

Phosphatidylinositol 4-kinase II β negatively regulates invadopodia formation and suppresses an invasive cellular phenotype

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ABSTRACT The type II phosphatidylinositol 4-kinase (PI4KII) enzymes synthesize the lipid phosphatidylinositol 4-phosphate (PI(4)P), which has been detected at the Golgi complex and endosomal compartments and recruits clathrin adaptors. Despite common mechanistic similarities between the isoforms, the extent of their redundancy is unclear. We found that depletion of PI4KII α and PI4KII β using small interfering RNA led to actin remodeling. Depletion of PI4KII β also induced the formation of invadopodia containing membrane type I matrix metalloproteinase (MT1-MMP). Depletion of PI4KII isoforms also differentially affected *trans*-Golgi network (TGN) pools of PI(4)P and post-TGN traffic. PI4KII β depletion caused increased MT1-MMP trafficking to invasive structures at the plasma membrane and was accompanied by reduced colocalization of MT1-MMP with membranes containing the endosomal markers Rab5 and Rab7 but increased localization with the exocytic Rab8. Depletion of PI4KII β was sufficient to confer an aggressive invasive phenotype on minimally invasive HeLa and MCF-7 cell lines. Mining oncogenomic databases revealed that loss of the PI4K2B allele and under-expression of PI4KII β mRNA are associated with human cancers. This finding supports the cell data and suggests that PI4KII β may be a clinically significant suppressor of invasion. We propose that PI4KII β synthesizes a pool of PI(4)P that maintains MT1-MMP traffic in the degradative pathway and suppresses the formation of invadopodia.

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INTRODUCTION

The two human type II phosphatidylinositol 4-kinase (PI4KII) enzymes display 68% sequence identity across their conserved

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Abbreviations used: CI-M6PR, cation-independent mannose 6-phosphate receptor; CNA, copy number alteration; ECM, extracellular matrix; EE, early endosome; EEA1, early endosomal autoantigen 1; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; GST, glutathione-S-transferase; LE, late endosome; MT1-MMP, membrane type I matrix metalloproteinase; NT, nontargeting siRNA; P4C, PI(4)P-binding domain of SidC; PI4KII α , phosphatidylinositol 4-kinase type II alpha; PI4KII β , phosphatidylinositol 4-kinase type II beta; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate.

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catalytic domains and exist in multiple subcellular membrane compartments (Balla and Balla, 2006; Minogue and Waugh, 2012). Since the molecular characterization of the PI4KIIs, a general model of PI4KII function in post-*trans*-Golgi network (TGN) traffic has gradually emerged in which the kinases generate phosphatidylinositol 4-phosphate (PI(4)P), which is required for the recruitment of clathrin adaptors needed in TGN-to-endosomal traffic. PI4KIIs have been most extensively described in endosomes (Balla et al., 2002), the TGN (Wang et al., 2003), and at the plasma membrane (Minogue et al., 2006), as has PI(4)P (Hammond et al., 2014). The PI4KII α isoform generates PI(4)P, which interacts with AP-1 (Wang et al., 2003), GGAs (Wang et al., 2007), and AP-3 (Salazar et al., 2005; Craige et al., 2008; Mossinger et al., 2012) and therefore has roles in TGN-to-endosome and TGN-to-late endosomal trafficking known to be important in transport to the lysosome. More recently, a mechanism has been described involving the redirection of PI from phosphatidylinositol 3-phosphate (PI(3)P) into PI(4)P synthesis, which is required for exocytic traffic from endosomes. This pathway requires PI4KII α and is significant because it represents an important step in

the recycling of plasma membrane cargoes such as $\beta 1$ -integrin and the transferrin receptor (Ketel *et al.*, 2016). PI4KII α has also been implicated in human cancer (Li *et al.*, 2010, 2014). PI4KII β functions in TGN-to-endosomal traffic by directly interacting with AP-1, playing a role in developmental signaling by controlling the endosomal traffic of Frizzled, a receptor for Wnt (Wieffer *et al.*, 2013). The PI4KII isoforms therefore produce lipids needed for different steps in post-TGN trafficking.

Protease-dependent matrix remodeling performs key physiological functions during embryo development and in the pathogenesis of metastatic disease. In metastatic cells, proteolytic degradation of extracellular matrix (ECM) is mediated by extracellular proteases such as the membrane type I metalloproteinase (MT1-MMP), whose increased expression is associated with a poor prognosis in a wide variety of human tumors (Egeblad and Werb, 2002). ECM invasion by tumor-derived cells is mediated in part by specialized actin-

phosphotyrosine-rich protrusions termed invadopodia, which are capable of degrading extracellular matrix through the directed membrane traffic of MMPs (Poincloux *et al.*, 2009). The overexpression of components of invadopodia such as cortactin in breast and squamous carcinomas and ovarian, bladder, and lung cancers is associated with a more aggressive phenotype and poorer patient prognosis (Paz *et al.*, 2014). MT1-MMP is a key component of mature invadopodia and has been implicated in breaching of basement membranes and invasion through interstitial collagen (Sabeh *et al.*, 2004; Hotary *et al.*, 2006). Trafficking to the plasma membrane from endosomal compartments is a recognized pathway for the delivery of MT1-MMP to invadopodia (Steffen *et al.*, 2008; Yu *et al.*, 2012). However, the molecular machinery controlling the complex trafficking of this key protease is not fully understood.

The PI4KII enzymes have been associated with both the development of breast cancer (Li *et al.*, 2014) and metastasis of hepatocellular carcinoma (HCC; Mazzocca *et al.*, 2008), but links between the PI4KII and cellular processes such as actin reorganization during oncogenesis are unclear. We therefore investigated the role of PI4KII α and PI4KII β in actin remodeling and found that depletion of the enzymes had starkly different effects on the actin cytoskeleton. We also found that PI4KII β depletion specifically induced the formation of invadopodia and was sufficient to convert HeLa and MCF-7 cells into an invasive phenotype. Loss of PI4KII α and PI4KII β affected different PI(4)P TGN pools and post-TGN traffic. The steady-state distribution of MT1-MMP was also shifted from an endosomal to an exocytic trafficking route. Finally, database analyses indicated that PI4KII β was lost in human cancers, indicating that PI4KII β may be a clinically significant tumor suppressor.

RESULTS

Depletion of PI4KII isoforms differentially affects the actin cytoskeleton

We used small interfering RNA (siRNA) to induce loss of PI4KII function. Gene silencing was confirmed by immunoblotting 72 h after transfection of HeLa cells with isoform-specific siRNA SMARTpools. We repeatedly achieved at least 85% reduction in levels of PI4KII α and PI4KII β compared with nontargeted controls (Figure 1, A and B, and Supplemental Figure S1, A and B). When we specifically depleted PI4KII isoforms in this way, we noted that monolayers of cells treated with PI4KII β siRNA were more dispersed than with PI4KII α and controls (unpublished results), as described previously (Mazzocca *et al.*, 2008). To investigate this further, we stained the actin cytoskeleton with fluorescently labeled phalloidin and found that, in comparison to controls, loss of PI4KII α led to increased actin stress fiber formation at leading

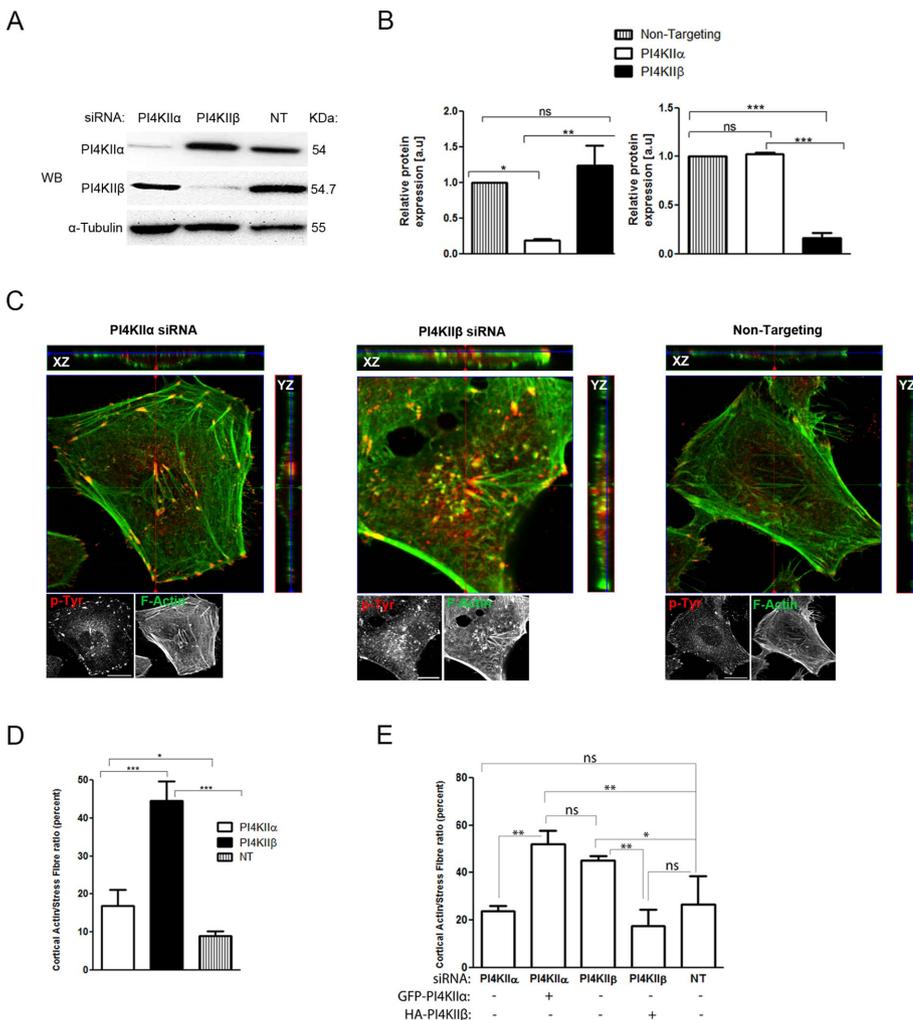


FIGURE 1: PI4KII α and PI4KII β exert different effects on the actin cytoskeleton. (A, B) Total cell lysates of PI4KII α , PI4KII β , and control siRNA-transfected HeLa cells were analyzed for protein expression by Western blotting; α -tubulin served as a control for protein levels. (C) HeLa cells transfected with the siRNA SMARTpool as indicated for 36 h were reseeded onto collagen matrix and then costained for tyrosine phosphorylated proteins (red) and the actin cytoskeleton (green). Confocal sections were acquired at 0.25- μ m intervals and rendered into orthogonal views. Single XY-sections of the basal surface. Single pY and F-actin channels are shown below. Scale bars, 10 μ m. (D) Ratio of cortical actin to stress fiber intensity from three independent experiments ($n = 20$ cells). (E) Cells were also transfected with single siRNA oligos and rescued by reexpression of siRNA-resistant PI4KII α and PI4KII β . Data are presented as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

edges (Figure 1C). Cells also showed some clear focal colocalization with phosphotyrosine (Figure 1C). Loss of PI4KII β , on the other hand, resulted in complete loss of actin stress fibers and increased the intensity of cortical actin staining (Figure 1C), as well as of the formation of scattered, actin-rich punctae staining positive for phosphotyrosine (Figure 1C), which were absent in PI4KII α and control siRNAs. Actin remodeling was confirmed by analyzing the ratio of cortical actin:stress fiber signals (Figure 1D), and normal actin phenotypes were restored by reexpression of siRNA-resistant PI4KII β (Figure 1E and Supplemental Figure S1, C–F). The same rescue experiment with overexpressed GFP-PI4KII α led to increased levels of cortical actin (Figure 1E), a likely consequence of increasing PI(4)P levels (Henmi *et al.*, 2016), which would be expected to alter the actin cytoskeleton. These data indicate that loss of PI4KII α and PI4KII β has markedly different effects on the actin cytoskeleton.

Loss of PI4KII β induces the formation of invadopodia

We sought to confirm the identity of the scattered actin-rich punctae in Figure 1C formed as a result of loss of PI4KII β . Initially, we costained F-actin along with the early endosomal marker EEA1; however, these showed minimal colocalization (Supplemental Figure S2A), indicating that these punctae were distinct from actin comets localized to endosomal membranes (Taunton *et al.*, 2000). Furthermore, these structures typically had average diameters of $\leq 0.25 \mu\text{m}$, mostly clustered in regions under the nucleus, and were thus suggestive of invadopodia (Garcia *et al.*, 2014). To confirm the identity of these punctae, we immunostained cells for actin and cortactin, the coincidence of which is diagnostic of invadopodia and related structures (Garcia *et al.*, 2014).

We found that loss of PI4KII β , but not of PI4KII α , induced the formation of punctae staining positive for both markers in HeLa cells (Figure 2A). When we acquired confocal z-series data from PI4KII β -

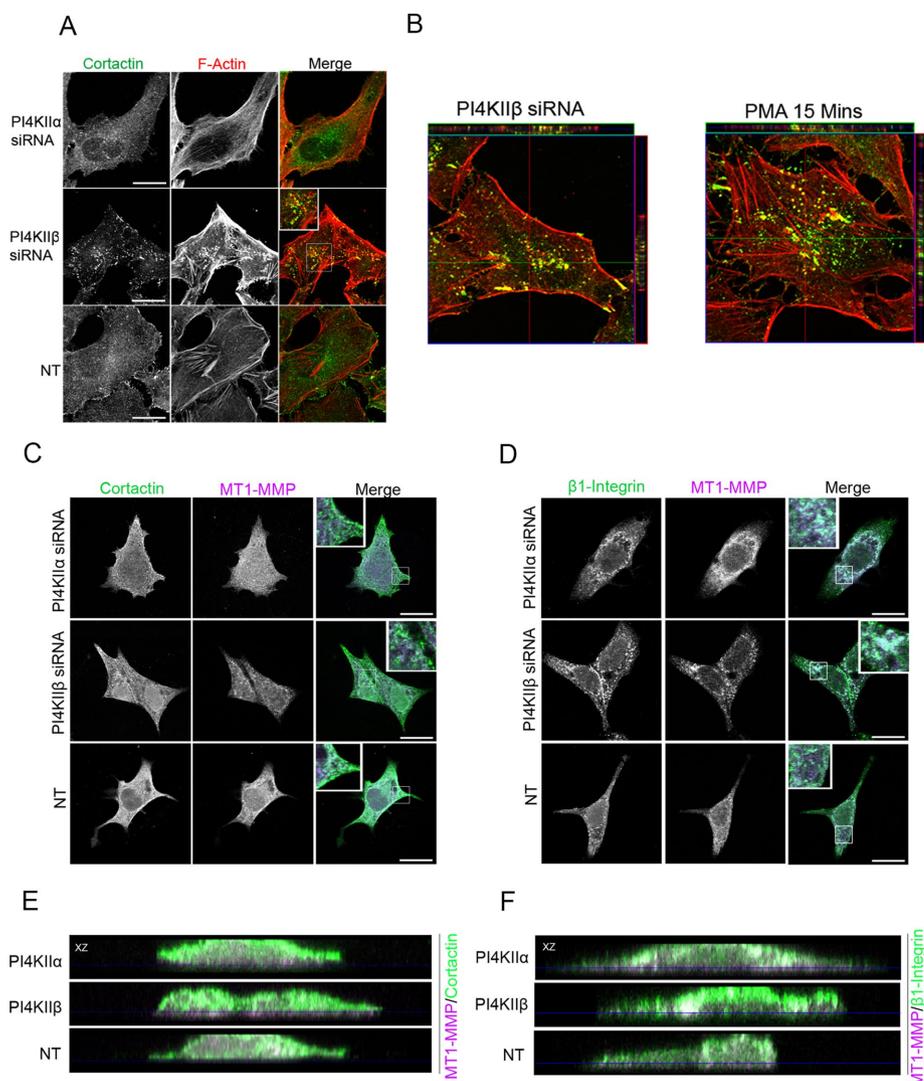


FIGURE 2: Depletion of PI4KII β induces invadopodia formation. (A) Confocal images showing immunostaining of cortactin (green) and F-actin (red). (B) Confocal images showing comparisons between HeLa cells transfected with PI4KII β siRNA and cells treated with 50 nM PMA. (C–F) Confocal images showing cells grown on thick layers of collagen and coimmunostained for MT1-MMP (magenta), (C) cortactin (green), or (D) β 1-integrin (green). (E, F) XZ rendering of 20 confocal sections acquired at 0.25- μm intervals, showing localization of MT1-MMP to ventral structures containing cortactin (E) or β 1-Integrin (F). Horizontal lines indicate the position of the ECM surface. Scale bars, 20 μm .

depleted cells, we observed the colocalization of actin and cortactin at the ventral surface of gelatin-coated coverslips (Figure 2B), thereby confirming that these structures were indeed invadopodia. As further confirmation, we performed a positive control by treating cells with the phorbol ester phorbol-12-myristate-13-acetate (PMA; Tatin *et al.*, 2006) and found that the actin- and cortactin-rich structures were indistinguishable from those obtained by depletion of PI4KII β (Figure 2B). Key constituents of invadopodia are the matrix metalloproteinase MT1-MMP (Poincloux *et al.*, 2009) and β 1-integrin (Destaing *et al.*, 2010). To determine whether the ventral structures contained known components of mature invadopodia, we stained siRNA-treated cells plated on coverslips coated with a three-dimensional (3D) layer of collagen for MT1-MMP and β 1-integrin or cortactin. Confocal microscopy demonstrated the colocalization of these markers with MT1-MMP (Figure 2, C and D), particularly on the ventral surface of PI4KII β -depleted cells (Figure 2, E and F). Furthermore, these structures were seen to penetrate the 3D ECM when we performed Z-series confocal microscopy (Figure 2, E and F).

Loss of PI4KII β causes increased matrix degradation and migration through collagen gel

MT1-MMP requires posttranslational processing to become active (Williams and Coppolino, 2011). We therefore determined whether the structures formed upon the loss of PI4KII β expression were capable of ECM degradation. We plated target and control siRNA-transfected HeLa cells on an ECM composed of cross-linked fluorescein isothiocyanate (FITC)-conjugated gelatin. In comparison with PI4KII α and control siRNA-transfected cells, loss of PI4KII β resulted in an approximately fourfold increase in fluorescent matrix degradation in comparison with PI4KII α (Figure 3, A and B). Areas of degradation also

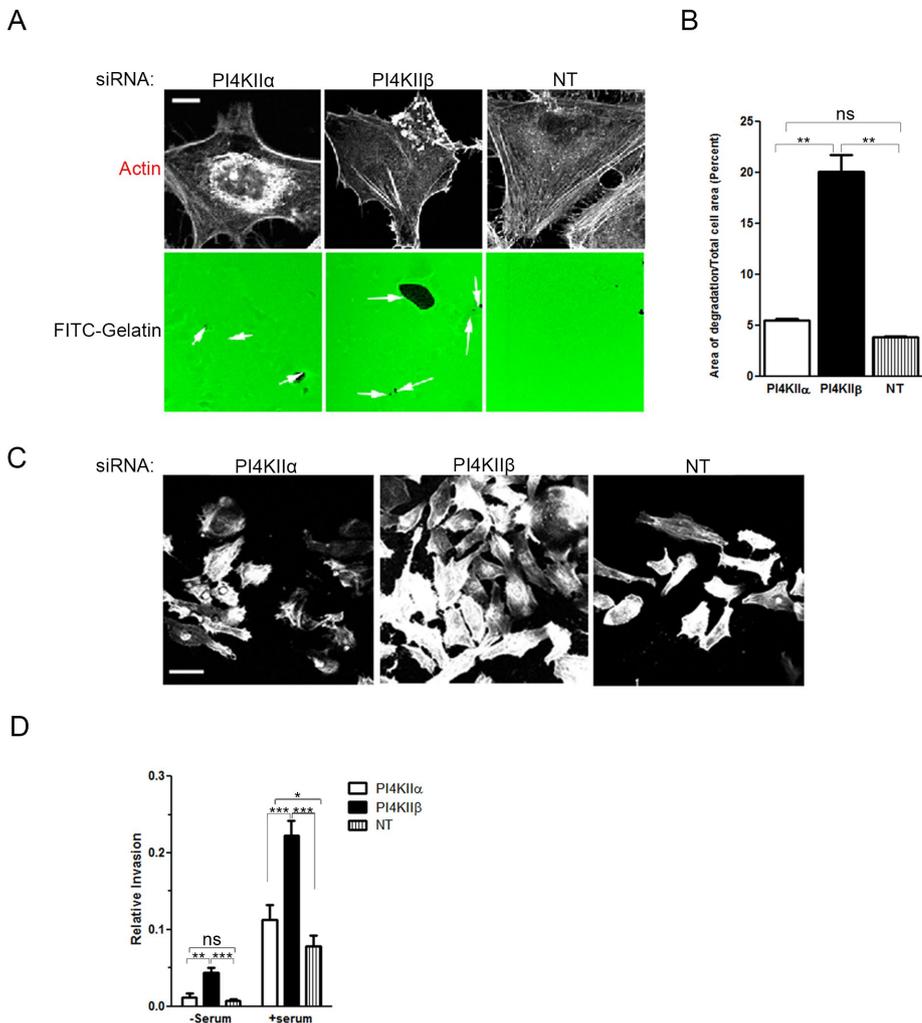


FIGURE 3: PI4KII β knockdown promotes ECM degradation and cell migration. (A) HeLa cells were reseeded onto FITC-gelatin-coated coverslips at 58 h posttransfection and then labeled for F-actin (red). Areas of proteolytic degradation are marked with arrows. (B) Percentage areas of degradation per total cell area in a $4 \times 10^4 \mu\text{m}^2$ field. Data are mean \pm SEM ($n = 30$; experiment performed in triplicate). (C, D) HeLa cells were reseeded onto Transwell inserts coated with type I collagen gel (100 μl of 2 mg/ml) 58 h posttransfection and then placed in a chemoattractant (10% FCS) for 12 h. (C) Representative fields of cells that traversed the collagen-coated membrane of a Transwell chamber in response to chemoattractant. (D) Quantitation of invasion expressed as the ratio of cells that migrated toward chemoattractant relative to the total number of cells seeded onto the serum-free upper chamber. Data are presented as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

corresponded to clusters of actin-rich punctae (Figure 3A), demonstrating that they were the source of the gelatinolytic activity.

We also performed Transwell invasion assays to measure the ability of PI4KII-depleted cells to invade a 3D collagen matrix in response to chemoattractant (serum). Invasion in the absence of serum was relatively low but significantly higher in PI4KII β siRNA cells. The introduction of serum to the lower chamber increased the number of migrating cells, with PI4KII β cells invading at approximately three times control values and two times that recorded for PI4KII α -depleted cells (Figure 3, C and D, and Supplemental Figure S2B).

PI4KII α and PI4KII β isoforms synthesize separate pools of TGN PI(4)P involved in TGN-to-endosome traffic

PI4KII isoforms are not known to directly influence the actin cytoskeleton; instead, they control trafficking from the TGN to endosomes

through the recruitment of clathrin adaptors. To address the question of how PI4KII β knockdown leads to increased matrix degradation, we asked whether the PI4KII isoforms synthesized separate pools of PI(4)P and whether loss of this PI(4)P affected post-TGN membrane traffic. We visualized the PI(4)P generated by each PI4KII isoform by inhibiting PI4KIII activities with wortmannin in control and siRNA-treated cells. When we stained siRNA-treated cells with the PI(4)P reporter glutathione S-transferase (GST)-FAPP-PH, we noted that the intensity of staining in the Golgi region was affected in both PI4KII α - and PI4KII β -knockdown cells but included signal from PI(4)P synthesized by the Golgi enzyme PI4KIII β (Supplemental Figure S3A). We therefore treated the same cells with wortmannin to inhibit PI4KIII β and fixed and stained the cells with recombinant GST-P4C, which is an unbiased PI(4)P reporter (Luo *et al.*, 2015). The residual GST-P4C signal allowed us to report the PI(4)P derived from the activity of PI4KII α (PI(4)P^{PI4KII α}) and PI4KII β (PI(4)P^{PI4KII β} ; Figure 4, A–C, and Supplemental Figure S4D), which in both cases localized to membranes in the Golgi region and on cytoplasmic vesicles (Figure 4, A–C). Cells in which PI4KII α was depleted showed residual PI(4)P^{PI4KII β} staining that colocalized with TGN46 (Figure 4, A and D). In contrast, PI(4)P^{PI4KII α} predominantly colocalized with the TGN marker syntaxin 6 (Figure 4, B and D). Colocalization of PI(4)P with syntaxin 6 and TGN46 was restored upon expression of plasmids containing silent mutations rendering them resistant to siRNA (Figure 4, E and F, and Supplemental Figure S3, E and F). This demonstrates that the wortmannin-insensitive PI(4)P^{PI4KII α} and PI(4)P^{PI4KII β} pools generated by the PI4KII isoforms exist in distinct subdomains of TGN/endosomal membranes and that knockdown of each isoform affects a metabolically separate PI(4)P compartment.

PI(4)P at the TGN is known to control the recruitment of the clathrin adaptor AP-1 in TGN-to-endosomal traffic (Wang *et al.*, 2003; Wiewfer *et al.*, 2013), and we therefore reasoned that loss of TGN PI(4)P would affect this trafficking route. We investigated how loss of TGN PI(4)P altered the steady-state distribution of the cation-independent mannose-6-phosphate receptor (CI-M6PR), finding that PI4KII α and PI4KII β led to a significant loss in colocalization between PI(4)P and this AP-1 cargo (Figure 4, C and D). This effect was particularly marked in PI4KII β cells, which also displayed a more compact staining pattern in the Golgi region and an absence of vesicular M6PR (Figure 4C). We also stained cells transfected with GFP-M6PR for AP-1 and found that PI4KII β siRNA cells showed reduced colocalization between them (Figure 4, G and H), indicating defective TGN-to-endosomal traffic. We tested the consequences on endolysosomal traffic by performing an epidermal growth factor receptor (EGFR) degradation assay and found that depletion of both PI4KII α and

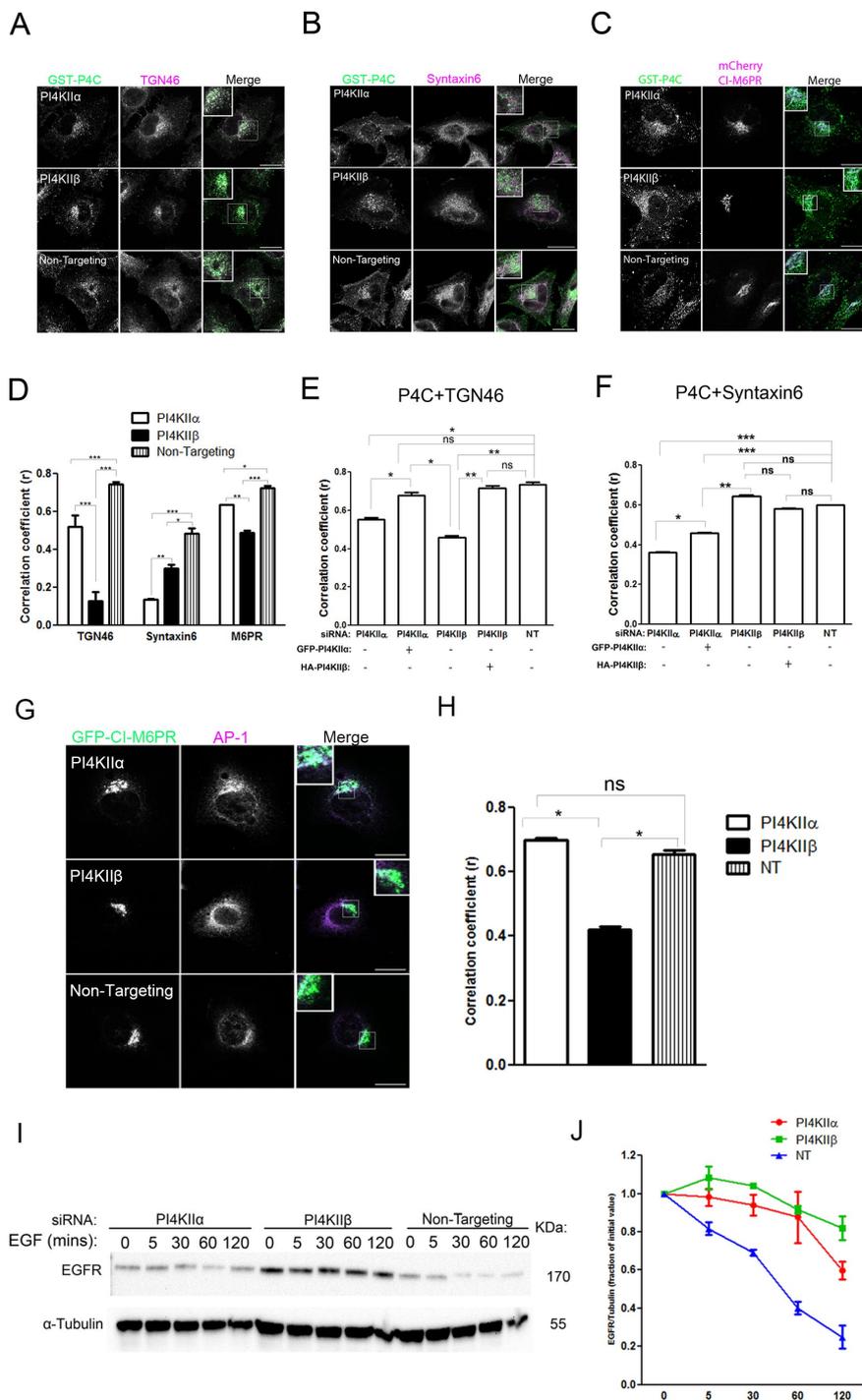


FIGURE 4: PI4KII α and PI4KII β siRNA affects separate pools of PI(4)P at the TGN and perturbs TGN-endosomal traffic. (A–C) Recombinant GST-tagged protein containing the P4C domain from the *Legionella pneumoniae* SidC gene (GST-P4C) was used to indirectly stain membrane pools of PI(4)P after wortmannin treatment. Samples were costained with (A) TGN46 or (B) syntaxin 6 or (C) transfected with mCherry-tagged (CI-M6PR). (D) Pearson's r calculated for colocalization between PI(4)P and different TGN markers (20 cells, three independent experiments). (E, F) Pearson's r for colocalization between PI(4)P and (E) TGN46 or (F) syntaxin 6 after siRNA-mediated silencing with or without subsequent transfection with siRNA-resistant constructs. (G) Confocal images showing immunostaining for GFP-tagged CI-M6PR and adaptor AP-1. Scale bars, 10 μ m. (H) Pearson's r for colocalized pixel intensities between the green (GFP) and magenta (AP-1) channels (20 cells, three independent experiments). (I) HeLa cells were transfected with the indicated siRNA oligos and then serum starved and stimulated with 100 ng/ml EGF for indicated times before analysis of EGFR levels by Western blotting. (J) Signal intensities were quantified by densitometry. Data are mean \pm SEM ($n = 3$; three independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

PI4KII β significantly impaired ligand-induced degradation of the EGFR (Figure 4, I and J). Given that loss of either PI4KII isoform impairs EGFR degradation, we coimmunostained MT1-MMP with the lysosomal marker CD63 and found that both PI4KII α and PI4KII β siRNA resulted in reduced colocalization (Supplemental Figure S4, A and B). Collectively these results show that loss of either PI(4)P pool affects TGN-to-endosome traffic and ultimately the degradation of cargo destined for lysosomes.

Loss of PI4KII β leads to increased exocytic trafficking of MT1-MMP

MT1-MMP is delivered to invadopodia from endosomal compartments (Steffen *et al.*, 2008; Wiesner *et al.*, 2013). We therefore considered that altered intracellular traffic might play a role in the increased localization of MT1-MMP to invadopodia. We analyzed cell surface MT1-MMP staining versus total cellular MT1-MMP using fluorescence-activated cell sorting (FACS) and found that loss of either PI4KII isoform led to an increase in total cellular MT1-MMP levels compared with controls (Supplemental Figure S4C); however, loss of PI4KII β led to an approximately threefold increase in MT1-MMP at the cell surface compared with control and PI4KII α siRNA-transfected cells (Figure 5A).

Increased surface MT1-MMP could result from a decreased rate of endocytosis or an increased rate of exocytic trafficking. We therefore analyzed changes to the complex trafficking itinerary of MT1-MMP by coimmunostaining MT1-MMP and a selective panel of Rab GTPases known to control traffic of cargo in endosomal and Golgi membranes (Wiesner *et al.*, 2013). On loss of PI4KII α and PI4KII β , MT1-MMP displayed decreased colocalization with early endosomal Rab5 and the late endosomal Rab7 (Figure 5, B and C). A significant increase in colocalization was observed with the post-Golgi Rab8, but meaningful differences were not detected in the colocalization of MT1-MMP with late endosomal Rab9 or the recycling endosomal Rab11 (Figure 5, B and C). This analysis demonstrates that MT1-MMP trafficking is redirected from an early endosomal (EE)–late endosomal (LE) route to the plasma membrane via a Rab8 exocytic route.

Loss of PI4KII β confers invasiveness on MCF-7 cells

We asked whether PI4KII β depletion was sufficient to convert the minimally invasive MCF-7 epithelial cell line into an invasive migratory phenotype. To test this, we evaluated the ability of PI4KII β -depleted MCF-7 cells

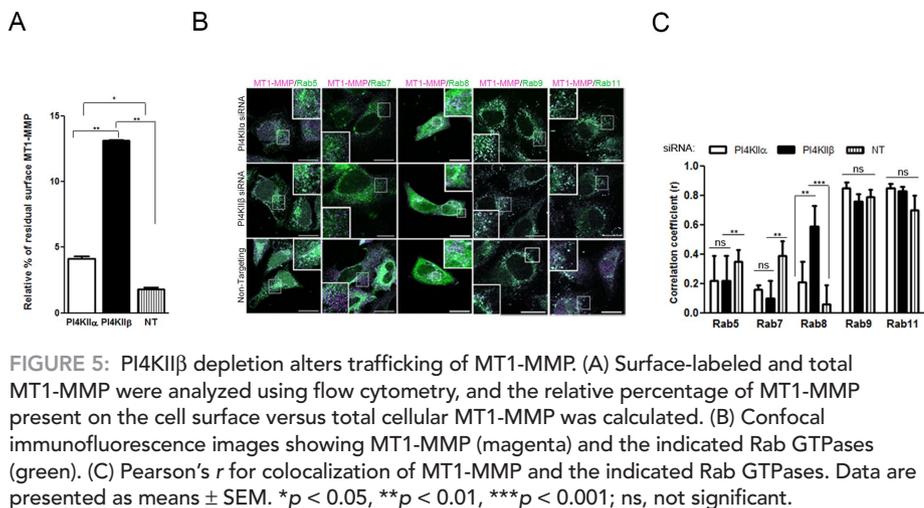


FIGURE 5: PI4KII β depletion alters trafficking of MT1-MMP. (A) Surface-labeled and total MT1-MMP were analyzed using flow cytometry, and the relative percentage of MT1-MMP present on the cell surface versus total cellular MT1-MMP was calculated. (B) Confocal immunofluorescence images showing MT1-MMP (magenta) and the indicated Rab GTPases (green). (C) Pearson's r for colocalization of MT1-MMP and the indicated Rab GTPases. Data are presented as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

to degrade FITC-gelatin matrix and invade a collagen layer in the Transwell chamber assay. PI4KII β -depleted MCF-7 cells degraded FITC-gelatin to an extent comparable to the highly invasive MDA-MDB-231 line (Figure 6, A and B). Furthermore, they displayed increased invasiveness and the ability to invade collagen ECM and cross the membrane toward a chemoattractant (Figure 6C). These findings indicate that PI4KII β suppresses cell invasion in vitro.

Loss of PI4KII β is associated with human cancers

A region of human chromosome 4 encompassing the gene for PI4KII β (PI4K2B) contains tumor suppressor genes associated with sporadic colorectal carcinoma (Zheng *et al.*, 2008). We therefore interrogated oncogenomic and transcriptomic data in the CBio-Portal and OncoPrint databases and found an association between heterozygous loss of PI4K2B and lung (squamous cell and adenocarcinoma), esophageal, pancreatic, prostate, breast, liver, and various other epithelial cancers (Figure 7A and Supplemental Figure S5). In pancreatic tumors, homozygous loss of PI4K2B was detected in 2.75% of samples, heterozygous loss in 43.1%, and

gain in 12.8%. Copy number was normal in 39.44% ($n = 109$ samples; Figure 7B). In HCC, both PI4K2B alleles were deleted in 0.52%, 23.68% displayed heterozygous deletion, 64.73% had normal copy numbers, and 10.52% showed gain of PI4K2B ($n = 190$; Figure 7C). Significant reductions in PI4KII β mRNA expression were detected in invasive breast carcinoma ($n = 59$; Figure 7D). We also analyzed survival data in the small number of cases available (Ciriello *et al.*, 2015) and found that loss of PI4KII β expression correlated with poor survival in patients diagnosed with carcinoma of the breast (Figure 7E). Together these data support the hypothesis that loss of PI4KII β is a risk factor in cancers.

DISCUSSION

Here we identify a post-TGN trafficking pathway that negatively regulates endolysosomal traffic and the formation of invadopodia. Loss of the PI4KII isoforms had dramatically different and unexpected effects on the actin cytoskeleton. An additional and striking feature of PI4KII β -depleted cells was the appearance of mature and functional invadopodia on the ventral surface.

PI4KII β plays a role in the development of a migratory phenotype in HCC (Mazzocca *et al.*, 2008), but there is limited evidence for the direct involvement of mammalian PI4KII isozymes in actin regulation. In contrast, several studies have described PI4KII functions in post-TGN traffic through PI(4)P biosynthesis and direct binding to clathrin adaptors AP-1 and AP-3 (Wang *et al.*, 2003, 2007; Salazar *et al.*, 2005; Craige *et al.*, 2008), both pathways that contribute to endolysosomal traffic. PI4KII α controls LE traffic of the EGFR (Minogue *et al.*, 2006), endolysosomal traffic mediated by AP-3 (Craige *et al.*, 2008), and that of lysosomal membrane proteins and hydrolases such as the enzyme β -glucocerebrosidase (Jovic *et al.*, 2012). This explains the similar effects of PI4KII α and PI4KII β depletion on EGFR degradation and LE traffic (Figure 4, I and J). PI4KII β

localizes to the TGN and recruits AP-1, where it controls the endosomal sorting of Frizzled by traffic to degradative compartments (Wieffer *et al.*, 2013). There is therefore a substantial amount of data from our study and in published work indicating that each PI4KII contributes to TGN-EE/LE traffic by acting at different points in a pathway leading to lysosomal degradation. The other principal way in which the PI4KII isoforms differ is in the regulation of their PI4KII activity. The reported low activity of PI4KII β (Balla *et al.*, 2002) and the need to recruit this isoform to membranes (Jung *et al.*, 2011) illustrate the importance of regulating its activity and suggest that this isoform is subject to tight regulation.

Few studies have investigated the role of PI4KII in TGN PI(4)P synthesis. Our data demonstrate for the first time that the PI4KII enzymes synthesize separate TGN pools of PI(4)P that are defined by the markers syntaxin 6 and TGN46. This is consistent with our previous description of a

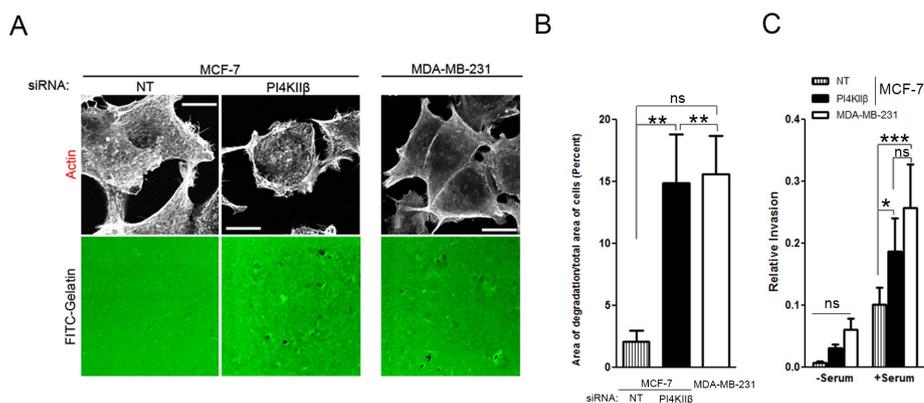


FIGURE 6: PI4KII β depletion is sufficient to confer a migratory and invasive phenotype. (A) siRNA-treated MCF-7 and untreated MDA-MB-231 cells were reseeded onto FITC-gelatin-coated coverslips at 58 h posttransfection and then labeled for F-actin (red). (B) Percentage areas of proteolytic degradation per total cell in a 4×10^4 μm^2 field. Data are mean \pm SEM ($n = 30$; experiments done in triplicate). (C) HeLa cells were reseeded onto collagen-coated Transwell inserts at 58 h posttransfection and placed in a chemoattractant (10% FCS) for 12 h. Histogram shows index of cell migration through an artificial barrier in response to chemoattractant. Data are mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

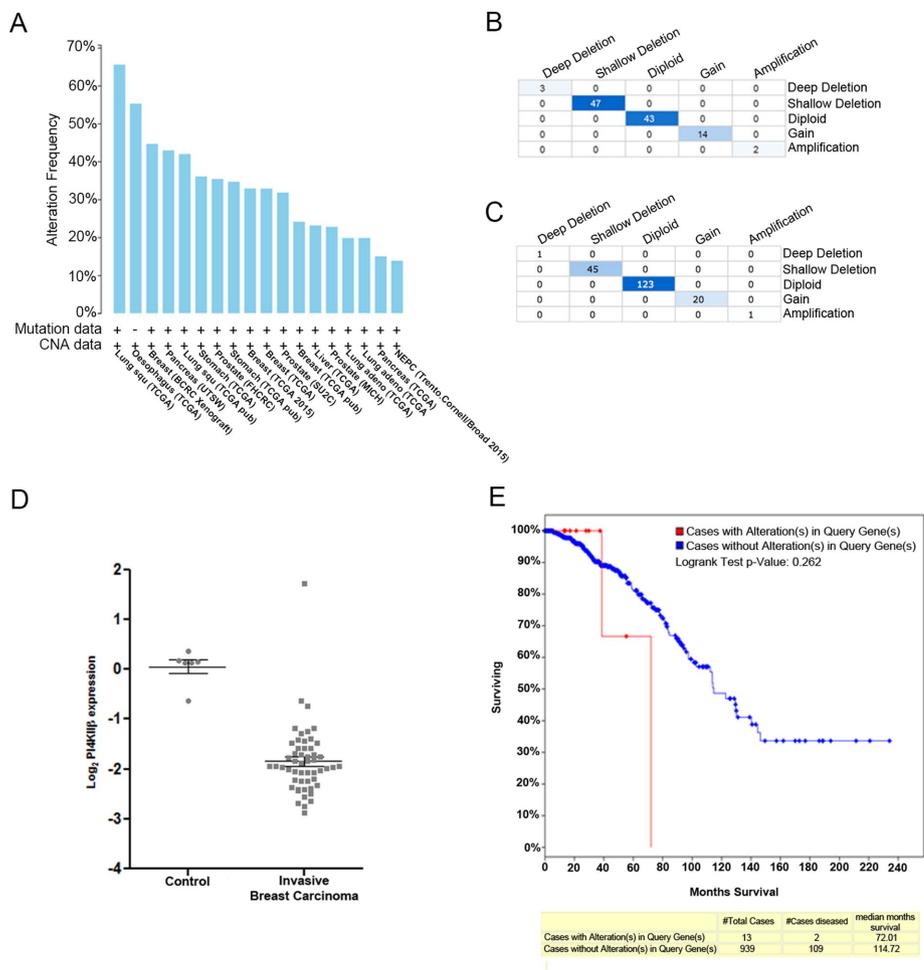


FIGURE 7: Database analysis shows that loss of PI4KII β expression is associated with cancers. (A) Cross-cancer copy number alteration (CNA) summary of 18 selected cancer studies (including lung, esophageal, breast, prostate, and pancreatic tumors) for heterozygous loss of the PI4K2B allele. Threshold: minimum 5% altered samples, cBioPortal analysis (Cerami *et al.*, 2012; Gao *et al.*, 2013). adeno, adenocarcinoma; NEPC, neuroendocrine prostate cancer; Squ, squamous cell carcinoma. Sources of the data are given in parentheses: TCGA, The Cancer Genome Atlas; BCRC, British Columbia Cancer Research Center; UTSW, University of Texas Southwestern; FHRC, Fred Hutchinson Cancer Research Center; SU2C, Stand Up To Cancer/Prostate Cancer Foundation (PCF); MICH, University of Michigan. (B) PI4K2B putative copy number alterations for pancreatic cancers (deletion or amplification) was analyzed using cBioPortal. (C) Overall change in PI4K2B copy number (deletion or amplification) in HCC was analyzed using cBioPortal. (D) Analysis of PI4KII β mRNA expression (OncoPrint) in recurrent breast carcinoma (Finak breast, reporter: A_23_P18598). (E) Overall Kaplan-Meier survival graph of breast carcinoma case analysis (cBioPortal) with PI4KII β expression alteration (Ciriello *et al.*, 2015).

TGN-endosomal compartment enriched in syntaxin 6 containing highly active PI4KII α (Simonsen *et al.*, 1999; Waugh *et al.*, 2003). The structurally unrelated, wortmannin-sensitive PI4KII β also contributes to PI(4)P synthesis in the Golgi of MDCK cells (Weixel *et al.*, 2005), indicating that the Golgi complex contains at least three metabolically separate PI(4)P pools.

Cells such as HeLa and MCF-7 normally display minimal invasive potential and typically have low levels of surface MT1-MMP, reflecting the importance of controlling surface metalloproteinase activity (Poincloux *et al.*, 2009). Loss of either PI4KII isoform led to increased levels of the metalloproteinase, consistent with a general impairment of endolysosomal degradative traffic; however, only depletion of PI4KII β caused increased levels of surface MT1-MMP, indicating that intracellular traffic of MT1-MMP was affected in an isoform-specific manner.

The reduced colocalization of MT1-MMP with Rab7, which acts at the EE-LE boundary, in both PI4KII-depleted cells demonstrates that the proteinase is not sorted to the LE. This is consistent with the elevated levels of MT1-MMP observed in both PI4KII knockdowns (Supplemental Figure S4C), the impaired degradation of the EGFR (Figure 4, I and J), and the failure of MT1-MMP to traffic to CD63-containing lysosomes (Supplemental Figure 4, A and B). The most dramatic change in colocalization was a PI4KII β -specific increase in association with Rab8 membranes. Rab8 operates in a post-Golgi exocytic pathway that determines directional traffic to protrusions of the plasma membrane (Hattula *et al.*, 2006) and is known to mediate the trafficking of MT1-MMP (Bravo-Cordero *et al.*, 2007). A mechanistic investigation of Rab8 was outside the scope of our study, but it is interesting to note that this GTPase was recently shown to regulate the cortical actin cytoskeleton (Bravo-Cordero *et al.*, 2016), suggesting that the mistrafficking of Rab8 may contribute to the actin phenotype.

We were unable to detect increased trafficking of MT1-MMP-GFP to invasive structures in live cell experiments; however, our attempts were fraught with technical difficulties arising from the substantial MT1-MMP signal present in multiple subcellular compartments that obscured invadopodia dynamics. The trafficking of MT1-MMP is known to be complex, and surface levels are controlled by endocytosis and exocytosis from several compartments (Poincloux *et al.*, 2009). The increased localization of MT1-MMP to the plasma membrane, increased association with invasive protrusions, loss of colocalization in Rab5- and Rab7-positive compartments, and increased colocalization with exocytic Rab8 membranes indicate that loss of PI4KII β and the associated pool of TGN PI(4)P causes disequilibrium between the endolysosomal and exocytic trafficking routes controlling plasma membrane levels of MT1-MMP. Our data support a model in which MT1-MMP traffic is directed away from the default endolysosomal pathway and forced into an exocytic pathway. One possible consequence of reduced MT1-MMP traffic into the endolysosomal pathway is increased recycling. Although we did not directly address this possibility, it remains likely because we also detected increased invadopodial localization of β 1-integrin, a molecule known to be recycled through a pathway involving Rab8 and cotrafficked with MT1-MMP (Macpherson *et al.*, 2014). Of interest, the recent finding that PI4KII α directs exocytic traffic of β 1-integrin from endosomes to the plasma membrane when endosomal PI(3)P is lost (Ketel *et al.*, 2016) suggests that endosomal PI(4)P^{PI4KII α} may play a role in β 1-integrin delivery. Further investigation is required to determine whether loss of PI(4)P^{PI4KII β} results in an increase in PI(4)P^{PI4KII α} and whether this is accompanied by increased β 1-integrin recycling.

Integrin traffic to the plasma membrane plays important roles in tissue organization and is known to be dependent on PI(3)P (Ribeiro *et al.*, 2011). Cross-talk between this PI monophosphate and the PI(4)P produced by the PI4Ks is an unanswered question.

The conversion of the minimally invasive HeLa and MCF-7 cell lines to an invasive phenotype and the fact that tumor suppressor genes map closely to the chromosomal location of the PI4K2B allele (Zheng *et al.*, 2008) led us to perform database searches. These showed that loss of heterozygosity of the PI4K2B allele and PI4KII β underexpression were associated with numerous cancers of epithelial origin. PI4KII β underexpression was also associated with poorer patient survival; however, the low statistical power of the studies led to a relatively low probability value ($p = 0.262$). Although we are cautious in drawing conclusions, we are aware that this finding may be highly significant and therefore speculate that PI4KII β acts as a metastasis suppressor by maintaining PI(4)P-dependent post-TGN traffic into the endolysosomal pathway. The association of PI4KII β with relatively common human cancers (lung, breast, liver, pancreas, and others) warrants further investigation, particularly because of the possibility that this putative suppressor pathway could be rescued by inhibition of PI(4)P phosphatases acting at the TGN.

It is notable that a developmental role for PI4KII β in zebrafish has been described (Wieffer *et al.*, 2013). The pathological process of tumor cell invasion has many similarities with important processes during embryogenesis along with neurite outgrowth and angiogenesis. It is therefore possible that PI4KII β activity is subject to regulation controlling physiological invasion. This hypothesis points to a PI(4)P-regulated switch that determines the decision between lysosomal degradation of cargoes and their traffic to the plasma membrane that may be fundamentally important in the intracellular traffic of key molecules such as integrins and matrix metalloproteinases that determine tissue remodeling during development.

MATERIALS AND METHODS

Cell lines, culture, and transfection

HeLa and MCF7 cell lines were cultured in DMEM (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Dorset, UK) and maintained at 37°C and 5% CO₂. MDA-MB-231 cells were cultured in Leibowitz's L-15 medium (Life Technologies) supplemented with 15% FBS and 2 mM L-glutamine (Life Technologies) and maintained at 37°C, 0.1% CO₂. All media were supplemented with antibiotics (1000 U/ml penicillin and 0.1 mg/ml streptomycin; Sigma-Aldrich). DharmaFECT (GE Healthcare Dharmacon, Lafayette, CO) and Lipofectamine (Life Technologies) were used to transfect siRNAs and plasmids, respectively.

Antibodies and reagents

All primary antibodies with corresponding dilutions are listed in Supplemental Table S1. Highly cross-adsorbed Alexa Fluor-conjugated secondary antibodies and Alexa Fluor-conjugated phalloidin (1:250 dilution) were purchased from Molecular Probes (Life Technologies). Hoechst 33342 trihydrochloride was also from Molecular Probes and was used at 1:5000 for nuclear staining. Human recombinant EGF was purchased from Sigma-Aldrich. Unless otherwise stated, other reagents were from Sigma Aldrich.

Plasmids

siRNA-resistant GFP-tagged PI4KII α and hemagglutinin-tagged PI4KII β were generous gifts from Volker Haucke (Leibniz Institut für Molekulare Pharmakologie, Berlin, Germany). mCherry and GFP-tagged Cl-M6PR were kindly provided by Mihaela Anitei (Technische Universität, Dresden, Germany).

Small interfering RNAs

We silenced PI4KII α and PI4KII β using ON-TARGET $plus$ SMART-pools siRNA oligonucleotides (GE Healthcare Dharmacon). Each comprised a mix of four siRNA duplexes targeting specific sequences within the named genes. We also used an ON-TARGET $plus$ nontargeting pool (D-001810-10-05) as control. Gene silencing was confirmed by Western blotting of whole-cell lysates. PI4KII-knockdown phenotypes were rescued using single siRNA duplexes and the ectopic expression of siRNA resistant plasmids as described previously (Mossinger *et al.*, 2012; Wieffer *et al.*, 2013). Further details are given in the Supplemental Materials and Supplemental Table S2.

Western immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 0.5 mM ethylene diamine tetraacetic acid [EDTA], 0.5 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid [EGTA], 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.2 mM Na₃VO₄) and 1 \times cComplete Protease Inhibitor (Roche, Mannheim, Germany). Protein content was determined using the Lowry method (Bio-Rad, Hertfordshire, UK), and lysates were solubilized in 2 \times Laemmli sample buffer, separated using SDS-PAGE, blotted, and probed with respective antibodies. Blots were imaged on a FluorChem M Multifluor imaging system (ProteinSimple, San Jose, CA) and analyzed by densitometry using its gel analysis function.

Immunofluorescence microscopy

Cells were grown on glass coverslips, fixed, and probed with antibodies of interest as previously (Minogue *et al.*, 2006). Unless otherwise stated, images were acquired using the 63 \times /1.4 numerical aperture lens on a Zeiss LSM 510 confocal microscope. Randomly chosen representative fields were selected and 12-bit images acquired using identical detector gain and offset settings. Images were quantitatively analyzed for fluorescence intensities, pixel correlations, area measurements, and cell densities using ImageJ software (National Institutes of Health, Bethesda, MD). Full details are available in the Supplemental Materials.

Fluorescent matrix degradation

The ability of cells to degrade the extracellular matrix was analyzed using fluorescent gelatin matrix-coated culture dishes prepared according to Bowden *et al.* (1999). Targeted or control siRNA-transfected cells were seeded onto FITC-gelatin-coated coverslips and cultured for 12–18 h. Matrix degradation was calculated as the total area of matrix degradation (black holes) per total cell area expressed as a percentage (full details are given in the Supplemental Materials).

MT1-MMP trafficking

We used an established method (Kean *et al.*, 2009). Briefly, MCF-7 cells were reseeded onto coverslips thinly coated with collagen (5 μ g/cm²) at 36 h posttransfection and grown in complete DMEM for 20 h at 37°C. Cells were then serum starved for 4 h to facilitate internalization of MT1-MMP. For trafficking of endogenous MT1-MMP, cells were pulsed with anti-MT1-MMP antibody (Abcam, Cambridge, UK) for 1 h at 4°C. Cells were subsequently fixed with 3.7% formaldehyde, permeabilized, and immunostained.

For flow cytometry experiments, we induced internalization of MT1-MMP by serum starvation and stimulated surface expression as described. Cells were lifted using FACS buffer (5 mM EDTA in phosphate-buffered saline [PBS], pH 7.4) and subsequent steps carried out at 4°C. Cells were probed with rabbit anti-MT1-MMP (Abcam)

for 1 h, washed three times with PBS, and then labeled with anti-rabbit Alexa Fluor antibody. Cells were analyzed using a FACS LSRII cell sorter (BD Biosciences, Oxford, UK), and ~10,000 cells were counted per sample. Postacquisition analysis was performed using FlowJo software, version V10 (FlowJo LLC, Ashland, OR).

Transwell migration assay

This was performed in 8- μ m-pore, collagen-coated Transwell chambers (Corning, Tewksbury, MA). Cells were transfected with target or control siRNA in six-well plates and, after 48 h, resuspended in serum-free medium and seeded at 5×10^4 into the upper chamber of the Transwell. Cells were allowed to migrate toward 10% FBS plus DMEM for 12 h. Migratory cells at the lower chamber were fixed and stained with Alexa 488-conjugated phalloidin, imaged using similar optical settings (20 \times Objective, Zeiss LSM 510 confocal microscope), and counted in five random fields using ImageJ. Histograms represent relative invasion of the Transwell chamber, calculated as the number of cells counted at the lower chamber relative to the total number of cells seeded onto the upper chamber (details are given in the Supplemental Materials).

Data analysis

Prism 5 (GraphPad, La Jolla, CA) was used for statistical analysis. Data are presented as mean \pm SEM from at least three independent experiments. Statistical comparisons were performed using one-way analysis of variance followed by Tukey's posttest at a 95% confidence interval.

Oncogenomic database analysis

The cBio Cancer Genomics Portal (<http://cbioportal.org/>; Cerami *et al.*, 2012; Gao *et al.*, 2013) and OncoPrint (Rhodes *et al.*, 2004) are open access resources for cancer genomics data sets containing data from 69 cancer genomics studies with 17,177 samples (cBio) and 715 data sets with 86,733 samples (OncoPrint). Heterozygous loss of the PI4K2B allele was analyzed in all cancers, as were copy number alterations in pancreatic cancers and hepatocellular carcinoma. In addition, correlations of PI4KII β mRNA expression, recurrence, metastatic events, or overall survival in breast cancer cases were investigated.

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