Epidermal growth factor (EGF) triggers nuclear calcium signaling through the intranuclear phospholipase Cδ4 (PLCδ4)

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Calcium (Ca\(^{2+}\)) signaling within the cell nucleus regulates specific cellular events such as gene transcription and cell proliferation. Nuclear and cytosolic Ca\(^{2+}\) levels can be independently regulated, and nuclear translocation of receptor tyrosine kinases (RTKs) is one way to locally activate signaling cascades within the nucleus. Nuclear RTKs, including the epidermal growth factor receptor (EGFR), are important for processes such as transcriptional regulation, DNA-damage repair, and cancer therapy resistance. RTKs can hydrolyze phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) within the nucleus, leading to Ca\(^{2+}\) release from the nucleoplasmic reticulum by inositol 1,4,5-trisphosphate receptors. PI(4,5)P\(_2\) hydrolysis is mediated by phospholipase C (PLC). However, it is unknown which nuclear PLC isoform is triggered by EGF. Here, using subcellular fractionation, immunoblotting and fluorescence, siRNA-based gene knockdowns, and FRET-based biosensor reporter assays, we investigated the role of PLCδ4 in epidermal growth factor (EGF)-induced nuclear Ca\(^{2+}\) signaling and downstream events. We found that EGF-induced Ca\(^{2+}\) signals are inhibited when translocation of EGFR is impaired. Nuclear Ca\(^{2+}\) signals also were reduced by selectively buffering inositol 1,4,5-trisphosphate (InsP\(_3\)) within the nucleus. EGF induced hydrolysis of nuclear PI(4,5)P\(_2\) by the intranuclear PLCδ4, rather than by PLCγ1. Moreover, protein kinase C, a downstream target of EGF, was active in the nucleus of stimulated cells. Furthermore, PLCδ4 and InsP\(_3\) modulated cell cycle progression by regulating the expression of cyclins A and B1. These results provide evidence that EGF-induced nuclear signaling is mediated by nuclear PLCδ4 and suggest new therapeutic targets to modulate the proliferative effects of this growth factor.

The spatial-temporal distribution of calcium (Ca\(^{2+}\)) signals contributes to the versatility of this second messenger (1). For example, increases in Ca\(^{2+}\) within the cell nucleus selectively promote cellular events such as gene transcription (2), cell proliferation, and tumor growth (3). Moreover, nuclear Ca\(^{2+}\) signals can be regulated independently of cytosolic Ca\(^{2+}\) signals (4). This is possible because the nucleus contains the machinery necessary for Ca\(^{2+}\) mobilization (5–8). Specifically, the nuclear envelope is contiguous with the endoplasmic reticulum (ER) (9) and has invaginations that can reach deep within the nucleoplasm (10). These invaginations express InsP\(_3\)Rs, store and release Ca\(^{2+}\) in an InsP\(_3\)-sensitive fashion (5, 11), and have been referred to as the nucleoplasmic reticulum (NR) (5, 10). The distribution of InsP\(_3\)Rs isoforms and also Ca\(^{2+}\) signaling in the nucleus become altered in various disease states, such as fatty liver disease (12) and cholangiocarcinoma (13). Intranuclear InsP\(_3\) is formed from PI(4,5)P\(_2\) hydrolysis, which is induced by growth factors such as hepatocyte growth factor (7) and insulin (8). Some RTKs undergo nuclear translocation upon activation, which appears necessary for initiation of nuclear Ca\(^{2+}\) signals (7, 8), so this could be one mechanism to regulate Ca\(^{2+}\) release locally.

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This article contains Fig. S1.

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3 The abbreviations used are: ER, endoplasmic reticulum; AVP, arginine vasopressin; CHC2, clathrin heavy chain 2; CFP, cyan fluorescent protein; DAG, diacylglycerol; EGF, epidermal growth factor; EGFR, EGF receptor; FRET, Förster resonance energy transfer; InsP\(_3\), inositol 1,4,5-trisphosphate; InsP\(_3\)-R, InsP\(_3\) receptor; mRFP, monomeric red fluorescent protein; NR, nucleoplasmic reticulum; PdBu, phorbol 12–13-dibutyrate; PI(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; RTK, receptor tyrosine kinase; YFP, yellow fluorescent protein; HGF, hepatocyte growth factor; ANOVA, analysis of variance.
Nuclear localization of EGFR is of clinical relevance, as it is correlated with cancer prognosis (14) and relates to resistance to various cancer therapies (15, 16). Nuclear EGFR function has been the subject of extensive investigation, as has been nuclear targets of EGFR. EGFR can shuttles from the cell surface to the nucleus (17, 18) where it acts as a transcriptional regulator (19, 20), transmits signals (21, 22), and is involved in processes such as cell proliferation, tumor progression, and DNA repair and replication (23). However, it is unknown whether EGFR is linked to nuclear calcium release.

PLCδ4 could be involved in primary nuclear Ca^{2+} signaling because activation of InsP3 receptors generally is involved in the release of Ca^{2+} in the nucleus (24). In mammals, the PLC family is composed of 13 isoforms, divided into six classes as follows: \( \beta, \gamma, \delta, \epsilon, \zeta, \) and \( \eta \) according to their structures (25). All of these isoforms catalyze the reaction of PI(4,5)P2 cleavage, generating InsP3 and diacylglycerol (DAG), but each one possesses unique physiological functions (25). Some PLCs are described within the nucleus in specific cell types such as PLCβ1, PLCγ1, PLCδ1, PLCδ4, and PLCγ2 (26, 27). The activity of these nuclear PLCs is related to the regulation of several cellular processes, such as proliferation and differentiation. These particular isoforms also have been linked to specific diseases, including myelodysplastic syndromes (PLCβ1), neurological diseases (PLCγ1), and infertility (PLCζ and PLCδ1–4) (28).

PLCδ4 has been identified as a nuclear protein in different cell types and may be involved in proliferative processes (27, 29–31). It was first purified from regenerating rat liver protein extracts (32), and its gene was cloned (33) from a regenerating rat liver cDNA library, indicating a possible role of PLCδ4 in cell proliferation. Despite its nuclear localization, it is unknown how human PLCδ4 is activated within the nucleus. This work investigates whether EGFR activates nuclear Ca^{2+} signaling via PLCδ4.

**Results**

**EGFR translocates to the nucleus and induces Ca^{2+} signals**

Stimulation with EGFR induces its RTK EGFR to translocate to the nucleus (18), similar to what has been shown for other RTKs, including the hepatocyte growth factor (HGF) receptor, c-Met, and the insulin receptor (7, 8). EGFR translocated to the nucleus in both primary rat hepatocytes and SKHep-1 cells, a human liver cancer cell line (Fig. 1A). EGFR protein levels in the nucleus fraction were higher than in nontreated control cells as soon as 2.5 min after stimulation with EGFR (Fig. 1A). In SKHep-1 cells, EGFR decreased in the non-nuclear fraction as it increased in the nuclear fraction. The peak of EGFR nuclear translocation in this cell line was at 10 min (Fig. 1A). Super-resolution immunofluorescence showed that EGFR accumulated in the nucleus within 10 min of exposure to EGFR (Fig. 1B). EGFR stimulation induced bigger EGFR clusters throughout the cells, including at the nucleus, as demonstrated previously (15). These data demonstrate that EGFR induces EGFR to translocate to the nucleus.

Translocation of EGFR to the nucleus depends on clathrin-mediated endocytosis (17), so we used clathrin heavy chain 2 (CHC2) siRNAs to disrupt this endocytic pathway and thereby inhibit internalization of EGFR. Fig. 1C shows that siRNA treatment reduced CHC2 expression by 94 ± 3% using the CHC2 siRNA 1 and by 87 ± 8% using the CHC2 siRNA 2, relative to control (\( p < 0.001 \)). Stimulation of control cells with EGFR led to a gradual Ca^{2+} increase with some superimposed oscillations, similar to the Ca^{2+} signal pattern induced by other growth factors (7, 8), but CHC2 knockdown diminished the peak of EGFR-induced Ca^{2+} signals by 85 ± 2% (\( p < 0.001 \)) using the CHC2 siRNA 1 and by 100 ± 2% (\( p < 0.001 \)) using the CHC2 siRNA 2, compared with control (Fig. 1D). This indicates that most of the Ca^{2+} signals induced by EGFR depend on EGFR internalization.

**EGF triggers intranuclear PI(4,5)P2 hydrolysis and InsP3 formation**

EGF induces intracellular Ca^{2+} transients through InsP3 (34), so we investigated whether EGF-induced InsP3 formation in the nucleus was responsible for increasing intranuclear Ca^{2+}. SKHep-1 cells were transfected with cytosolic or nuclear InsP3-buffer constructs whose targeting was confirmed by subcellular localization of the monomeric red fluorescent protein (mRFP) (Fig. 2A). Fluo-4/AM was used to monitor Ca^{2+} release in response to EGF. Cytosolic InsP3-buffer decreased the Ca^{2+} response by 50.5 ± 6.1% in the nucleus and by 53.8 ± 4.5% in the cytoplasm (Fig. 2B), but EGF-induced Ca^{2+} signals were decreased by 86 ± 2.7% in the nucleus and by 96 ± 3.9% in the cytoplasm in cells expressing the nuclear InsP3-buffer (Fig. 2B). This suggests that EGF triggers nuclear Ca^{2+} signals by inducing the formation of InsP3 within the nucleus, similar to what has been observed in liver cells stimulated with HGF (7) or insulin (8). To investigate whether EGF induces nuclear PI(4,5)P2 hydrolysis to produce InsP3, PI(4,5)P2 was measured in nuclear fractions of hepatocytes. Nuclei from EGF-stimulated cells contained 64 ± 1.5% less PI(4,5)P2 than nuclei from control cells (Fig. 2D). These findings provide evidence that EGF triggers nuclear PI(4,5)P2 hydrolysis and local InsP3 production to generate Ca^{2+} signals.

**EGF stimulates intra-nuclear PKC activity**

PI(4,5)P2 hydrolysis generates not only InsP3 but also DAG, which can activate PKC (24). Increases in nuclear DAG can either participate in translocation of PKCs, from the cytosol to the nucleus, or can directly activate PKCs that reside in the nucleus (35). To determine whether EGF triggers nuclear PKC activity, we used a Förster resonance energy transfer (FRET) reporter based on PKC activity and tagged to a nuclear localization signal (NucCKAR) (36). The nuclear localization of this construct was confirmed by intra-nuclear detection of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorescence by confocal microscopy (Fig. 3A). Phosphorylation of the reporter by PKC results in decreased rather than increased FRET (36), so the CFP/YFP ratio was monitored to reflect PKC activity. NucCKAR-expressing SKHep-1 cells were stimulated with EGF, phorbol 12–13-dibutyrate (PdBu) as a positive control for PKC activation, or arginine vasopressin (AVP) to reflect PKC activity. NucCKAR-expressing SKHep-1 cells contained 64 ± 8% of PI(4,5)P2 than nuclei from control cells (Fig. 2D). These findings provide evidence that EGF triggers nuclear PI(4,5)P2 hydrolysis and local InsP3 production to generate Ca^{2+} signals.
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A

|                | SKHep-1 cells | Hepatocytes |
|----------------|---------------|-------------|
|                | Non-nuclear fraction | Nuclear fraction | Non-nuclear fraction | Nuclear fraction |
| EGFR           | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. |
| Lamin B        | 250 kDa 150 kDa 75 kDa 50 kDa | 150 kDa 75 kDa 50 kDa | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. |
| α-Tubulin      | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. | 0 2.5 5 10 20 min. |

B - EGF

C

|                | Control | Lipofectamine | Control siRNA 1 | CHC2 siRNA 1 | CHC2 siRNA 2 |
|----------------|---------|---------------|-----------------|--------------|--------------|
| CHC2           | 150 KDa | 100 KDa  |
| α-Tubulin      | 50 KDa  | 37 KDa       |

D

- EGF

+ EGF

Nuclear Fluorescence Intensity (A.U.)

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PLCγ1 is localized to the cytosol and PLC6γ4 is in the nucleus

PLC mediates PI(4,5)P₂ hydrolysis and formation of InsP₃ and DAG (29, 30), so the relative role played by several candidate PLC isoforms during EGF stimulation was investigated. PLCγ1 is the primary isoform typically thought to bind to and be activated by RTKs (1). PLC6γ4 has been recognized as a predominantly nuclear PLC isoform, and its expression is increased in liver regeneration (33), so we compared the localization of these two isoforms in SKHep-1 cells. Confocal immunofluorescence showed that PLCγ1 is mostly localized to the cytosol, and this pattern does not change upon EGF stimulation (Fig. 4A). In contrast, PLC6γ4 was detected exclusively in the nucleus of SKHep-1 cells, demonstrated by both confocal microscopy (Fig. 4B) and Western blotting (data not shown). The subcellular localization of these PLC isoforms was also assessed in primary rat hepatocytes. Similar to what was observed in SKHep-1 cells, PLCγ1 was found in non-nuclear fractions of both control and EGF-stimulated cells, whereas PLC6γ4 was only detected in the nuclear fractions (Fig. 4C). The amount of PLCγ1 or PLC6γ4 in each cell fraction was not altered by stimulation with EGF (Fig. 4C), indicating that there is no translocation of these PLCs between nuclear and non-nuclear compartments in response to EGF. Further examination showed that EGF stimulation for up to 10 min does not induce PLCγ1 to translocate from the cytoplasm to the nucleus (Fig. 4D).

PLC6γ4 participates in EGF-induced nuclear PI(4,5)P₂ hydrolysis, InsP₃ signaling, Ca²⁺ signals, and PKC activity

An siRNA approach was employed to determine the relative roles of PLC6γ4 and PLCγ1 in EGF-induced nuclear PI(4,5)P₂ hydrolysis and Ca²⁺ signaling. Specific siRNAs reduced PLCγ1 expression by 90 ± 14 and 91 ± 27% using siRNAs 1 and 2, respectively. PLC6γ4 expressions were reduced by 75 ± 7 and 86 ± 5% using siRNAs 1 and 2, respectively (p < 0.01) (Fig. 5A). Nuclear fractions were isolated from these cells after EGF stimulation, and PI(4,5)P₂ was quantified. EGF significantly increased nuclear PI(4,5)P₂ hydrolysis in control siRNAs and PLCγ1 siRNA-treated cells, but not in PLC6γ4 siRNA-treated cells (Fig. 5B). Furthermore, cells treated with PLC6γ4 siRNAs significantly reduced nuclear InsP₃ production, nuclear PKC activity, and Ca²⁺ signals, but this was not seen in PLCγ1 siRNA-treated cells (Fig. 5, C–E). These results suggest that PLC6γ4 but not PLCγ1 participates in nuclear PI(4,5)P₂ hydrolysis, InsP₃ production, PKC activation, and Ca²⁺ signaling triggered by EGF stimulation.

PLC6γ4 participates in cell cycle progression

Nuclear Ca²⁺ is important for cell proliferation (3), an important downstream effect of EGF. Therefore, we investigated whether PLC6γ4 is involved in EGF-mediated cell proliferation. Cell proliferation was measured by either cell counting (Fig. S1) or BrdU incorporation (Fig. 6A) was decreased by over 67% in cells in which PLC6γ4 expression was reduced. SKHep-1 proliferation was restored when PLC6γ4 expression was recovered (Fig. S1). Cell death does not mediate the decreased proliferation because annexin V–FITC and propidium iodide assays did not reveal cell death between control and siRNA-treated groups (Fig. 6B). Next, the role of PLC6γ4 in cell cycle progression was investigated. To understand the basis for this, the effects of PLC6γ4 on the expression of various cyclins were studied (Fig. S1). Expression of cyclin B1, a G₂/M-phase checkpoint protein, and cyclin A, an S-phase checkpoint protein, was decreased in PLC6γ4 knockdown cells compared with cells treated with control siRNA (Fig. 6C). A nuclear InsP₃-buffer also decreased the expression of cyclins A and B. These findings suggest that nuclear PLC6γ4 affects cell cycle progression, in part by affecting cyclin expression.

Discussion

EGFR has several direct effects on signaling in the nucleus, including interaction with transcription factors (37) and phosphorylation (21). This work extends this repertoire of intranuclear actions by showing that EGFR translocates to the nucleus of hepatic cells to initiate InsP₃-mediated Ca²⁺ signals. EGFR likely translocates from the plasma membrane to the nucleus via the ER (20, 23), and a similar trafficking route has been observed for other EGFR family members.

Figure 1. EGF induces EGFR nuclear translocation and clathrin-mediated endocytosis-dependent Ca²⁺ signals. A, Western blot analysis of EGFR in non-nuclear and nuclear fractions isolated from resting (control) or EGF-stimulated hepatocyte and SKHep-1 cells. α-Tubulin and lamin B1 were used as purity controls for non-nuclear and nuclear fractions, respectively. Indicated kDa values represent the position of the referenced bands from pre-stained protein standard. Densitometric measurements of cellular fractions show that nuclear EGFR is maximal within 10 min of stimulation in SKHep-1 cells and within 2.5–5 min (p < 0.01) in hepatocytes when compared with the control (0 min). Values are scaled relative to the initial amount in the non-nuclear fraction and relative to the amount at 2.5 min in the nuclear fraction (mean ± S.E.; n = 3) (nuclear SKHep-1 fractions, one-way ANOVA, F(4,10) = 3.215; p = 0.061; nuclear hepatocyte fractions, one-way ANOVA, F(4,10) = 8.530, p = 0.0029). B, gated STED super-resolution images of control SKHep-1 cells (right image) and 10 min after EGF stimulation (middle image). Serial optical sections were collected for three-dimensional reconstruction; y-z sections are shown at the right of each image. EGFR is represented in green; lamin B1 is in red, and the nuclear compartment marked in blue. Note that EGFR redistributes to the region of the nuclear envelope as well as within the nuclear interior (arrows). Bar = 10 μm. Scatter plot (right panel) showing the quantification of EGFR that co-localizes with the nucleus before and after EGF stimulation. n = 7–12 cells for each group. The image collection settings for fluorescence quantification was adjusted according to the cells stimulated with EGF to avoid nuclear-saturated pixels of EGFR clusters. ***, p < 0.001 (Student’s t-test). C, Western blotting (top) analysis of clathrin heavy chain 2 (CHC2) expression in nontreated, Lipofectamine only, control siRNAs-treated, or CHC2 siRNAs-treated SKHep-1 cells. α-Tubulin was used as a loading control. Bar graph shows the summary of the Western blottings (n = 4); ***, p < 0.001. (One-way ANOVA, F(5,18) = 34.41; p < 0.0001.) D, tracings represent Fluoro-4/AM fluorescence intensity changes, normalized by the baseline, by the time of EGF stimulation. Lower panel, bar graph compiling peaks of Fluoro-4/AM fluorescence intensity in EGF stimulated at 15 min. Control siRNA-treated (n = 15), Lipofectamine only (n = 14), control siRNA 1 (n = 10), control siRNA 2 (n = 10), CHC2 siRNA 1 (n = 13), or CHC2 siRNA 2-treated (n = 13) SKHep-1 cells. Fluorescence changes over time from whole cells were normalized and represented as fluorescence intensity (F₀) by baseline fluorescence (F₀) multiplied by 100. ***, p < 0.001. (One-way ANOVA, F(5,69) = 18.78; p < 0.0001.) Experiments were performed on at least 3 different days.
described for both viruses and toxins as well (38–40). Trafficking of other RTKs from the plasma membrane to the nucleus has also been described. For example, c-Met that is biotinylated at the plasma membrane can be recovered from the nucleus after cells are stimulated with HGF (7). Similarly, fluorescently-labeled EGF can be tracked from the cell membrane to the cell nucleus over a 10-min period (18). Furthermore, super-resolution imaging can be used to quantify the amount of EGF/EGFR clusters that accumulate in the nucleus (18). The peak in nuclear translocation of EGFR coincides with the peak in Ca²⁺ signals (Fig. 1, A–C). This work builds on the previous observation that translocation of EGFR to the nucleus depends on dynamin and clathrin-mediated endocytosis (17) by showing that knockdown of clathrin reduces EGF-induced Ca²⁺ signals. These findings are consistent with the idea that EGFR must translocate to the nucleus in order to initiate nuclear Ca²⁺ signals, similar to what has been shown for c-Met (7).

Ca²⁺ signaling in the nucleus rather than cytosol is important for proliferation and tumor growth (3). Nuclear Ca²⁺ can also regulate gene expression (2, 4). Thus, increases in nuclear

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**Figure 2.** EGF triggers Ca²⁺ release by nuclear InsP₃ and nuclear PI(4,5)P₂ hydrolysis. A, confocal images of SKHep-1 cells loaded with Fluo-4/AM expressing cytosolic InsP₃-buffer, nuclear InsP₃-buffer, or mRFP (control). Images show cells before EGF stimulus (baseline) and the peak (15 min) of EGF-induced Fluo-4/AM fluorescence intensity changes. Expression of constructs was checked by detection of mRFP. Bar = 10 μm. B, bar graph showing Fluo-4/AM fluorescence intensity peak in cytosolic and nuclear regions of control, cytosolic, or nuclear InsP₃-buffer expressing cells upon EGF stimulus (8–11 cells in each group; *, p < 0.05; **, p < 0.01; and ***, p < 0.001) (cytosol: one-way ANOVA, F(2,24) = 17.17, p < 0.0001; nuclear, one-way ANOVA, F(2,26) = 28.03; p < 0.0001.). C, average traces of SKHep-1 expressing cytosolic or nuclear InsP₃-buffer or control cells stimulated with EGF. Cytosolic or nuclear regions for fluorescent intensity measurements were selected using the assistance of the digital image of contrast (D.I.C) images, see squares at the nucleus and non-nuclear regions. Nuclear but not cytosolic InsP₃-buffer blocked the peak of EGF response in both compartments. D, bar graph represents the amount of PI(4,5)P₂ of nucleus isolated from control or EGF-stimulated hepatocytes (n = 6). 5 min of EGF stimulation reduces nuclear PI(4,5)P₂ by 64 ± 1.5% (p < 0.05) (Student's t test).
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**Figure 3.** EGF induces nuclear PKC activity. A, confocal images of a representative SKHep-1 cell expressing NucCKAR shows CFP and YFP emission as well as a transmitted image of the cell, confirming nuclear localization of the construct. Bar = 10 μm. B, representative tracings of NucCKAR CFP/YFP emission normalized by baseline show changes in emission ratio during 30 min of perfusion with 200 nM phorbol 12-13-dibutyrate (PdBu) (n = 6), 100 ng/ml EGF (n = 10), or 500 nM AVP (n = 3). C, bar graph compiling the average results shown in B. NucCKAR FRET ratio change in EGF-stimulated cells in the presence (n = 8) or absence (n = 10) of the PKC inhibitor Go6983 (250 nM). The PKC inhibitor significantly attenuates EGF-induced NucCKAR FRET changes: *, p < 0.05 (one-way ANOVA, F(3,23) = 5.645; p = 0.0002).

Ca\(^{2+}\) could be one mechanism for EGF to promote those cellular processes. The nucleus contains the machinery necessary for InsP\(_3\) receptor-mediated Ca\(^{2+}\) release (5, 7, 8, 24). Although PLC-mediated P(4,5)P\(_2\) hydrolysis and formation of InsP\(_3\) and DAG within the cytoplasm have been described extensively (1), there are fewer studies showing that P(4,5)P\(_2\) hydrolysis also occurs within the nucleus. This work provides evidence that EGF triggers P(4,5)P\(_2\) hydrolysis and InsP\(_3\) formation within the nucleus (Figs. 2 and 5), similar to what has been observed in response to stimulation with HGF (7) or insulin (8).

PLC within the nucleus is involved in signal-transduction pathways that are separate from those activated by isoforms localized in other compartments (41). The most common par-
tively inhibit PLC\(\gamma\)4 could be important for selective inhibition of liver cancer, which is the third leading cause of cancer death worldwide.

**Experimental procedures**

**Cell culture and EGF stimulus**

The SKHep-1 is a human liver cancer cell line (ATCC, Manassas, VA). It was cultured at 37 °C in 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.).

Hepatocytes were isolated from healthy male Sprague-Dawley rats (190–200 g; Charles River Laboratories), as described previously (56). Primary cells were cultured at 37 °C in 5% CO\(_2\) in Williams’ medium E (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and plated on collagen-coated coverslips (50 μg/ml) (BD Biosciences). Hepatocytes were used within 24 h of isolation. All animal studies were approved by the Yale Univer-

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**Figure 4. PLC\(\gamma\)1 is a cytosolic and PLC\(\delta\)4 is a nuclear protein.**

A, representative confocal images of SKHep-1 cells before and after EGF stimulation showing PLC\(\gamma\)1 in red, EGFR in green, and TO-PRO nuclear dye in blue. Merged images reveal that PLC\(\gamma\)1 is predominantly expressed outside of the nucleus in both conditions (n = 15 cells). B, representative confocal images of control and EGF-stimulated SKHep-1 cells showing PLC\(\delta\)4 in red, which is exclusively intra-nuclear (n = 15 cells). C, Western blotting showing PLC\(\gamma\)1 and PLC\(\delta\)4 expressions in non-nuclear and nuclear fractions from control or EGF-stimulated hepatocytes. α-Tubulin and lamin B1 were used as purity controls for nuclear and non-nuclear fractions. Indicated kDa values represent the position of the reference bands from pre-stained protein standard. Subcellular localizations of PLC\(\gamma\)1 and PLC\(\delta\)4 in hepatocytes are not altered by EGF stimulation for 10 min (n = 3). D, tracings of normalized amounts of PLC\(\gamma\)1 in non-nuclear and nuclear fractions of SKHep-1 cells upon EGF stimulation with different time points, quantified from Western blotting experiments (n = 3).
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Subcellular fractionation and Western blotting

Cells were washed twice with ice-cold 10 mM PBS, pH 7.4 (PBS) (Sigma), harvested by scraping, and lysed in a buffer with 20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl\(_2\), 0.5% Nonidet P-40. After incubation on ice for 10 min, the cells were homogenized by vortex. The homogenate was centrifuged at 15,000 \(\times\) g for 5 min to sediment the nuclei. The supernatant was then centrifuged at a maximum speed of 16,100 \(\times\) g for 20 min, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellet was washed three times with lysis buffer to remove any contamination from cytoplasmic membranes. The isolated nuclei were resuspended in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40), and the mixture was sonicated briefly to aid nuclear lysis. Nuclear lysates were collected after centrifugation at 16,100 \(\times\) g for 20 min at 4 °C. Protease and phosphatase inhibitors (Sigma) were added to all buffers. Proteins were quantified by Bradford assay (Sigma).

Western blotting was performed and detected as described previously (7). In brief, the primary antibodies used were as follows: rabbit polyclonal anti-EGFR (1:500, Santa Cruz Biotechnology, catalog no. SC3); mouse monoclonal anti-EGFR clone 8G6.2 (1:200, Millipore/Sigma, catalog no. ab16048); monoclonal anti-\(\alpha\)-tubulin (1:2000, Sigma, catalog no. T6199); rabbit polyclonal anti-lamin B1 (1:2000, Abcam, catalog no. ab16048); monoclonal anti-clathrin (1:500, Abcam, catalog no. b21679); mouse monoclonal anti-PC\(\lambda\)y1 (1:1000, Cell Signaling Technology, catalog no. 12822); rabbit polyclonal anti-PC\(\lambda\)y4 (1:500, Santa Cruz Biotechnology, catalog no. H-250); rabbit polyclonal Erk1/2 (1:1000; Cell Signaling, catalog no. 9101); rabbit monoclonal anti-phospho-Erk1/2 Thr-202/Tyr-204 (1:1000; Cell Signaling, catalog no. 4370); rabbit monoclonal anti-AKT (1:1000, Cell Signaling, catalog no. 4691); rabbit monoclonal anti-phospho-AKT Thr-308 (1:1000; Cell Signaling, catalog no. 13038); cyclins A, B1, D1, D2, D3, E2 and H (1:500 to 1000, Cell Signaling Technology. Kit catalog no. 9869). The membranes were developed using ECL Plus (GE Healthcare). Subsequently, the films were scanned and analyzed using ImageJ software. All antibodies used were found to have the correct molecular weight by Western blotting. The antibodies for clathrin, PCφ4, and PC\(\lambda\)y1 were validated with at least two specific siRNAs.

Immunofluorescence

Confocal and super-resolution immunofluorescence imaging were performed as described previously (18). In brief, cells were labeled with rabbit polyclonal anti-EGFR (1:250, Santa Cruz Biotechnology, catalog no. SC3) or mouse monoclonal anti-EGFR clone 8G6.2 (1:200, Millipore/Sigma, catalog no. 05-1047) and rabbit polyclonal anti-PC\(\lambda\)y1 (1:200, Cell Signaling, catalog no. 12822) and polyclonal anti-PC\(\lambda\)y4 (Santa Cruz Biotechnology, catalog no. H-250) and then incubated with secondary antibodies conjugated to Alexa Fluor 488 or 546 (Life Technologies, Inc.). Hoechst 33342 (Life Technologies, Inc.) was used as a marker for the nuclear compartment. Images were collected using a Leica SP8 Gated STED super-resolution microscope with a \(\times\) 100, 1.4 NA objective lens.

siRNA transfection

Cells were transfected using Lipofectamine RNAiMax reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. 50 nm siRNAs were used for clathrin heavy chain knockdown (CHC2 siRNA 1, 5’-GCAAUAUAUAAGGCCGU-ACCGUATT-3’, Life Technologies, Inc., catalog no. s223263; CHC2 siRNA 2, 5’-AGGGGCUUCAUAUAUCAUGAC-3’, IDT, catalog no. hs.Ri.CLT13.1). 25 nm siRNAs were used for PC\(\lambda\)y1 knockdown (PC\(\lambda\)y1 siRNA 1, 5’-GAAUGCU-GAGAGCUAUATT-3’, Life Technologies, Inc., catalog no. h10632; and PC\(\lambda\)y1 siRNA 2, 5’-GACCUCAUAGCUCUA-UGAGAACAC-3’, IDT, catalog no. hs.Ri.PCG1.13.1). For PC\(\lambda\)y4 knockdown, 50 nm of the following siRNAs were used: ON-TARGETplus SMARTpool (PC\(\lambda\)y4 siRNA 1, 5’-CAAGAG-
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Figure 6. Knockdown of PLCδ4 diminishes cell growth and decreases cyclins A and B1 without affecting cell death. A, effects of PLCδ4 depletion on cell proliferation. BrdU measurements were performed 48 h after transfection. *, p < 0.05 (EGF−, one-way ANOVA, F(5,35) = 8.042; p < 0.0001; EGF+, one-way ANOVA, F(5,35) = 9.570; p < 0.0001). B, quantification of cells labeled with annexin V-FITC and propidium iodide. The bar graph shows the percentages of cells labeled with either annexin V-FITC or propidium iodide or double-labeled with annexin V-FITC plus propidium iodide compared with all cells from each field. Each treatment was made in triplicate, and for each one, three different images were counted using ImageJ software; then, the means were used for statistical analysis. All groups were compared with control groups (control, Lipofectamine, and control siRNAs). Doxorubicin (Dox) 10 μg/ml was used as positive control, and it differs from all experimental groups. ***, p < 0.001. Annexin V staining did not reveal differences in the level of cell death between control and siRNA-treated groups (one-way ANOVA, F(6,71) = 20.64; p < 0.0001). C, Western blotting showing cyclins A and B1 expression in SKHep-1 cells under controls or siRNA-treated conditions. α-Tubulin was used as a loading control. Representative image of three independent experiments. Middle and right graphs, quantification of the Western blottings of cyclins that were normalized with the expression of α-tubulin (n = 3). *, p < 0.05, and ***, p < 0.001 (cyclin A, one-way ANOVA, F(6,14) = 17.92; p < 0.0001; cyclin B1, one-way ANOVA, F(5,12) = 69.17; p < 0.0001). D, Western blotting showing the effects of nuclear InsP₃ buffer on the expression of the cyclins A and B1. Right graph, quantification of the Western blottings of cyclins that were normalized with the expression of α-tubulin (n = 3). **, p < 0.01 (cyclin A and B1 used a Student’s t test).

AGUUCAGCGGUUAU-3’, 5’-GCUCAAUCCCAUACCCGACA-3’, 5’-GACCAUUGCGUAGGCGAUU-3’, and CAACAAGGUUACCCGCCACA; Dharmacon, catalog no. L-005065-01 (PLCδ4 siRNA 2, 5’-AAGGCAGGUUGCAACUAGAAAUUCAC-3’) (IDT, catalog no. hs.Ri.PLCD4.13.3). 50 nm of the following nontargeting siRNAs were used: Silencer Select Negative Control No. 2 siRNA (control siRNA 1, Life Technologies, Inc., catalog no. 4390846) and IDT Scrambled negative control.
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Figure 7. Proposed model: EGF triggers nuclear PI(4,5)P_{2} hydrolysis, local InsP_{3} production, and Ca^{2+} release into the nucleoplasm through intra-nuclear PLCγ4, which mediates cell proliferation. Scheme of the proposed EGF-induced nuclear Ca^{2+} signaling pathway. Upon EGF binding EGFR is activated and initiates signal transducer cascades. EGFR is shuttled to the nucleus to induce downstream targets. PLCγ4 is a nuclear enzyme induced by EGF stimulus and leads to PI(4,5)P_{2} hydrolysis that generates InsP_{3} and DAG. InsP_{3} binds to InsP_{3}R present on the inner nuclear envelope triggering Ca^{2+} release in the nucleoplasm. DAG can activate nuclear-resident PKCs. Ca^{2+} and PKCs can activate downstream targets which ultimately result in cell proliferation.

DsiRNA (control siRNA 2, 5’- CUUCCUCUUCUUUCUCC-CUUGUGA-3’, catalog no. 51-01-19-08). Further analysis was performed 48 h after transfection.

InsP_{3}-buffer constructs and calcium imaging

Generation of InsP_{3}-buffer constructs was as described previously (7). Briefly, the InsP_{3}-binding domain (residues 224–605) of the human type I InsP_{3} receptor was tagged with an mRFP and with a nuclear localization signal or nuclear exclusion signal to generate the nuclear and cytosolic constructs, respectively. The adenoviruses were built by ViraQuest Inc. Cells were infected with 10 multiplicities of infection, and analysis was performed 48 h. For calcium imaging, cells were incubated with 5 μM Fluo-4/AM (Life Technologies, Inc.) and incubated with anti-BrdU–POD for 90 min at room temperature. After three washing steps, 3,3’-Tetramethylbenzidine was added, and the plate was read at 370 and 492 nm (Synergy 2, Biotek).

For proliferation curve assay, SKHep-1 cells were transfected with control siRNA 1 or PLCγ4 siRNA 1. After 48 h, cells were collected by trypsinization and counted in a Neubauer chamber using trypan blue for viability exclusion in triplicate.

For the proliferation recovery experiment, the Tet-pLKO-neo plasmid (Addgene ID. 21916) was used to express shRNAs under the control of a doxycycline promoter. The PLCγ4 shRNAs target sequences were obtained from “The RNAi Consortium Collection” (https://portals.broadinstitute.org/gpp/public/), and the shRNAs were designed and cloned according to Addgene instructions. In summary, shRNA sequences were subcloned into Tet-pLKO-neo after digestion with AgeI and EcoRI. We used the following target sequences for the shRNAs: PLCγ4 shRNA 1 (5’-AGAGCAGCGTCGAGGGATATA-3’); PLCγ4 shRNA 2 (5’-ACTACCACCTCTACGAGATAT-3’);

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scramble shRNA 1 (5′-CTAAGGTTAAGTGCCCTCG-3′); and scramble shRNA 2 (5′-CUUCUCUCUUUCUCUCCCU-UGUGA-3′). SKHeP-1 cells were transfected and selected with 500 ng/ml G418. Stable cells were treated with 1 μg/ml doxycycline for 48 h and then were kept without doxycycline for another 48 h for PLCδ4 expression recovery. BrdU assay was performed as described above.

**Cell death assay**

For this experiment, the cells were incubated with 10 μg/ml doxorubicin for 24 h, as a positive control for cell death. The cells were incubated with annexin V–FITC (Life Technologies, Inc.) and propidium iodide (Life Technologies, Inc.) for 1 h. Images were collected with a ×10 and ×20 objective lens on an Olympus IX70 inverted fluorescence microscope (Olympus America, Melville, NY), and the data obtained were quantified by counting cell labeled for annexin V–FITC, propidium iodide, double-labeled, and crossed with bright-field images used to obtain total cell count. Each treatment was made in triplicate, and three different images were counted, and the means were used for statistical analysis.

**Statistical analysis**

The significance of changes in treatment groups was determined by Student’s t test or one-way analysis of variance with Tukey’s multiple comparison tests if not otherwise stated, using GraphPad Prism software. Data are represented as mean ± S.E.

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