, KRAS, and NRAS as baits, we found strong and specific interaction among all three RAS proteins and p110α (Figure 1A). In contrast, p110β bound to neither of the RAS proteins, but did bind to RAB5, a previously identified GTPase interactor of p110β (Christoforidis et al., 1999). Mutating key residues within the RBD of p110α (T208D/K227A) abrogated RAS binding, whereas introduction of analogous mutations (see below for details) into p110β did not affect RAB5 binding. Similar results were obtained when we used recombinant full-length GST-p110/p85 complexes to pull down active RAS or RAB5 proteins (Figure 1B). Moreover, when we expressed constitutively active RAS or RAB5, along with p110α/p85 or p110β/p85 in COS7 cells, and measuredPIP2 levels (Figure 1C) or steady-state phospho-AKT (Figure S1A available online) as indicators of PI3K activity, HRAS and KRAS strongly enhanced p110α activity, whereas p110β was not stimulated by either RAS proteins or RAB5.

**An Intact RBD Is Essential for p110β Activity in Cells**

The modest RBD sequence similarity among the four paralogs of type I PI3K is shown in Figure 1D. Even though the overall structural organization of the p110β RBD is conserved (Zhang et al., 2011), we speculated that because we cannot detect any interaction with RAS, it might have lost or altered its function. We therefore mutated two highly conserved key residues within the p110β RBD to generate a p110β-S205D/K224A double mutant (p110β-RBD-DM; Figure 1E). Analogous mutations in p110α and p110γ disrupt RAS binding (Gupta et al., 2007; Piacold et al., 2000). In vitro, the basal lipid kinase activity of purified recombinant p110β-RBD-DM protein was indistinguishable from its wild-type counterpart prepared in parallel (Figure 1F). Moreover, p110β-RBD-DM was still stimulated by the addition of purified recombinant Gβγ subunits, alone or in combination with a platelet-derived growth factor receptor (PDGFR)-derived phosphotyrosine peptide (pY740), indicating that the RBD mutations had no intrinsic effect on p110β lipid kinase activity or RBD-independent stimulatory input (Figure 1G). However, RBD mutant p110β was much less active than wild-type when expressed in COS7 cells (Figures 1H and S1B) even when Gβγ subunits were coexpressed or a myristoylation signal was added, pointing to a critical role of the RBD for p110β activity in living cells.

**p110β Interacts with Distinct RAS Subfamily GTPases**

To identify RBD interactors of p110β, we probed all 34 murine members of the RAS subfamily of small GTPases (RFGs) for binding to p110β/p85 (Figure S1C). Parallel experiments were performed with p110α/p85, p110γ/p101, and p110δ/p85, respectively (Figure S1D). Strikingly, whereas all non-β isoforms interacted with the three prototypical RAS proteins and a partially overlapping subset of closely related RFGs (RRAS1, RRAS2, MRA, and ERAS), p110β bound to none of those (Figure 2A). Instead, p110β exclusively bound to the more distantly related DiRAS1 and DiRAS2 proteins in a GTP-dependent manner (Figure S1C). DiRAS selectively bound wild-type and not RBD mutant p110β (Figure S2B), suggesting binding to the RBD. However, DiRAS failed to stimulate p110β lipid kinase activity in vitro (Figure 2C) and in cells, where constitutively active DiRAS proteins seemed to repress rather than elevate phospho-AKT when coexpressed along with p110β (Figure 2B), making DiRAS an unlikely in vivo activator of p110β.

**p110β Is a Direct RAC/CDC42 Target Protein**

When comparing DiRAS with RAS, an obvious difference is the substitution of Asp33 within the G2 box of RAS with Ile37 in DiRAS (Figure 2D). This substitution is relevant to PI3K binding because an HRAS-D33I mutant showed attenuated binding to p110α and DiRAS1-I37D showed reduced binding to p110β, even though exchange of this residue did not enable RAS binding to p110β or DiRAS binding to p110α (Figure S2A), pointing to additional, G2-box-independent determinants of PI3K isoform specificity. Several members of the RHO subfamily of small GTPases harbor a hydrophobic isoleucine or valine residue in this position (Figure S2C), which prompted us to test p110β for binding to representative RHO family GTPases (Figure S2D). Surprisingly, p110β bound to both RAC1 and CDC42 in a GTP-dependent manner. Weaker binding to ROH and minimal
binding to RHOA was also observed (Figure 2E). Importantly, RAC1, CDC42, RHOG, and RHOA preparations bound similar amounts of GTP, indicating proper folding and functionality (Figure 2E, right lower graph), and a RAC1-I33D mutant showed reduced binding to p110β (Figure 2E), confirming a key role of this residue in GTPase binding to p110β.

The RAC1/CDC42-p110β interaction was isoform specific because neither RAC1 nor CDC42 significantly bound non-β isoforms under parallel conditions (Figures 2F and S2F). Strikingly, GTPγS-loaded RAC1 or CDC42 strongly stimulated p110β lipid kinase activity in vitro (Figure 2G), alone and in cooperation with a phosphotyrosine peptide (pY740), or when p110β was complexed with a less inhibitory, truncated p85 (Δp85 schematic in Figure 3C). Stimulation of p110β by active RAC1 and CDC42 was dose dependent (Figure 2H). Coexpression of constitutively active RAC1 or CDC42 (Figure S2G, lanes 4–6), but not RHOG (Figure S2H), along with p110β/p85 in COS7 cells strongly elevated cellular phospho-AKT and PIP3 levels (Figure 2I), indicating that both GTPases activate p110β in transfected cells. PIP3 levels were further enhanced by coexpression of Gβγ/Gγ2 subunits or by myristoylation of p110β (Figure 2J). In contrast, GTPγS-loaded RAC1/CDC42 did not stimulate p110α in vitro (Figure S2J), nor did V12-RAC1/CDC42 cooperate with p110α, p110γ, or p110δ to elevate cellular phospho-AKT levels (Figure S2J). Taken together, these data show that the RHO family GTPases RAC1 and CDC42 bind to p110β in an isoform-specific manner and potently and directly stimulate its lipid kinase activity.

**RAC and CDC42 Are RBD Interactors of p110β**

We next aimed to confirm that RAC and CDC42 are RBD interactors of p110β. Purified recombinant wild-type p110β/p85 bound to RAC1 and CDC42 in a concentration-dependent manner, whereas p110β-RBD-DM/p85 complexes showed no binding (Figure 3A). Similarly, RBD mutant p110β was not stimulated by active RAC1 or CDC42 in vitro (Figure 3B) or in cells (Figure S2E, lanes 7–9). To test whether the BCR homology domain (BHD) on p85, which had previously been shown to bind RAC and CDC42 (Bokoch et al., 1996; Zheng et al., 1994), is required for RAC/CDC42 binding to p110β, we truncated p85 (Δp85 schematic in Figure 3C) and probed for binding of p110β/Δp85 to RAC1 and CDC42 in vitro. Intriguingly, binding was unaffected by removal of the BHD but completely disrupted when full-length p85 was in complex with RBD mutant p110β, strongly arguing for the RBD as the RAC/CDC42-binding site. To further corroborate these findings, we generated 43 single point mutations covering 37 residues across the p110β RBD and assayed these mutants for binding to RAC1 and CDC42 (Figures 3D, S3A, and S3B). Of those, 17 mutations of 14 RBD residues affected binding to both GTPases without affecting p110β protein stability. Several of these residues were part of the RBDβ1 and β2 sheets or the loop adjacent to the RBDα1 helix (Zhang et al., 2011), areas known to be important for RAS binding in non-β isoforms (Pacold et al., 2000). Finally, we employed isothermal titration calorimetry (ITC) to study thermodynamics of the RAC1/CDC42-p110β interaction. In solution, RAC1-GTP·S bound to p110β/Δp85 with a molar ratio close to 1 and an average Kd of 1.42 μM, whereas the affinity measured for CDC42-GTP·S was 3.1 μM (Figures 3E and 3F). Similar affinities have been reported for the RAS-p110α and RAS-p110γ interactions (Pacold et al., 2000; Rodriguez-Viciana et al., 1996), indicating that RAC1 and CDC42 are plausible RBD interactors of p110β. No binding was observed between GTPγS-loaded RAC1/CDC42 and p110α or p110β-RBD-DM, respectively (Figure S3C).

**p110β-RBD-DM Mice Show Signs of Reduced PI3K Activity**

To study the role of interactor binding to the p110β RBD for PI3K signaling in vivo, we generated mice harboring the two p110β RBD point mutations (S205D/K224A) within their germline. Homologous recombination in embryonic stem (ES) cells was employed to replace exon 6 of the murine Pik3cb gene (Figure S4A), and germline transmission was achieved by eight-cell embryo injection (Figures S4B and S4C). p110β-RBD-DM mice were viable and fertile, although numbers of homozygous animals at the time of biopsy (around day 14) were moderately reduced (73 where 105 were expected; p < 0.02; Figure 4A), indicating incomplete lethality for undetermined reasons. Newborn homozygous p110β-RBD-DM pups were smaller than their wild-type littermates (Figure 4B). The size difference in adult mice was subtle but remained significant when same-sex

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**Figure 1. PI 3-Kinase p110β Is Unable to Interact with RAS**

(A) p110β does not bind to GTP-loaded RAS proteins. Purified recombinant, GTP-S-loaded HRAS, KRAS, NRAS, and RAB5 were incubated with lysates from COS7 cells expressing FLAG-p110α/p85 or FLAG-p110β/p85, wild-type (WT), or RBD double mutant (DM).

(B) Active RAS proteins do not bind to immobilized p110β/p85. Purified recombinant GST-p110α/p85 and GST-p110β/p85 were incubated with lysates from COS7 cells expressing Myc-tagged, constitutively active HRAS, KRAS, NRAS, and RAB5.

(C) Active RAS proteins do not stimulate p110β in cells. Lipids were extracted and PIP3 levels measured from COS7 cells expressing constitutively active mutants of HRAS, KRAS, NRAS, and RAB5, alone or in combination with p110α/p85 or p110β/p85 (n = 3; mean with SD; one-way ANOVA).

(D) Type I PI3K RBDs show moderate sequence similarity. Alignment of amino acid sequences of all four type I PI3K RBDs (PIK3CC = p110α, PIK3CD = p110β, PIK3CA = p110α, PIK3CG = p110γ) of RAS represents RAS-binding residues in p110α, and arrows represent conserved “RAS-binding” residues.

(E) Mutation of RBD key residues in p110β. The two point mutations are shown together with hypothetical interactor residues modeled on the RAS-p110γ interaction.

(F) Unaltered lipid kinase activity of recombinant p110β-RBD-DM protein. Lipid kinase assay assessing basal activities of purified recombinant p110β/p85 complexes (n = 3; mean with SEM).

(G) p110β-RBD-DM protein remains sensitive to Gβγ and phosphotyrosine. A representative lipid kinase assay assessing effect of recombinant Gβγ and a PDGFR-derived phosphotyrosine peptide (pY740) on the activity of purified recombinant p85/p110β-WT and p85/p110β-RBD-DM is shown.

(H) Activity of p110β-RBD-DM in living cells is reduced. Lipids were extracted and PIP3 levels measured from COS7 cells expressing wild-type or RBD mutant p110β/p85. Gβγ, coexpression of Gβ2 and Gγ1; Myr, myristoylated p110β (n = 3; mean with SEM; paired t test).

See also Figure S1.
litter- and cage-mates were compared (Figure 4C). MEFs homozygous for the p110β RBD mutation proliferated at a significantly slower rate than their wild-type counterparts (Figure 4D), which was reflected by a higher percentage of cells in G1 (1% fetal calf serum [FCS]: 56.7% ± 0.32% versus 64.2% ± 0.53%, n = 4, p < 0.001; 10% FCS: 47.5% ± 3.0% versus 52.2% ± 3.9%, n = 4, p < 0.05), fewer cells in G2 (1% FCS: 13.4% ± 1.4% versus 21.0% ± 1.9%, n = 4, p < 0.05; 10% FCS: 23.9% ± 1.4% versus 21.1% ± 1.6%, n = 4, p < 0.05) and fewer cells in S phase for 1% FCS (1% FCS: 13.4% ± 1.4% versus 11.0% ± 169%, n = 4, p < 0.05) (Figure 4E). Moreover, p110β-RBD-DM MEFs showed lower steady-state phospho-AKT levels (Figure 4F), suggesting that stimulatory signaling to p110β via its RBD contributes to PI3K activity in vivo. Expression levels of p110β, p110α, and AB

See also Figure S2.
p85 were indistinguishable among the genotypes (Figure S4D), and the stoichiometry of p110 subunit binding to p85 was undisturbed (Figure S4E).

**RAC and CDC42 Regulate p110β In Vivo**

To determine whether RAC and CDC42 are upstream activators of p110β in vivo, we transfected wild-type and p110β-RBD-DM MEFs with small interfering RNA (siRNA) pools (Dharmacon On-target plus) targeting these GTPases (Figure 4G). Although single knockdowns had only minor effects, combined knockdown of p85 were mutually exclusive in vitro and in vivo. Purified recombinant p85/p110β (WT) and p85/p110β-RBD-DM (DM) protein complexes, at the indicated concentrations, were incubated with GST-tagged, GDP/GTPγS-loaded RAC1 and CDC42. (B) RAC1 and CDC42 do not stimulate p110β-RBD-DM lipid kinase activity. Purified recombinant GTPγS-loaded RAC1/CDC42 (1 μM) were added to purified recombinant p85/p110β-RBD-DM in lipid kinase assays (n = 2; mean with SD). Data are part of experiment shown in Figure 2G. (C) The N terminus of p85 is not required for p110 binding to RAC1/CDC42. (D) Single RBD point mutations disrupt p110β binding to RAC1/CDC42. (E) Thermodynamic characterization of the RAC1/CDC42-p110β interaction by ITC. Binding of purified recombinant GTPγS-loaded RAC1 and CDC42 to purified recombinant p110β/p85 in solution was studied. Top: differential power over time; bottom: thermal energy (H) over molar ratio. (F) Table summarizes results from ITC experiments. Numbers represent K_d values determined in independent experiments. n.b., no binding. See also Figures S3A and S3B.

RAC1 and CDC42 significantly lowered phospho-AKT levels in wild-type, but not in p110β-RBD-DM cells, closing the gap in steady-state phospho-AKT levels between the genotypes and suggesting that endogenous RAC1 and CDC42 cooperatively activate p110β via its RBD. We next acutely expressed constitutively active mutants of RAC1 and CDC42 in wild-type, p110α-, and p110β-knockout MEFs (Figures 4H and S4F). Both RAC1 and CDC42 increased steady-state phospho-AKT levels in wild-type and p110α-deleted cells but not in p110β-knockout MEFs. Moreover, expression of V12-RAC1 and V12-CDC42 failed to elevate phospho-AKT levels in p110β-RBD-DM MEFs (Figure 4I). Taken together, these findings indicate that RAC1 and CDC42 activate PI3K in living cells by isoform-specific regulation of p110β through its RBD.

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**Figure 3. RAC and CDC42 Are Interactors of the p110β RAS-Binding Domain**

(A) RAC1 and CDC42 do not bind p110β-RBD-DM in vitro. Purified recombinant p85/p110β (WT) and p85/p110β-RBD-DM (DM) protein complexes, at the indicated concentrations, were incubated with GST-tagged, GDP/GTPγS-loaded RAC1 and CDC42. (B) RAC1 and CDC42 do not stimulate p110β-RBD-DM lipid kinase activity. Purified recombinant GTPγS-loaded RAC1/CDC42 (1 μM) were added to purified recombinant p85/p110β-RBD-DM in lipid kinase assays (n = 2; mean with SD). Data are part of experiment shown in Figure 2G. (C) The N terminus of p85 is not required for p110β binding to RAC1/CDC42. (D) Single RBD point mutations disrupt p110β binding to RAC1/CDC42. (E) Thermodynamic characterization of the RAC1/CDC42-p110β interaction by ITC. Binding of purified recombinant GTPγS-loaded RAC1 and CDC42 to purified recombinant p110β/p85 in solution was studied. Top: differential power over time; bottom: thermal energy (H) over molar ratio. (F) Table summarizes results from ITC experiments. Numbers represent K_d values determined in independent experiments. n.b., no binding. See also Figures S3A and S3B.
Activation of p110β Downstream of GPCRs Requires an Intact RBD

To study whether the p110β RBD is required for coupling p110β to GPCRs, we stimulated wild-type and p110β-RBD-DM MEFs with the lipid growth factors and GPCR agonists LPA and S1P.
pathway, we performed a small candidate siRNA screen.

Figure S5b

Downstream of GPCRs Requires

Activation of p110β Downstream of GPCRs Requires RAC

To test whether the identified p110β RBD interactors are required for linking p110β to GPCRs, we knocked down RAC1 and CDC42 in wild-type MEFs. Knockdown of RAC1 strongly impacted LPA/S1P-induced AKT phosphorylation, knockdown of CDC42 had only minor effects, and combination knockdown induced by tyrosine kinase receptor agonists (EGF, PDGF, and insulin; Figure S5E). Similarly, EHT1864, a direct inhibitor of RAC but not CDC42 activation, dose-dependently inhibited AKT phosphorylation induced by LPA/S1P (Figure 5E), but not EGF, PDGF, or insulin (Figure S5F). Therefore, acute loss or inhibition of RAC phenocopied the signaling defect observed in p110β-RBD-DM MEFs. In line with this, RAC was activated very rapidly upon LPA stimulation, reaching its peak activity within 20 s (Figure 5F).

Dock180/Elmo1 Activates RAC Downstream of GPCRs and Upstream of p110β

To provide further mechanistic insight into the GPCR-RAC-p110β pathway, we performed a small candidate siRNA screen to identify the guanine nucleotide exchange factor (RAC-GEF) involved. Transfection of wild-type MEFs with siRNA pools of both RAC1 and CDC42 had little additional effect compared to RAC1 knockdown alone (Figure 5D). Deconvolution experiments using single siRNA oligonucleotides confirmed the leading role of RAC1 in this pathway (Figure S5D). Neither RAC1 nor CDC42 knockdown affected LPA/S1P-induced phosphorylation of ERK or activation of either pathway.

(Figure 5B and not shown). In contrast, p110β-RBD-DM MEFs responded normally to epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin in dose-response (Figure S5C) and time course experiments (data not shown). Notably, in p110β-knockout cells, AKT phosphorylation in response to LPA was completely abolished (Figure 5C), indicating that the RBD is essential for much but not all p110β activation downstream of GPCRs.
targeting the Dbl family RAC-GEFs Vav1-3, PREX1/2, and α/β-PIX had no clear effect on LPA/S1P-induced AKT phosphorylation (Figures 6A and S6A). In contrast, knockdown of the Dock family RAC-GEF Dock180 or its adaptor protein Elmo1 interfered with AKT phosphorylation induced by LPA and S1P (Figure 6B and S6B), but not by EGF, PDGF, and insulin (Figure S6C). Specificity of results was confirmed in deconvolution experiments using individual siRNA oligonucleotides targeting Dock180 and Elmo1 (Figures S6D and S6E). Knockdown of Elmo2 had no effect (Figure S6F). Moreover, knockdown of Dock180 abolished LPA- but not EGF-induced RAC activation (Figure 6C), firmly placing Dock180/Elmo1 downstream of the LPA receptor and upstream of RAC and p110β. The DHR-1 domain of Dock180 has been shown to bind Pip3 (Côté et al., 2005), raising the possibility of a Pip3-driven feedback loop, in which RAC would be upstream and downstream of p110β. However, whereas sensitive to pertussis toxin, LPA-induced RAC activation was entirely insensitive to Pip3 inhibition by GDC0941, a pan

**Figure 6. Dock180/Elmo1 Activate RAC Downstream of GPCRs and Upstream of p110β**

(A) siRNA pools targeting Dbl family RAC-GEFs fail to affect LPA-induced AKT phosphorylation. Immortalized wild-type MEFs were transfected with scrambled duplex or gene-specific siRNA pools targeting indicated Dbl family RAC-GEFs. A total of 48 hr after transfection, serum-starved cells were stimulated with LPA (10 μM) for 5 min.

(B) Dock180 and Elmo1 are essential for LPA-induced AKT phosphorylation. Immortalized wild-type MEFs were transfected with scrambled duplex or Dock180-specific siRNA pools targeting indicated Dock family RAC-GEFs. Then 48 hr after transfection, serum-starved cells were stimulated with LPA (10 μM) for 5 min.

(C) Dock180 is essential for LPA-induced RAC activation. Immortalized wild-type MEFs, transfected with scrambled duplex or Dock180-specific siRNA pools, were stimulated with LPA (10 μM) or EGF (10 ng/ml) for 20 s and active RAC was quantified (n = 4; mean with SEM; t test; bottom: a representative experiment).

(D) LPA-induced RAC activation is Pip3 independent. Immortalized MEFs were preincubated with PTX (200 ng/ml, 16 hr) or GDC0941 (10 μM, 1 hr) and stimulated with LPA (10 μM) for the indicated time periods (n = 4; mean with SEM; one-way ANOVA; bottom: representative lysates).

(E) Gβγ subunits directly bind to the N terminus of Elmo1. GST-tagged full-length Elmo1 and fragments as shown (schematic) were incubated with lysates from COS7 cells expressing Gβ2 and Gγ1. Bound Gβγ was detected by western blot.

(F) Model of GPCR-induced p110β activation. See text for details.

See also Figure S6.
in Figure 6F). We could not detect any tyrosine phosphorylation on p85 in response to LPA (Figure S6G), and the tyrosine kinase inhibitors dasatinib, erlotinib, and FP2 (all at 1 μM) had no effect on the signaling pathway studied (data not shown).

**DISRUPTION OF THE LPA-DOCK180/ELMO1-RAC-P110α AXIS AFFECTS FIBROBLAST CHEMOTAXIS**

To assess the functional importance of the Dock180/Elmo1-Rac-p110α signaling axis for fibroblast chemotaxis, we employed transwell filter assays to study migration in gradients of LPA and PDGF. p110α-RBD-DM MEFs showed significantly reduced migration in LPA but not PDGF gradients (Figure 7A). Similarly, wild-type MEFs transfected with siRNA pools targeting Dock180, Elmo1, or Rac1 showed defective migration in gradients of LPA but not PDGF (Figure 7B), pointing to the specificity of this pathway for GPCR-induced chemotaxis. In contrast, PI3K activity was required for normal migration in either gradient, because pretreatment of wild-type cells with GDC0941 strongly affected migration toward LPA and PDGF, whereas pretreatment of cells with pertussis toxin selectively blocked migration in LPA gradients (Figure S7A).

In agreement with a key role of Rac upstream of p110α in fibroblast migration, acute expression of constitutively active Rac (V12-Rac1) stimulated migration of wild-type but not p110α-RBD-DM MEFs in the absence of chemotactant and in the presence of a low concentration of LPA (10 nM) in the lower chamber, whereas migration toward 1% FCS was largely unaffected (Figure S7B).

**P110α-RBD-DM MICE ARE PROTECTED FROM BLEOMYCIN-INDUCED LUNG FIBROSIS**

LPA has been identified as important fibroblast chemoattractant in bleomycin-induced lung fibrosis, a well-studied mouse model of human fibrotic lung disease, and was found to be elevated in patients with idiopathic lung fibrosis (Tager et al., 2008). We therefore wondered whether the disruption of p110α activation by LPA in p110α-RBD-DM mice would be sufficient to affect experimental lung fibrosis. Following a single intratracheal application of bleomycin, a quarter of all wild-type animals died or had to be culled according to local animal welfare regulations, whereas all p110α-RBD-DM mice survived (Figure 7C). Also, wild-type but not p110α-RBD-DM mice significantly lost body weight (Figure 7D) upon bleomycin treatment. Lung weights increased in both groups, but to a significantly lesser extent in p110α-RBD-DM mice (Figure S7C). Histology of lungs 14 days after bleomycin challenge revealed extended areas of fibrotic changes in wild-type animals (Figure 7E, hematoxylin and eosin staining [H&E], top), characterized by accumulation of activated, smooth muscle antigen-positive fibroblasts (Figure 7E, middle) and deposition of crosslinked collagen fibers (Figure 7E, Sirius red, bottom). Changes in p110α-RBD-DM mice were milder, with some mice showing almost normal lungs and others showing more limited areas of fibrosis. Morphometric analysis of multiple nonoverlapping lung areas confirmed the differences between the genotypes: transparent lung areas were significantly reduced (Figures 7F and S7D) and SMA-positive areas significantly increased (Figure 7G and S7E) in wild-type but not p110α-RBD-DM mice when compared to saline controls.

**DISCUSSION**

**RAS PROTEINS DO NOT REGULATE THE UBIQUITOUS P110α ISOFORM**

In this study, we show that, in contrast to what has been widely presumed, Ras is not a general regulator of type I PI3Ks. We find that out of the two ubiquitously expressed PI3K isoforms, only p110α is regulated by Ras, whereas p110β is a direct Rac and CDC42 target protein, indicating that key members of the pivotal Ras and Rho families of small G proteins directly regulate type I PI3Ks, with each family controlling their own distinct ubiquitous p110 isoform.

That p110β proved unable to physically and functionally interact with Ras is unexpected given the presence of a moderately conserved PI3K-type RBD in all four type I PI3K p110 catalytic subunits (Pacold et al., 2000). Comparison between the published structures of the four RBDs in their interferor-free states reveals little pointing to the distinct interactor specificity of p110β (R. Chaleil and P. Bates, personal communication). p110β is not only unable to interact with Ras under conditions readily revealing the transient, low-affinity interactions of Ras with other isoforms, but it also has an entirely distinct Ras superfamily GTPase interactor profile with a subfamily switch from Ras to Rho at the core of it, making any, for whatever reason, undetectable interaction with Ras unlikely. RBDs, classified on grounds of a ubiquitin fold structure with interactor specificities distinct from Ras, are not uncommon, as exemplified by the human formin FHOD1, a Rac interactor (Schulte et al., 2008), or the N terminus of Elmo1, shown to bind RHOG and the ARF family member ARL4A (Patel et al., 2011).

**RAC AND CDC42 ARE ISOFORM-SPECIFIC RBD INTERACTORS OF P110α**

Our biochemical experimentation identifies Rac1 and CDC42 as RBD interactors of p110α. An association of PI3K with Rac and CDC42 was first noticed nearly 20 years ago (Tolias et al., 1995), but was attributed to Rac/CDC42 binding to the amino-terminal BHD on p85, which has sequence homology to RHO-GAP domains (Bokoch et al., 1996; Zheng et al., 1994). These studies left the epistasis of information transfer between Rac/CDC42 and PI3K unclear. In retrospect, all functional data from these studies can be explained by the presence of p110α in the cell lysates and PI3K preparations used. Only the reported binding of Rac and CDC42 to recombinant p85 remains puzzling. We did not study monomeric p85, which has not been found in living cells (Geering et al., 2007), but our biochemical data strongly argue against an involvement of p85 in the Rac/CDC42-p110α interaction, because (1) Rac1 and CDC42 do not interact with p110α/ p85 or p110β/p85, (2) Rac1 and CDC42 bind normally to p110β in the absence of the p85 BH domain, and (3) RBD point mutations abrogate Rac1 and CDC42 binding to p110β in complex with full-length p85. A body of literature has accumulated identifying Rac or CDC42 as essential upstream activators of PI3K in various systems (Keely et al., 1997; Srinivasan et al., 2003; Weiner et al., 2002), but straightforward analysis of the relationship between Rho family GTPases and PI3K has been difficult, mainly because Rac and CDC42 also act downstream of PI3K, activated through PIP3-dependent GEFs (Welch et al., 2003). A very recent study
Figure 7. p110β-RBD-DM Mice Are Protected from Bleomycin-Induced Lung Fibrosis

(A) p110β-RBD-DM fibroblasts show reduced migration in gradients of LPA. Migration of wild-type and p110β-RBD-DM MEFs in gradients of LPA and PDGF was assessed in transwell filter assays (n = 3; mean with SD; one-way ANOVA).

(B) Dock180, Elmo1, and RAC1 are required for fibroblast migration in gradients of LPA. Immortalized wild-type MEFs were transfected with scrambled duplex or gene-specific siRNA pools targeting Dock180, Elmo1, or RAC1. Migration in gradients of LPA and PDGF was assessed in transwell filter assays and cell numbers were normalized to control conditions (n = 4; mean with SEM; one-way ANOVA).

(C) p110β-RBD-DM mice are protected against death from bleomycin-induced lung damage. Wild-type and homozygous p110β-RBD-DM mice were treated with a single intratracheal dose of bleomycin (1.25 U/kg) and observed for 14 days (n = 16 mice per genotype; Mantel-Cox test).

(D) p110β-RBD-DM mice are protected against weight loss following bleomycin instillation. Wild-type and p110β-RBD-DM mice received a single intratracheal dose of saline (n = 3 per genotype) or bleomycin (n = 10 per genotype) and weights were taken 14 days later (mean ± SEM; one-way ANOVA).

(E) p110β-RBD-DM mice are protected from bleomycin-induced lung fibrosis. Representative lung areas from wild-type and homozygous p110β-RBD-DM mice 14 days after treatment with intratracheal bleomycin (×4 magnification). Top: H&E; middle: IHC for α-SMA; bottom: Sirius red.

(F) p110β-RBD-DM mice are protected against loss of transparent lung areas following bleomycin instillation. Lungs were analyzed by H&E 14 days after bleomycin challenge. Multiple nonoverlapping areas of representative sections from each lung were photographed and transparent (white) areas were quantified using Nikon NIS elements software (mean ± SEM; one-way ANOVA; see Figure S7C for raw data).

(G) p110β-RBD-DM mice accumulate fewer activated lung fibroblasts following bleomycin instillation. Lungs were analyzed by immunohistochemistry for smooth muscle antigen (α-SMA) 14 days after bleomycin challenge. Multiple nonoverlapping areas of representative sections from each lung were photographed and SMA-positive (brown) areas were quantified using Nikon NIS elements software (mean ± SEM; one-way ANOVA; see Figure S7D for raw data). See also Figure S7.
using microscopy-based assays in transfected cells revisited the interaction of small GTPases and PI3K, confirming that both active RAS and RHO family GTPases can activate PI3K in living cells. However, based on experimentation exclusively with a p110β reporter construct, the authors interpreted PI3K regulation by RHO family members as indirect (Yang et al., 2012).

**RAC/CDC42 Binding to p110β Controls PI3K Activity In Vivo**

Our findings in MEFs suggest that RAC1 and CDC42 cooperatively control steady-state PI3K activity in living cells by isoform-specific activation of p110β and that this requires the p110β-RBD. A model in which p110β provides basal, low-level PI3K activity has been proposed in the context of insulin signaling (Knight et al., 2006), and data showing PTEN-loss-driven prostate cancers to be entirely dependent on p110β, as well as metabolic findings in p110β kinase-dead mice, have been interpreted in the same way (Ciraolo et al., 2008; Jia et al., 2008). Our data point to RAC1 and CDC42 as drivers of such a basal, p110β-controlled activity, an idea consistent with a previous report finding that ectopic expression of wild-type but not RBD mutant p110β is sufficient to transform chicken embryo fibroblasts (Kang et al., 2006). It will be interesting to explore the oncogenic potential of the RAC/CDC42-p110β interaction in the setting of PTEN loss and also in the context of the recently discovered activating mutations in RAC (Hodis et al., 2012; Krauthammer et al., 2012) in human melanomas, where p110β could be an important downstream target.

**GPCRs Activate p110β through its RBD**

A firm body of in vivo evidence (Ciraolo et al., 2008; Guillermet-Guibert et al., 2008; Jia et al., 2008) has established p110β as a GPCR-regulated PI3K isoform. We find that mutation of the p110β-RBD strongly attenuates p110β activation downstream of GPCRs, highlighting the importance of the RBD for p110β key signaling functions. The residual p110β activity in p110β-RBD-DM MEFs argues for a second, RBD-independent activation route, for which direct binding of Gβγ to p110β (Dbouk et al., 2012) is the most obvious candidate. A puzzling question is whether a cooperative effect of RBD interactors and Gβγ binding to p110β can suffice to fully activate p110β, or if additional phosphotyrosine input is required to overcome the strong inhibition of lipid kinase activity imposed by p85 (Zhang et al., 2011). Trans-activation of receptor tyrosine kinases has been suggested to activate p110β downstream of GPCRs (Yart et al., 2002). Although we found no effect of tyrosine kinase inhibitors and p85 phosphotyrosine phosphorylation in response to LPA in support of such a mechanism in MEFs, there is evidence for cooperative GPCR and phosphotyrosine signaling to p110β in leukocytes (Kulkarni et al., 2011), and such a scenario appears possible in thrombocytes, where p110β is activated by integrins, ITAM-bearing receptors, and GPCRs (Martin et al., 2010).

**Dock180/Elmo1 Couples GPCRs to RAC and p110β**

RAC1 is essential for p110β activation downstream of the GPCRs for LPA and S1P, and the RAC-GEF Dock180/Elmo1 is upstream of both RAC and p110β in this pathway. The Dock/Elmo-RAC signaling axis is a highly conserved pathway controlling RAC-dependent key functions such as actin remodeling, migration, and phagocytosis (Côté et al., 2005). Recent findings in Dictyostelium have directly linked Gβγ subunits from GPCRs to Dock/Elmo-RAC and the cytoskeleton (Yan et al., 2012). Reminiscent of Dictyostelium ElmoE, we find the N terminus of human Elmo1 to directly bind Gβγ subunits, suggesting conservation of this pathway in mammals. In line with this, PI3K activity is not required for RAC activation by LPA, placing p110β entirely downstream of RAC in fibroblasts, which contrasts with a proposed PI3γ-driven feedback loop controlling RAC activity upstream and downstream of PI3K in leukocytes (Weiner et al., 2002). Although PI3K is not required to activate RAC, it is still essential for fibroblast migration in gradients of LPA, indicating that PI3γ-regulated pathways distinct from RAC activation contribute to GPCR-driven chemotaxis. Importantly, whereas our findings identify Dock180/Elmo1 downstream of LPA/S1P in MEFs, our experiments cannot rule out involvement of other RAC-GEFs within this pathway. We also cannot directly prove that the role of Dock180/Elmo1 in controlling fibroblast migration is exclusively and directly through activation of RAC upstream of p110β. Finally, different GPCRs and other cell types may signal through RAC-GEFs other than Dock180/Elmo1, and further studies will be required to determine whether the Dock180/Elmo1-RAC-1-p110β axis is a fixed signaling module or just one example of how RAC/CDC42 is activated upstream of p110β.

Overall, the picture that emerges from these studies is one in which p110β regulation by GPCRs operates through a two-track signaling pathway with direct and indirect input into p110β. The direct route involves Gβγ interaction with p110β, whereas the indirect route goes through stimulation of RAC via Dock180/Elmo1, possibly also recruited through Gβγ (see Figure 6F). Such two-track wiring is reminiscent of receptor tyrosine kinase regulation of p110α via p85 interaction with tyrosine-phosphorylated receptor or adaptor protein and Grb2-Sos-RAS-p110α interaction. It can be speculated that this signaling logic might provide an improved ability to amplify a weak signal input or might increase the possibility for fine-tuning the signal through crosstalk of other pathways onto components such as Dock180/Elmo1, RAC itself, or RAC-GAPs terminating RAC activity. Another interesting issue is whether such Gβγ-Elmo1 and Gβγ-p110β interactions are mutually exclusive or can occur in a heterotrimeric complex. At present, we cannot distinguish between a model in which one Gβγ protein heterodimer binds directly to both Elmo1 and p110β simultaneously and one in which Elmo and p110β are engaged by two different Gβγ protein heterodimers (as shown in Figure 6F).

See Supplemental Information online for the Extended Discussion, including information on other GTPase interactors of p110β, the phenotype of p110β-RBD mutant mice, and resistance of these mice to bleomycin-induced lung fibrosis.

**EXPERIMENTAL PROCEDURES**

Detailed procedures are described in the Extended Experimental Procedures.

**Isothermal Titration Calorimetry**

Purified recombinant soluble PI3K protein complexes were loaded into the cell of a Microcal iTC200 microcalorimeter at concentrations of approximately 20 μM. Nucleotide-loaded GTPases were loaded into the syringe at 200 μM.
In a typical experiment, 16 injections of GTPase into the cell were recorded at 15°C, and relevant thermodynamic parameters were analyzed and calculated

**Bleomycin-Induced Lung Fibrosis**

All animal experimentation was carried out in compliance with UK Home Office animal welfare regulations. Age- and sex-matched wild-type and homozygous p110β–/– mice from intercrosses of heterozygous p110β–/– mice were used to study bleomycin-induced lung fibrosis. Mice (8–12 weeks old) were anaesthetized with isoflurane, and bleomycin in sterile normal saline (1.25 U/kg) or saline alone (50 μl) was given by intratracheal instillation. After 14 days, mice were culled and lungs were recovered for further analysis.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Discussion, Extended Experimental Procedures, seven figures, and one table and can be found with this article online at [http://dx.doi.org/10.1016/j.cell.2013.04.031](http://dx.doi.org/10.1016/j.cell.2013.04.031).

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