Citation: Cell Death and Disease (2010) 1, e106; doi:10.1038/cddis.2010.84

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Differential effects of the phosphatidylinositol 4-kinases, PI4KIIα and PI4KIIIβ, on Akt activation and apoptosis

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In this study, we investigated the role of PI4P synthesis by the phosphatidylinositol 4-kinases, PI4KIIα and PI4KIIIβ, in epidermal growth factor (EGF)-stimulated phosphoinositide signaling and cell survival. In COS-7 cells, knockdown of either isozyme by RNA interference reduced basal levels of PI4P and PI(4,5)P_2, without affecting receptor activation. Only knockdown of PI4KIIα inhibited EGF-stimulated Akt phosphorylation, indicating that decreased PI(4,5)P_2 synthesis observed by loss of either isoform could not account for this PI4KIIα-specific effect. Phospholipase Cγ activation was also differentially affected by knockdown of either PI4K isozyme. Overexpression of kinase-inactive PI4KIIα, which induces defective endosomal trafficking without reducing PI(4,5)P_2 levels, also reduced Akt activation. Furthermore, PI4KIIα knockdown profoundly inhibited cell proliferation and induced apoptosis as evidenced by the cleavage of caspase-3 and its substrate poly(ADP-ribose) polymerase. However, in MDA-MB-231 breast cancer cells, apoptosis was observed subsequent to knockdown of either PI4KIIα or PI4KIIIβ and this correlated with enhanced proapoptotic Akt phosphorylation. The differential effects of phosphatidylinositol 4-kinase knockdown in the two cell lines lead to the conclusion that phosphoinositide turnover is inhibited through PI4P substrate depletion, whereas impaired antiapoptotic Akt signaling is an indirect consequence of dysfunctional endosomal trafficking.
Cell Death and Disease (2010) **1,** e106; doi:10.1038/cddis.2010.84; published online 9 December 2010
Subject Category: Cancer

Receptor-dependent phosphoinositide signaling pathways control cell survival and are frequently deregulated in cancer.[1,2] In this report, we focus on phosphoinositide signaling mediated by the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase that is overexpressed in a variety of solid tumors and is the target for multiple cancer therapeutics.[3] Ligand stimulation of EGFR leads to activation of two important phosphoinositide signaling cascades. The first pathway operates via phospholipase Cγ (PLCγ) isozymes, which hydrolyze phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P_2) to produce the second messengers, inositol-trisphosphate and diacylglycerol (reviewed in Bunney and Katan[2]). Enhanced PLCγ signaling has been implicated in upregulated cell motility and metastasis.[4,5] The second pathway is mediated by phosphoinositide 3-kinase (PI3K) isozymes, which phosphorylate PI(4,5)P_2 on the D3 position to produce phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P_3) (reviewed in Bunney and Katan[2]). PI(3,4,5)P_3 stimulates the activity of phosphoinositide-dependent kinase 1, which in turn phosphorylates and activates Akt (also known as PKB), a serine/threonine kinase that regulates a range of proteins such as BAD that are essential for cell survival.[6] Dysfunctional PI3K signaling is associated with defective cell proliferation and motility, and this pathway is a major target for chemotherapeutic intervention in a diverse range of malignancies.[1] However, there is also a growing awareness that the proliferation of cancer cells with normal PI3K signaling is insensitive to Akt inhibition.[7]

Compared with PI(4,5)P_2, cellular levels of PI(3,4,5)P_3, even in stimulated cells, are very low.[8] Nevertheless, there is evidence that PI3K and PLCγ can compete for a common pool of PI(4,5)P_2, substrate, for example, during VEGF-stimulated angiogenesis.[9] Given that PI4Ks can potentially supply substrate into both phosphoinositide signaling pathways, we sought to explore the roles of these enzymes as possible upstream regulators of PLC and Akt activation. Furthermore, as the syntheses of both PI(4,5)P_2[10,11] and PI(3,4,5)P_3 have been associated with antiapoptotic signaling, we investigated whether targeting PI4P synthesis could modulate cell survival.

There are four PI4K isozymes in mammalian cells: the type III PI4Ks, PI4KIIIα and PI4KIIIβ, which can be inhibited by wortmannin and are structurally homologous to the PI3K family, and the type II PI4Ks, PI4KIIα and PI4KIIβ, which are wortmannin insensitive and structurally distinct from other phosphoinositide kinases.[12] Despite their similar catalytic activity, the PI4Ks have non-overlapping localizations within cells and perform isoform-specific functions.[12] In this study,

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Keywords: PI 4-kinase; EGFR; apoptosis; phosphatidylinositol 4-phosphate; Akt; PLC
Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI4K, phosphatidylinositol 4-kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate
Received 08.6.10; revised 29.10.10; accepted 02.11.10; Edited by P Salomoni

we concentrate on PI4KIIα and PI4KIIIβ that localize to different regions of the *trans*-Golgi network[13,14] and to endosomes.[14–16]

PI4KIIα[14–16] and PI4KIIIβ have been implicated in Golgi-endosomal trafficking,[17–20] and PI4KIIα has been shown to regulate Wnt3a[21] and proangiogenic vascular endothelial growth factor signaling.[22] However, investigations in to the roles of different PI4K isoforms[23] and Golgi-associated PI4P synthesis[24] in G-protein-coupled receptor (GPCR)-mediated signaling indicated only minor roles for PI4KIIα and PI4KIIIβ. There are precedents for PI4K-dependent cell survival in yeast[25] and zebrafish,[26] and we recently reported that PI4KIIα homozygous knockout mice develop late-onset neurodegeneration.[27] Although PI4KIIα and PI4KIIIβ are known to be important for phosphoinositide-dependent intracellular membrane trafficking, the roles of these enzymes in the regulation of antiapoptotic signaling have not been explored. On the basis of what is already established for these enzymes, it is most likely they modulate phosphoinositide signaling through effects on phosphoinositide substrate supply and/or vesicular trafficking.

Our main aim in this study was to investigate whether decreased supply of PI4P substrate was important for epidermal growth factor (EGF)-stimulated PLC and Akt signaling, and consequently cell survival. A previous study using LY294002 at concentrations sufficient to inhibit PI4KIIIs indicated that the EGF-stimulated calcium response required both PI4KII and PI4KIII activity.[28] This suggests a key difference between EGFR and GPCR signaling, and possibly a more prominent role for PI4KIIs in EGF-dependent phosphoinositide signaling. However, nothing is known about the PI4K isoform dependency of EGF-stimulated PI3K signaling. We investigated the relationships between PI4Ks, PI(4,5)P_2 levels, Akt activation and cell proliferation using COS-7 and MDA-MB-231 cell lines, which have contrasting dependencies on Akt activation for cell survival.[29,30] COS-7 cells are an SV40-transformed African green monkey kidney fibroblast cell line that proliferates in response to EGF treatment through a mechanism requiring Akt activation.[29] On the other hand, proliferation of MDA-MB-231 breast cancer cells is insensitive to EGF stimulation[30] or Akt inhibition.[7] Moreover, enhanced Akt phosphorylation is associated with impaired MDA-MB-231 cell survival.[31] MDA-MB-231 are also an example of a triple-negative breast cancer cells, which express neither hormone receptors nor HER2 and are therefore difficult to target with current chemotherapeutic strategies. Another important feature of MDA-MB-231 cells is that they are normal for class 1A PI3K and PTEN, and are not addicted to Akt signaling.[7] We found that RNA interference (RNAi)-mediated PI4KIIα knockdown induced apoptosis and reduced cell proliferation in both cell lines. PI4KIIα depletion led to cell-specific effects on Akt phosphorylation that were independent of changes to total cellular PI4P and PI(4,5)P_2 levels. However, loss of the PI4KIIIβ isoform induced apoptosis only in MDA-MB-231 cells and this correlated with enhanced Akt phosphorylation and not with phosphoinositide depletion. These data reveal novel insights into the functions of PI4KIIα and PI4KIIIβ in antiapoptotic signaling and cell survival.

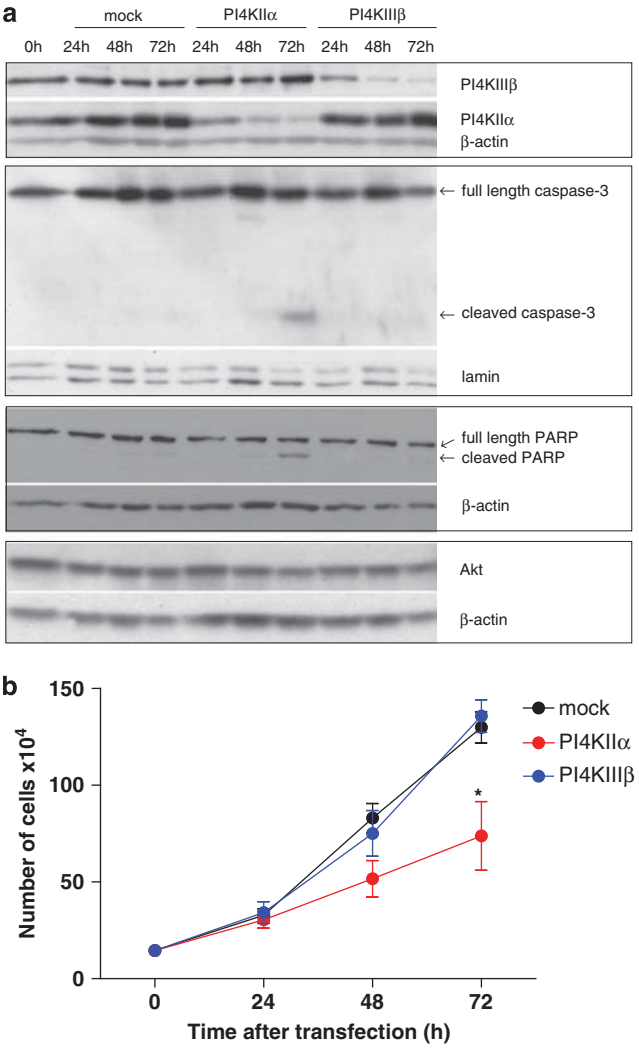

Figure 1 PI4KIIα is required for cell survival in COS-7 cells. COS-7 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. (**a**) Time course of PI4K knockdown. Effects of PI4KIIα or PI4KIIIβ depletion on caspase-3 activation and cleavage of its substrate PARP. Akt levels over RNAi time course. Cells were analyzed by immunoblotting at 0, 24, 48 and 72 h after transfection. Representative blots from 2–5 independent experiments are shown. (**b**) Effects of PI4KIIα or PI4KIIIβ depletion on cell proliferation. Live cell counts were determined at 0, 24, 48 and 72 h after transfection. Data presented are means ± S.E.M. of four independent experiments. $^*P < 0.05$

Results

Loss of PI4KIIa results in activation of caspase-3 and cleavage of PARP. We employed established RNAi methodology[14,15,32] to deplete PI4KIIα or PI4KIIIβ, and investigated whether loss of either PI4K had consequences for COS-7 cell survival and proliferation. We found that loss of PI4KIIα but not PI4KIIIβ resulted in proteolytic activation of caspase-3 and cleavage of its substrate poly(ADP-ribose) polymerase (PARP) at 72 h, when there is maximum depletion of PI4KIIα (Figure 1a). In addition, cell proliferation was profoundly reduced by loss of PI4KIIα (Figure 1b). Compared with mock-transfected cells, knockdown of PI4KIIα reduced cell proliferation

43.2 ± 10.4% at 72 h after transfection ($n = 4$, $P < 0.05$ at 72 h). In contrast to PI4KIIα, knockdown of PI4KIIIβ had no effect on cell proliferation. These results indicate an isoform-specific role for PI4KIIα in protecting COS-7 cells from caspase-mediated apoptosis. We also found that there were no changes in total Akt levels over the 72 h time course (Figure 1a), indicating that PI4KIIα does not directly regulate Akt stability or expression.

RNAi knockdown of PI4KIIα inhibits EGF activation of Akt. Given that knockdown of PI4KIIα inhibited COS-7 cell proliferation, we examined the relationship between PI4K depletion and antiapoptotic EGF-stimulated phosphoinositide signaling. Maximum knockdown of PI4KIIα and PI4KIIIβ were observed at 72 h after transfection (Figure 1a), therefore this time point was chosen for stimulations with EGF. siRNA-treated cells were serum starved and stimulated with 100 ng/ml EGF or vehicle control for 10 min, then harvested in SDS-PAGE sample buffer. The cell lysates were analyzed by immunoblotting against PI4KIIα and PI4KIIIβ to verify target knockdown, and phospho-specific antibodies for EGFR, PLCγ and Akt to investigate signaling activation. Phosphorylation of Akt reports receptor-stimulated synthesis of $PI(3,4,5)P_3$ through the PI3K pathway, whereas phosphorylation of PLCγ depends on EGFR activation. PI4P produced by PI4KIIα has the potential to supply substrate to both the PI3K and PLC pathways.

As shown in Figure 2b, loss of PI4KIIα but not PI4KIIIβ resulted in reduced basal and EGF-stimulated levels of phospho-Akt. This suggests a specific requirement for the PI4KIIα isoform in EGF-dependent PI3K signaling. We observed no reduction in agonist-stimulated EGFR phosphorylation or EGFR levels (Figure 2b), thus the diminished levels of phospho-Akt activation were not due to inhibition of receptor activation or reduced receptor levels. Furthermore, depletion of the PI4KIIα isoform did not inhibit EGF-stimulated PLCγ phosphorylation (Figure 2b). However, loss of PI4KIIIβ resulted in enhanced basal PLCγ phosphorylation and reduced responsiveness to EGF. These results demonstrate that loss of the PI4KIIα isoform specifically affected EGF-dependent Akt signaling.

Interestingly, following serum starvation, knockdown of PI4KIIα but not PI4KIIIβ resulted in a major fall in Akt levels, a small decrease in PLCγ and no change to total EGFR levels (Figure 2b). Although reduced total Akt levels accounted for decreased Akt activation in COS-7 cells, the residual Akt protein was still phosphorylated in response to EGF addition (Figure 2b). These results indicate that EGFR coupling to the PI3K pathway was not inhibited through loss of PI4KIIα and suggest that PI4KIIα may regulate Akt activation independently of $PI(4,5)P_2$ substrate provision to PI3K.

PI4KIIα and PI4KIIIβ supply PI4P and $PI(4,5)P_2$ for EGF-stimulated phosphoinositide metabolism. We investigated whether the diminished EGF-dependent Akt activation with PI4KIIα knockdown is a result of reduced phosphoinositide synthesis. [^{32}P]phosphate metabolic radiolabeling studies were performed on siRNA-treated COS-7 cells. EGF (100 ng/ml) stimulation for 10 min resulted in a significant fall in [^{32}P]PI4P to 57.5 ± 14.1%

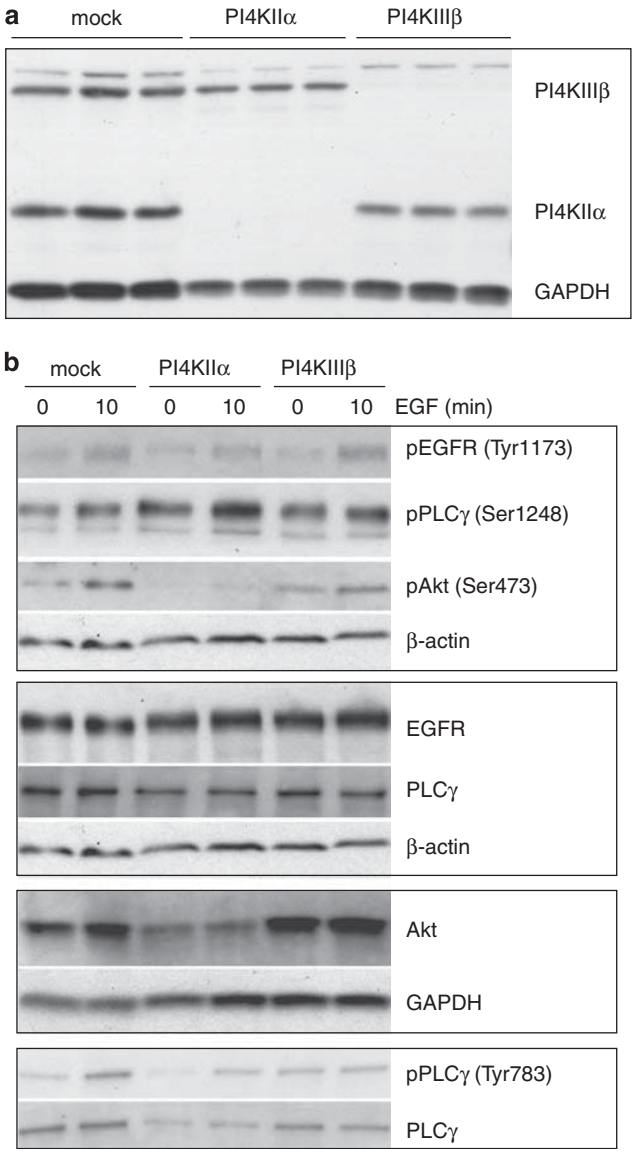

Figure 2 Knockdown of PI4KIIα inhibits EGF-induced Akt activation. COS-7 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. After 72 h of transfection, the cells were stimulated with 100 ng/ml EGF or vehicle control for 10 min and analyzed by immunoblotting. (**a**) Levels of knockdown of PI4KIIα or PI4KIIIβ with siRNA treatment. Triplicate wells for each condition are shown. (**b**) PI4KIIα knockdown inhibits Akt phosphorylation but not EGFR or PLCγ phosphorylation. Loss of PI4KIIα leads to a large reduction in Akt levels following serum starvation. Representative blots from two 2–10 independent experiments are shown

($n = 4$, $P < 0.05$) of basal levels in mock-transfected cells (Figure 3a). Cells treated with siRNA against PI4KIIα or PI4KIIIβ had reduced basal levels of [^{32}P]PI4P, which were, respectively, 36.4 ± 13.9 ($n = 4$, $P < 0.01$) and 27.5 ± 4.8% ($n = 4$, $P < 0.0001$) of control levels (Figure 3a). In addition, the EGF-stimulated [^{32}P]PI4P response was inhibited by RNAi depletion of PIKIIα or PI4KIIIβ ($n = 4$, Figure 3a). Loss of either PI4K isoform also led to substantial falls in basal [^{32}P]$PI(4,5)P_2$, to 50.2 ± 11.3% ($n = 4$, $P < 0.01$) and 41.2 ± 15.5% ($n = 4$, $P < 0.01$) of control levels for PI4KIIα

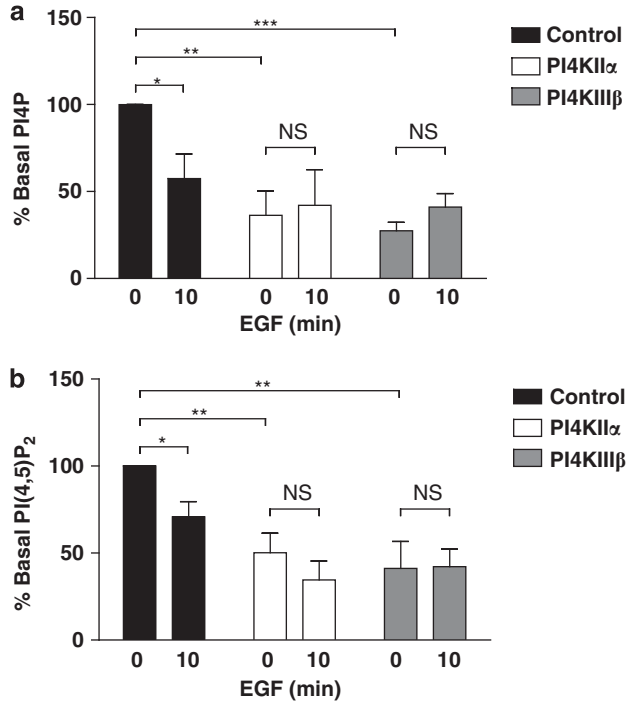

Figure 3 Loss of PI4KIIα or PI4KIIIβ reduces pools of signaling lipids and affects EGF-induced phosphoinositide metabolism. COS-7 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. After 72 h, they were labeled with [^{32}P]phosphate and stimulated with 100 ng/ml EGF or vehicle control for 10 min. Cells were harvested and [^{32}P]-labeled lipids were analyzed by TLC. Effects of PI4K RNAi on (**a**) [^{32}P]-labeled PI4P and (**b**) [^{32}P]-labeled PI(4,5)P_2. Data presented are means ± S.E.M. from four independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$; NS, not significant

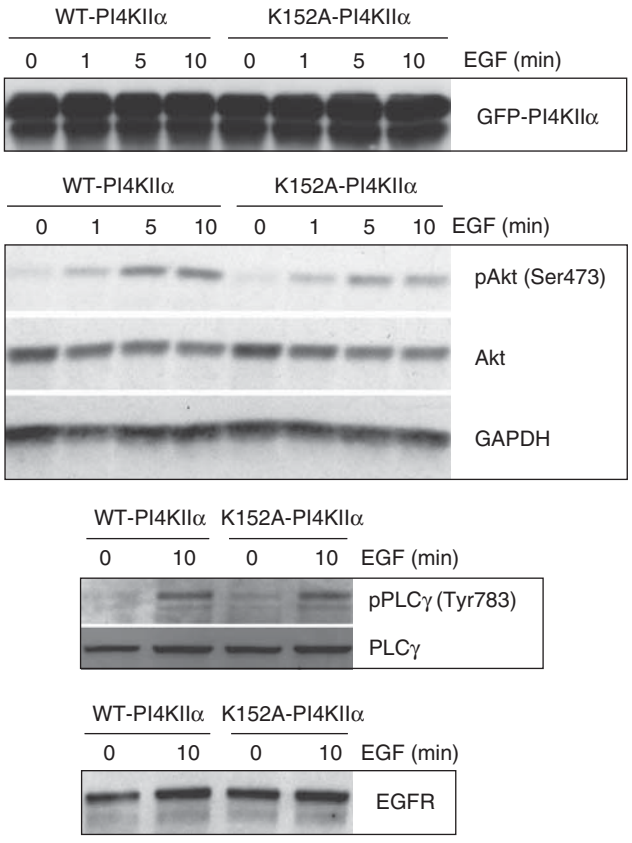

Figure 4 Kinase-inactive (K152A)PI4KIIα selectively inhibits Akt activation. COS-7 cells were transfected with GFP-PI4KIIα or catalytically inactive GFP-(K152A)PI4KIIα. Cells were serum starved, stimulated with 100 ng/ml EGF and cell lysates were analyzed by immunoblotting. Representative blots from three independent experiments are shown

and PI4KIIIβ respectively (Figure 3b). Notably, knockdown of PIKIIα or PI4KIIIβ abolished the [^{32}P]PI(4,5)P_2 response to EGF (Figure 3b). Thus, knockdown of either PI4K isozyme blocks PI(4,5)P_2 turnover without any inhibition of EGF-stimulated PLCγ phosphorylation (Figure 3). These results demonstrate that both PI4KIIα and PI4KIIIβ can supply PI4P to PI4P5K during EGFR activation.

Kinase-inactive PI4KIIα inhibits EGF activation of Akt. To confirm the finding that regulation of Akt by PI4KIIα is not due to changes in total cellular PI4P and PI(4,5)P_2 levels, we utilized the lipid kinase-inactive mutant version (K152A)PI4KIIα.[15] It has been shown that overexpression of kinase-inactive PI4KIIα does not lead to any measurable changes to either total cellular PI4P or PI(4,5)P_2 levels,[14] but results in defective endosomal trafficking.[16,33] Overexpression of catalytically inactive PI4KIIα leads to reduced transferrin uptake,[33] and compared with wild-type enzyme, the inactive enzyme colocalizes less with AP-3 and LAMP1, and more with AP-1 and transferrin receptor.[16] COS-7 cells were transiently transfected with constructs encoding GFP-(WT)PI4KIIα or GFP-(K152A)PI4KIIα, serum starved and stimulated with 100 ng/ml EGF or vehicle control for 0–10 min. The cells were harvested and analyzed by immunoblotting. Compared with wild-type PI4KIIα-expressing cells, (K152A)PI4KIIα inhibited EGF-stimulated Akt phosphorylation but not PLCγ phosphorylation (Figure 4). EGFR levels were unchanged, consistent with the observation that kinase-inactive PI4KIIα does not inhibit EGFR endocytosis.[15] These data suggest that PI4KIIα activity is important for EGF-stimulated Akt activation, but likely acts via endosomal trafficking changes[16,33] rather than depletion of PI(4,5)P_2.[14]

PI4K-dependent cell survival in MDA-MB-231 cells. We repeated these experiments in the triple-negative breast cancer cell line MDA-MB-231. As in COS-7 cells, RNAi knockdown of PIKIIα or PI4KIIIβ was maximal at 72 h after transfection (Figure 5a). Relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both PI4K isoforms were expressed at higher levels in COS-7 cells, illustrating a cell type difference (Figure 5b).

We investigated whether MDA-MB-231 cell survival depended on PI4K levels, by assessing PARP cleavage in RNAi-treated cells. Unlike COS-7 cells, there was a low level of PARP cleavage evident in mock-transfected cells, which was augmented by knockdown of either PI4KIIα or PI4KIIIβ (Figure 6a). In addition, the onset of cleavage was earlier, and was evident by 48 h after transfection (Figure 6a). The lack of

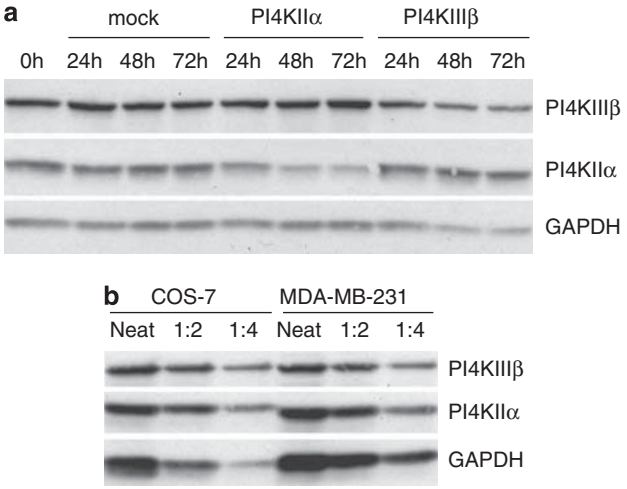

Figure 5 Knockdown of PI4KIIα or PI4KIIIβ in MDA-MB-231 cells. MDA-MB-231 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. (**a**) Time course of PI4K knockdown. Cells were analyzed by immunoblotting against PI4KIIα, PI4KIIIβ or GAPDH at 0, 24, 48 and 72 h after transfection. Representative blot from five experiments are shown. (**b**) Western blot of dilution series of non-transfected COS-7 or MDA-MB-231 cell lysates showing relative levels of PI4Ks compared with GAPDH. Blot shown is representative of two independent experiments. Note that differences in expression are most evident at 1 : 4 dilution

isoform selectivity in protecting MDA-MB-231 cells from apoptosis was confirmed by the results of TUNEL assays, which allows quantification of cell death by labeling of DNA strand breaks. Loss of either PI4K isoform induced large fivefold increases in cell death at 72 h after transfection (Figure 6b and c). In all, $5.6 \pm 0.7\%$ of mock-transfected cells were apoptotic, compared with $26.1 \pm 3.2\%$ for PI4KIIα knockdown and $22.7 \pm 2.9\%$ for PI4KIIIβ knockdown (Figure 6c).

Similar to the apoptosis data, proliferation assays showed that loss of either PI4K isoform led to decreased cell proliferation, with a 28.4% reduction for PI4KIIα and 26.1% for PI4KIIIβ ($P<0.05$ for either PI4K compared with mock at 72 h, Figure 6d). Taken together, the results indicate an antiapoptotic role for PI4KIIα in both cell types, whereas the antiapoptotic activity of PI4KIIIβ is cell type dependent.

Role of PI4K isoforms in MDA-MB-231 EGFR signaling. Unlike COS-7 cells, MDA-MB-231 cells do not proliferate in response to EGF[30] and their proliferation is insensitive to Akt inhibition.[7] With this in mind, we investigated whether there were any correlations between MDA-MB-231 cell survival, EGF signaling and PI4K-dependent phosphoinositide levels. As might be expected,[30] we observed high basal levels of EGFR phosphorylation in MDA-MB-231 cells, which were only slightly increased following agonist stimulation. However, EGF addition augmented Akt and PLCγ phosphorylation, indicating that receptor-activated phosphoinositide signaling was functional in MDA-MB-231 cells (Figure 7a). Knockdown of either PI4KIIα or PI4KIIIβ resulted in increased Akt activation in response to EGF (Figure 7a). EGF-stimulated PLCγ activation was not enhanced following loss of either PI4K isoform (Figure 7a). Similar to COS-7 cells, we found that knockdown of either PI4KIIα or PI4KIIIβ in MDA-MB-231 cells markedly reduced basal levels of $[^{32}P]$PI4P and $[^{32}P]$PI(4,5)P_2, which makes it very unlikely that EGF-stimulated Akt phosphorylation is directly dependent on PI4P synthesis by either isozyme. The recent finding that Akt activation in MDA-MB-231 cells induces apoptosis[31] leads us to conclude that loss of either PI4K in this cell line results in cell death through enhanced proapoptotic signaling.

Discussion

The aim of this study was to examine the roles of PI4KIIα and PI4KIIIβ in EGF-dependent phosphoinositide signaling and cell survival. As exemplified by the effects of PI4KIIIβ loss in COS-7 cells, large-scale and sustained depletion of cellular PI(4,5)P_2 does not necessarily lead to caspase-dependent apoptosis or inhibited Akt activation. This differs from a published study, indicating an antiapoptotic role for PI(4,5)P_2 through direct caspase-3 inhibition.[11] Additionally, we demonstrate that depletion of PI4P and PI(4,5)P_2 by either PI4K decreases EGF-stimulated phosphoinositide turnover without affecting PLC activation. This is consistent with previous work on EGF-stimulated calcium signaling, suggesting roles for type II and type III PI4Ks in PLC signaling[28] but differs from GPCR signaling where PI4KIIα loss has only minor effects on the PLCβ-mediated response.[23] Therefore, our results reveal that both PI4K isoforms can supply phosphoinositide substrate during receptor tyrosine kinase activation of PLCγ. Furthermore, our results in COS-7 cells do not indicate any correlation between reduced PLC signaling and inhibited cell proliferation.

On the other hand, we found a correlation between Akt phosphorylation and cell survival, both of which are under the control of PI4Ks but not through PI(4,5)P_2 synthesis. RNAi studies showed that when PI4K loss induced apoptosis, this was always accompanied by a change in Akt phosphorylation status known to correlate with decreased survival of that particular cell line. Specifically, decreased Akt phosphorylation in COS-7 cells in response to PI4KIIα loss resulted in caspase-mediated apoptosis, whereas in MDA-MB-231 cells, enhanced Akt phosphorylation induced by loss of either PI4KIIα or PI4KIIIβ led to substantial cell death. These opposing effects on Akt phosphorylation cannot be explained by depletion of PI(4,5)P_2 levels, which were reduced in both cell types by loss of either PI4K isoform.

We were able to further differentiate the effects of changing total cellular PI4P and PI(4,5)P_2 from effects on vesicular trafficking by overexpression of kinase-inactive PI4KIIα. This well-characterized approach induces defective endosomal trafficking without measurable changes to total cellular PI(4,5)P_2 levels.[14,16,33] Overexpression of kinase-inactive PI4KIIα in COS-7 cells resulted in reduced Akt activation in response to EGF. Although it is not yet technically possible to visualize the pool of PI4P synthesized by PI4KIIα, there is evidence for microdomains of PI4KIIα activity on intracellular membranes.[34–36] These are not recognized by available PI4P-specific PH domain reporters or current anti-PI4P antibody-staining protocols,[37] but are nevertheless important

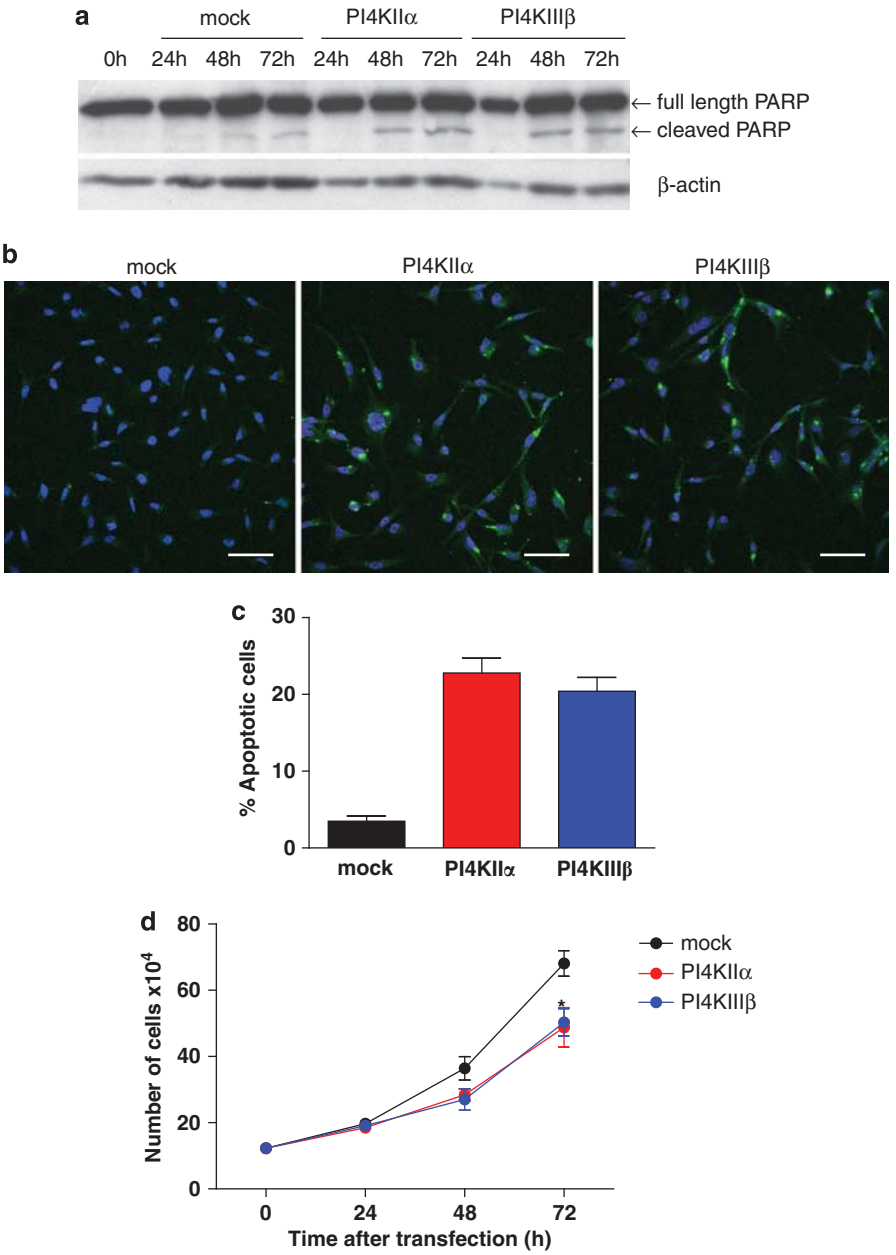

Figure 6 Loss of PI4KIIα or PI4KIIIβ induces apoptosis and reduces proliferation in MDA-MB-231 cells. MDA-MB-231 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. (**a**) Effects of PI4KIIα or PI4KIIIβ depletion on PARP cleavage as analyzed by immunoblotting. Representative blot from two independent experiments is shown. (**b** and **c**) Analysis of RNAi-treated cells for apoptosis using the TUNEL assay. Representative confocal images and apoptotic cell counts from two independent experiments. Scale bar = 50 μm. (**d**) Effects of PI4KIIα or PI4KIIIβ depletion on cell proliferation. Live cell counts were determined at 0, 24, 48 and 72 h after transfection. Data presented are means ± S.E.M. of four independent experiments. *$P<0.05$ for PI4KIIα and PI4KIIIβ knockdown

in the maintenance of PI4K-dependent vesicular trafficking.[15,16] This leads us to infer that cell-specific antiapoptotic vesicular trafficking pathways rather than gross changes to phosphoinositide metabolism underlie PI4K-dependent cell survival. In concordance with this idea, there are now several examples of sustained prosurvival signaling that are dependent on endosomal signaling outputs (reviewed in Murphy *et al.*[38]).

We show here that PI4KIIα inhibition may be an effective way of generally inhibiting cell survival. This differs from the scenario with PI3Ks where all p110 subunits can contribute toward cell survival,[39,40] and where class 1A PI3K and PTEN status are also important.[7] Although the antiapoptotic function of PI4KIIIβ appears to be more cell type-dependent, the results presented here are significant in that MDA-MB-231 are a triple-negative breast cancer cell line that expresses neither hormone receptors nor HER2 and are normal for class 1A PI3K and PTEN.[7] Interestingly, enhanced Akt phosphorylation in MDA-MB-231 cells is associated with reduced cell proliferation.[31] This subclass of breast cancers is not targeted by hormonal therapies or anti-HER2 Herceptin antibody, and their proliferation is insensitive to Akt inhibition.[7] Hence,

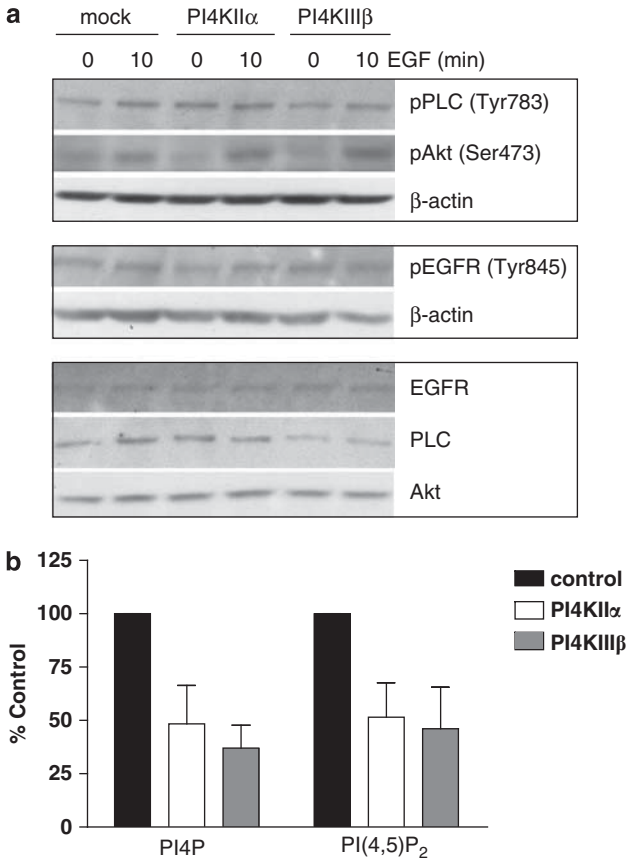

Figure 7 Effects of PI4KIIα or PI4KIIIβ knockdown in MDA-MB-231 cells on EGF signaling. MDA-MB-231 cells were mock transfected or transfected with PI4KIIα or PI4KIIIβ siRNA. (**a**) PI4KIIα or PI4KIIIβ knockdown enhances Akt phosphorylation without affecting EGFR phosphorylation, PLCγ phosphorylation or EGFR levels. After 72 h of transfection, the cells were stimulated with 100 ng/ml EGF or vehicle control for 10 min and analyzed by immunoblotting. Representative blots from at least two independent experiments are shown. (**b**) Effects of PI4K siRNA on [^{32}P]-labeled PI4P and [^{32}P]-labeled PI(4,5)P_2. After 72 h of siRNA transfection, cells were labeled with [^{32}P]phosphate and harvested. [^{32}P]-labeled lipids were analyzed by TLC. Data presented are from two independent experiments

targeted inhibition of PI4KIIIβ represents a potential chemotherapeutic strategy to treat such malignancies. Therefore, PI4Ks indirectly modulate Akt signaling in a manner consistent with cell survival, but the isoform dependency needs to be considered on a cell- or tissue-specific basis.

Materials and Methods

Materials. Mouse EGF was purchased from Sigma-Aldrich (Dorset, UK). Anti-PI4KIIα monoclonal antibody 1C4 was described previously.[27] Anti-PI4KIIIβ was obtained from BD Biosciences (Oxford, UK). Anti-EGFR was from Cell Signaling Technologies (Hertfordshire, UK) and from BD Biosciences. The following primary antibodies were from Cell Signaling Technologies: anti-pEGFR(Tyr1173), anti-pEGFR(Tyr845), anti-PLCγ1, anti-pPLCγ1(Ser1248), anti-pPLCγ1(Tyr783), anti-Akt, anti-pAkt(Ser473), anti-caspase-3, anti-PARP, anti-β-actin, anti-lamin and anti-GAPDH. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were from Cell Signaling Technologies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from PAA Laboratories (Somerset, UK). [^{32}P]phosphate was from Perkin Elmer (Cambridge, UK). Other reagents were from Sigma-Aldrich. Lipofectamine was from Invitrogen (Paisley, UK).

Cell culture. Cells were maintained at 37°C in a humidified incubator at 5% CO_2. Cells were cultured in DMEM containing stable glutamine, 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. For experiments involving overexpression of eGFP-PI4KIIα or kinase-inactive eGFP-(K152A)PI4KIIα, cells were transfected using lipofectamine 24 h before EGF addition, according to the manufacturer's instructions.

RNAi. Three siRNA duplexes targeting human PI4KIIα were used in combination (oligos 1 and 2 are described in Wang *et al.*,[14] and oligo 3 was used in Minogue *et al.*[15]). The siRNA duplex targeting human PI4KIIIβ was described in Balla *et al.*[32] Oligos were synthesized by Eurofins MWG Operon (London, UK). Cells were seeded in penicillin/streptomycin-free medium the day before transfection, onto 12-well plates for EGF stimulation experiments (4.0×10^4 cells, 1 ml medium per well) or onto 6-well plates (9.9×10^4 cells, 2.5 ml medium per well) for proliferation assays. Transfections were performed using Oligofectamine (Invitrogen) according to the manufacturer's instructions. In all, 3 μl Oligofectamine and 120 pmol total siRNA were added per well of a 12-well plate. Equivalent amounts were used for cells on 6-well plates. After 24 h, cells were transfected a second time in the same manner, and were analyzed up to 72 h after the first transfection.

Stimulation with EGF. After 72 h of transfection, cells were serum starved in DMEM with 25 mM HEPES (pH 7.4) for at least 3 h before stimulation with 100 ng/ml EGF in the same medium, in a 37°C water bath. Stimulations were stopped by adding the same volume of hot 2X SDS-PAGE sample buffer onto the cells. The samples were scraped, harvested, heated to 70°C for 5 min and sonicated.

Immunoblotting. Samples were resolved by SDS-PAGE, transferred onto PVDF membrane, which was blocked with 5% Marvel or 5% bovine serum albumin, probed with primary antibodies in blocking buffer. After washing and incubation with HRP-conjugated secondary antibody, protein bands were visualized by chemiluminescence (ECL from GE Healthcare, Buckinghamshire, UK, or Immobilon Western Chemiluminescent HRP Substrate from Millipore, Hertfordshire, UK) and exposed to X-ray film. For quantification of western blots, films were scanned with a Bio-Rad GS-800 Calibrated Densitometer into image software PD Quest 7.3.0 (Bio-Rad, Hertfordshire, UK) at the highest resolution.

Phospholipid analysis. Cell monolayers growing in 12-well plates were transfected with siRNA. After 72 h, they were labeled with [^{32}P]phosphate (0.7–1 MBq/well) for 2 h at 37°C in phosphate-free and serum-free DMEM containing 25 mM HEPES (pH 7.4). Cells were stimulated by the addition of EGF and reactions were stopped by the addition of an equal volume of ice-cold 1 M HCl. Following two washes with 1 M HCl, the cell monolayers were scraped and transferred to an eppendorf tube. Phosphoinositides were extracted and separated by thin layer chromatography (TLC) as previously described.[34] TLC plates were imaged and radioactive spots quantified using a Typhoon phosphorimager (GE Healthcare) within the linear range of the instrument. To correct for RNAi-induced differences in cell number, a matched non-radiolabeled well from each RNAi condition was harvested in SDS-PAGE sample buffer. As a measure for protein content, the samples were analyzed by western blotting and densitometry for GAPDH. This permitted the normalization of [^{32}P]phospholipid content against protein content.

Proliferation assay. Cells treated with siRNA were stained with trypan blue and viable cells were counted in a hemocytometer at 0, 24, 48 and 72 h after the first transfection. Separate wells were lysed in 2X SDS-PAGE sample buffer, scraped and harvested for immunoblotting.

TUNEL assay. MDA-MB-231 cells grown on glass coverslips were treated with siRNA. After 72 h, the cells were fixed with 4% (v/v) formaldehyde for 10 min on ice. They were then permeabilized and TUNEL labeled for DNA strand breaks using the *in situ* Cell Death Detection Kit (Roche, West Sussex, UK) according to the manufacturer's protocol. After counterstaining with Hoechst 33342 (Invitrogen), the coverslips were mounted in ProLong Gold anti-fade reagent (Invitrogen). Cells were imaged using a Zeiss LSM 510 Meta laser-scanning confocal microscope system (Carl Zeiss Ltd, Hertfordshire, UK) under a $\times$20 objective as described previously.[15] Fields of cells were picked at random and gain settings were identical for each condition. A total of 1100–1400 cells were counted from 10–11 fields for each condition.

Data analysis. [^{32}P]phospholipid data were analyzed by two-tailed Student's *t*-test and live cell counts from proliferation assays by one-way ANOVA with Dunnett's post test, using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. We acknowledge support from the Cancer Research UK grant C25540/A8562 (MGW, KMEC and JJH) and BBSRC grant BB/G021163/1 (SM and MGW). We also acknowledge the Royal Free NHS trust support of the Advanced Microscopy Unit used in these studies.

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