PI3Kβ links integrin activation and PI(3,4)P_2 production during invadopodial maturation

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Running title: PI3Kβ required for invadopodial maturation

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Abstract

The invasion of tumor cells from the primary tumor is mediated by invadopodia, actin rich protrusive organelles that secrete matrix metalloproteases and degrade the extracellular matrix. This coupling between protrusive activity and matrix degradation facilitates tumor invasion. We previously reported that the PI3Kβ isoform of PI 3-kinase, which is regulated by both receptor tyrosine kinases and GPCRs, is required for invasion and gelatin degradation in breast cancer cells. We have now defined the mechanism by which PI3Kβ regulates invadopodia. We find that PI3Kβ is specifically activated downstream of integrins, and is required for integrin-stimulated spreading and haptotaxis as well as integrin-stimulated invadopodia formation. Surprisingly, these integrin-stimulated and PI3Kβ-dependent responses require the production of $PI(3,4)P_2$ by the phosphoinositide 5'-phosphatase SHIP2. Thus, integrin activation of PI3Kβ is coupled to the SHIP2-dependent production of $PI(3,4)P_2$, which regulates the recruitment of PH domain-containing scaffolds such as lamellipodin to invadopodia. These findings provide novel mechanistic insight into the role of PI3Kβ in the regulation of invadopodia in breast cancer cells.

Introduction

Invadopodia are actin rich protrusions that mediate the secretion of matrix metalloproteases (MMPs), which degrade the extracellular matrix (ECM) and facilitate tumor cell invasion (Murphy and Courtneidge, 2011; Beaty *et al.*, 2014). By coupling actin-mediated protrusion to the enzymatic digestion of ECM barriers, invadopodia promote tumor cell dissemination from the primary tumor into surrounding tissue, and are critical for traversing endothelial layers during intravasation into the blood and extravasation at distal sites.

Invadopodia form in a step-wise manner in response to the activation of integrins and growth factor receptors (Artym *et al.*, 2006; Hoshino *et al.*, 2013). Formation of a degradation-incompetent precursor structure, containing actin, cortactin, Tks5, cofilin, and N-WASP, is followed by recruitment of β1 integrin and talin, which form a ring around the invadopodia core, and by the activation of Src-family kinases (Branch *et al.*, 2012; Beaty *et al.*, 2013). This leads to the tyrosine phosphorylation of cortactin, which promotes cofilin-dependent barbed end formation and Arp2/3-mediated actin polymerization (Oser *et al.*, 2009; Rosenberg *et al.*, 2017). Mature invadopodia interact with the microtubule cytoskeleton, which deliver vesicular cargo containing both membrane bound (MT1-MMP) and soluble MMPs (Castro-Castro *et al.*, 2016).

A key step in the maturation process is the production of $PI(3,4)P_2$, through the action of the phosphoinositide 5'-phosphatase SHIP2 (Sharma *et al.*, 2013). Production of $PI(3,4)P_2$ leads to the recruitment of proteins whose PH domains bind selectively to this lipid, including Tks5 and lamellipodin (Krause *et al.*, 2004; Saini and Courtneidge, 2018). SHIP2 produces $PI(3,4)P_2$ by dephosphorylating phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), which is the product of the Class I phosphoinositide 3-kinases (PI3K). The coupling between PI3K signaling and SHIP2 in invadopodia has not been defined. Of the four isoforms of Class I PI3K (PI3Kα, PI3Kβ, PI3Kδ

and PI3Kγ), the PI3Kα isoform has been previously implicated in invadopodia formation and matrix degradation (Yamaguchi *et al.*, 2011). More recently, we showed that the PI3Kβ isoform is required for invasion and gelatin degradation by MDA-MB-231 breast cancer cells (Khalil *et al.*, 2016). PI3Kβ is unique among Class I PI3Ks in several respects: (a) it is activated by both receptor tyrosine kinases (through SH2 domains in the p85 regulatory subunit) and G-protein coupled receptors (through direct binding of Gβγ to the p110 catalytic subunit (Dbouk *et al.*, 2012)), (b) whereas the Ras Binding Domains (RBDs) of all other Class I PI3Ks bind to activated Ras, the RBD of PI3Kβ binds to Cdc42 and Rac1 (Fritsch *et al.*, 2013); and (c) PI3Kβ binds directly to activated Rab5 (Heitz *et al.*, 2019). Importantly, we showed a pronounced defect in experimental metastasis in breast cancer cells expressing a PI3Kβ that is unable to bind Gβγ (Khalil *et al.*, 2016).

This study explores the mechanism by which PI3Kβ contributes to tumor cell invasion and identifies a key role for PI3Kβ in invadopodial maturation. We show that signaling by activated integrins specifically requires coupling between PI3Kβ and SHIP2 to produce PI(3,4)P_2, which recruits lamellipodin to maturing invadopodia. Our data show that PI3Kβ links two previously unrelated components of invadopodia maturation – integrin activation and PI(3,4)P_2 production – and therefore provides novel mechanistic insight into the regulation of invadopodia in breast cancer cells.

Results

Gβγ signaling to PI3Kβ is required for invadopodia maturation and matrix degradation.

We tested the role of the Class I PI3-kinase isoforms in invadopodia formation in the triple negative human breast cancer cell line MDA-MB-231, which expresses all four p110 catalytic subunits (p110α, β, δ and γ) (Sawyer *et al.*, 2003; Yamaguchi *et al.*, 2011; Brazzatti *et al.*,

2012). Our lab previously described MDA-MB-231 cells in which endogenous p110β was knocked down and rescued with physiological levels of murine wild type p110β or two previously described mutants: ^{526}KK-DD (referred to as KKDD), which abolishes p110β binding to Gβγ (Dbouk *et al.*, 2012), or ^{799}K-R (referred to as KR), which abolishes lipid kinase activity (Ciraolo *et al.*, 2008). We have remade these lines using lentiviral tranduction of HA-tagged constructs; the cells all express similar amounts of p110β, at levels that are somewhat higher than seen in parental MDA-MB-231 (Fig 1A). We have previously reported that both kinase activity as well as Gβγ coupling to PI3Kβ is required for gelatin degradation in the MDA-MB-231 cell line (Figure 1B and (Khalil *et al.*, 2016)). However, PI3Kα has also been implicated in invadopodia formation in MDA-MB-231 cells (Yamaguchi *et al.*, 2011). We therefore compared the effect of isoform selective inhibitors to all four Class I PI3Ks on gelatin degradation. Treatment of cells with the p110β selective inhibitor (TGX221) caused a nearly 65% decrease in gelatin degradation (Figure 1C). In contrast, p110-selective inhibitors for the other class I isoforms (PI3Kα, BYL719 ; PI3Kδ, IC-87114; PI3Kγ, AS604850) caused small decreases in gelatin degradation that did not reach statistical significance. These data show a selective requirement for PI3Kβ during invadopodia-mediated matrix degradation.

Invadopodia form and mature in a step-wise manner (Murphy and Courtneidge, 2011; Beaty *et al.*, 2014). Previous studies have identified invadopodial components such as Tks5, Arg and β1 integrin, whose knockdown or inhibition blocks invadopodial maturation to degradative-competent organelles, but does not affect the number of invadopodial precursors (Beaty *et al.*, 2013; Sharma *et al.*, 2013). To determine the step affected by the loss of PI3Kβ activity, we used MDA-MB-231 cells knockdown cells expressing wild type or mutant p110β. Co-localization of actin, cortactin and degraded gelatin was used to identify mature invadopodia, and actin-cortactin punctae that did not co-localize with degraded gelatin were defined as precursors. As

compared to MDA-MB-231 cells expressing wild type p110β, expression of the KKDD and KR mutants had no effect on the number of precursor invadopodia, but significantly reduced the number of mature invadopodia (Figure 1D and E). These data show that mutation of PI3Kβ leads to an inhibition of invadopodial maturation.

Integrin stimulated cell migration and spreading selectively requires PI3Kβ signaling.

PI3Kβ acts downstream from activated integrins in platelets and neutrophils (Cipolla *et al.*, 2013; Houslay *et al.*, 2016). Furthermore, like PI3Kβ, β1 integrin signaling is required for invadopodia maturation but not precursor formation in MDA-MB-231 cells (Beaty *et al.*, 2013). Therefore, we asked whether PI3Kβ is required for integrin signaling in these cells. First, we analyzed the role of PI3Kβ in a Boyden chamber haptotaxis assay. MDA-MB-231 cells expressing wild type p110β showed robust migration when the undersides of the filters were coated with collagen I; this migration was integrin-specific, as it was not observed in control experiments with poly-l-lysine coated filters (Supplemental Figure 1). As compared to wild type cells, cells expressing the KKDD and KR mutants of PI3Kβ showed a 75% reduction in the number of cells migrating toward collagen I (Figure 2A). The requirement for PI3Kβ in haptotaxis was specific; as TGX221 inhibited haptotaxis but other Class I isoform-selective inhibitors had no effect (Figure 2B). Next, we tested whether integrin-driven cell spreading on collagen I requires PI3Kβ signaling. Consistent with the haptotaxis assay, cell spreading was inhibited in cells expressing kinase dead (KR) or Gβγ-uncoupled (KKDD) p110β (Figure 2C). Moreover, cell spreading was inhibited by selective inhibition of PI3Kβ with TGX221, but not by other Class I isoform-selective inhibitors (Figure 2D). These data demonstrate a specific requirement for PI3Kβ in integrin-stimulated responses in breast cancer cells.

During platelet activation, PI3Kβ activity has been implicated in both inside-out integrin activation as well as outside-in integrin signaling (Bresnick and Backer, 2019). To test whether PI3Kβ signaling is required for outside-in signaling downstream of integrin activation in breast cancer cells, we used the TS2/16 β1 integrin activating antibody; this antibody bypasses inside-out signaling by forcing cell surface β1 integrins into their active high-affinity conformation (Su *et al.*, 2016). Treatment of cells with the TS2/16 antibody significantly increased cell spreading area in DMSO treated cells (Figure 2E). However, this increase was abolished in cells treated with TGX221; cells treated with TS2/16 or control IgG spread to the same extent in the presence of the PI3Kβ inhibitor. These data show that PI3Kβ activity is required downstream from activated β1 integrins.

Gβγ signaling to p110β is required for integrin-stimulated matrix degradation.

High-density fibrillar collagen (HDFC), a 4–5-μm-thick layer of densely packed fibrillar collagen I, is a potent inducer of invadopodia (Artym *et al.*, 2015). When plated on HDFC, cells form invadopodia via an integrin-stimulated signaling pathway that does not require growth factors or serum. To determine whether PI3Kβ is required for integrin-stimulated invadopodia formation, we measured invadopodia formation and matrix degradation on HDFC. Cells expressing wild type and mutant PI3K showed abundant invadopodia (Figure 3A, top panels). Matrix degradation, measured by immunostaining for the ¾ cleavage fragment of collagen I, was readily apparent in cells expressing wild type PI3Kβ (Figure 3A, lower left panel and Figure 3B). However, in cells expressing mutant PI3Kβ (Figure 3A, lower center and right panels and Figure 3B) or treated with TGX221 (Figure 3C), collagen I degradation was almost completely abolished. Inhibitors selective for other PI3K isoforms had no effect (Figure 3C). These data are consistent with a specific requirement for Pi3Kβ during integrin-stimulated invadopodia maturation.

Gβγ signaling but not Akt is required for matrix degradation, cell spreading and cell migration.

Our data show that the KKDD mutation, which uncouples PI3Kβ from Gβγ, disrupts gelatin degradation as well as integrin-stimulated haptotaxis, spreading and invadopodia maturation. Consistent with a requirement for Gβγ stimulation of PI3Kβ in these processes, we found that treating MDA-MB-231 cells with pertussis toxin (PTX) significantly inhibits gelatin degradation, as well as cell spreading and haptotaxis in response to collagen I (Figures 4A, 4B, 4C). Collagen degradation on HDFC was also significantly reduced in PTX treated cells as compared to DMSO controls (Figure 4D). These data show that activation of trimeric G-proteins is required for these integrin-stimulated responses.

Next, we examined whether Akt acts as a downstream effector of PI3Kβ during integrin stimulated responses. It has been previously reported that Akt is required for gelatin degradation in MDA-MB-231 cells (Yamaguchi *et al.*, 2011). However, we found that treating cells with an Akt inhibitor (MK2206) had no effect on invadopodia maturation and gelatin degradation (Figure 4A). Moreover, the Akt inhibitor had no effect on cell spreading, haptotaxis or invadopodia maturation on HDFC (Figure 4B, C, D). Control experiments verified that the inhibitor blocks Akt phosphorylation (Supplemental Figure 2).

PI(3,4)P_2 is required for gelatin degradation, integrin-mediated cell spreading and migration, and integrin-stimulated invadopodia maturation.

PI(3,4)P_2 can be formed either by the dephosphorylation of PIP_3 by 5'-phosphatases such as synaptojanin or SHIP2 or by phosphorylation of PI(4)P by Class II PI3-kinases. PI(3,4)P_2 has recently emerged as a signaling lipid with unique downstream effectors (Hawkins and Stephens,

2016). Previous studies have shown that PI(3,4)P_2, is required for invadopodia maturation and gelatin degradation (Sharma *et al.*, 2013; Malek *et al.*, 2017). Consistent with these findings, expression of a membrane targeted PI(3,4)P_2 4'-phosphatase, mCherry-INPP4B-CAAX, profoundly inhibited gelatin degradation in MDA-MB-231 cells (Figure 5A).

Given that the canonical PIP_3 effector Akt is not required for PI3Kβ-dependent integrin-stimulated responses, we considered whether PI3Kβ might be coupled to the production of PI(3,4)P_2. If this were correct, we would expect integrin signaling in MDA-MB-231 cells to also require PI(3,4)P_2. We therefore reduced PI(3,4)P_2 levels by treating cells with a SHIP2 inhibitor, or by expressing mCherry-INPP4B-CAAX. We found that both cell spreading (Figure 5B) and haptotaxis (Figure 5C) were significantly inhibited. The number of cells in the INPP4B experiment is reduced as compared to other haptotaxis experiments because only the transfected (mCherry-positive) cells were counted. In addition, integrin-stimulated collagen degradation on HDFC was inhibited by both the SHIP2 inhibitor and mCherry-INPP4B-CAAX (Figure 5D). These data show that PI3Kβ-dependent integrin signaling requires the production of PI(3,4)P_2.

We also tested the relationship between PI3Kβ and PI[3,4]P_2 during integrin signaling and matrix degradation in two other triple-negative breast cancer cell lines, BT549 and MDA-MB-468. In both cell lines, inhibition of PI3Kβ, but not PI3Kα, blocked cell spreading on collagen and collagen-stimulated haptotaxis (Supplemental Figure 3A and B). Gelatin degradation was also blocked by inhibition of PI3Kβ, but not PI3Kα in both cell lines (Supplemental Figure 3C). In all cases, the effects of PI3Kβ inhibition were mimicked by inhibition of PI[3,4]P_2 levels, either through inhibition of SHIP2 or overexpression of INPP4B (Supplemental Figures 3A, 3B and 3C).

PI3Kβ regulates PI(3,4)P_2 production in invadopodia.

Integrin signaling in MDA-MB-231 cells requires both PI3Kβ and PI(3,4)P_2. To test whether PI3Kβ is coupled to PI(3,4)P_2 production in invadopodia, we sought to measure PI(3,4)P_2 in these structures. While excellent probes for PI(3,4)P_2 are available (Goulden *et al.*, 2018), we observed that their expression in MDA-MB-231 cells markedly inhibits invadopodia formation on HDFC (Supplemental Figure 4). We were also unable to detect PI[3,4]P_2 in invadopodia by staining with antibodies against PI[3,4]P_2, although the lipid could be detected at the cell periphery. This presumably reflects the binding of invadopodial proteins to PI[3,4]P_2, which blocks antibody binding.

Instead, we used the recruitment of endogenous lamellipodin as an indicator of PI(3,4)P_2 levels in invadopodia. Lamellipodin is an actin regulatory protein whose PH domain binds specifically to PI(3,4)P_2 (Krause *et al.*, 2004), and lamellipodin localizes to invadopodia (Carmona *et al.*, 2016). When cells expressing wild type PI3Kβ were plated on HDFC, lamellipodin co-localized extensively with actin-cortactin punctae; this co-localization was much less apparent in cells expressing kinase dead or Gβγ-uncoupled p110β (Figure 6A). Quantitation of lamellipodin localization to invadopodia showed a 60% decrease in cells expressing mutant PI3Kβ (Fig. 6B), which was similar to the reduction in lamellipodin localization when PI(3,4)P_2 production was blocked by the SHIP2 inhibitor (Figure 6C). Neither mutation of PI3kβ nor inhibition of SHIP2 affected the number of invadopodial precursors (Figure 6D). These data show that PI3Kβ is required for invadopodial PI(3,4)P_2 production and for the recruitment of lamellipodin, a key regulator of invadopodial maturation.

Discussion

The maturation of invadopodia from cortactin/Tks5 precursors to degradation-competent mature invadopodia is a multi-step process (Murphy and Courtneidge, 2011; Beaty *et al.*, 2014). Early studies have implicated the PI3Kα-dependent activation of Akt in invadopodia-mediated matrix degradation (Yamaguchi *et al.*, 2011), consistent with an important role for canonical PI3K-PIP_3 signaling in invadopodia. More recent studies have shown that dephosphorylation of PIP_3 to $PI(3,4)P_2$, by the 5'-phosphatase SHIP2, is required for invadopodial maturation (Sharma *et al.*, 2013). $PI(3,4)P_2$ acts in part through the recruitment of proteins whose PH domains bind to $PI(3,4)P_2$, such as Tks5 and lamellipodin (Krause *et al.*, 2004; Murphy and Courtneidge, 2011; Sharma *et al.*, 2013). In addition, activation of β1 integrins, which form a ring around the invadopodial core (Branch *et al.*, 2012), as well as integrin recruitment and stimulation of Src family kinases (Beaty *et al.*, 2013), have been implicated in invadopodial maturation.

Our work suggests that the PI3Kβ isoform of Class I PI3Ks plays a unique role in linking integrin signaling, $PI(3,4)P_2$ production, lamellipodin recruitment, and invadopodia maturation. Our data show a general requirement for $PI(3,4)P_2$ in integrin signaling, and we define PI3Kβ and SHIP2 as the source of this signaling lipid. Moreover, we show that PI3Kβ and SHIP2 are required for the maturation of invadopodia formed in response to integrin activation. Finally, we directly demonstrate a role for PI3Kβ in the $PI(3,4)P_2$-mediated recruitment of lamellipodin to invadopodia. Taken together, these data establish PI3Kβ as the linchpin that links integrin activation to $PI(3,4)P_2$ production and invadopodial maturation.

Our finding that $PI(3,4)P_2$ is required for integrin-stimulated haptotaxis and cell spreading has not to our knowledge been previously reported. Integrin activation leads to the production of both PIP_3 and $PI(3,4)P_2$ in osteoclasts (Chellaiah *et al.*, 1998) and in Cos cells (King *et al.*,

1997). However, a more general role for PI(3,4)P_2 downstream of integrin activation has not been described. Given that β1 integrin signaling is required for invadopodia maturation, we propose that the coupling of PI3Kβ to integrin activation provides a mechanism for the requirement for PI3Kβ in invadopodia maturation. This hypothesis is supported by the finding that inhibition of PI3Kβ and inhibition of PI(3,4)P_2 signaling, using a SHIP2 inhibitor or overexpression of INPP4B, have similar effects on spreading, haptotaxis, and integrin-stimulated invadopodia maturation on HDFC. Of note, although our data using activating integrin antibodies clearly places PI3Kβ downstream from β1 integrins, earlier studies in platelets suggest that both PI3Kβ and PI(3,4)P_2 are required for ADP-stimulated inside-out signaling (Schoenwaelder *et al.*, 2007). The role of PI(3,4)P_2 in inside-out integrin signaling in breast cancer cells is currently under investigation.

We do not yet fully understand the mechanism of PI3Kβ activation by integrins. While integrins are known to activate Class I PI3Ks, this is primarily thought to occur through the activation of FAK and Pyk2 in focal adhesions or invadopodia, respectively, and the recruitment and tyrosine phosphorylation of Cbl (King *et al.*, 1997; Horne *et al.*, 2005; Manganaro *et al.*, 2015; Genna *et al.*, 2018). FAK and Cbl possess tyrosine phosphorylation sites that bind to p85 SH2 domains, which could recruit and activate Class I PI3Ks (Chen *et al.*, 1996; Hunter *et al.*, 1999). Interestingly, our data also show a requirement for Gβγ signaling, as integrin-stimulated cell responses are inhibited by a mutation of PI3Kβ that disrupts binding to Gβγ and by treatment of cells with pertussis toxin, which blocks trimeric G-protein activation and Gβγ release by $G\alpha_{i/o}$-coupled GPCRs (Kehrl, 2016). The source of Gβγ in the HDFC experiments, in which there are no added growth factors or serum, is not yet known. Possible sources include GPCRs responding to autocrine stimulation by secreted ligands, adhesion GPCRs (Purcell and Hall, 2018), and non-receptor GEFs for trimeric G-proteins, including Girdin/GIV and its family

members (Ghosh *et al.*, 2017). We do not think that Girdin itself is the source of Gβγ, as Girdin knockdown inhibits haptotaxis in MDA-MB-231 cells, as previously reported (Leyme *et al.*, 2015), but has no effect on cell spreading or HDFC degradation (Supplemental Figure 5).

Our data suggests that selective coupling of PI3Kβ to SHIP2 leads to production of $PI(3,4)P_2$. This coupling could be mediated by the preferential binding of PI3Kβ to the CRKL adapter, an interaction that was originally identified in PTEN-null tumor cells (Zhang *et al.*, 2017). CRKL binds via its SH2 to tyrosine phosphorylated p130Cas, an integrin-associated adapter that is present in invadopodia (Alexander *et al.*, 2008). p130Cas also binds to SHIP2 (Prasad *et al.*, 2001). Thus, integrin-stimulated recruitment and phosphorylation of Cas could mediate the formation of a p130Cas/SHIP2/CRKL/PI3Kβ complex that would efficiently divert PI3Kβ's production of PIP_3 into $PI(3,4)P_2$. Alternatively, integrin activation could independently but simultaneously recruit p130Cas/SHIP2 and target PI3Kβ to membranes by producing Gβγ.

A role for PI3Kα in matrix degradation by MDA-MB-231 cells plated on gelatin has been previously reported (Yamaguchi *et al.*, 2011). This assay was performed in the presence of serum, which complicates the potential signaling pathways involved. While we observed a small decrease in gelatin degradation in cells treated with a PI3Kα selective inhibitor, only loss of PI3Kβ signaling had a pronounced and statistically significant effect. In contrast, invadopodia maturation in response to integrin activation is highly selective for PI3Kβ. We also did not detect a role for canonical PI3K signaling through Akt in the assays for integrin activation and invadopodia maturation. This does not mean that Akt is not important for cell motility and invasion, but suggests that it functions at other steps. In fact, Akt is activated in response to plating of MDA-MB-231 cells on collagen I, by a mechanism that depends primarily on PI3Kα and to a lesser extent on PI3Kβ (Supplemental Figure 6). However, Akt activity is not required

for spreading, haptotaxis or invadopodia maturation in breast cancer cells. In fibroblasts, Akt has been implicated in the function of podosomes in cells expressing activated Src (Eves *et al.*, 2015), Interestingly, Src-mediated Akt activation requires SHIP2 (Hakak *et al.*, 2000) and presumably involves $PI(3,4)P_2$ binding to the Akt PH domain. Akt has also been linked to activation of mTORC2 (Liu *et al.*, 2015), which regulates adhesion and invasion through effects on protein expression (Wang *et al.*, 2018) and through the regulation of the actin cytoskeleton (Liu and Parent, 2011). Akt1 also phosphorylates the actin bundling protein paladin, although the role of paladin as an activator or inhibitor of breast cancer invasion has been controversial (Goicoechea *et al.*, 2009; Chin and Toker, 2010; Najm and El-Sibai, 2014; Mansour *et al.*, 2015).

In summary, our data define PI3Kβ as a critical regulator of invadopodia maturation. PI3Kβ is selectively coupled to $PI(3,4)P_2$ production downstream from integrin activation (Supplemental Figure 7). This provides a mechanism for the specific requirement for PI3Kβ during integrin activation in other systems, and also shows how PI3Kβ links integrin signaling and $PI(3,4)P_2$ signaling in the context of invadopodial maturation. The selective role for PI3Kβ in these processes suggests that drugs targeting PI3Kβ could be useful in clinical approaches to metastatic disease.

Materials and Methods

Antibodies and Reagents

The lamellipodin antibody (cat# 91138) was purchased from Cell Signaling Technology. The β1 integrin antibody 9EG7, which recognizes the active conformation, was purchased from BD Bioscience (cat# 553715). The TS2/16 β1 integrin activating antibody (cat# MA2910) was purchased from Invitrogen. The Tks5 antibody (cat# M300) was purchased from Santa Cruz

Biotechnology. The cortactin (p80/85) antibody was purchased from Millipore Sigma (cat# 05-180). The collagen type I (¾ fragment) antibody (cat# AG-25T-0116) was purchased from AdipoGen Life Sciences. Alexa Fluor-488 phalloidin, Alexa Fluor-555 phalloidin and all secondary antibodies for immunofluorescence studies were purchased from Invitrogen. Lipofectamine 3000 was purchased from Invitrogen. 0.01% poly-L-lysine solution was purchased from Sigma. Rat tail collagen I (cat# 354249) for coating glass coverslips and for the preparation of HDFC matrixes was purchased from Corning. Transwells with an 8.0 µm pore size membrane were purchased from Corning. Glutaraldehyde and paraformaldehyde solutions were purchased from Electron Microscopy Sciences. For mounting immunofluorescence coverslips, DAPI Fluoromount-G was purchased from Southern Biotech. TGX221 and IC-87114 were a gift from Peter Shepherd, Univ. Auckland. BYL719 was a gift from Novartis. AS604850 was purchased from Selleckchem. Pertussis toxin (PTX) was purchased from Millipore. AS1949490 was purchased from Santa Cruz Biotechnology, and MK2206 was purchased from Santa Cruz Biotechnology.

Cell Culture

The human breast cancer cell lines MDA-MB-231, MDA-MB-468 and BT549 were obtained from ATCC. MDA-MB-231 and MDA-MB-468 cells were cultured in DMEM containing 10% FBS, and BT549 cells were cultured in RPMI 1640 medium supplemented with 0.023 U/ml insulin and 10% FBS.

Stable p110β knockdown cell lines were previously reported (Khalil *et al.*, 2016); HA-tagged murine wild type, Gβγ-uncoupled (p110β^{KKDD}) or kinase dead (p110β^{KD}) p110β lentiviruses were used to express p110β in the knockdown cells. Stable cell lines were selected with blasticidin (10 µg/ml, InVivoGen).

For experiments with inhibitors, the following concentrations were used: p110β inhibitor TGX221, 100 nM; p110α inhibitor BYL719, 1 μM; p110γ inhibitor AS604850, 2 μM; p110δ inhibitor IC-87114, 1 μM; PTX, 200 ng/mL; SHIP2 inhibitor AS1949490, 10 μM; Akt inhibitor MK2206, 10 μM.

DNA Constructs and Transfection

The mCherry-INPP4B-CAAX construct was a gift from Dr. Volker Haucke (Leibniz-Forschungsinstitut für Molekulare Pharmakologie). The mCherry-CAAX construct was a gift from Dr. Dianne Cox (Albert Einstein College of Medicine). Transfections with Lipofectamine 3000 were performed according to the manufacturer's protocol.

Gelatin Degradation Assay

The gelatin degradation assay was performed as described previously (Khalil *et al.*, 2016). Briefly, coverslips were coated with 0.01% poly-L-lysine followed by crosslinking with 0.5% glutaraldehyde. The coverslips were then coated with 200 μg/mL Oregon Green 488-conjugated gelatin and quenched with a 0.1 M glycine. For experiments with inhibitors, cells were pretreated for 15 minutes before seeding for the assay. 5 x 10^4 cells were plated for 16 hours, and then fixed and permeabilized with 0.05% Triton X-100. Cells were stained with Alexa Fluor-555 phalloidin. Alternatively, cells were stained with Alexa Fluor-555 phalloidin and cortactin antibodies to analyze the number of precursor and mature invadopodia. For inhibitor studies, images were taken with a 60X 1.4 N.A. objective on an Olympus IX70 microscope. For the analysis of precursor/mature invadopodia, 0.77 μm sections were taken with a 63X 1.4 N.A. objective on a Leica SP5 AOBS confocal microscope. For quantitation of the fluorescent gelatin images, the background was subtracted with rolling ball radius of 20. The images were

thresholded to define areas of degradation per cell, which were measured in ImageJ. For counting invadopodia, local maxima were selected on the cortactin image with a noise tolerance of 250 for epifluorescence images or 80 for confocal images. The selected points were transformed to ROIs, which were then copied to the gelatin and phalloidin images. ROIs containing coincident actin-cortactin punctae were defined as invadopodia. Precursor invadopodia were defined as coincident actin-cortactin punctae lacking corresponding gelatin degradation, and mature invadopodia were defined as coincident actin-cortactin punctae that colocalized with gelatin degradation. All the invadopodia in each cell (at least 15 cells per condition) were counted. The reported values are the mean ± SEM from three independent experiments.

Gelatin degradation by MDA-MB-468 and BT549 cells was measured as above, except that crosslinking with glutaraldehyde was omitted. 8 x 10^4 cells were seeded, and gelatin degradation was measured after 24 hours (MDA-MB-468) or 16 hours (BT549).

Spreading Assay

Glass coverslips were coated with 15 µg/cm^2 collagen I for 1 hour. MDA-MB-231 cells were starved for 16 hours in serum free DMEM supplemented with 0.5% BSA. Starved cells were detached from plates with 10 mM EDTA/PBS, centrifuged and re-suspended for 1 h in starvation media containing inhibitors or antibody (TS2/16 or IgG, 5 µg/ml final) as indicated. 5 x 10^4 cells were then seeded on the collagen I coated coverslips for 30 minutes and then fixed and stained with Alexa Fluor-488 phalloidin. Images were taken using a 20X 0.50 N.A. objective with 1.5X magnification on an Olympus IX70 microscope. Where indicated, cells were transiently transfected with mCherry-INNP4B-CAAX or mCherry-CAAX constructs 48 hour prior to plating. Cells were imaged expressing using a 40X 0.75 N.A. objective. Images were thresholded to select the cell area, which was measured using ImageJ software. For each

experiment at least 15 cells were imaged. The reported values are the mean ± SEM from three independent experiments.

For experiments with MDA-MB-468 and BT549 cells, cells grown in complete media were detached with EDTA/PBS and starved for 30 min. 8 x 10^4 cells were seeded for 1 hour, fixed and imaged as described above.

Haptotaxis Assay

Cell migration was evaluated using transwell inserts. The lower sides of 8 µm pore membranes were coated with 1.5 µg/cm^2 rat tail collagen I and washed with serum free media. A 100 µl of suspension containing 3x10^4 starved MDA-MB-231 cells were detached and treated with inhibitors as described above, and seeded in the upper chamber. Cells were allowed to migrate for 4 hours. Cells remaining in the upper chamber were removed with a cotton tip applicator before fixing the membranes with 4% paraformaldehyde. The membranes were mounted on coverslips using DAPI Fluoromount-G and imaged with a 20X 0.5 N.A. objective on an Olympus IX70 microscope. The image was thresholded to select the nuclei, and number of cells per field was analyzed by counting the number of nuclei using ImageJ. Experiments were performed in triplicates and at least 15 fields per condition were imaged. The reported values are the mean ± SEM from three independent experiments. Where indicated, cells were transiently transfected with mCherry-INNP4B-CAAX or mCherry-CAAX constructs 48 hours prior to seeding. Transfected cells were imaged using a 40X 0.75 N.A. objective. Only cells that exhibited mCherry fluorescence were counted.

For experiments with MDA-MB-468 and BT549 cells, cells grown in complete media were detached with EDTA/PBS and starved for 30 min. 8 x 10^4 cells were seeded in the upper

chamber and allowed to migrate for 24 hours. The inserts were then processed and imaged as described above.

HDFC Degradation Assay

High Density Fibrillar Collagen (HDFC) matrices were prepared in 10 mm diameter MatTek dishes according to the method of Artym et al. ((Artym, 2016). Briefly, rat tail collagen I was neutralized by adding a $1/10^{th}$ volume of 10X DMEM supplemented with 0.22 g/L sodium bicarbonate and 0.48 g/L HEPES. pH paper was used to measure the pH of the collagen solution, and 2N HCl and 2N NaOH solutions were used to adjust the pH to 7.1–7.4. 6 µl of ice cold neutralized collagen solution was used to coat pre-chilled MatTek dishes. The dishes were then placed in a 37°C/5% CO_2 incubator for 30 minutes to polymerize the fibrillar collagen. The MatTek dishes were placed on a microplate centrifuge adapter and centrifuged at 3,500 X g for 20 min to flatten the collagen meshwork into a 2D layer. HDFC matrices were treated with PBS containing 4% paraformaldehyde and 5% sucrose for 20 minutes to crosslink the HDFC. After washing with DMEM, $4x10^4$ cells were starved, detached and treated with inhibitors as described above, and plated for 3 hours. The cells were fixed and stained with the collagen ¾ fragment antibody and Alexa Fluor-488 phalloidin without permeabilization. Images were taken with 60X 1.4 N.A. objective on an Olympus IX70 microscope. For each experiment at least 15 cells were imaged. To quantify the collagen ¾ images, the background was subtracted using a rolling ball radius of 50 and the degradation area per cell was quantified. The reported values are the mean ± SEM from three independent experiments.

Lamellipodin Localization

Serum starved cells were detached and treated with inhibitors as described above, and seeded on HDFC matrixes for 2 hours. Samples were fixed, permeabilized and immunostained with cortactin and lamellipodin antibodies and Alexa 488-phalloidin. Images were obtained with a

63X 1.4 N.A. objective on Leica SP5 confocal microscope. For image analysis, the background was subtracted from the actin and cortactin images using a rolling ball radius of 10. Using the mathematical function “AND” in ImageJ, the area showing coincident actin and cortactin staining was transformed into a new image. Invadopodia were defined by finding the local maxima in the “AND” image using ImageJ. Lamellipodin intensity was measured in ROIs containing coincident actin/cortactin staining (defined as an invadopod) and in an area immediately adjacent to the invadopod, to measure background staining. The ratio of lamellipodin intensity in invadopodia versus background was calculated for each invadopod, and all invadopodia in each cell were measured. The ratios were averaged per cell. In each experiment, at least 5 cells were analyzed per condition, and 17-54 invadopodia per cell (average of 30) were counted. The reported values are the mean ± SEM from three independent experiments.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism version 7. Based on the number of groups, data were analyzed using one-way ANOVA or a Student’s t test. For all analyses a *p* value of ≤ 0.05 was considered statistically significant.

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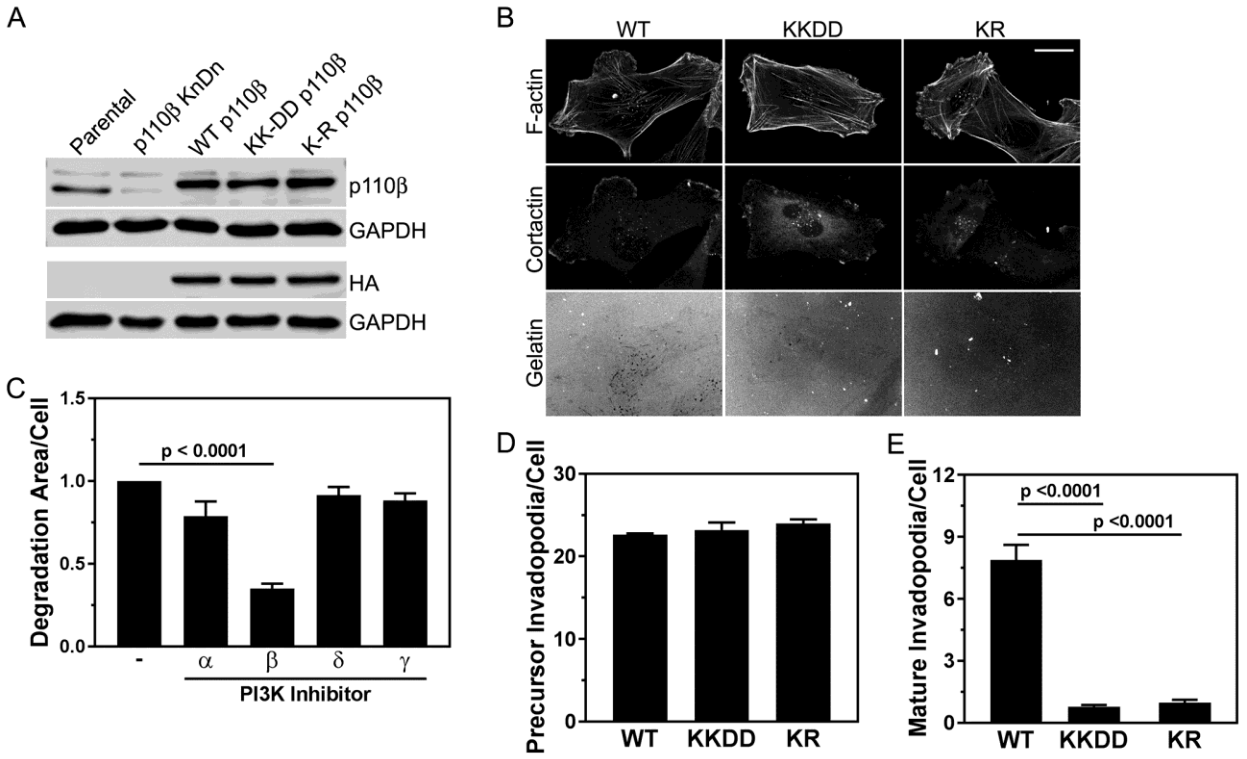

Figure 1. Gβγ signaling to PI3Kβ is required for invadopodia maturation. (A) Lysates from parental and MDA-MB-231 knockdown/rescue cell lines were blotted for p110β, HA and GAPDH. (B) Representative images of p110β knockdown MDA-MB-231 cells expressing wild type, Gβγ-uncoupled (KKDD) or kinase dead (KR) murine p110β. Cells were seeded on Oregon Green-labeled gelatin for 16 hours and stained with Alexa Fluor-555 phalloidin and cortactin antibodies as markers of invadopodia. Precursor invadopodia were defined by the colocalization of actin and cortactin without corresponding gelatin degradation. Mature invadopodia were defined by the colocalization of actin and cortactin with corresponding gelatin degradation. Scale bar = 20 μm. (C) Gelatin degradation was measured in parental MDA-MB-231 cells treated with isoform-selective inhibitors against the class I PI3K isoforms: α (BYL719, 1 μM), β (TGX221, 100 nM), γ (AS604850, 2 μM) and δ (IC-87114, 1 μM). The degradation area per cell was normalized to the DMSO control. Quantification of invadopodia precursors (D) and mature invadopodia (E) in MDA-MB-231 cells expressing wild type, KKDD or KR p110β. ≥15 cells were imaged per condition per experiment. For each assay the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using one-way ANOVA.

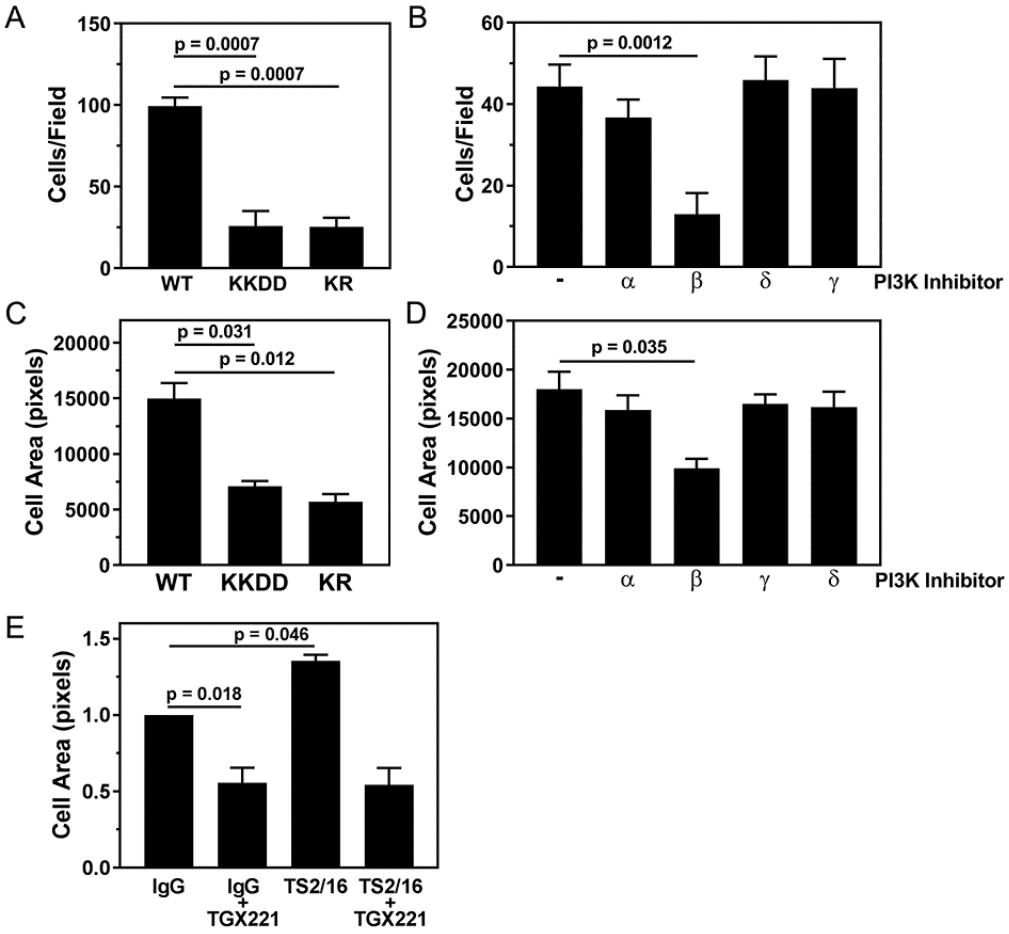

Figure 2. Integrin mediated cell migration and spreading requires Gβγ signaling to PI3Kβ. (A) Haptotaxis of p110β knockdown MDA-MB-231 cells expressing murine wild type, KKDD or KR p110β. Cells were plated in transwells in which the bottom of the membrane was coated with collagen I. After 4 hours, cells that had migrated through the membrane were fixed and stained, and the number of cells per field was quantified. (B) Haptotaxis of parental MDA-MB-231 cells treated with the class I PI3K isoform-selective inhibitors as in Figure 1. (C) Quantification of cell spreading in p110β knockdown MDA-MB-231 cells expressing murine wild type, KKDD or KR p110β. (D) Quantification of parental MDA-MB-231 cell spreading on collagen I in the absence or presence of class I PI3K isoform-selective inhibitors. (E) Quantification of MDA-MB-231 cell spreading in cells pre-treated with the activating β1 integrin antibody TS2/16 or IgG control, along with TGX221 or DMSO. For each assay, the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using one-way ANOVA.

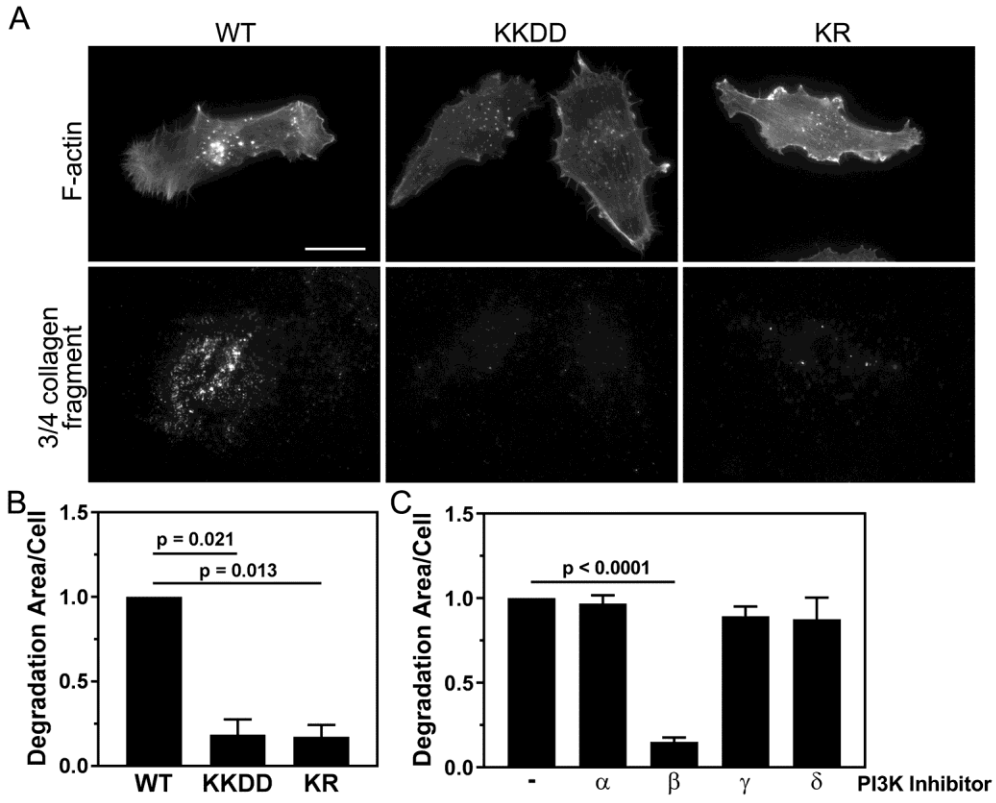

Figure 3. Gβγ signaling to PI3Kβ is required for matrix degradation on HDFC. (A) Representative images of serum-starved p110β knockdown MDA-MB-231 cells expressing murine wild type, KKDD or KR p110β, plated on crossed-linked HDFC for 3 hours. Cells were stained with phalloidin and the collagen I ¾ fragment antibody. Scale bar = 20 μm. (B) Quantification of degradation area/cell for cells expressing wild type, KKDD or KR p110β on HDFC. Values were normalized to cells expressing wild type p110β. (C) Quantification of degradation area/cell in parental MDA-MB-231 cells treated with the class I PI3K isoform-selective inhibitors on HDFC. Values were normalized to the DMSO control. For each assay, the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using one-way ANOVA.

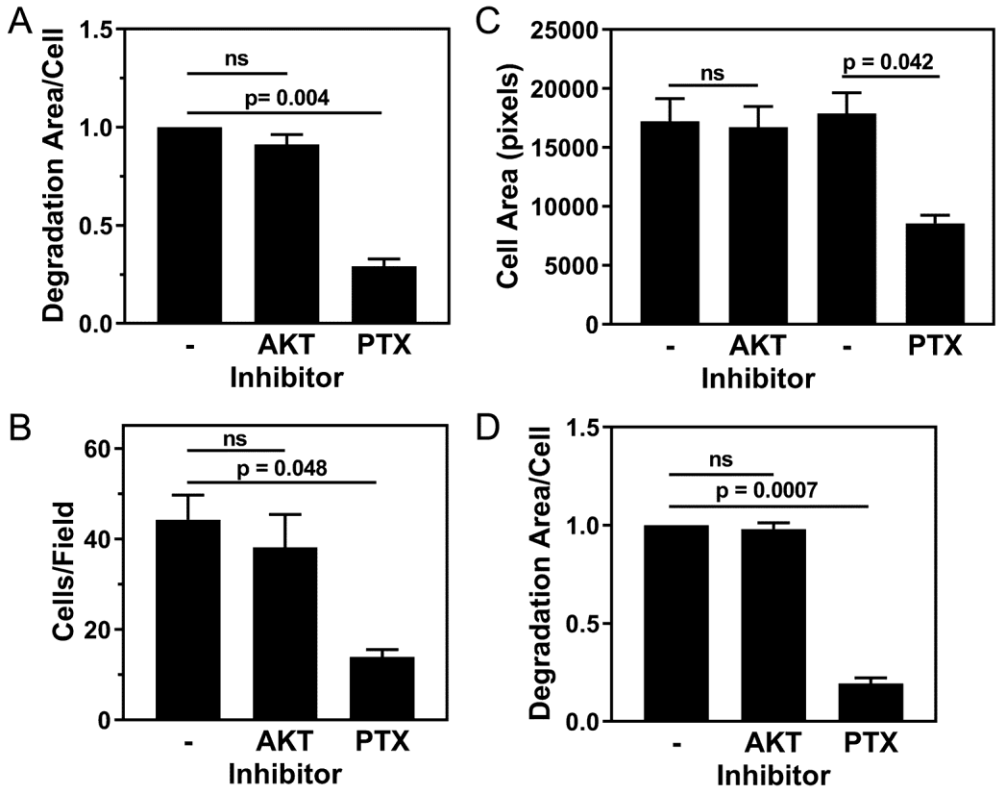

Figure 4. Gβγ but not Akt is required for integrin-mediated cell spreading, cell migration, and invadopodia formation. (A) Quantification of degradation area/cell in parental MDA-MB-231 cells plated overnight on Oregon green-labeled gelatin in the presence of pertussis toxin (PTX) or the Akt-selective inhibitor MK2206. Values were normalized to the DMSO control. (B) Quantification of parental MDA-MB-231 cell spreading on collagen I in cells treated with PTX or MK2206. (C) Quantitation of parental MDA-MB-231 haptotaxis towards collagen I in cells treated with PTX or MK2206. (D) Quantification of degradation area/cell in parental MDA-MD-231 cells plated on HDFC in the presence of PTX or MK2206. Data were normalized to the DMSO control. For each assay, the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using one-way ANOVA.

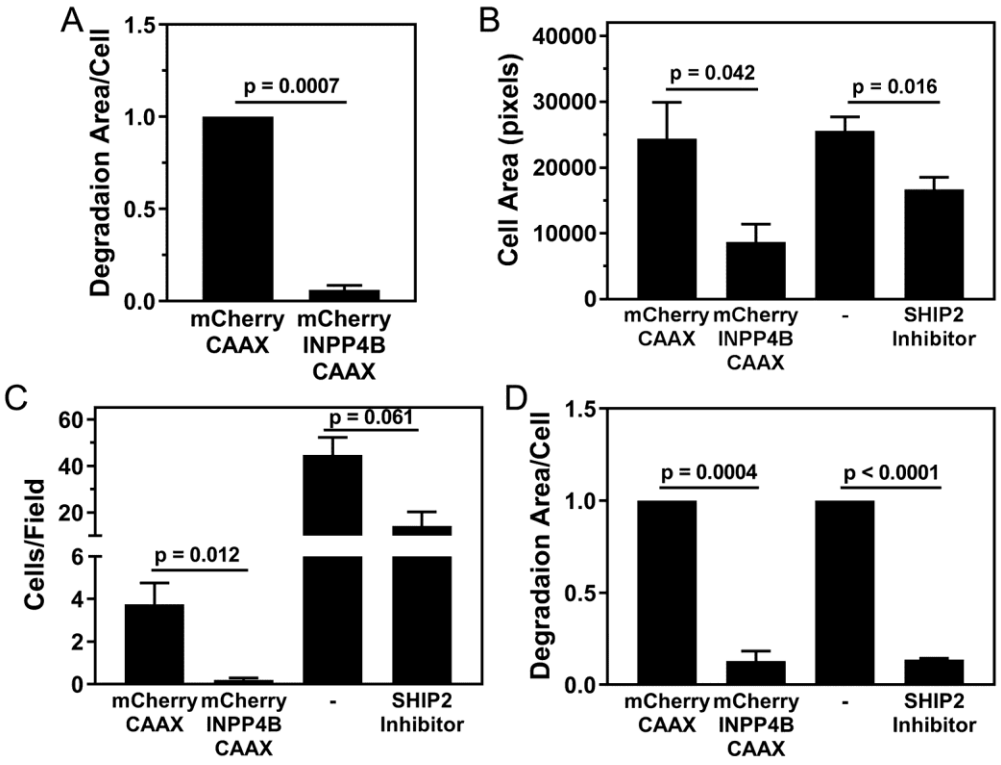

Figure 5. PI(3,4)P_2 is required for matrix degradation, integrin-mediated cell spreading and migration, and integrin-stimulated invadopodia maturation. (A) Degradation area/cell in MDA-MB-231 cells transiently transfected with mCherry-CAAX or mCherry-INPP4B-CAAX and plated overnight on Oregon green-labeled gelatin. Values were normalized to the degradation by cells expressing mCherry-CAAX. (B) Cell spreading on collagen I in parental MDA-MB-231 cells transiently transfected with mCherry-CAAX or mCherry-INNP4B-CAAX, or treated with the SHIP2 inhibitor AS1949490. (C) Haptotaxis to collagen I in parental MDA-MB-231 cells transiently transfected with mCherry-CAAX or mCherry-INNP4B-CAAX, or treated with AS1949490. (D) Degradation area/cell for parental MDA-MD-231 cells transiently transfected with mCherry-CAAX or mCherry-INNP4B-CAAX, or treated with AS1949490, and plated on HDFC. For each assay, the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using a Student's t-test.

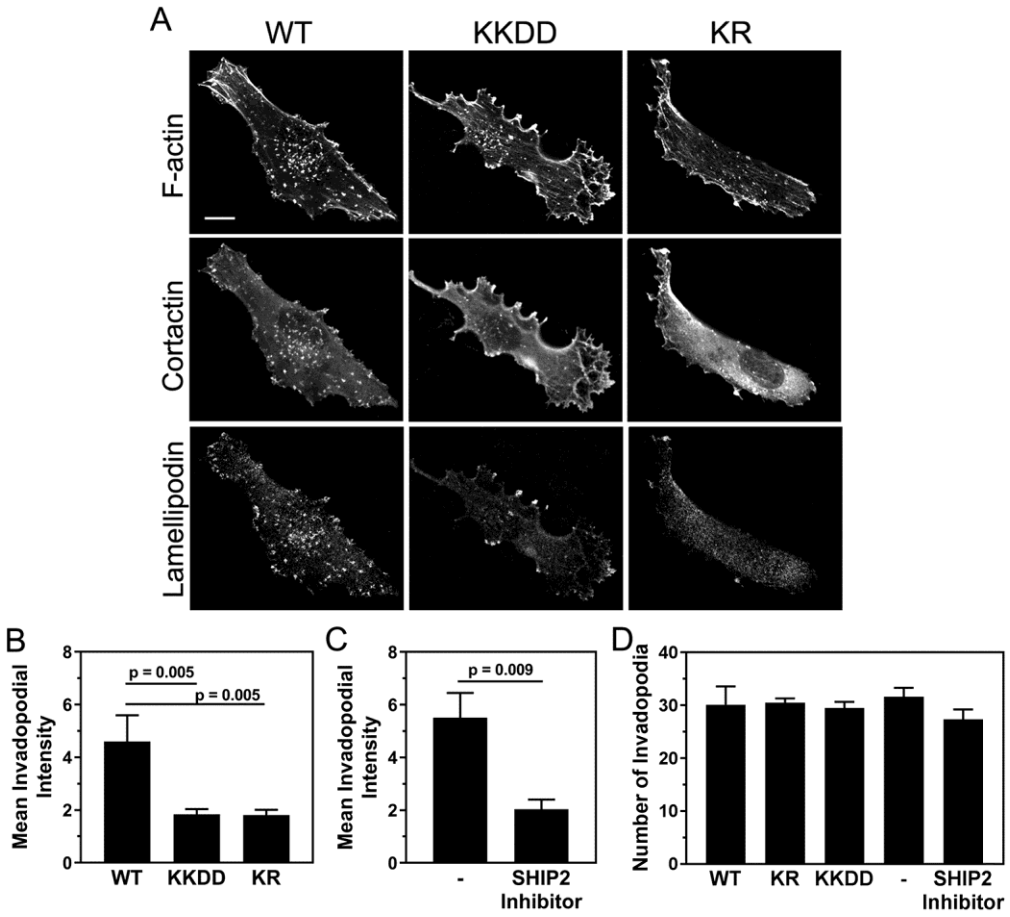

Figure 6. Gβγ activation of PI3Kβ is required for lamellipodin localization to invadopodia. (A) Representative images of serum starved MDA-MB-231 seeded on HDFC for 2 hours and stained with phalloidin, cortactin and lamellipodin antibodies. Scale bar = 20 µm. Quantification of lamellipodin localization to invadopodia in (B) p110β knockdown MDA-MB-231 cells expressing murine wild type KKDD or KR p110β, or (C) in parental cells treated with the SHIP2 inhibitor AS1949490. (D) Total number of invadopodia in cells expressing mutant PI3Kβ or treated with the SHIP2 inhibitor. For each assay, the data represent the mean ± SEM from 3 independent experiments. Statistical analyses were performed using one-way ANOVA.