

A novel regulatory mechanism links PLC γ 1 to PDK1

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Summary

3-Phosphoinositide dependent protein kinase-1 (PDK1) and phospholipase C (PLC) γ 1 are two key enzymes in signal transduction that control several intracellular processes. Despite the fact that PLC γ 1 has been investigated for several years, the mechanisms of activation of this enzyme are still not completely clear. Similarly, although PDK1 has been mostly investigated for its role in activation of Akt, a crucial enzyme in regulation of several cellular processes, it has become recently evident that the role of PDK1 in physiological and pathological conditions is not limited to Akt activation.

Here we demonstrate that PDK1 regulates PLC γ 1 activation in a mechanism involving association of the two enzymes and modulation of PLC γ 1 tyrosine phosphorylation. We further show that this novel PDK1-PLC γ 1 pathway is important for cancer cell invasion. The identification of a PDK1-PLC γ 1 pathway reveals the existence of a previously undetected link between two of the most important enzymes in signal transduction. This is likely to have profound consequences in our understanding of several cellular functions dependent on phosphoinositides and controlled by PDK1 and PLC γ 1.

Keywords: Cell invasion/ cell signalling/ 3-Phosphoinositide dependent protein kinase-1/ phosphoinositide 3-kinase/ phospholipase C γ 1

Introduction

Phosphoinositides (PIs) play a pivotal role in signal transduction and they are extensively studied both as precursors and as second messengers themselves. Members of the family of the phosphoinositide specific phospholipases C (PLC) converts the phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (Rhee, 2001). PI-PLCs have been involved in regulation of several intracellular functions through PtdIns(4,5) P_2 hydrolysis. In particular, PLC γ 1 has a well established role in mediating cellular signalling upon activation of several receptor tyrosine kinases (RTK), including epidermal growth factor (EGF) receptor, platelet-derived growth factor receptor and many more (Bunney and Katan, 2011). Many cellular functions regulated by PLC γ 1 activation have been discovered, including a central role in actin cytoskeleton remodelling and cell migration (Kolsch et al., 2008). Importantly, several studies have now demonstrated the key role of PLC γ 1 in metastasis (Wells and Grandis, 2003; Sala et al., 2008), revealing the involvement of this enzyme in cancer and suggesting that PLC γ 1 may represent an important therapeutic target for development of anti-metastatic drugs. Despite the fact that PLC γ 1 is one of the central enzymes in regulation of signalling cascades downstream of growth factors stimulation and that it has been investigated for several years, the mechanisms of activation of this enzyme are still not completely clear (Maffucci and Falasca, 2007). Although it is well established that tyrosine phosphorylation upon recruitment of the enzyme to RTKs is critical for PLC γ 1 activation, this event is not sufficient to induce full activation of the enzyme (Sekiya et al., 2004). Additional mechanisms of activation exist and they can be necessary for full activation of PLC γ upon RTK stimulation or complementary (Maffucci and Falasca, 2007). For instance PI3K product phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3] induces strong activation of PLC γ 1 in vitro and constitutively active PI3K induces PLC γ 1 activation in vivo (Bae et al., 1998; Falasca et al., 1998). This alternative and/or complementary mechanism can become particularly relevant in the context of specific PLC γ 1-mediated cellular functions. In particular we demonstrated that a PI3K-dependent PLC γ 1 activation is involved in regulation of cell migration upon EGF stimulation in breast cancer cells (Piccolo et al., 2002) and upon fibroblast growth factor 2 stimulation in human umbilical vein endothelial cells (Maffucci et al., 2009). The mechanism underlying the PI3K-

dependent regulation of PLC γ is not fully elucidated. Binding of PLC γ 1 pleckstrin homology (PH) domain to PtdIns(3,4,5) P_3 is involved in PLC γ 1 activation (Falasca et al., 1998) but it is not known whether other PI3K effectors can mediate the PI3K-dependent PLC γ 1 activation. In this respect it is worth mentioning that a dual PI3K-dependent regulation of PLC γ 2 isoform has been proposed, mediated by the interaction between the PH domains of Btk and PLC γ 2 and PtdIns(3,4,5) P_3 (Clayton et al., 2002; Kim et al., 2004). This leads to the possibility that PI3K may also control PLC γ 1 activation through an intermediate, PI3K-dependent effector.

3-Phosphoinositide dependent protein kinase-1 (PDK1) is a major regulator of PI3K-dependent signalling and it regulates activation of several AGC kinases, including protein kinase B/Akt (Bayascas, 2010; Pearce et al., 2010). Although PDK1 has been previously mostly studied because of its role in regulation of Akt and Akt-dependent cellular functions, including cell proliferation and survival, a huge interest has recently risen on investigating more extensively the wider contribution of PDK1 to signal transduction and cellular processes. This interest has been particularly fuelled by the identification of PDK1-dependent, Akt-independent signalling pathways that can be activated downstream of oncogenic PI3K (Vasudevan et al., 2009), indicating that PDK1-deregulated signalling in disease may have more profound consequences than simply affecting Akt-dependent processes. These data have highlighted the relevance of PDK1 as a potential therapeutic target in cancer and the potential advantages of specific PDK1 inhibitors as more efficient anti cancer drugs compared to drugs targeting only Akt (Raimondi and Falasca, 2011).

PDK1 and PLC γ 1 are two central enzymes in signal transduction, regulating several intracellular functions. Despite the general assumption that these two enzymes control parallel and distinct signalling pathways, it has been reported that downregulation of either PDK1 (Liu et al., 2009) or PLC γ 1 (Sala et al., 2008) inhibits migration and experimental metastasis of breast cancer cells. This observation led us to the hypothesis that the two enzymes could either co-operate or act in the same signalling cascade to control these processes.

Here we demonstrate that PDK1 regulates EGF-induced PLC γ 1 activation, identifying for the first time PLC γ 1 as a downstream target of PDK1 action. The mechanism of PDK1-dependent PLC γ 1 activation involves association of the two enzymes, specifically at the level of cell protrusions, and modulation of PLC γ 1 tyrosine phosphorylation. We further show that this novel PDK1-PLC γ 1 pathway is important for cancer cell invasion,

suggesting that blockade of this interaction may have a therapeutic potential to counteract metastasis dissemination. The identification of a PDK1-PLC γ 1 pathway reveals the existence of a previously undetected link between two of the most important enzymes in signal transduction and it is likely to have profound consequences in our understanding of several PIs-dependent cellular processes.

Results

PDK1 Regulates PLC γ 1 Activity

PDK1 and PLC γ 1 have both been reported to be involved in regulation of migration and experimental metastasis of breast cancer cells (Sala et al., 2008; Liu et al., 2009). Whether these two enzymes are involved in the same signalling cascade is not known. This possibility is supported by evidence indicating a role for PI3K or PI3K-dependent signalling in PLC γ 1 activation (Bae et al., 1998; Falasca et al., 1998; Maffucci and Falasca, 2007) and in regulation of PLC γ 1-dependent cell migration (Piccolo et al., 2002; Maffucci et al., 2009).

We therefore decided to investigate whether PDK1 and PLC γ 1 could be activated in the same intracellular signalling cascade. Using a tetracycline-inducible system of PLC γ 1 downregulation in breast cancer cells MDA-MB-231 we first observed that downregulation of PLC γ 1 did not affect the EGF-induced Akt phosphorylation at Thr308, an event dependent on PDK1 activation (supplementary material Fig. S1). These data indicated that PLC γ 1 does not regulate PDK1 activation.

We then tested the hypothesis that PDK1 could be involved in mediating the PI3K-dependent PLC γ 1 activation. To investigate this hypothesis we analysed the increase in intracellular calcium and accumulation of inositol phosphates induced by treatment of MDA-MB-231 cells with EGF. Both these processes are dependent on PLC γ 1 activation in this cell line since they are strongly inhibited upon tetracycline-induced downregulation of this enzyme (supplementary material Fig. S2A,B) and therefore these assays allowed us to monitor the specific activation of PLC γ 1 over other PLC isoforms.

When intracellular calcium was monitored in MDA-MB-231 transiently transfected with a control, scrambled siRNA we observed a clear increase of intracellular calcium upon EGF stimulation (Fig. 1A, left panel). In contrast downregulation of PDK1 completely inhibited the EGF-induced increase in intracellular calcium in these cells (Fig. 1A, right panel). Cells lacking PDK1 were as responsive as control cells to ATP treatment, confirming the specific inhibitory effect towards EGF-induced calcium increase (Fig. 1A). As a complementary assay to investigate PLC γ 1 activation we analysed the EGF-induced accumulation of total inositol phosphates in stable MDA-MB-231 cells expressing a control shRNA (5MUT) and in two distinct clones expressing a shRNA specifically targeting PDK1 (Fig. 1B). PDK1 knock down strongly inhibited the EGF-induced accumulation of inositol phosphates, further indicating that PDK1 regulates PLC γ 1

activation. Furthermore chemical inhibition of PDK1 using the specific inhibitor GSK2334470 (Najafov et al., 2010) also resulted in total abrogation of the EGF-induced intracellular calcium increase and inositol phosphates accumulation in MDA-MB-231 cells (Fig. 1C). These data demonstrate that PDK1 regulates PLC γ 1 activation upon EGF stimulation in breast cancer cells.

We next investigated whether PDK1 had a role in PLC activation in other, distinct cell lines. Both transient PDK1 downregulation (Fig. 2A) and chemical PDK1 inhibition (Fig. 2B) strongly inhibited the EGF-induced but not the ATP-mediated calcium increase in prostate cancer cells PC3. Similar results were obtained in melanoma cell line A375M upon transient PDK1 downregulation (Fig. 2C). Downregulation of PLC γ 1 also inhibited the EGF-mediated calcium increase in PC3 (supplementary material Fig. S2C) and A375M cells (supplementary material Fig. S2D), supporting the conclusion that PDK1 specifically regulates PLC γ 1 activity in different cancer cell lines. Finally, a clear inhibition of EGF-induced calcium increase was detected in PDK1-deficient embryonic stem cells (ES) compared to control cells (Fig. 2D).

Taken together these data demonstrate that PDK1 regulates PLC γ 1 activity in several distinct cell lines.

PDK1 Regulates The EGF-induced PLC γ 1 Tyrosine Phosphorylation

Since one of the key events for PLC γ 1 activation is phosphorylation of its residue Tyr783 (Rhee, 2001), we investigated whether PDK1 was involved in this process. Stable cell populations expressing the same PDK1-specific shRNA as clones shown in Fig. 1B or the control 5MUT were generated by retroviral infection and selected as described in the Material and Methods section. Cells were then serum starved overnight and stimulated with EGF for different times. Western blotting analysis indicated that EGF stimulation induced Tyr783 phosphorylation of PLC γ 1 in control (5MUT) MDA-MB-231 (Fig. 3A) and A375M (Fig. 3B) cells. Downregulation of PDK1 in these cells completely abrogated the EGF-induced PLC γ 1 tyrosine phosphorylation. As expected, inhibition of the PDK1-dependent Akt phosphorylation at its residue Thr308 was also detected in cells lacking PDK1 upon EGF stimulation (Fig. 3A,B). Inhibition of PLC γ 1 Tyr783 phosphorylation was also detected in EGF-stimulated MDA-MB-231 treated with the PDK1 inhibitor GSK2334470 (Fig. 3C). These data indicate that PDK1 modulates PLC γ 1 activation by regulating phosphorylation of the key residue Tyr783. Interestingly, treatment with the PDK1 specific inhibitor only partially inhibited whereas downregulation of PDK1

completely blocked PLC γ 1 Tyr783 phosphorylation. This was not due to partial PDK1 inhibition by GSK2334470 since the inhibitor completely abrogated Akt Thr308 phosphorylation upon EGF stimulation (Fig. 3C). This observation suggested an additional role for PDK1 in regulation of PLC γ 1 Tyr783 phosphorylation that is independent from its kinase activity.

PDK1 Associates With PLC γ 1

To gain further insight into the mechanisms of PDK1-dependent PLC γ 1 activation we first analysed the intracellular localisation of overexpressed PDK1 and PLC γ 1 in unstimulated and EGF-stimulated MDA-MB-231 cells. Confocal microscopy analysis indicated that EGF induced a strong co-localisation of the two proteins at the plasma membrane, in particular at the level of cell protrusions (Fig. 4A). We therefore decided to investigate whether the two enzymes could associate in a protein complex. Lysates containing a GST-tagged PDK1 or GST alone (expressed in HEK293 cells) were incubated with lysates from HEK293 overexpressing PLC γ 1. GST or GST-PDK1 were then pulled down by glutathione beads. Western blot analysis of the proteins associated with the beads revealed that PLC γ 1 was specifically pulled down by GST-PDK1 but not GST alone (Fig. 4B), indicating that the two enzymes can associate in a protein complex. In parallel experiments we analysed PLC γ 1 association with distinct GST-tagged PDK1 constructs. First we observed that a GST-tagged isolated PDK1 PH domain was not able to bind PLC γ 1 and that a GST-tagged Δ PH PDK1 associated with PLC γ 1 (Fig. 4B) indicating that PLC γ 1 does not bind to PDK1 PH domain *in vitro*. This however does not exclude the possibility that the PH domain of PDK1 contributes to the interaction of the two proteins *in vivo*, likely by targeting PDK1 to the plasma membrane. On the other hand, neither a kinase dead PDK1 nor PIF pocket inactive PDK1 mutant (L155E) were able to associate with PLC γ 1 (Fig. 4B), suggesting that alteration of PDK1 catalytic domain or possibly of PDK1 conformation impairs PDK1/PLC γ 1 interaction.

To further investigate the hypothesis that PDK1 and PLC γ 1 can associate in a protein complex and to investigate whether the association is modulated by EGF stimulation, co-immunoprecipitation experiments were performed using lysates from serum starved, unstimulated or EGF-stimulated MDA-MB-231 using an anti-PLC γ 1 antibody. Association of endogenous PDK1 and PLC γ 1 upon different times of EGF stimulation was analysed in the immunocomplexes by Western blotting. Data showed that endogenous PDK1

associated with endogenous PLC γ 1 specifically upon EGF stimulation (Fig. 4C), with a peak at 3 minutes of stimulation (Fig. 4D).

Taken together these data indicate that PDK1 and PLC γ 1 can form a complex, in particular upon EGF stimulation, and provide some indication of the mechanisms by which PDK1 through its association with PLC γ 1 can regulate the activity of the lipase.

FRET Analysis of PDK1 And PLC γ 1 Interaction

To further investigate the dynamic of PDK1 and PLC γ 1 interaction, steady state Förster Resonance Energy Transfer (FRET) analysis was performed in MDA-MB-231 by FACS analysis. Cells were co-transfected with plasmids to simultaneously overexpress PLC γ 1 and PDK1. As controls, MDA-MB-231 cells were co-transfected with GFP-vector and either the PLC γ 1- or the PDK1-expressing vector alone. The methodology used to detect steady state FRET using FACS analysis relies on increased fluorescence of the acceptor following donor excitation, without direct excitation of the acceptor itself (details of the experimental procedures and controls are discussed in the Materials and Methods section). As shown in Fig. 5A, unstimulated cells showed the same emission profile as the single positive sample. A shift in the acceptor emission profile together with increased acceptor fluorescence was detected upon EGF stimulation (Fig. 5A). Analysis of the acceptor emission profiles showed that stimulation with EGF induced 30% increase of acceptor fluorescence due to FRET occurring between PLC γ 1 and PDK1 (Fig. 5B). No increase in fluorescence was detected in cells expressing either PLC γ 1 or PDK1 together with a GFP-containing vector (Fig. 5B). These data indicate that EGF stimulation induces association of PLC γ 1 and PDK1, consistent with data obtained with co-immunoprecipitation studies.

To better investigate the mechanisms of PLC γ 1 and PDK1 association and to determine the specific intracellular localisation of the PLC γ 1/PDK1-containing protein complex, we performed FRET experiments using confocal microscopy analysis. Cells were starved overnight and then left untreated or stimulated with EGF for different times before being fixed. Increase in fluorescence in the donor channel was detected after acceptor's photobleaching. Donor fluorescence was measured at the level of membrane protrusions (induced by EGF stimulation) and in more cytoplasmic regions. Images presented in Fig. 5C and supplementary material Fig. S3A show the fluorescence intensity of the donor before and after photobleaching for each time point in chosen regions. The merged channel shows where the acceptor was photobleached. Arrows indicate representative regions

where donor fluorescence change was measured and value collected for statistical analysis. Charts (Figs. 5D, supplementary material S3B) show the donor fluorescence (green line) before and after photobleaching of the acceptor (red line) of the corresponding regions. FRET occurred after 1 minute and 3 minutes of EGF stimulation where drop of fluorescence of the acceptor following photobleaching and increased fluorescence of the donor were detected. Specifically, photobleaching of the acceptor caused a significant increase of fluorescence in the donor channel after 3 minutes of stimulation with EGF, indicative of FRET (Fig. 5E), consistent with results obtained in co-immunoprecipitation experiments of endogenous PLC γ 1 and PDK1 (Fig. 4C,D). No change in donor fluorescence was detected in internal cytoplasmic region after acceptor photobleaching (Fig. 5E). No FRET was detected in cells overexpressing either PLC γ 1 or PDK1 alone and co-stained with both Alexa488 and Alexa555 (Fig. 5E) or stained with the corresponding secondary Alexa488 antibody (supplementary material Fig. S3C). Similarly no FRET was observed in cells overexpressing both PLC γ 1 and PDK1 and stained with Alexa488 alone (supplementary material Fig. S3C). These data support the previous conclusion that PLC γ 1 and PDK1 associate upon EGF stimulation and further indicate that the association occurs in well-defined regions corresponding to cellular protrusions induced by EGF.

The PDK1-dependent PLC γ 1 Activation Regulates Cell Invasion

We next investigated the functional relevance of this newly-identified PDK1-PLC γ 1 signalling cascade. As mentioned before, both PLC γ 1 and PDK1 have been implicated in migration and experimental metastasis of breast cancer cells, therefore we first used this cellular model to investigate a potential role for PDK1 in this process. We observed that stable downregulation of PDK1 reduced invasion of MDA-MB-231 on Matrigel (Fig. 6A). Similar results were obtained upon downregulation of PDK1 in melanoma cells A375M (Fig. 6B) and when invasion was monitored in both cell lines using organotypic cultures as an alternative and complementary assay (Fig. 6C,D). These data demonstrate that PDK1 has a role in invasion in distinct cellular models, consistent with data in literature (Pinner and Sahai, 2008; Liu et al., 2009). Stable PDK1 knock down did not affect MDA-MB-231 cell proliferation (supplementary material Fig. S4A), consistent with previous data obtained in the same cell line upon PDK1 downregulation (Nagashima et al., 2011) whereas it inhibited proliferation of A375M (supplementary material Fig. S4B). Similar results were obtained in MDA-MB-231 and A375M upon treatment with GSK2334470

(supplementary material Fig. S4C,D). No difference in ERK phosphorylation was detected between stable knock down and control cells (supplementary material Fig.S4E).

To confirm the specific inhibitory effect of PDK1 silencing, cell invasion was monitored in cells upon transient downregulation of PDK1 using specific siRNA. Consistent with data obtained with stable knock down, transient silencing of PDK1 inhibited invasion of MDA-MB-231 (Fig. 6C) and A375M (Fig. 6D). Transient downregulation of PLC γ 1 also reduced invasion of A375M (Fig. 6D). Taken together these data indicate that PDK1 and PLC γ 1 are both required to promote invasion of MDA-MB-231 and A375M cells.

To determine whether the two enzymes act in the same cellular signalling pathway, we tested the effect of the PDK1 inhibitor GSK2334470 on stable cell lines expressing a shRNA targeting PLC γ 1 or a mutated (3MUT) shRNA unable to target PLC γ 1. Treatment of control “3MUT” cells with GSK2334470 significantly inhibited invasion of MDA-MB-231 and A375M (Fig. 7A,B), consistent with results obtained using stable and transient PDK1 downregulation. Stable downregulation of PLC γ 1 inhibited cell invasion in both cellular models (Fig. 7A,B). Importantly, treatment of cells lacking PLC γ 1 with GSK2334470 did not further reduce cell invasion (Fig. 7A,B) indicating that inhibition of PDK1 in cells lacking PLC γ 1 has no additive inhibitory effect on cell invasion. As a complementary approach, we then investigated the effect of transient downregulation of PLC γ 1 on invasion of stable PDK1 knock down (shPDK1) and control (5MUT) cells. MDA-MB-231 and A375M “5MUT” and “shPDK1” cells were transiently transfected with either the specific siRNA targeting PLC γ 1 or with the non-targeting, scrambled siRNA. A clear inhibition of cell invasion was detected in “5MUT” MDA-MB-231 and “5MUT” A375M cells transfected with the PLC γ 1-targeting siRNA compared to “5MUT” cells transfected with the scrambled siRNA (Fig. 7C,D). Invasion was similarly inhibited in shPDK1 MDA-MB-231 and shPDK1 A375M cells transfected with the non-targeting scrambled siRNA compared to the corresponding 5MUT/scrambled siRNA cells (Fig. 7C,D). Importantly, no further reduction in cell invasion was detected in shPDK1 cells transfected with PLC γ 1 siRNA compared to shPDK1 cells transfected with the scrambled siRNA (Fig. 7C,D) indicating that downregulation of PLC γ 1 in cells lacking PDK1 has no additive inhibitory effect on cell invasion.

These data suggest that PDK1 and PLC γ 1 act on the same signalling cascade and that the PDK1/PLC γ 1 pathway is required for regulation of cell invasion.

Discussion

This study demonstrates for the first time that PDK1 can regulate PLC γ 1 activation in a mechanism involving assembly of a complex between the two enzymes upon growth factor stimulation. These results shed a brand new and unexpected light on two of the main enzymes involved in signal transduction.

Since its identification as the kinase responsible for phosphorylation and activation of Akt, the role of PDK1 in signalling and regulation of cellular processes has been almost exclusively focused on its involvement in Akt activation. With the accumulation of data demonstrating the key role of Akt in several cellular functions and in cancer development and progression, for several years PDK1 has been completely shadowed by the huge interest on Akt. Less attention has been focussed on the role of PDK1 in activation of several other enzymes which also control many important cellular functions. More recently a renewed attention has been paid to PDK1 and it has become increasingly evident that the importance of PDK1 in physiological conditions goes far beyond Akt activation. Moreover, it has become clear that the role of PDK1 in cancer is not limited to Akt activation which has also fuelled a novel interest in developing specific PDK1 inhibitors as potential anti-cancer drugs (Raimondi and Falasca, 2011).

Results presented in this manuscript fit into this renewed interest towards PDK1 and add very important and brand new information, with relevance from both a physiological and therapeutic point of view. We demonstrate that inhibition of PDK1 by siRNA- or shRNA-mediated downregulation of the enzyme or by using a specific PDK1 inhibitor blocks PLC γ 1 activation in distinct cell lines. In this respect our data identify a novel and unexpected target for PDK1. While enzymes activated by PDK1 mostly belong to the AGC kinases family our data reveal for the first time that PDK1 can also regulate activation of the lipase PLC γ 1, a complete distinct enzyme from AGC kinases. Although chemical inhibition of PDK1 affects PLC γ 1 tyrosine phosphorylation and activation, the mechanism of PDK1-dependent PLC γ 1 activation appears to mainly involve assembly of a protein complex between the two enzymes which is regulated by growth factor stimulation and occurs in particular at the level of cellular protrusions. Whether other enzymes can be controlled by PDK1 in a similar manner is an interesting question that now needs to be answered.

Our results also add new information towards our understanding of the mechanism of PLC γ 1 activation. PLC γ 1 has been extensively investigated over the years because of its

role in mediating activation of key signalling pathways downstream of several growth factor receptors stimulation. It has been long known that tyrosine phosphorylation of the enzyme is critical but not sufficient for the full activation of PLC γ 1 (Sekiya et al., 2004; Maffucci and Falasca, 2007). Among the alternative and/or complementary mechanisms of activation, PI3K has been shown to be important for PLC γ 1 activation (Bae et al., 1998; Falasca et al., 1998) although the precise contribution of PI3K to this event has been elusive for long time. Our data here reveal that PDK1 can represent the missing link between PI3K and PLC γ 1 in a mechanism likely involving the PI3K-dependent translocation of both PDK1 and PLC γ 1 to the plasma membrane and assembly of the PDK1/PLC γ 1 complex (Fig. 8). This mechanism would guarantee a double control of PLC γ 1 by PI3K, through the PH domain/PtdIns(3,4,5) P_3 interaction and the PDK1/PLC γ 1 assembly. The consequences of this discovery are multiple since PLC γ 1 can control many cellular functions therefore it will be important to understand how many of the PLC γ 1-dependent cellular functions are controlled through a PDK1-dependent mechanism.

We demonstrate here that this novel PDK1/PLC γ 1 pathway is specifically involved in regulation of cell invasion, in particular of breast cancer and melanoma cells. A role for PDK1 in control of cancer cell migration was previously demonstrated by data indicating that co-overexpression of PDK1 and of the active mutant rat homolog of ERBB2 in MCF10A increased cell motility (Maurer et al., 2009) and that downregulation or inhibition of PDK1 inhibited Matrigel cell invasion in breast cancer cell lines, prostate cancer and melanoma (Pinner and Sahai, 2008). The precise mechanisms by which PDK1 regulates this process are not fully understood. It has been reported that PDK1 can control cancer cell motility by regulating cortical acto-myosin contraction in a mechanism involving activation of ROCK1 (Pinner and Sahai, 2008). Recently it has been shown that PDK1 regulates invadopodia formation in breast cancer cells (Yamaguchi et al., 2011). Interestingly, although a role for Akt in this process has also been reported in this study, downregulation of PDK1 reduced the number of cells presenting invadopodia much strongly than downregulation of Akt (Yamaguchi et al., 2011). Our results add important information towards a full understanding of the contribution of PDK1 to cell invasion by identifying PLC γ 1 as a novel downstream effector that PDK1 can activate to control this cellular process. Whether the PDK1/PLC γ 1 pathway regulates invadopodia formation or ROCK1 activation or distinct processes involved in cell invasion remains to be investigated.

More importantly accumulating evidence now suggests that PLC γ 1 plays a key role in metastasis development (Wells and Grandis, 2003; Sala et al., 2008) and progression (Sala et al., 2008). Reduced primary tumour spreading and invasion was detected in mice injected with DU145 prostate cancer cells expressing a dominant negative mutant of PLC γ 1. Furthermore, the expression of this mutant specifically in the breast and prostate decreased the invasiveness of primary tumours and reduced formation of distant lung metastasis without affecting the size of the primary tumour size (Shepard et al., 2007). Consistent with these data, we demonstrated that downregulation of PLC γ 1 inhibited the development of breast cancer MDA-MB-231 cells-derived lung metastases without affecting the primary tumour (Sala et al., 2008). The identification of PDK1 as a key upstream regulator of PLC γ 1 strongly suggests that PDK1 has also an important role in metastasis development and progression through regulation of PLC γ 1 activation.

In this respect it is worth mentioning that increased copy number of PDK1 is often present in human breast cancer compared to normal breast epithelia and correlates with the presence of activating mutation of class I PI3K p110 α or with ERBB2 overexpression (Maurer et al., 2009). Similarly, increased PLC γ 1 expression was reported in breast carcinoma samples compared to healthy tissue (Arteaga et al., 1991) and we recently reported that the enzyme accumulates in metastasis compared to the primary tumour (Sala et al., 2008). Importantly, we demonstrated that downregulation of PLC γ 1 after metastasis had developed was able not only to block metastasis spreading but also to revert it (Sala et al., 2008). These data, together with evidence indicating that pharmacological inhibition of PLC γ 1 is able to inhibit in vitro Matrigel invasion of breast and prostate cancer cells (Kassis et al., 1999) strongly suggest that inhibition of the PDK1/PLC γ 1 pathway may represent a novel strategy to counteract metastasis progression. In this respect, breast and melanoma cancer cells downregulated for PLC γ 1 show no additive inhibition of cell invasion in vitro when treated with the PDK1-specific inhibitor GSK2334470. These data suggest PLC γ 1 as one of the main downstream target of PDK1 to promote cancer cell invasion. Therefore pharmacological inhibition of the novel PDK1/PLC γ 1 pathway may have important therapeutic relevance.

In conclusion this study has demonstrated for the first time that PDK1 can control PLC γ 1 activation and regulate cell invasion. The identification of a direct link between two of the most studied enzymes in signal transduction is likely to have profound consequences and redefine our understanding of several cellular processes controlled by PDK1 and PLC γ 1.

Materials and methods

Cell Culture And Transfections

MDA-MB-231 and HEK293 were cultured in DMEM (Invitrogen, UK); A375M and PC3 were cultured in RPMI 1640 (Invitrogen or PAA, UK). Media were supplemented with 10% (v/v) Foetal Bovine Serum (Invitrogen), Sodium Pyruvate 1% (v/v), 1X L-glutamine/penicillin/streptomycin (Invitrogen) and 0.1% gentamycin (v/v) (Invitrogen, UK). PDK1^{+/+} and PDK1^{-/-} ES cells were kindly provided by Prof Dario Alessi (University of Dundee, UK) and cultured as previously described (Williams et al., 2000). siRNA transfection was performed using Oligofectamine (Invitrogen, UK) for MDA-MB-231 and PC3 or Hiperfect (Qiagen, UK) for A375M. SMARTpool siRNA targeting PDK1 and PLC γ 1 were purchased from Dharmacon, USA. Stable or inducible cell lines knockdown for PLC γ 1 were previously described (Sala et al., 2008). For stable downregulation of PDK1 a sequence targeting the human PDK1 mRNA was subcloned into pSUPERIOR.retro.puro vector (CAAGAGACCTCGTGGAGAA). Control vector pSUPERIOR.retro.puro 5MUT contains a five-point mutated sequence unable to target the human PDK1 mRNA (GACAGGAGGTCAAGGATTTGA). Single MDA-MB-231 clones (Fig. 1B) were selected in 1 μ g/ml puromycin upon transfection of the pSUPERIOR-based constructs using LipofectAMINE (Invitrogen, UK). MDA-MB-231 and A375M used in the rest of the study were cell populations generated by retroviral infection as previously described (Sala et al., 2008).

Western Blot Analysis

Cells were lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 0.1% NP40, 250 mmol/L NaCl, and proteases inhibitors cocktail 2 (Sigma-Aldrich, UK). Lysates were then centrifuged at 13,000 rpm for 10 min at 4°C, and the protein concentration was determined using a Bio-Rad assay kit (Bio-Rad). Forty micrograms of proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, USA). Membranes were probed with the indicated primary antibodies. Antibodies were as follows: phosphoTyr783 PLC γ 1, phosphoThr308 Akt and PDK1 from Cell Signaling Technology, USA; PLC γ 1, ERK2 and Actin from Santa Cruz Biotechnology, USA.

Intracellular Calcium Measurement

Cells were seeded on bottom-glassed chambers (Labtek, UK) and serum starved overnight. For experiments with siRNAs, cells were detached 24 hours after transfection, seeded on the bottom-glassed chambers and serum starved overnight the following day. Each well was then incubated with 200 μ l of HBSS (Invitrogen, UK), containing 0.5% BSA, 2mM CaCl_2 , 4 μ M Fluo-4-AM (Invitrogen, UK) for 45 minutes at 37°C. After loading, cells were washed twice in HBSS 0.5% BSA 2mM CaCl_2 and left in the same solution for 30 minutes for de-esterification of the Fluo-4-AM dye. Where indicated inhibitors were added during the de-esterification step for 30 minutes. Samples were then analysed using the LSM 510 inverted confocal microscope equipped with a chamber for live imaging at 37°C supplied with 5% CO_2 using a 20X objective. After recording basal fluorescence, cells were then stimulated with EGF (20ng/ml) and fluorescence was measured for 6 minutes. Thirty cells were gated and variation of fluorescence intensity was measured for each sample. The average of intensity per time point was calculated.

Analysis of Inositol Phosphates Accumulation

Cells were labelled with [^3H]myo inositol (PerkinElmer) in inositol free medium M199. After 24 h, cells were incubated in M199 containing 10 mM HEPES and 20 mM LiCl for 15 min before stimulation with 50ng/ml EGF for the indicated times, then lysed in 0.1 M HCOOH. In the case of GSK2334470 treatment, cells were pre-incubated with 1 μ M of GSK2334470 for 30 minutes and stimulated in the presence of the inhibitor. Cells treated with DMSO were used as control. Total inositol phosphates were separated by anion exchange chromatography using AG 1-X8 resin (formate form, 200–400 mesh, BioRad) and the total amount assessed by liquid radioactivity counting.

Confocal Microscopy Analysis

MDA-MB-231 cells were co-transfected with PRK5-PLC γ 1 and pOZ-PDK1 or with pEGFP and PRK5-PLC γ 1 or with pEGFP and pOZ-PDK1. Twenty four hours after transfection cells were serum deprived overnight. The following day, cells were stimulated with serum free DMEM containing EGF (50 ng/ml) for the indicated times before being fixed in paraformaldehyde 4% (v/v) for 30 minutes at RT. Cells were permeabilised in PBS/0.25% Triton-X-100 for 2.5 minutes at RT. Unspecific staining was prevented by blocking the coverslips with a solution PBS/0.1% of BSA for 30 minutes at RT. Coverslips were then incubated overnight with primary antibodies (anti-mouse-PLC γ 1 and

anti-rabbit-PDK1 diluted 1:50 in PBS/0.1% BSA). Coverslips were then washed 3X with PBS/0.1% BSA, incubated with secondary antibodies anti-mouse-Alexa488 (Invitrogen, UK) and anti-rabbit Alexa555 (Invitrogen, UK) and analysed using a Carl Zeiss LSM 510 Meta confocal microscope using a Zeiss plan apochromat 63X 1.4 NA(oil) lens. Co-localisation analysis was performed using ImageJ (Macbiophonics). Pseudo-colored “product of the differences from the mean” (PDM) image was calculated by the program as: $PDM = (\text{red intensity} - \text{mean red intensity}) \times (\text{green intensity} - \text{mean green intensity})$.

GST-Pull down Assay

Three sets of three 6-cm petri dishes of HEK293 were transfected with PRK5-PLC γ 1, pEBG2T-MYC-PDK1, pEBG2T-MYC-PDK1-L155E, pEBG2T-MYC-PDK1-D223A (PDK1 Kinase-Dead), pEBG2T-MYC-PDK1-(2–450) (PDK1- Δ PH-PDK1), pEBG2T-PDK1 (408 - end) (PH-PDK1) and pEBG2T-GST empty vector respectively. All GST constructs were kindly provided by Prof. Dario Alessi. Twenty four hours after transfection cells were lysed in lysis buffer (50mM Tris-pH 8.0, 50mM KCl, 1% NP-40) containing protease and phosphatases inhibitors (Sigma-Aldrich, UK). Lysates were cleared by centrifugation at 10,000g for 3 minutes at 4°C. 500 μ g of HEK293 overexpressing GST-empty vector and 500 μ g of HEK293 lysate overexpressing GST-PDK1 were incubated separately with 20 μ l of G4510 glutathione-Sepharose-beads (Sigma-Aldrich) for 1 hour at 4°C. Beads were washed 3X in washing buffer for 5 minutes at 4°C and incubated with 500 μ g of protein lysates from HEK293 overexpressing PLC γ 1, overnight on a rotating wheel at 4°C in a total volume of 1 ml. The following day beads were collected, washed 3X with lysis washing buffer, resuspended in 50 μ l of denaturing sample buffer and heated at 95°C for 5 minute. Supernatant was analysed by SDS-PAGE and Western blot.

Co-immunoprecipitation

Three confluent 10 cm petri dishes of MDA-MB-231 cells were used for these experiments. Serum starved cells were left untreated or stimulated with EGF for the indicated times. Cells were lysed using NP-40 lysis buffer [50mM Tris-pH 8.0, 50mM KCl, 1% (v/v) NP-40] containing protease and phosphatase inhibitors (Sigma-Aldrich, UK). One mg of protein lysates were mixed with 3 μ g of anti-PLC γ 1 antibody (Santa Cruz Biotechnology, USA) or control mouse IgG and incubated overnight at 4°C. The following day the mix was centrifuged at 10,000g for 3 minutes and incubated with 30 μ l of protein G Sepharose 4 fast flow (GE Healthcare, UK) on a rotating wheel at 4°C for 1 hour. Beads

were centrifuged at 2,000g for 1 minute. Supernatant was removed, beads were washed three times with lysis buffer on a rotating wheel at 4°C for 5 minutes and finally resuspended in 50 µl of 2X sample buffer and heated at 95°C for five minutes. The supernatant was analysed by SDS-PAGE and Western blot.

FRET Measurement by FACS

MDA-MB-231 cells were transfected with pOZ-PDK1 and PRK5-PLCγ1 either individually or in combination. In addition cells were cotransfected with pEGFP and pOZ-PDK1 vectors or with pEGFP and PRK5-PLCγ1. Cells transfected with pCDNA empty vector were used as control. Twenty four hours after transfection cells were serum starved over night and then detached by incubation with PBS + 0.2% EGTA (w/v) solution for 20 minutes. Cells were centrifuged at 1,200 RPM for 5 minutes and resuspended in 1 ml of media serum free supplemented with EGF (50 ng/ml) for 5 and 10 minutes. Cells were then fixed in 2% paraformaldehyde solution for 15 minutes and permeabilised using PBS/0.25% Triton-X-100 solution for 2.5 minutes at RT. Cells were centrifuged and resuspended in PBS/0.1% BSA blocking solution for 30 minutes at RT, then pelleted and resuspended in 400 µl blocking solution containing anti-PLCγ1 or anti-PDK1 antibodies either individually or in combination. Cells were incubated overnight at 4°C. The following day cells were centrifuged at 1,200 RPM for 5 minutes and washed three times for 5 minutes in blocking solution before being resuspended in 500 µl of blocking solution containing anti-mouse or anti-rabbit Alexa555-conjugated secondary antibodies and incubated for 1 hour at room temperature. Cells were washed three times and resuspended in PBS for FACS analysis with a Canto II FACS (Becton, Dickinson BD; UK). Cells were gated according to forward and sideward scatter (FSC/SSC). All samples were excited with 488 nm laser and emission measured at 555 nm. The single positive samples stained with Alexa-555 conjugated secondary antibodies allowed to measure the background emission of the dye when excited with the 488 nm laser. The single positive samples stained with Alexa-488 conjugated secondary antibodies allowed removing of bleed-through fluorescence of the donor (Alexa-488) in the FRET channel adjusting the photomultiplier tube (PMT) voltages and compensation for Alexa-488 and Alexa-555 dyes to specifically assess FRET in double positive cells. Cells co-transfected with pEGFP and PLCγ1 or PDK1 were used as negative control of FRET and acquired using FACS Diva software (Becton, Dickinson BD; UK). Data were analysed with FlowJo Software (Treestar, US).

FRET by Acceptor Photo-bleaching

Cells were treated as for confocal microscopy analysis. Spectral imaging analysis was performed on Carl Zeiss LSM 510 Meta. Cells were imaged with excitation $\lambda=488$ nm and $\lambda=543$ laser line and emission spectra were collected respectively in two different channels of the PMT detector. Reference spectra were generated from coverslips incubated separately with the two Alexa-conjugated secondary antibodies alone in order to generate single positive sample and all data were corrected for crosstalk and background fluorescence. Furthermore cells overexpressing PLC γ 1 or PDK1 or co-expressing PLC γ 1 and PDK1 were stained with Alexa-555-conjugated antibodies in order to examine crosstalk and background fluorescence in overexpressing conditions. The FRET efficiency was measured by acceptor photo-bleaching as previously described (Guillaud et al., 2008). A selected area of the cell was repeatedly photobleached with $\lambda = 543$ nm laser line at full power for 1 minute and FRET efficiency was measured as the increase (or dequenching) of donor fluorescence after photo-bleaching in the selected area. All fluorescence measurements were performed in MetaMorph software (Molecular Devices Inc., US). Two independent areas in the bleached zone and one area in the non-bleached zone were analysed in parallel to ensure that the FRET efficiency values were not artefacts due to the photo-bleaching protocol. FRET efficiency was calculated using the following equation:

$$E=[1-(\text{Donor.before}-\text{BCK.before})/(\text{Donor.after}-\text{BCK.after})] \times 100$$

Donor.before = fluorescence intensity of PLC γ 1 before photobleaching

BCK.before = background intensity before photobleaching

Donor.after = fluorescence intensity of PLC γ 1 after photobleaching

BCK.after = background intensity after photobleaching. As a negative control, GFP was used as donor to validate the system.

Invasion Assay

MDA-MB-231 and A375M were serum starved overnight before the experiment. The following day Matrigel pre-coated insert (8.0 μ m pores, 10mm diameter, BD Bioscience, UK) were re-hydrated. Where indicated, cells were pre-treated with 1 μ M GSK22334470 (GlaxoSmithKline, UK) for 30 minutes and then allowed to invade in presence of the inhibitor. After 36 hours the upper face of the porous membrane was cleaned with a cotton bud and cells that invaded the Matrigel were fixed with paraformaldehyde and stained with 0.1% crystal violet solution for 10 minutes. A Leica phase-light microscope using a 10X magnification objective was used for manual counting. A minimum of five fields was counted

per insert. Each invasion experiment was performed in duplicate and the average of invading cells/fields was calculated.

Organotypic cultures

Organotypic cultures on collagen:Matrigel gels were performed as previously described with some modifications (Nystrom et al., 2005) in triplicate. Collagen: Matrigel (70:30) gels were prepared mixing type I collagen (First Link, UK), Matrigel (BD Biosciences, UK), 1 volume of 10x DMEM, 1 volume of FBS and 1 volume of DMEM with 10% FBS containing human fibroblasts (re-suspended at a density of 5×10^6 /ml). One ml of the gel mixture was placed into each well of a 24-well plate and allowed to polymerase at 37°C for 2 hour. After polymerization, 1 ml of DMEM or RPMI 10%FBS, containing MDA-MB-231 or A375M at a density of 5×10^5 per gel respectively, expressing PDK1 shRNA or 5MUT shRNA non-targeting sequence was added on top of the gels. After 24 hours, the gels were raised to the air-liquid interface on stainless steel grids. The gels were harvested at day 10, fixed and H&E staining was performed.

Statistical Analysis

Statistical analyses were performed using the one-tailed, paired Student's t-test using Microsoft Excel®.

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Author Contributions

C.R. performed all the experiments, A.C. contributed to generation of the stable cell lines and performed organotypic cultures, C.R., T.M. and M.F. analysed the data, T.M. and M.F. wrote the manuscript, M.F. supervised the study.

Competing Interests

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1 Inhibition of PDK1 blocks PLC γ 1 activity. (A) Analysis of intracellular calcium variation in MDA-MB-231 transiently transfected with scrambled siRNA (left panel) or with siRNA targeting PDK1 (right panel) upon stimulation with EGF or ATP at the indicated time points (arrows). Data are means from four independent experiments. Representative images of unstimulated and EGF-stimulated cells and western blot analysis of PDK1 levels are also shown. (B) Inositol phosphates accumulation was measured in two clones of MDA-MB-231 expressing shRNA targeting PDK1 or 5MUT control shRNA at the indicated times of stimulation with 50 ng/ml EGF. Data are means \pm s.e.m. from four independent experiments. * p <0.05. Levels of PDK1 in the indicated clones assessed by Western blot analysis are also shown. Equal loading was determined using an anti-ERK2 antibody. (C) Intracellular calcium mobilisation (left panel) and inositol phosphates accumulation (right panel) in MDA-MB-231 treated with 1 μ M GSK2334470 and stimulated with EGF at the indicated time points. Left panel shows a representative experiment from three independent experiments. Representative images of unstimulated and EGF-stimulated cells are shown in left panel. Data in the right panel are means \pm s.e.m. from five independent experiments. * p <0.05.

Fig. 2 Inhibition of PDK1 impairs EGF-induced calcium increase in different cell lines. (A) Analysis of intracellular calcium variation in PC3 transiently transfected with scrambled siRNA or with siRNA targeting PDK1 upon EGF or ATP stimulation. Levels of PDK1 assessed by Western blot analysis are shown. Equal loading was determined using an anti-ERK2 antibody. (B) Analysis of intracellular calcium variation in PC3 treated with 1 μ M GSK2334470 upon stimulation with EGF or ATP at the indicated time points (arrows). (C-D) Analysis of intracellular calcium variation in A375M transiently transfected with scrambled siRNA or with siRNA targeting PDK1 (C) and in PDK1^{+/+} or PDK1^{-/-} ES cells (D) upon stimulation with EGF or ATP at the indicated time points (arrows). Levels of PDK1 assessed by Western blot analysis are also shown. Equal loading was determined using an anti-ERK2 antibody. All panels show a representative experiment from three independent experiments. Representative images of unstimulated and EGF-stimulated cells are shown.

Fig. 3 Phosphorylation of PLC γ 1 Tyrosine 783 is inhibited upon PDK1 inhibition. (A,B) Representative blot of lysates from stable MDA-MB-231 and A375M cell

populations expressing the shRNA specifically targeting PDK1 or the “5MUT” control shRNA untreated or stimulated with 50 ng/ml EGF for the indicated times. Phosphorylation of Threonine 308 Akt in the same lysates was analysed in parallel gels. Anti-actin was used as equal loading control. In panel B, PDK1 levels were determined after stripping of membranes previously incubated with the anti-phosphoThr308 Akt. Anti-actin antibody was used as equal loading control. (C) MDA-MB-231 were pre-treated with 1 μ M GSK2334470 for 30 minutes before stimulation with EGF for the indicated times. PLC γ 1 phosphorylation was determined using a specific anti-phosphoTyr783 antibody. Levels of PDK1 and phosphorylation of AKT threonine 308 were also analysed. Equal loading was determined using an anti-actin antibody. Data are representative of four independent experiments.

Fig. 4 PDK1 associates with PLC γ 1 in a protein complex. (A) Serum starved MDA-MB-231 cells overexpressing PLC γ 1 and PDK1 were left untreated or stimulated with EGF for 10 minutes before being fixed and stained with anti-PLC γ 1 (green) and anti-PDK1 (red) antibodies. Co-localisation was analysed by confocal analysis. Pseudo-coloured “product of the differences from the mean” (PDM) image where each pixel is equal to the PDM value at that location is shown with a PDM scale. (B) Western blot analysis of PDK1 and PLC γ 1 recovered in GST-pull-down experiments performed from lysates containing the indicated GST-tagged PDK1 constructs or GST alone (expressed in HEK293 cells) upon incubation with lysates from HEK293 overexpressing PLC γ 1. GST or GST-PDK1 proteins were pulled down by glutathione beads. Loading control was assessed by incubating with anti GST antibody. Blot is representative of three independent experiments. (C) Serum starved MDA-MB-231 cells were left untreated or stimulated with EGF for the indicated times. Co-immunoprecipitation experiments of the endogenous proteins were performed using PLC γ 1 antibody and western blot analysis was performed using anti-PLC γ 1 and anti-PDK1 antibodies. Parallel co-immunoprecipitation with mouse-IgG was performed as control. (D) Densitometry analysis performed on three independent co-immunoprecipitation experiments.

Fig. 5 PDK1 interacts with PLC γ 1. FRET analysis was performed by FACS (A,B) or confocal microscopy (C-E) as described in the Materials and Methods section. (A) Histogram displays acceptor fluorescence of single positive (control) and unstimulated cells (left), and single positive and EGF-stimulated cells (right) measured by FACS; chart

shows acceptor fluorescence intensity over time of acquisition of single positive, unstimulated and EGF stimulated cells. **(B)** Quantification of fluorescence in the FRET channels measured by FACS in MDA-MB-231 transfected with PLC γ 1 or pOZ-PDK1 plasmid and labelled with acceptor-dye-conjugated antibody, or expressing a GFP-containing vector (donor) together with PLC γ 1 plasmid or pOZ-PDK1 plasmid labelled with acceptor-dye-conjugated antibody. Graph is the mean \pm s.e.m. from three independent experiments. **(C)** Confocal microscopy images of FRET analysis performed in serum starved MDA-MB-231 cells co-expressing PLC γ 1 (green) and PDK1 (red) untreated or stimulated with EGF. **(D)** The green and red lines in the charts show the fluorescence of donor and acceptor respectively, before and after acceptor photobleaching measured in cells along the indicated red arrow shown in panel C. **(E)** Quantification of FRET measured by acceptor photobleaching in serum-starved MDA-MB-231 overexpressing PLC γ 1 alone (+PLC γ 1) or PDK1 alone (+PDK1) or coexpressing PLC γ 1 and PDK1 (+PLC γ 1/+PDK1), untreated or treated with EGF for the indicated times and co-stained with anti-mouse-Alexa-488 and anti-rabbit-Alexa-555 conjugated antibodies. Graph shows the mean \pm s.e.m. from three independent experiments. * $p < 0.05$

Fig. 6 Inhibition of PDK1 blocks cancer cell invasion (A,B) Results from invasion assay on Matrigel performed in the indicated MDA-MB-231 and A375M stable cell populations. **(C,D)** H&E staining of organotypic cultures of the indicated cells. Arrows indicate invading cells. Pictures are representative of two independent experiments for each cell line. **(E)** Results from invasion assay of MDA-MB-231 transiently transfected with siRNA targeting PDK1 or scrambled siRNA. **(F)** Results from invasion assay of A375M transiently transfected with scrambled siRNA or siRNAs targeting PDK1 or PLC γ 1. Representative pictures of Transwell stained with crystal violet and Western blots confirming proteins downregulation are also shown. All graphs indicate number of invaded cells/field expressed as percentage of control (5MUT or scrambled cells respectively) and are mean \pm s.e.m. from three independent experiments. * $p < 0.05$.

Fig. 7 Simultaneous inhibition of PDK1 and PLC γ 1 does not enhance the effect of inhibition of PDK1 or PLC γ 1 alone on cell invasion. (A,B) Cell invasion on Matrigel of MDA-MB-231 and A375M expressing shRNA targeting PLC γ 1 or a 3MUT non targeting sequence and treated with 1 μ M GSK2334470. **(C,D)** Results from invasion assay performed in the indicated MDA-MB-231 and A375M stable cell populations transiently

transfected with scrambled siRNA or siRNA targeting PLC γ 1. In all panels data indicate number of invaded cells/field expressed as percentage of control (5MUT or 3MUT cells respectively). Blots in figure show the levels of PDK1 or PLC γ 1 in the indicated cells. Anti-ERK2 antibody was used as equal loading control. Representative images of inserts stained with crystal violet are shown. In all panels data are means \pm s.e.m. from three independent experiments performed in duplicate. * p <0.05. ** p <0.01.

Fig. 8 Schematic representation of the potential mechanism of PDK1-dependent PLC γ 1 regulation. In unstimulated conditions, EGF receptor is not phosphorylated, PI3K is not activated and both PDK1 and PLC γ 1 are confined in the cytosol. Upon EGF stimulation, EGF receptor is phosphorylated, PI3K is activated and PtdIns(4,5) P_2 is converted to PtdIns(3,4,5) P_3 . Binding of the PH domain of PDK1 to PtdIns(3,4,5) P_3 recruits the enzyme to the plasma membrane. PLC γ 1 is also recruited to the plasma membrane through a similar PH domain/PtdIns(3,4,5) P_3 interaction and it is further associated to the phosphorylated tyrosine residues of the receptor through its SH2 domains. The PDK1-dependent-PLC γ 1 activation guarantees a further level of interaction and further stabilises the association of PLC γ 1 to the receptor, which allows PLC γ 1 tyrosine phosphorylation at Tyr783.















