

The p110 β isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110 γ

Julie Guillermet-Guibert*, Katja Bjorklof*, Ashreena Salpekar*, Cristiano Gonella*, Faruk Ramadani[†], Antonio Bilancio*, Stephen Meek[‡], Andrew J. H. Smith[‡], Klaus Okkenhaug[†], and Bart Vanhaesebroeck*[§]

*Center for Cell Signaling, Institute of Cancer, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom; [†]Laboratory of Lymphocyte Signaling and Development, Babraham Institute, Cambridge CB2 3AT, United Kingdom; [‡]Gene Targeting Laboratory, The Institute for Stem Cell Research, University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ, United Kingdom

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The p110 isoforms of phosphoinositide 3-kinase (PI3K) are acutely regulated by extracellular stimuli. The class IA PI3K catalytic subunits (p110 α , p110 β , and p110 δ) occur in complex with a Src homology 2 (SH2) domain-containing p85 regulatory subunit, which has been shown to link p110 α and p110 δ to Tyr kinase signaling pathways. The p84/p101 regulatory subunits of the p110 γ class IB PI3K lack SH2 domains and instead couple p110 γ to G protein-coupled receptors (GPCRs). Here, we show, using small-molecule inhibitors with selectivity for p110 β and cells derived from a p110 β -deficient mouse line, that p110 β is not a major effector of Tyr kinase signaling but couples to GPCRs. In macrophages, both p110 β and p110 γ contributed to Akt activation induced by the GPCR agonist complement 5a, but not by the Tyr kinase ligand colony-stimulating factor-1. In fibroblasts, which express p110 β but not p110 γ , p110 β mediated Akt activation by the GPCR ligands stromal cell-derived factor, sphingosine-1-phosphate, and lysophosphatidic acid but not by the Tyr kinase ligands PDGF, insulin, and insulin-like growth factor 1. Introduction of p110 γ in these cells reduced the contribution of p110 β to GPCR signaling. Taken together, these data show that p110 β and p110 γ can couple redundantly to the same GPCR agonists. p110 β , which shows a much broader tissue distribution than the leukocyte-restricted p110 γ , could thus provide a conduit for GPCR-linked PI3K signaling in the many cell types where p110 γ expression is low or absent.

gene targeting | signaling | tyrosine kinase | Akt | insulin

The lipid second messengers generated by phosphoinositide 3-kinases (PI3Ks) regulate a wide variety of cellular functions such as cell growth, proliferation, differentiation, and survival and have been implicated in cancer, inflammation, and diabetes. Mammals have eight isoforms of PI3K, which have been divided in three classes (1). Thus far, attention has focused mainly on the class I PI3Ks that are acutely activated by extracellular ligands. These heterodimers consist of a p110 catalytic subunit in complex with a regulatory subunit and have further been subdivided into class IA and IB PI3Ks. The class IA catalytic subunits (p110 α , p110 β , and p110 δ) are in complex with an Src homology 2 (SH2) domain-containing regulatory subunit (of which there are five species, often referred to as p85s) that binds phosphoTyr in intracellular proteins, allowing recruitment of p85/p110 complexes to the membrane. The class IB regulatory subunits (p84, p101) do not have SH2 domains and link the single class IB PI3K catalytic subunit (p110 γ) to G protein-coupled receptors (GPCRs).

Over the last few years, the cellular signaling contexts and physiological roles of p110 α , p110 δ , and p110 γ have become clearer, because of the generation of gene-targeted mice and small-molecule inhibitors for these PI3K isoforms. In contrast, very little is known about p110 β , both at the cellular and organismal level. One confounding factor has been the very early

embryonic lethality of the p110 β KO mice and the fact that proliferating cells could not be derived from these embryos (2). Recent progress has been made by the generation of small-molecule inhibitors with selectivity for p110 β , allowing scientists to define a role for p110 β in platelet function and thrombus formation (3). Some evidence has also been presented for the coupling of p110 β to GPCRs, either by *in vitro* studies that documented activation of p110 β by G $\beta\gamma$ subunits (4, 5) or in cellular experiments where p110 β function was probed by microinjection of neutralizing antibodies to p110 β (6, 7), RNAi against p110 β (8), or overexpression of p110 β (8, 9). Here, we have used pharmacological tools with selectivity for p110 β (3), in conjunction with cells derived from a mouse line in which p110 β has been inactivated by gene targeting, to investigate the role of p110 β in PI3K signaling downstream of Tyr kinase and GPCR ligands.

Results

Conditional Genetic Inactivation of p110 β . We have created a mouse line in which exons 21 and 22 of the kinase domain of p110 β are flanked by *loxP* sites [supporting information (SI) Fig. S1]. This floxed p110 β allele, which is further referred to as p110 β^{flox} , is schematically shown in Fig. 1A. Exon 22 contains the DFG motif and the activation loop, which are critical for the activity of kinases. The *loxP* sites were positioned in such a way that, after treatment with Cre, the locus is expected to give rise to an mRNA in which exon 20 is spliced in-frame onto exon 23, encoding an internally truncated p110 β protein with a predicted M_r of ≈ 112 kDa (further referred to as p110 $\beta^{\Delta 21,22}$) instead of the ≈ 122 -kDa WT protein.

From crosses with heterozygous parents, we obtained $\approx 30\%$ of expected numbers of viable mice, homozygous for the p110 $\beta^{\Delta 21,22}$ allele (p110 $\beta^{\Delta 21,22/\Delta 21,22}$ mice), indicating lethality with incomplete penetrance, for reasons that are unclear at this point of time. Mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages (BMMs) from viable p110 $\beta^{\Delta 21,22/\Delta 21,22}$ mice were used for further study. RT-PCR and DNA sequencing on mRNA prepared from p110 $\beta^{\Delta 21,22/\Delta 21,22}$ MEFs showed that the mutant p110 β locus gave rise to the expected truncated mRNA (Fig. S1I and data not shown).

In control p110 $\beta^{\text{flox/flox}}$ MEFs, lipid kinase activity in p110 β

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[§]To whom correspondence should be addressed. E-mail: bart.vanh@qmul.ac.uk.

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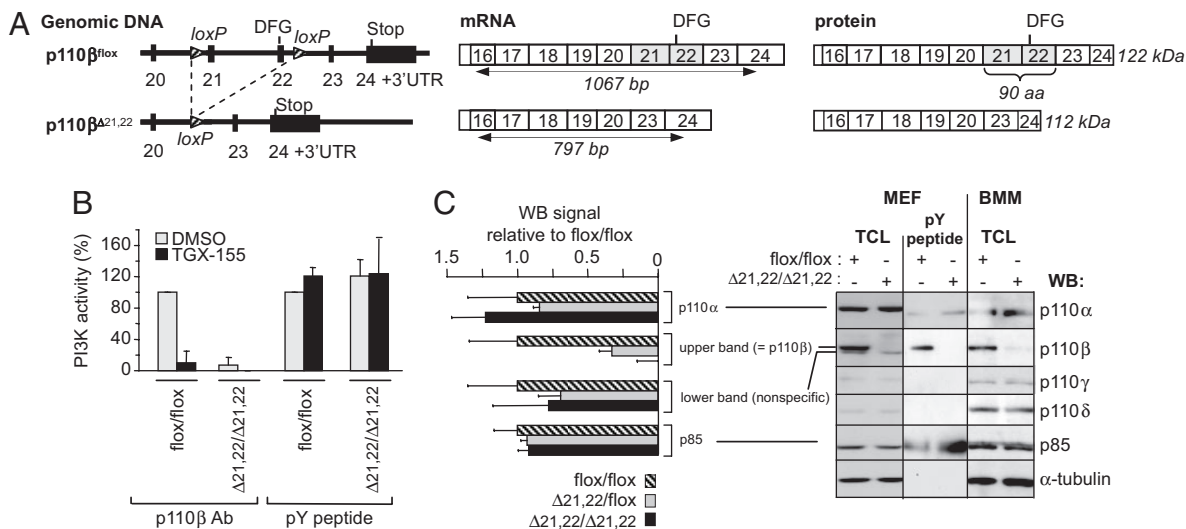


Fig. 1. Genetic inactivation of p110β in mice. (A) Schematic representation of genomic DNA, mRNA, and protein primary structure of the p110β^{flox} and p110β^{Δ21,22} locus. Exons 22 and 23 of *Pik3cb* were flanked by loxP sites as described in Fig. S1, creating a floxed p110β allele that is further referred to as p110β^{flox}. Exon 22 encodes the DFG domain and activation loop that are critical for p110β kinase activity. Constitutive deletion of exons 21 and 22 in mice was achieved by intercrossing p110β^{flox/flox} mice with Cre deleter mice, creating the p110β^{Δ21,22} allele. The loxP sites were positioned in such a way that, after treatment with Cre, the locus is expected to give rise to mRNA in which exon 20 is spliced in-frame onto exon 23, now encoding an internally truncated p110β protein lacking amino acids 886–975 in the catalytic domain. This p110β^{Δ21,22} protein is predicted to be kinase-inactive, with a ≈10-kDa reduction in *M_r* relative to the WT p110β protein and to retain reactivity with antisera raised against the extreme C terminus of p110β. (Left) Exon sequences are represented by filled black rectangles, and intron sequences are indicated by a horizontal black line. The loxP sites are shown as hatched triangles with the pointed end indicating orientation. (Center) Exon boundaries are represented from exons 16 to 24. The positions of the primers used for PCR screening are designated by arrows together with the expected amplification products and their size (bp). (Right) Schematic representation and predicted *M_r* of WT and mutant p110β proteins, based on exon boundaries from exons 16 to 24. (B) Effect of p110β deletion on *in vitro* lipid kinase activity. Homogenates of the indicated MEFs were immunoprecipitated by using p110β Abs or adsorbed onto PDGF receptor phosphoTyr peptide (pY peptide) immobilized to Sepharose (which binds all class IA PI3K regulatory subunits), followed by *in vitro* lipid kinase assay with or without 100 nM TGX-155. The level of p110β and p85 in the indicated cell fractions was verified by immunoblotting (data not shown). (C) Effect of p110β deletion on PI3K isoform expression. (Left) Analysis of p110β protein expression in MEFs from p110β^{flox/flox}, p110β^{flox/Δ21,22}, and p110β^{Δ21,22/Δ21,22} mice by Western blotting. Immunoblot signal intensities obtained with Abs to p110α, p110β, and p85 were expressed relative to signals obtained with antibodies to α-tubulin for at least three different embryos and expressed relative to the signals in p110β^{flox/flox} MEFs. Quantification of the signals detected by the sc-602 p110β Abs revealed that only the intensity of the upper band is decreased upon Cre treatment, whereas the intensity of the lower band is unaffected. p110α expression was not affected in each genotype. p110γ and p110δ were hardly detectable in fibroblasts and could not be reliably quantified. (Right) Total cell lysates or pY-peptide pull-downs were immunoblotted by using the indicated Abs. In NIH 3T3 cells and MEFs (but not in BMMs), the anti-p110β Abs used for Western blotting (sc-602) recognize a nonspecific protein just below the specific p110β signal, indicated as lower band (aspecific) and upper band (p110β), respectively. It is only the upper band that disappears upon p110β deletion.

immunoprecipitates, made using Abs against the C terminus of p110β, was sensitive to the p110β inhibitor TGX-155 (Fig. 1B). In contrast, no significant lipid kinase activity could be recovered in p110β immunoprecipitates (IPs) from p110β^{Δ21,22/Δ21,22} MEFs (Fig. 1B). PI3K activity associated with a phosphoTyr peptide matrix (which binds all p85 species; referred to as pY peptide) was unaffected in p110β^{Δ21,22/Δ21,22} MEFs (Fig. 1B), indicating that p110β contributes minimally to p85-associated PI3K activity in unstimulated fibroblasts.

Transient overexpression in HEK293 cells of the p110β^{Δ21,22} cDNA cloned from p110β^{Δ21,22} MEFs revealed that the p110β^{Δ21,22} protein could be translated from this mRNA (Fig. S1K). As expected, the ≈122-kDa WT p110β protein could no longer be detected in p110β^{Δ21,22/Δ21,22} MEFs and BMMs upon immunoblotting with C-terminal (Fig. 1C Right) or N-terminal antibodies to p110β (Fig. S1J). However, no evidence for the ≈112-kDa p110β^{Δ21,22} protein could be found (Fig. 1C and Fig. S1J), indicating that this truncated protein is either not produced, is further truncated, or is unstable [note that the lower *M_r* signal observed with the p110β Cter Abs in MEFs is a nonspecific signal that is not altered in p110β^{Δ21,22/Δ21,22} cells (Fig. 1C); this lower MW signal is not observed in BMMs]. The levels (Fig. 1C) and activity (Fig. S1L) of p110α were unaffected. The observation that expression of the p85 regulatory subunit and the other PI3K isoforms is not affected in MEFs and BMMs (Fig. 1C) argues against the degradation of the entire p110β protein,

which would most likely lead to a reduction in p85 levels (10). Thus, in p110β^{Δ21,22/Δ21,22} cells, p110β activity is absent, yet the p85/p110 stoichiometry is unaltered.

p110β and p110γ Signal Downstream of the Same GPCR Ligands in Cells Expressing All Class I PI3Ks. We first assessed the role of p110β in early PI3K signaling in BMMs that express all class I p110 isoforms, including p110γ (Fig. 1C).

Phosphorylation of Akt and Erk induced by the Tyr kinase ligand colony-stimulating factor-1 (CSF-1) was not affected by pharmacological or genetic inactivation of p110β or p110γ (Fig. S2 A-C; p110γ was inhibited by using AS604850, a small-molecule inhibitor with selectivity for p110γ (11)). These data indicate that p110β and p110γ do not play a major role in Tyr kinase-induced PI3K activation in BMMs. This was previously reported for p110γ (11–13), but is somewhat unexpected for p110β, given that injection of neutralizing Abs to p110β has been shown to block CSF-1-induced chemotaxis in a macrophage cell line (14).

In contrast to Tyr kinase ligands, Akt phosphorylation induced by the GPCR agonist complement 5a (C5a) was substantially, but not completely, inhibited by inactivation of p110β, either genetically (Fig. 2 A and C) or pharmacologically (Fig. 2 D and F). The residual C5a-induced Akt phosphorylation in the absence of p110β activity could be blocked by the p110γ inhibitor AS604850 (Fig. 2 G and I), indicating that p110γ controlled this component of the signal.

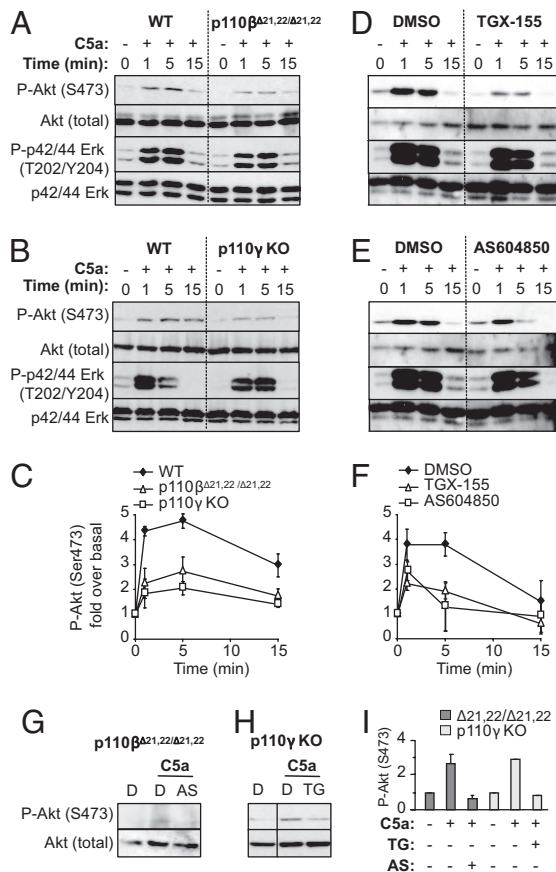


Fig. 2. Role of p110 β and p110 γ in cell signaling in macrophages. (A and B) Starved BMMs of the indicated genotype were stimulated with C5a, followed by immunoblotting using the indicated Abs. (D and E) Starved WT BMMs were treated for 30 min with TGX-155 (0.5 μ M) (D) or AS604850 (1 μ M) (E), followed by C5a stimulation and immunoblotting using the indicated Abs. (C and F) Quantification of at least two independent experiments was performed, and data are shown as fold over P-Akt under unstimulated conditions. (G–I) Starved BMMs of the indicated genotype were treated for 30 min with AS604850 (1 μ M) or TGX-155 (0.5 μ M) and stimulated for 5 min with C5a, followed by immunoblotting using the indicated Abs. I shows the quantification of the experiments in G and H. Data are expressed as fold over P-Akt under unstimulated conditions.

In line with previous reports (12), C5a-induced Akt phosphorylation was decreased in p110 γ KO BMMs (Fig. 2B and C) and in WT BMMs upon treatment with AS604850 (Fig. 2E and F). However, residual C5a-stimulated Akt phosphorylation was also observed under those conditions (see quantification of Western blot signal in Fig. 2C and F). This activity appears to be mediated by p110 β , because TGX-155 treatment inhibited the residual p-Akt signal in C5a-stimulated p110 γ KO BMMs (Fig. 2H and I).

Pertussis toxin (PTx) treatment blocked the C5a-induced pAkt signal in p110 γ KO and p110 $\beta^{\Delta 21,22/\Delta 21,22}$ cells (Fig. S2D and E), indicating that C5a-stimulated Akt phosphorylation mediated by p110 β and p110 γ , respectively, is G protein-dependent.

Taken together, these data show that p110 β signals effectively downstream of a GPCR in cells and that p110 β and p110 γ are activated downstream of the same GPCR ligand to mediate Akt phosphorylation. Genetic or pharmacological inactivation of p110 β or p110 γ did not affect CSF-1 or C5a-induced Erk phosphorylation (Fig. 2A, B, D, and E).

p110 β Controls GPCR-Induced Early PI3K Signaling in Fibroblasts. We next tested the role of p110 β in Tyr kinase and GPCR signaling

in NIH 3T3 and MEFs, which mainly express p110 α and p110 β and have undetectable or low levels of p110 γ and p110 δ , respectively (Fig. 1C and Fig. S3A). Absolute quantification of class IA p110 isoforms in NIH 3T3 cells showed that p110 β is expressed at much higher levels than p110 α and p110 δ [2,000 versus 2,000 and 1,000 molecules per cell, respectively (15)]. However, given that barely detectable levels of p110 γ can have a major biological impact [such as in cardiomyocytes (16)], we first tested the potential role of p110 γ in these cells. The p110 γ inhibitor AS604850 did not impair lysophosphatidic acid (LPA)-induced phosphorylation of Akt in NIH 3T3 cells (Fig. S3B). Moreover, in p110 γ KO MEFs, GPCR stimuli such as sphingosine-1-phosphate (S1P) and LPA could still induce Akt phosphorylation (Fig. S3C). This Akt phosphorylation was sensitive to TGX-155 but not to AS604850 (Fig. S3C), suggesting that p110 β is the main GPCR-coupled PI3K isoform in fibroblasts.

To further substantiate these findings, a broader range of agonists was tested in NIH 3T3 cells. Akt phosphorylation induced by PDGF, insulin, or insulin-like growth factor 1 (IGF1) was sensitive to LY294002 but was not blocked by TGX-155 (Fig. 3A Top). This finding is in stark contrast to Akt phosphorylation induced by each of the GPCR ligands tested [S1P, stromal cell-derived factor (SDF-1 α), LPA], which were equally sensitive to TGX-155 and LY294002 in each case (Fig. 3A Middle; for quantification of the results of three independent experiments with all six stimuli, see Fig. 3A Bottom). Similar results were obtained in WT MEFs upon stimulation with PDGF or GPCR ligands such as S1P and LPA (Fig. S3D) and upon using TGX-115 and TGX-221, two alternative inhibitors from the same chemical series as TGX-155 (3) (data not shown).

LPA-induced, but not PDGF-induced, phosphorylation of Akt was also inhibited by TGX-155 when tested at different time points (Fig. S3E). TGX-155 inhibited LPA-induced T308 and S473 phosphorylation of Akt in a dose-dependent manner, with an IC₅₀ of 0.1 μ M (Fig. S3F), in line with the PI3K isoform specificity of this compound (see Materials and Methods). Occasionally, an increase in LPA-induced S473 phosphorylation of Akt was observed at very low doses of TGX-155 (0.01 μ M; data not shown) for reasons that are unclear. PTx pretreatment decreased LPA-induced Akt phosphorylation in NIH 3T3 cells, but had no effect on PDGF-induced Akt phosphorylation (Fig. S3G).

In p110 $\beta^{\Delta 21,22/\Delta 21,22}$ MEFs, Tyr kinase (PDGF, insulin, IGF1, EGF)-induced Akt phosphorylation was largely unaffected, in contrast to Akt phosphorylation induced by the GPCR ligands LPA, SDF-1, and IL-8, which was almost completely blocked (Fig. 3B). Similar results were obtained with MEFs derived from mice that are homozygous for a kinase-dead allele of p110 β (p110 $\beta^{\text{D931A/D931A}}$; data not shown). Also acute genetic deletion of p110 β [by 4-hydroxytamoxifen treatment of MEFs from p110 $\beta^{\text{flox/flox}}$ mice crossed onto the ROSA-CreERT2 mice (17), resulting in activation of Cre by its translocation from the cytosol to the nucleus], reduced LPA-induced Akt phosphorylation, with the residual pAkt signal being no longer sensitive to TGX-155 (Fig. S3H).

Taken together, these data show that p110 β is critical for early PI3K signaling induced by GPCRs but is not a major transducer downstream of receptor Tyr kinases.

p110 γ Can Complement p110 β Function in GPCR-Induced PI3K Signaling in Fibroblasts. To test whether p110 γ could in principle signal downstream of GPCRs in fibroblasts, we stably transfected p110 γ , together with its p101 regulatory subunit, into NIH 3T3 cells (Fig. 3C Left). In control transfectants, the p110 γ inhibitor AS604850 did not decrease LPA-induced Akt phosphorylation (Fig. 3C). In contrast, NIH 3T3 cells expressing p110 γ became sensitive to AS604850 and also showed reduced sensitivity to the p110 β inhibitor TGX-155 (Fig. 3C). These data further substan-

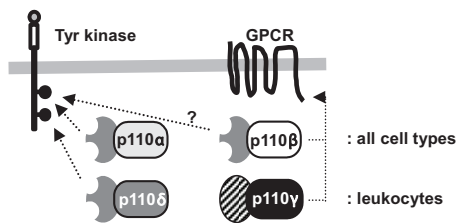


Fig. 4. Model for class IA PI3K signaling downstream of Tyr kinase receptors and GPCRs. The ubiquitously expressed p110 α plays an important role in Tyr kinase-driven PI3K signaling in all cell types, with p110 δ providing additional Tyr kinase-driven PI3K signaling in cell types in which it is expressed at a high level, such as in leukocytes. A similar scenario could be envisaged for the broadly expressed p110 β that could control GPCR-driven PI3K in all cell types, with p110 γ providing additional GPCR-PI3K signaling capacity in white blood cells.

aged for the ubiquitously expressed p110 β that could control GPCR-driven PI3K in all cell types, with p110 γ providing additional GPCR-PI3K signaling capacity in white blood cells.

Materials and Methods

Reagents. Small-molecule inhibitors were dissolved in DMSO, with final concentration of DMSO in the assays maximally 0.2%. LY294002 was from Calbiochem. The p110 β inhibitor TGX-155 [*in vitro* IC₅₀ for p110 α : >20 μ M; p110 β : 0.03 μ M; p110 γ : >20 μ M; p110 δ : 0.34 μ M (3, 33)] and p110 γ inhibitor AS604850 [*in vitro* IC₅₀ for p110 α : 3.4 μ M; p110 β : >20 μ M; p110 γ : 0.19 μ M; p110 δ : >20 μ M (11)] were a gift from Merck/Serono (Geneva). Abs to class IA PI3Ks were generated in-house. p110 β Ab used for IP was generated by using a C-terminal peptide of p110 β (KVNWMAHTVRKDYRS) as immunogen, affinity-purified over this peptide, and further purified over GST-p110 α and GST-p110 δ to deplete cross-reactivity with p110 α and p110 δ . p110 β Abs used for immunoblot were from Santa Cruz Biotechnology (sc-602). These recognize the last 19 aa of p110 β (SWTTKVNWMAHTVRKDYRS) and are referred to as p110 β C-ter Abs. Anti-N-terminal p110 β Abs used for Western blotting were generated in-house with p110 β peptides present in exon 3, 5, 9, and 12 (QLEVPREATISY, EFRRKMRKFSEAKIQ, CKTKKSTKINPSKYQ, and CLASGDSANVSSRGGK, respectively) as immunogen and used as a mix at 1 μ g/ml each; this mix is referred as p110 β Nter Abs. Additional PI3K Abs were from Upstate (p85pan; catalogue no. 06-195) or Alexis (p110 γ ; clone H1). Abs to Akt, α -tubulin, and GAPDH were from Cell Signaling, Sigma, and Abcam, respectively. Cell culture reagents were from Invitrogen. IGF1, EGF, SDF-1 α , PDGF-BB, S1P, LPA, CSF-1, and C5a were from Preprotech. PTx (*Bordetella pertussis*) and 4-hydroxytamoxifen were from Sigma.

Mice. Mice were kept in individually ventilated cages and cared for according to U.K. Home Office regulations. Constitutive conversion of the p110 β ^{fllox} gene into the p110 β ^{Δ 21,22} allele was achieved by crossing p110 β ^{fllox/fllox} mice with the Cre deleter strain. Cre-negative p110 β ^{Δ 21,22/fllox} mice from a second generation

were intercrossed to generate p110 β ^{Δ 21,22/ Δ 21,22} and p110 β ^{fllox/fllox} mice. Embryos from timed pregnant mice were used to prepare MEFs.

Fibroblast Culture and Stimulation. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. MEFs were generated from embryonic day 13.5 embryos that were minced and dissociated with trypsin, and cells were allowed to adhere on dishes. NIH 3T3 cells and MEFs were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. For stimulation, NIH 3T3 cells and early-passage (P2–P5) MEFs were seeded in 10-cm dishes (10⁶ cells per dish), starved in DMEM without FBS and antibiotics for 24 h, treated for 1 h with the indicated doses of TGX-155 (TG), AS604850 (AS), LY294002 (LY), or vehicle (DMSO), followed by stimulation with PDGF (30 ng/ml), insulin (1 μ M), EGF (20 ng/ml), IGF1 (30 ng/ml), S1P (200 nM), SDF (30 ng/ml), or LPA (1 μ M) for 10 min, unless stated otherwise. PTx (100 ng/ml) was added for 15 h in DMEM without FBS before cell stimulation.

Macrophage Culture and Stimulation. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. BMMs were derived from bone marrow precursor cells isolated from femurs as detailed (34). PTx (active or heat-inactivated) was added 4 h before cell stimulation. PI3K inhibitors were included 30 min before cell stimulation. Cells were stimulated with CSF-1 (30 ng/ml) or C5a (100 ng/ml) for 5 min, unless stated otherwise.

Cell Transfection. NIH 3T3 cells were transfected with pcDNA3 without or with the coding sequences for human p110 γ (untagged) and porcine p101 (HA-tagged) by using Superfect (Qiagen), followed by selection in 800 μ g/ml geneticin (Invitrogen) for at least 15 days. Geneticin-resistant clones from a single well were pooled and maintained under continuous selection in 200 μ g/ml geneticin.

Immunoblotting. Cell lysis and immunoblotting of NIH 3T3 cells and MEFs was performed as described (34). Total cell lysates of macrophages were prepared by directly harvesting cells in 2 \times Laemmli sample buffer, followed by shearing of DNA by passing five times through a 0.45-mm needle. Samples were resolved by 10% SDS/PAGE.

Lipid Kinase Assay. PI3K activity assays were performed as described with PIP₂ as a substrate.

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