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# Temporal production of the signaling lipid phosphatidic acid by phospholipase D2 determines the output of ERK signaling in cancer cells

Feng Zhang[a], Ziqing Wang[a], Maryia Lu[a], Yoshiya Yonekubo[a], Xiao Liang[ab], Yueqiang Zhang[a], Ping Wu[a], Yong Zhou[a], Sergio Grinstein[c], John F. Hancock[a] and Guangwei Du[a, #]

[a]Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin St, Houston, TX 77030, United States.

[b]Shanghai Institute of Digestive Disease, Shanghai Renji Hospital, Shanghai Jiao Tong University School of Medicine, 145 Shan-dong Zhong Rd, Shanghai 200001, China

[c]Division of Cell Biology, Hospital for Sick Children
555 University Ave., Toronto, M5G 1X8, Canada

**Running title:** Phosphatidic acid regulation of ERK signaling

[#]**To whom correspondence should be addressed**: Guangwei Du, Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin St, Houston, TX 77030, United States. Tel: (713)500-7055; FAX: (713)500-7444; Email: guangwei.du@uth.tmc.edu

**Abbreviations:** DAG (diacylglycerol), DGK (diacylglycerol kinase), EGF (Epidermal growth factor), ERK (extracellular signal-regulated kinases), FIPI (5-fluoro-2-indolyl des-chlorohalopemide), FLIM-FRET (fluorescence lifetime imaging microscopy-Förster resonance energy transfer), GEF (guanine exchange factor), PA (phosphatidic acid), PASS (Phosphatidic Acid biosensor with Superior Sensitivity), $PI(4,5)P_2$ (phosphatidylinositol *4*,*5*-bisphosphate), $PI(3,4,5)P_2$ (phosphatidylinositol 3,*4*,*5*-bisphosphate), PLD (phospholipase D), PM (plasma membrane), PMA (phorbol-12-myristate-13-acetate).

**Abstract**

The Ras-extracellular signal-regulated kinases (ERK) cascade is an important signaling module in cells. One regulator of the Ras-ERK cascade is phosphatidic acid (PA) generated by phospholipase D (PLD) and diacylglycerol kinase (DGK). Using a newly developed PA biosensor, PASS (Phosphatidic Acid biosensor with Superior Sensitivity), we found that PA was generated sequentially by PLD and DGK in epidermal growth factor (EGF)-stimulated HCC1806 breast cancer cells. Inhibition of PLD2, one of the two PLD members, was sufficient to eliminate most of the PA production, whereas inhibition of DGK decreased PA production only at the later stages of EGF stimulation, suggesting that PLD2 is prior to DGK activation. The temporal production of PA by PLD2 is important for the nuclear activation of ERK. While inhibition of both PLD and DGK had no effect on the overall ERK activity, inhibition of PLD2, but not PLD1 or DGK, blocked the nuclear ERK activity in several cancer cell lines. The decrease of active ERK in the nucleus inhibited the activation of Elk1, an ERK nuclear target, leading to decreased proliferation of HCC1806 cells. Together, these findings reveal that PA production by PLD2 determines the output of ERK in cancer cell growth factor signaling.

**Key words:** ERK, PASS, phosphatidic acid, phospholipase D, PLD2.

## Introduction

Phosphatidic acid (PA) has attracted increasing attention in recent years due to its roles as a signaling molecule and as a central intermediate in the synthesis of membrane lipids (1-3). PA can be produced by multiple enzymes, including two well-known families of enzymes: phospholipase D (PLD) and diacylglycerol kinase (DGK) (4-7). In mammalian cells, there are two PLD family members, PLD1 and PLD2, which differ strikingly in subcellular localization and function (5,7). The mammalian DGK family consists of 10 members, classified into five different subtypes characterized by different regulatory domains (6). It has been proposed that activation of distinct PA-generating enzymes at different times and in different subcellular compartments determines the specific cellular functions of PA, including cell proliferation, survival and migration (1,5).

One of the most important intracellular signaling pathways involves the cascade of Ras, Raf, MEK, and the extracellular signal-regulated kinases 1 and 2 (ERK1/2, referred to as ERK herein) (8,9). Activated ERK can either remain in the cytoplasm or translocate to the nucleus, where it phosphorylates and activates a number of proteins that control proliferation, differentiation, survival, apoptosis and development (8-10). The precise outcome of stimulating the Ras-ERK cascade depends on the duration, strength, and localization of the signals (8,10,11). It has been reported that PA is involved in the regulation of the Ras-ERK pathway in fibroblasts and lymphocytes (4,12-14). However, the mechanisms whereby PA regulates the Ras-ERK cascade appear to be very distinct in different cell types. Moreover, it remains unknown how growth factors activate different PA-generating enzymes, *i.e.*, PLD and DGK, and whether PA generated from different sources regulates the Ras-ERK cascade in the same manner. Importantly, signaling by growth factors such as epidermal growth factor receptor (EGFR) and the Ras-ERK cascade is frequently upregulated in many types of cancer (15,16). Interestingly, the PA-generating enzymes, PLD and DGK, have also been reported to be critical for proliferation, migration and survival of

cancer cells (6,7,17). It is not clear how and why dysregulation of the Ras-ERK cascade by PA contributes to cancer initiation and progression.

To study the functions of PA, it is critical to faithfully monitor its spatiotemporal production. Traditionally, PA levels have been measured using biochemical methods such as thin layer chromatography (TLC) and high-performance liquid chromatography (18). In recent years, identification and quantification of various lipids, including PA, have become more simple and sensitive with substantially improved mass spectrometry analyses (19,20). However, all these biochemical techniques measure only the total cellular PA level, and cannot reveal the intracellular locations of PA production. In addition, when PA is measured by biochemical methods, the relatively high level of PA on the surface of the ER, where it is used as a precursor for the synthesis of phospholipids and triglycerides (TAG) (3,21), may mask the changes of the comparatively less abundant PA generated during signaling at the plasma membrane and other intracellular organelles. As an alternative method, changes in phospholipid levels can be detected by using fluorescently-tagged protein domains that bind specifically to certain lipids. For example, PH domains from PLCδ and AKT have been used widely to monitor phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), respectively (18,22). Such reagents have greatly advanced our understanding of the dynamics and functions of phosphatidylinositides. However, despite great interest (23), we still lack a PA biosensor with the specificity and sensitivity comparable to those of the phosphatidylinositide probes.

In the present study, we report the development of a specific and sensitive PA biosensor. Using this new tool, we demonstrate that PA production is differentially controlled by PLD and DGK in epidermal growth factor (EGF) signaling, and that PA generated by PLD2 is critical for the nuclear activity of ERK and proliferation in cancer cells. Our findings reveal that PLD2-generated PA determines the signaling output of ERK.

## Experimental procedures

*General reagents and antibodies* –α-tubulin, Flag, phospho- and total ERK1/2 antibodies, Phosphatidylserine (PS), and the PLD inhibitor 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) (PLDi) were from Sigma-Aldrich (St. Louis, MO) (24). The inhibitors for PLD1/2 (VU0155056), PLD1 (PLD1i, VU0359595), and PLD2 (PLD2i, VU0285655-1) were from Avanti Polar Lipids (Alabaster, AL) (25). The DGK inhibitor R59022 (DGKi) was from Calbiochem (La Jolla, CA). EGFR, phospho- and total Elk1, phospho- and total Fra1, total c-Fos and phospho-Rsk antibodies, were from Cell Signaling Technology (Danvers, MA). Mouse GST, phospho-c-Fos and PLD1 rabbit monoclonal antibodies were from Abcam (Cambridge, MA). PLD2 rabbit polyclonal antibody was kindly provided by Dr. Y. Banno (Gifu University of Tokyo, Gifu, Japan). Goat anti-mouse and anti-rabbit IgGs conjugated with Alexa 680 were from Invitrogen. Goat anti-mouse and anti-rabbit IgGs conjugated with IRDye 800CW were from Rockland Immunochemicals (Gilbertsville, PA). 1,2-Dilauroyl-sn-glycero-3-phosphate (DLPA) was from Echelon (Salt Lake City, UT).

*Construction of plasmids* – To generate EGFP-PASS, a nuclear export sequence (NES) derived from protein kinase A inhibitor (PKI)-α (26) was added between EGFP and Spo20-PABD cloned in pEGFP-C1 (27,28). PASS tagged with monomeric GFP or RFP (mGFP or mRFP) was generated by replacing EGFP in the EGFP-PASS with mGFP or mRFP using *Age* I and *BsrG* I sites. Different tags did not change the localization of PASS. All PASS constructs used in the current study are tagged with monomeric GFP and RFP. GST-PASS-His was generated by inserting PASS and a pair of oligos encoding 6xHis into the *BamH* I site of pGEX-4T1 from GE Healthcare Biosciences (Pittsburgh, PA). The 4E mutant was generated by site-directed mutagenesis using the QuikChange kit from Agilent Technologies (Santa Clara, CA). The lentiviral vectors carrying GFP-PASS and

RFP-PASS were subcloned into the *Nhe* I and *BamH* I sites of pCDH-CMV-MCS and pCDH-CMV-MCS-EF1-Puro from System Biosciences (Mountain View, CA), respectively. The shRNAs for PLD1 and PLD2 characterized in the previous publications (12,28-31) were subcloned into the lentiviral vector pLKO.1.

*Bacterial expression, purification of recombinant PASS, and liposome pulldown assay* - Expression and purification of GST-PASS-His using $Ni^{2+}$-chelate chromatography from Qiagen (Valencia, CA) were as described before (32). All purified recombinant proteins used in our experiments are >95% pure, as judged by Coomassie Blue stained SDS-PAGE (polyacrylamide gel electrophoresis) gels. The purified proteins were used within one week. The preparation of liposomes has been previously described (32,33). POPC (phosphatidylcholine), DOPE (Phosphatidylethanolamine), and POPA (phosphatidic acid) were from Avanti Polar Lipids, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) were purchased from Matreya (Pleasant Gap, PA).

*Cell culture and treatment* – Cell culture media were from Thermo Fisher (Waltham, MA). Sera were from Invitrogen (Carlsbad, CA). All cell lines were from ATCC (Manassas, VA). CHO cells were grown in Ham's F12 medium. HCC1806 and HCC827 cells were grown in RPMI-1640 medium. MDA-MB-468 and A431 cells were grown in Dulbecco's Modified Eagle's Medium. BT-20 cells were grown in Eagle's Minimum Essential Medium. All media were supplemented with 10% fetal bovine serum. For inhibitor treatment, cells were pre-incubated with dimethyl sulfoxide (DMSO) or inhibitors for 1 hr. Transient transfection in CHO cells was done using the deacylated polyethylenimine reagent (PEI) (34). Unless specified, cells were serum-starved overnight and then stimulated with 100 nM PMA, 100 μM PA (DLPA), 100 μM PS, or 100 ng/ml EGF for the indicated time. For cell proliferation measurements, cells were seeded in 6-well plates in RPMI-

1640 media containing 0.1 % FBS and 5 ng/ml EGF in the presence of DMSO or inhibitors, or in the presence of control and PLD shRNAs. Viable cells were measured by trypan blue exclusion.

*Lentivirus production and transduction* - The delivery of expression constructs in HCC1806 cells was through lentiviral infection. Viruses were generated in TLA-293T cells from Thermo Fisher by co-transfecting four plasmids including the lentiviral vector, pMDLg/pRRE, pRSV-Rev and pMD2.G using Lipofectamine and Plus reagent from Invitrogen. At 48 hours and 72 hours post-transfection, virus-containing supernatants were collected for infection. The cells were used for experiments 2-3 days postinfection.

*Western blot analysis* – Fluorescently labeled secondary antibodies were used for Western blot, and detected by the LI-COR Odyssey Infrared Imaging System from LI-COR Biotechnology (Lincoln, NE). Band intensity was quantified using the program supplied by the manufacturer. The bands on Western blots were defined by identical region of interest, and measured as integrated pixel intensity for all samples. The background was determined by measuring the average pixel intensity of a user-defined area.

*Confocal microscopy* – All images were captured with a 60X oil-immersion objective on a Nikon A1 confocal microscope (Melville, NY). Fields with moderate cell confluence were selected for measurement. For visualizing GFP and RFP fluorescence of PASS, cells grown on coverslips were transiently transfected (CHO cells) or infected with lentiviruses (HCC1806 cells), and then directly fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. After several PBS washes, the coverslips were mounted onto slides using 4% propyl gallate mounting solution. The fluorescent images of GFP and RFP of random fields were collected. For each treatment, at least five fields, including more than 50 cells, were quantified. Cells with saturated exposure were not used for quantification. The change in the plasma membrane localization was quantified using the line pixel intensity histogram in NIH Image J as described previously (22). A line was randomly

drawn to span a large area of the cytoplasm, but not the nucleus. The pixel intensity of the two most outside peaks represented the PASS on the plasma membrane, while the average value between represented that in the cytoplasm.

For pERK staining, cells were stained with a pERK1/2 antibody/Alexa 594-conjugated goat anti-mouse secondary antibody and DAPI after cold methanol fixation at -20°C for 10 minutes. After defining the nuclei with DAPI staining, the cytoplasmic and nuclear areas were manually marked, and then measured for the fluorescent intensity of pERK staining using NIH Image J. The change of pERK localization was represented as the ratio of pERK in the nucleus and cytoplasm.

*Statistics* – All statistical differences were evaluated between control and each of the treatments using two-tailed student's t-test. All data are shown as mean ± SD.

## Results

### Development of a sensitive PA biosensor

To study the regulation and functions of PA in distinct cellular compartments, it is important to precisely monitor the spatial and temporal production of PA. Currently, the most widely used PA biosensor is the PA-binding domain derived from a yeast protein Spo20 (Spo20-PABD) (23,28,35). However, green fluorescent protein (GFP)-tagged Spo20-PABD only responded to strong stimuli such as phorbol-12-myristate-13-acetate (PMA) (24) and depolarizing concentrations of potassium (35), or some cellular processes such as cell replating (28). We were unable to detect changes of PA levels in cells stimulated with ligands for membrane receptors using this PA biosensor. Even in cells stimulated with PMA, a potent activator of PLD activity (36,37), the membrane localization of GFP-Spo20-PABD was barely visible in most cells unless the images were overexposed. These results seem contradictory to the original reports that Spo20-PABD binds to PA with exceptional

sensitivity and specificity *in vitro* (23,38,39). Since GFP-Spo20-PABD predominantly localizes to the nucleus, we reason that the nuclear localization prevents it from accessing PA molecules on the plasma membrane or other cytoplasmic organelles. To improve the sensitivity of Spo20-PABD, we added a nuclear export sequence (NES) derived from protein kinase A inhibitor-α (PKI-α) (26) to the N-terminus of the Spo20-PABD (Fig. 1A), and named the resulting construct PASS (Phosphatidic Acid biosensor with Superior Sensitivity) due to its exceptional sensitivity to detect the cellular PA levels (further demonstrated in Figs. 2 and 3). We first tested whether the addition of NES affected the binding specificity of PA *in vitro* using recombinant PASS protein purified from *E. coli* and synthetic liposomes. As controls, we also included liposomes containing $PI(4,5)P_2$ or $PI(3,4,5)P_3$, since the original Spo20-PABD also showed some binding to these lipids (23,38). As expected, we found that PASS specifically bound to PA-containing liposomes in a concentration-dependent manner (Fig. 1B). In sharp contrast, PASS bound to $PI(4,5)P_2$- or $PI(3,4,5)P_3$-containing liposomes with much lower affinity (Fig. 1B). These results are consistent with the previous finding that Spo20-PABD binds to PA selectively, and confirm that addition of the NES does not change the PA-binding specificity of PASS.

We next tested whether PASS can be used to measure PA levels in intact CHO cells when fused with either a green fluorescent protein (GFP) or red fluorescent protein (RFP). PLD activity is very low-to-undetectable in unstimulated (basal) CHO cells, but can be elevated by stimulation with PMA or by overexpression of PLD2 (24,28,40). As observed before, the original GFP-Spo20-PABD mainly localized to the nucleus in both basal and PMA-stimulated conditions ((28,35) Fig. 2A). Two other PA biosensors, GFP-Raf1-PABD (27,41) and GFP-Sos1-PH (12), localized to the cytoplasm in basal conditions, and only partially translocated to membranes in PMA-stimulated conditions (Fig 2A). In addition, GFP-Raf1-PABD often aggregated in some cells (Fig 2A), and induced cell toxicity when it was expressed for more than two days (not shown). In contrast, both

GFP- and RFP-tagged PASS localized diffusely in the cytoplasm under nonstimulatory conditions, but translocated to the plasma membrane and endosome-like vesicles upon PMA stimulation (Fig. 2B) when PLD was activated (29,33,42). Furthermore, the plasma membrane translocation of PASS induced by PMA was blocked when PLD activity was inhibited by FIPI (24) (Fig. 2B). The mutation of four lysine residues to glutamic acid (K66E, K68E, R71E, and K73E, called 4E hereinafter) on the original Spo20-PABD disrupted its binding to PA (38). We generated the same 4E mutations in RFP-PASS. As expected, PMA stimulation caused the plasma membrane translocation of the wild-type GFP-PASS, but not that of the PA-binding deficient mutant RFP-PASS-4E (Fig. 2C). Another way to increase the cellular PA level is through overexpression of PLD2, which has a high basal activity (42,43). As before, GFP-PASS, but not the RFP-PASS-4E mutant, was recruited to the plasma membranes in CHO cells stably overexpressing mouse PLD2 (29) (Fig. 2D). In contrast, PASS localized diffusely in the cytoplasm, and only translocated to the plasma membrane and intracellular vesicles upon PMA treatment in CHO cells stably overexpressing human PLD1 (Fig 2D), which has a low basal activity and is activated by external stimulation (30,36). The increased PASS localization on intracellular vesicles in PLD1-overexpressing cells suggests the activation of PLD1 on these organelles, where the majority of PLD1 is localized (7,33). Finally, we tested whether addition of exogenous PA to culture medium was able to recruit GFP-PASS to the plasma membrane; PA can be rapidly incorporated into the plasma membrane and can induce cellular responses (12,44,45). As expected, exogenously added PA stimulated translocation of GFP- and RFP-PASS to the plasma membrane, but failed to induce the translocation of RFP-PASS-4E (Fig. 2E). In contrast, external addition of another negatively charged phospholipid, phosphatidylserine (PS), did not cause recruitment of GFP- and RFP-PASS to the plasma membrane (Fig. 2E), further supporting the specificity of this biosensor.

**EGF regulation of temporal PA production in HCC1806 breast cancer cells**

Our ultimate goal was to study PA dynamics during the course of growth factor stimulation. Due to their poor sensitivity, we were not able to observe consistent membrane translocation of several previously generated PA biosensors, *i.e.*, GFP-Raf1-PABD, GFP-SOS-PH and GFP-Spo20-PABD, in cells stimulated with EGF or other growth factors. Having established the specificity and improved sensitivity of PASS, we used this probe to examinutee growth factor signaling in cancer cells. We initially used HCC1806 cells, a good experimental model for EGF signaling and aggressive triple-negative breast cancer (46,47). GFP-PASS diffusely localized in the cytoplasm of serum-starved HCC1806 cells, but quickly translocated to the plasma membrane within 1 minute of EGF stimulation (Fig. 3A and 3B), reaching maximal levels at 3 minutes. Most of the GFP-PASS returned to the cytoplasm after 30 minutes (Fig. 3A and 3B). To our knowledge, PASS is the only probe capable of detecting such dynamic changes in PA subcellular distribution in response to physiological stimuli like growth factors.

Two major sources of PA are phosphatidylcholine and diacylglycerol (DAG), which are used as the substrates by PLD and DGK, respectively. To determine the source of PA in response to EGF, we treated cells with PLD or DGK inhibitors. Interestingly, PLD and DGK inhibitors showed different effects on the EGF-dependent translocation of GFP-PASS. The PLD inhibitor FIPI almost completely abolished the translocation of GFP-PASS to the membrane (Fig. 3A and 3B). In contrast, the DGK inhibitor R59022 only blocked the late (≥ 3minutes), but not the early (1 minute) component of GFP-PASS translocation (Fig. 3A and 3B). These results suggest that upon EGF stimulation, PA is sequentially generated by PLD and DGK. To determine the PLD isoform responsible for PA generation in response to EGF, we examined the localization of PASS in HCC1806 cells infected with lentiviruses carrying either control luciferase (Luc) small hairpin RNAs (shRNAs) or shRNA targeting PLD1 or PLD2 (12,28,29), or treated with PLD isoform-

specific inhibitors (25). Only PLD2 shRNA or inhibitor, but not the control, PLD1 shRNA or inhibitor, blocked the plasma membrane translocation of PASS triggered by EGF stimulation (Fig. 3C-E). In addition, PLD2 was mainly localized on the plasma membrane in HCC1806 cells before and after EGF stimulation, whereas PLD1 was mainly localized to perinuclear vesicles (Fig. 3F). Taken together, these results suggest that PLD2 is the predominant source of PA.

**PLD2-generated PA is important for EGF-triggered nuclear ERK activation**

Since PA regulates the Ras-ERK cascade in fibroblasts and lymphocytes (4,12-14), we investigated whether PA generated from PLD and DGK also regulates ERK activation in cancer cells. ERK activity (as measured by phosphorylated ERK1/2, pERK) was low in non-stimulated HCC1806 cells, and was not changed by treatments with the PLD and DGK inhibitors (Fig. 4A and 4B). EGF stimulation triggered a rapid increase in pERK levels, which reached a maximum at 3 minutes and was maintained for more than 2 hours. However, inhibition of either PLD or DGK did not affect the formation of pERK level in EGF-stimulated HCC1806 cells, as measured by Western blot analysis (Fig. 4A and 4B). Downregulation of the expression of PLD isoforms using shRNAs also failed to affect ERK phosphorylation (Fig. 4C and 4D). We next assessed ERK activation and localization by monitoring pERK in the nucleus and cytoplasm using quantitative fluorescence microscopy. The staining of activated ERK (pERK) in untreated cells was already strong in the cytoplasm 3 minutes after EGF stimulation, and by 6 minutes activated ERK became abundant in the nucleus (Fig. 4E and 4F). Addition of PLD and DGK inhibitors did not affect ERK activation or alter its kinetics of appearance in the cytoplasm. However, the PLD inhibitor significantly blocked the appearance of nuclear pERK staining after 3 minutes of EGF stimulation. In contrast, the DGK inhibitor only slightly decreased the nuclear pERK staining at all the time points tested (Fig. 4E and 4F).

We then examined which PLD isoform was responsible for regulating nuclear ERK activity using shRNAs. While expression of Luc and PLD1 shRNAs did not change the nuclear and cytoplasmic distribution of pERK in cells stimulated with EGF, PLD2 shRNA inhibited the appearance of nuclear pERK staining (Fig. 4G and 4H), as found in cells treated with pan-PLD inhibitor. To further confirm that PA is responsible for PLD2 regulation of nuclear ERK activity, we treated HCC1806 cells with both PLD2 inhibitor and different concentrations of PA. Addition of exogenous PA rescued the decrease in pERK staining in a dose-dependent manner (Fig. 4I and 4J). These results suggest that PLD2 activity is required for the nuclear translocation of activated ERK. Activations of EGFR on the plasma membrane and endosomes lead to selective activation of ERK on different subcellular compartments or distinct biological activities (8,10,48,49). Previous studies in fibroblasts found that overexpression of catalytically inactive mutants for PLD1 and PLD2 blocked the activation of ERK by inhibiting the endocytosis of EGFR (50). To determine if PLD2 regulates ERK activation in the nucleus through modulating EGFR endocytosis in HCC1806 cells, we also examined the localization of EGFR in the presence and absence of PLD2 inhibitor. PLD2 inhibitor treatment did not seem to affect the localization of EGFR before and after EGF stimulation (Fig 4K). This result, together with the dominant plasma membrane localization of PLD2 and PASS (Fig 3), suggest that PLD2 regulation of the nuclear ERK activity is independent of EGFR endocytosis.

We then addressed the question whether PLD2 also controls the activation of ERK in the nucleus in other cancer cells treated with EGF. Both PLD2 inhibitor and shRNAs significantly blocked the nuclear staining of pERK in all four cancer cell lines we tested: HCC827 (lung cancer), MDA-MB-468 and BT-20 (breast cancer), and A431 (skin cancer) (Fig. 5), suggesting that PLD2 regulation of nuclear ERK activity is a universal phenomenon in cancer cells.

**Inhibition of PLD2 suppressed the activity of Elk1 and cell proliferation in HCC1806 cells**

After translocation into the nucleus, active ERK phosphorylates several proteins involved in transcriptional regulation. One of the best studied ERK targets in the nucleus is Elk1 (11,51). Consistent with the decrease in pERK in the nucleus, we found that the PLD inhibitor caused a decrease in Elk1 phosphorylation, whereas the DGK inhibitor did not (Fig. 6A and 6B). Moreover, only PLD2 shRNA but not PLD1 shRNA, inhibited Elk1 phosphorylation (Fig. 6C and 6D), supporting our findings that PLD2 is specifically required for ERK activity in the nucleus in HCC1806 cells. We also checked the phosphorylation of other ERK substrates. Without phosphorylation, c-Fos and Fra1, the other two nuclear substrates of ERK, undergo rapid degradation; c-Fos and Fra1 are stabilized when phosphorylated by ERK (52-54). As expected, EGF stimulation increased both phosphorylation and total protein levels of c-Fos and Fra1, which were inhibited by PLD but not DGK inhibitor treatment (Fig. 6E and 6F). In contrast, inhibition of PLD and DGK had no effect on phosphorylation and total protein levels of a cytoplasmic substrate of ERK, Rsk (8,11) (Fig. 6E and 6F).

Phosphorylation of ERK nuclear targets increases their affinity for the serum response factor, and thus enhances transcription of proliferation-promoting (16,51). The observation that PLD2 inhibition decreased the activity of Elk1, c-Fos and Fra1, implies that PLD2 activity is required for cell proliferation. To test this hypothesis, we examined cell proliferation in HCC1806 cells in the presence of inhibitors or shRNAs. As expected, the rate of cell proliferation corresponded to the phosphorylation and protein levels of Elk1, c-Fos and Fra1. Cells treated with a PLD1 inhibitor, DGK inhibitor, or expressing PLD1 shRNA showed similar proliferation rates as the controls (Fig. 7). However, cells treated with the PLD2 inhibitor or expressing PLD2 shRNA displayed marked decrease in cell proliferation (Fig. 7). These results suggest that PLD2 regulates cell proliferation through modulating the activity of nuclear ERK and its nuclear substrates in

HCC1806 breast cancer cells.

## Discussion

### The new biosensor PASS offers a useful tool to study phospholipid signaling

The ability to precisely monitor the dynamics of PA in different subcellular locations is important for understanding the role of PA signaling in cells. We and others have developed several PA biosensors, *i.e.*, the PA-binding region of Raf-1 (Raf1-PABD) (41) and Spo20-PABD (28,35), the Sos1 PH domain (12), and the Pii FRET biosensors derived from Dock2 and Sos1-PH (55). However, compared to the phosphoinositide probes, none of these PA biosensors has been widely used, because they either lack sufficient sensitivity, require a special microscopy setup, or only monitor PA production in certain membrane compartments. The PA biosensor PASS developed in the current study has circumvented these problems. The specificity of PASS as a PA biosensor has been validated by a series of *in vitro* and *in vivo* experiments: (1) it bound to PA with high affinity and specificity, as shown by a liposome-pull-down assay using the purified recombinant protein; (2) increasing cellular PA levels by PMA stimulation, exogenous addition of PA, or by overexpression of PLD2 promoted the plasma membrane binding of PASS tagged with either GFP or RFP; (3) association of PASS with the membrane was abolished by treatment with PLD inhibitors and expression of PLD2 shRNA; (4) point mutations that disrupt PA binding *in vitro* inhibited the recruitment of PASS to the membrane.

The most significant improvement of PASS is its exceptional sensitivity. We have attempted to use several previously developed PA biosensors, *i.e.* Raf1-PABD, Spo20-PABD and Sos1-PH, to monitor PA levels in various cells. However, only a small fraction of these biosensors, if any, translocated to membranes upon PMA (Fig 2A) and growth factor stimulation. PASS is by far the only PA biosensor that quickly responds to stimulation and binds to the plasma membrane of the

352 various cell types that we have tested. In addition, GFP- Raf1-PABD also aggregates in cells and
353 causes cell toxicity. The binding of Sos1-PH to both PI(4,5)$P_2$ and PA also limits its use as a PA-
354 specific biosensor (12). To increase PA detection, previous PA biosensors derived from Spo20-
355 PABD contain tandem repeats of Spo20-PABD (35,56). We find that PASS described in our current
356 work (contain one copy of Spo20-PABD fused to NES) responds to stimulation equally well or
357 better while having lower background membrane binding. In addition, we also observed that PASS
358 labeling of some intracellular vesicles, presumably endosomes, are further increased by PLD1
359 overexpression in PMA-stimulated cells (Fig. 2). These results support the previous findings that
360 PLD and DGK function on both the plasma membrane and endosomes (4-7). Moreover, a tandem
361 repeat version of PASS labels also the endoplasmic reticulum in macrophages, HeLa, and HEK293
362 cells (57). This is particularly interesting since PASS is the only PA biosensor currently known to
363 label the PA pool at the endoplasmic reticulum, which is the site of phospholipid and triglyceride
364 synthesis (3). Finally, when paired with specific membrane markers, the new biosensor may also be
365 used for fluorescence lifetime imaging microscopy-Förster resonance energy transfer (FLIM-FRET)
366 or FRET analysis to examine the production of PA in different membrane domains. Compared to
367 the Pii FRET sensors (55), the pairing of PASS with an independent membrane marker should
368 provide a more reliable assessment of the sites of PA production. It is noteworthy that, to increase
369 their sensitivity, the Pii biosensors are targeted to the plasma membrane through the C-terminus of
370 K-Ras (55). Since the C-terminus of K-Ras only resides in a subdomain of the plasma membrane
371 (58,59), the Pii biosensors cannot, in principle, monitor the PA generated in other plasma membrane
372 domains or in intracellular organelles.
373
374 **Differential activation and functions of PLD and DGK in growth factor signaling in cancer**
375 **cells**

In mammalian cells, there are two PLD family members and ten members in the DGK family (4-7). It has been unclear how different PA-generating enzymes contribute to PA production upon activation of a particular signaling pathway, and whether different sources of PA perform the same functions. An important finding from our experiments is that EGF triggers a sequential activation of PLD and DGK in distinct membrane nanodomains. Moreover, our results also suggest that DGK activation may be dependent on PLD activity. It is likely that, as an early event in EGF signaling, PLD2 activation is required for the activation of DGK or for the production of DAG, the substrate of DGK. More work is still needed to further investigate the crosstalk between PLD and DGK. These include monitoring the production of both PA and DAG using PASS and DAG biosensors, and examining the spatiotemporal activation of their downstream signaling pathways, in cells where PLD or DGK is inhibited individually, or simultaneously. In combination with small molecule inhibitors and RNA interference, the newly developed PASS should be a useful tool in determining the regulation of individual PA-generating enzymes and of their cellular functions in different physiological and pathological conditions.

**PLD and DGK differentially regulate Ras-ERK signaling in cancer cells**

Various mechanisms are thought to ensure the specific outcomes of Ras-ERK signaling (60). However, little is known about how and why the activation of the Ras-ERK components in separate cellular compartments might be regulated. Previous studies have suggested that the site where Ras is activated dictates the nature of the downstream signals and of subsequent biological outputs elicited (60-62). For example, Ras activated on the plasma membrane led to the translocation of activated ERK to the nucleus, whereas active ERK was restricted to the Golgi when Ras was activated in that compartment (63). These findings are also consistent with the observation that Ras activated on the Golgi was fully dispensable for proliferation and transformation (61). Interestingly,

we found that PLD2 activity is important for the translocation of activated ERK to the nucleus in cancer cells upon growth factor stimulation. In this regard, it is important to note that at the early stages of EGF stimulation PA is generated by PLD2, but not by DGK. Growth factor activation of ERK on the plasma membrane requires the binding of Raf, MEK and ERK to the scaffold protein KSR1 (9,64). However, only activated MEK and ERK transport to the nucleus (9,64), suggesting that disassembly of the Raf/MEK/ERK/KSR1 complex is prerequisite to pERK nuclear translocation. It is therefore conceivable that the stability of the Raf/MEK/ERK/KSR1 complex determines the cytoplasmic and nuclear destination of the pERK, a mechanism similar to those used to retain pERK on the endosomes by β-arrestin-2 and on the Golgi by another scaffold protein Sef (63,64). Interestingly, PA binds to several components of the ERK cascade on the plasma membrane, such as Sos1, Raf1 and KSR1 (12,41,65). It is likely that PLD2-generated PA association to Raf1, KSR1, or other scaffold proteins, potentially weakens their binding to MEK and ERK, therefore allowing MEK/ERK nuclear translocation in cancer cells.

There are clear differences between the mechanisms through which PLD2-generated PA regulates the Ras-ERK cascade in cancer cells and other cell types. In EGF-stimulated fibroblasts, PLD2-generated PA directly regulates the activity of total Ras and ERK levels though Sos1, a Ras guanine exchange factor (12), or Raf1 (41). In lymphocyte function-associated antigen-1-stimulated lymphocytes, PLD2-generated PA activates Ras indirectly when being converted to DAG by PA-phosphatases, which then activates a different Ras guanine exchange factor–RasGRP1 (14). Our findings that the precise control of PA production by PLD2 is critical for the delivery of active ERK to the nucleus of cancer cells contribute a mechanistic insight into many previous observations. It is possible that different expression levels of MAPK components, such as Sos1, Raf1 and scaffold proteins, and PA-generating enzymes, such as PLD and DGK, in different cell types, may affect the duration, magnitude, and compartmentalization of ERK, leading to different biological outcomes.

PLD expression and activity are elevated in a variety of human cancer tissues (66-71) and human cancer cell lines (72-76). Interestingly, high levels of PLD2 activity also correlate with more aggressive cancer phenotypes (77). Moreover, it has been proposed that PLD2 could be a "driver" in breast tumorigenesis, based on the relatively high rate of somatic mutation of PLD2 found in a sequencing analysis of human breast cancer patients (78). Finally, consistent with elevated PLD expression and activity, PLD has been reported to be important for cell proliferation, survival and migration in many cancer cell lines (79,80). It is therefore important to clarify how upregulation of PLD2 expression and activity may "rewire" the MAPK pathway in cancer cells. Further understanding the unique role of PLD2 in cancer cell signaling may help us design strategies for the treatment of human malignancies.

In summary, the present studies demonstrate that the new PA biosensor PASS is capable of recognizing PA with unprecedented specificity and sensitivity, which allows us to monitor the spatiotemporal changes of PA at the single-cell level. Analysis of cellular PA with this biosensor revealed that EGF stimulation activates PLD2 and DGK sequentially. Because the spatiotemporal production of PA by PLD2 is important for the nuclear ERK activity and proliferation, the use of this novel probe should facilitate our research into the biology of both normal and cancerous cells.

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444 **References**


445 1. Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006) Signaling functions of
446 phosphatidic acid. *Prog Lipid Res* **45**, 250-278

447 2. Hancock, J. F. (2007) PA promoted to manager. *Nat. Cell. Biol.* **9**, 615-617

448 3. Carman, G. M., and Han, G. S. (2011) Regulation of phospholipid synthesis in the yeast
449 Saccharomyces cerevisiae. *Annu Rev Biochem* **80**, 859-883

450 4. Zhang, Y., and Du, G. (2009) Phosphatidic acid signaling regulation of Ras superfamily of
451 small guanosine triphosphatases. *Biochim Biophys Acta* **1791**, 850-855

452 5. Selvy, P. E., Lavieri, R. R., Lindsley, C. W., and Brown, H. A. (2011) Phospholipase D:
453 enzymology, functionality, and chemical modulation. *Chemical reviews* **111**, 6064-6119

454 6. Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011) Regulation and functions of
455 diacylglycerol kinases. *Chemical reviews* **111**, 6186-6208

456 7. Peng, X., and Frohman, M. A. (2012) Mammalian phospholipase D physiological and
457 pathological roles. *Acta Physiol (Oxf)* **204**, 219-226

458 8. Murphy, L. O., and Blenis, J. (2006) MAPK signal specificity: the right place at the right
459 time. *Trends Biochem. Sci.* **31**, 268-275

460 9. McKay, M. M., and Morrison, D. K. (2007) Integrating signals from RTKs to ERK/MAPK.
461 *Oncogene* **26**, 3113-3121

462 10. Ebisuya, M., Kondoh, K., and Nishida, E. (2005) The duration, magnitude and
463 compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling
464 specificity. *J. Cell. Sci.* **118**, 2997-3002

465 11. Mebratu, Y., and Tesfaigzi, Y. (2009) How ERK1/2 activation controls cell proliferation and
466 cell death: Is subcellular localization the answer? *Cell Cycle* **8**, 1168-1175

12. Zhao, C., Du, G., Skowronek, K., Frohman, M. A., and Bar-Sagi, D. (2007) Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat. Cell. Biol.* **9**, 706-712

13. Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T.-C., Frohman, M. A., Watkins, S. C., and Romero, G. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the MAP kinase pathway. *J. Biol. Chem.* **274**, 1131-1139

14. Mor, A., Campi, G., Du, G., Zheng, Y., Foster, D. A., Dustin, M. L., and Philips, M. R. (2007) The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nat. Cell. Biol.* **9**, 713-719

15. Dhillon, A. S., Hagan, S., Rath, O., and Kolch, W. (2007) MAP kinase signalling pathways in cancer. *Oncogene* **26**, 3279-3290

16. Roberts, P. J., and Der, C. J. (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**, 3291-3310

17. Park, J. B., Lee, C. S., Jang, J. H., Ghim, J., Kim, Y. J., You, S., Hwang, D., Suh, P. G., and Ryu, S. H. (2012) Phospholipase signalling networks in cancer. *Nat Rev Cancer* **12**, 782-792

18. Rusten, T. E., and Stenmark, H. (2006) Analyzing phosphoinositides and their interacting proteins. *Nat Methods* **3**, 251-258

19. Ivanova, P. T., Milne, S. B., Myers, D. S., and Brown, H. A. (2009) Lipidomics: a mass spectrometry based systems level analysis of cellular lipids. *Current opinion in chemical biology* **13**, 526-531

20. Oliveira, T. G., Chan, R. B., Tian, H., Laredo, M., Shui, G., Staniszewski, A., Zhang, H., Wang, L., Kim, T. W., Duff, K. E., Wenk, M. R., Arancio, O., and Di Paolo, G. (2010)

Phospholipase d2 ablation ameliorates Alzheimer's disease-linked synaptic dysfunction and cognitive deficits. *J. Neurosci.* **30**, 16419-16428

21. Athenstaedt, K., and Daum, G. (1999) Phosphatidic acid, a key intermediate in lipid metabolism. *Eur. J. Biochem.* **266**, 1-16

22. Balla, T., and Varnai, P. (2002) Visualizing cellular phosphoinositide pools with GFP-fused protein-modules. *Sci STKE* **2002**, pl3

23. Kassas, N., Tryoen-Toth, P., Corrotte, M., Thahouly, T., Bader, M. F., Grant, N. J., and Vitale, N. (2012) Genetically encoded probes for phosphatidic acid. *Methods in cell biology* **108**, 445-459

24. Su, W., Yeku, O., Olepu, S., Genna, A., Park, J. S., Ren, H., Du, G., Gelb, M. H., Morris, A. J., and Frohman, M. A. (2009) 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Mol. Pharmacol.* **75**, 437-446

25. Scott, S. A., Selvy, P. E., Buck, J. R., Cho, H. P., Criswell, T. L., Thomas, A. L., Armstrong, M. D., Arteaga, C. L., Lindsley, C. W., and Brown, H. A. (2009) Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. *Nat Chem Biol* **5**, 108-117

26. Henderson, B. R., and Eleftheriou, A. (2000) A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp. Cell. Res.* **256**, 213-224

27. Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N. T., Frohman, M. A., Vitale, N., Bader, M. F., and Grant, N. J. (2006) Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis. *Traffic* **7**, 365-377

28. Du, G., and Frohman, M. A. (2009) A lipid-signaled myosin phosphatase surge disperses cortical contractile force early in cell spreading. *Mol. Biol. Cell.* **20**, 200-208

29. Du, G., Huang, P., Liang, B. T., and Frohman, M. A. (2004) Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. *Mol. Biol. Cell.* **15**, 1024-1030

30. Su, W., Chardin, P., Yamazaki, M., Kanaho, Y., and Du, G. (2006) RhoA-mediated Phospholipase D1 signaling is not required for the formation of stress fibers and focal adhesions. *Cell Signal* **18**, 469-478

31. Mor, A., Wynne, J. P., Ahearn, I. M., Dustin, M. L., Du, G., and Philips, M. R. (2009) Phospholipase D1 regulates lymphocyte adhesion via upregulation of Rap1 at the plasma membrane. *Mol. Cell. Biol.* **29**, 3297-3306

32. Roach, A. N., Wang, Z., Wu, P., Zhang, F., Chan, R. B., Yonekubo, Y., Di Paolo, G., Gorfe, A. A., and Du, G. (2012) Phosphatidic acid regulation of PIPKI is critical for actin cytoskeletal reorganization. *J Lipid Res* **53**, 2598-2609

33. Du, G., Altshuller, Y. M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A. J., Bader, M. F., and Frohman, M. A. (2003) Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. *J. Cell. Biol.* **162**, 305-315

34. Thomas, M., Lu, J. J., Ge, Q., Zhang, C., Chen, J., and Klibanov, A. M. (2005) Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc. Nat. Acad. Sci. USA* **102**, 5679-5684

35. Zeniou-Meyer, M., Zabari, N., Ashery, U., Chasserot-Golaz, S., Haeberle, A. M., Demais, V., Bailly, Y., Gottfried, I., Nakanishi, H., Neiman, A. M., Du, G., Frohman, M. A., Bader, M. F., and Vitale, N. (2007) Phospholipase D1 production of phosphatidic acid at the plasma

membrane promotes exocytosis of large dense-core granules at a late stage. *J. Biol. Chem.* **282**, 21746-21757

36. Xiao, N., Du, G., and Frohman, M. A. (2005) Peroxiredoxin II functions as a signal terminator for H2O2-activated phospholipase D1. *Febs J* **272**, 3929-3937

37. Du, G., Morris, A. J., Sciorra, V. A., and Frohman, M. A. (2002) G-protein-coupled receptor regulation of phospholipase D. *Methods Enzymol.* **345**, 265-274

38. Nakanishi, H., de los Santos, P., and Neiman, A. M. (2004) Positive and negative regulation of a SNARE protein by control of intracellular localization. *Mol. Biol. Cell.* **15**, 1802-1815

39. Liu, S., Wilson, K. A., Rice-Stitt, T., Neiman, A. M., and McNew, J. A. (2007) In vitro fusion catalyzed by the sporulation-specific t-SNARE light-chain Spo20p is stimulated by phosphatidic acid. *Traffic* **8**, 1630-1643

40. Du, G., Altshuller, Y. M., Kim, Y., Han, J. M., Ryu, S. H., Morris, A. J., and Frohman, M. A. (2000) Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. *Mol. Biol. Cell.* **11**, 4359-4368

41. Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G. (2000) The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J. Biol. Chem.* **275**, 23911-23918

42. Jenkins, G. M., and Frohman, M. A. (2005) Phospholipase D: a lipid centric review. *Cell. Mol. Life. Sci.* **62**, 2305-2316

43. Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altshuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191-201

44. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294**, 1942-1945

45. Nishikimi, A., Fukuhara, H., Su, W., Hongu, T., Takasuga, S., Mihara, H., Cao, Q., Sanematsu, F., Kanai, M., Hasegawa, H., Tanaka, Y., Shibasaki, M., Kanaho, Y., Sasaki, T., Frohman, M. A., and Fukui, Y. (2009) Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. *Science* **324**, 384-387

46. Volk-Draper, L. D., Rajput, S., Hall, K. L., Wilber, A., and Ran, S. (2012) Novel model for basaloid triple-negative breast cancer: behavior in vivo and response to therapy. *Neoplasia (New York, N.Y* **14**, 926-942

47. Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., and Pietenpol, J. A. (2011) Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Invest.* **121**, 2750-2767

48. Kranenburg, O., Verlaan, I., and Moolenaar, W. H. (1999) Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. *J. Biol. Chem.* **274**, 35301-35304

49. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274**, 2086-2089

50. Shen, Y., Xu, L., and Foster, D. A. (2001) Role for phospholipase D in receptor-mediated endocytosis. *Mol. Cell. Biol.* **21**, 595-602

51. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**, 403-407

52. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C., and Blenis, J. (2002) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell. Biol.* **4**, 556-564

584 53. Okazaki, K., and Sagata, N. (1995) The Mos/MAP kinase pathway stabilizes c-Fos by
585 phosphorylation and augments its transforming activity in NIH 3T3 cells. *EMBO J.* **14**,
586 5048-5059
587 54. Basbous, J., Chalbos, D., Hipskind, R., Jariel-Encontre, I., and Piechaczyk, M. (2007)
588 Ubiquitin-independent proteasomal degradation of Fra-1 is antagonized by Erk1/2 pathway-
589 mediated phosphorylation of a unique C-terminal destabilizer. *Mol. Cell. Biol.* **27**, 3936-
590 3950
591 55. Nishioka, T., Frohman, M. A., Matsuda, M., and Kiyokawa, E. (2010) Heterogeneity of
592 phosphatidic acid levels and distribution at the plasma membrane in living cells as
593 visualized by a Foster resonance energy transfer (FRET) biosensor. *J. Biol. Chem.* **285**,
594 35979-35987
595 56. Ariotti, N., Liang, H., Xu, Y., Zhang, Y., Yonekubo, Y., Inder, K., Du, G., Parton, R. G.,
596 Hancock, J. F., and Plowman, S. J. (2010) Epidermal growth factor receptor activation
597 remodels the plasma membrane lipid environment to induce nanocluster formation. *Mol.*
598 *Cell. Biol.* **30**, 3795-3804
599 57. Bohdanowicz, M., Schlam, D., Hermansson, M., Rizzuti, D., Fairn, G. D., Ueyama, T.,
600 Somerharju, P., Du, G., and Grinstein, S. (2013) Phosphatidic acid is required for the
601 constitutive ruffling and macropinocytosis of phagocytes. *Mol. Biol. Cell.* **24**, 1700-1712
602 58. Hancock, J. F. (2006) Lipid rafts: contentious only from simplistic standpoints. *Nat Rev Mol*
603 *Cell Biol* **7**, 456-462
604 59. Harding, A. S., and Hancock, J. F. (2008) Using plasma membrane nanoclusters to build
605 better signaling circuits. *Trends Cell Biol* **18**, 364-371
606 60. Prior, I. A., and Hancock, J. F. (2012) Ras trafficking, localization and compartmentalized
607 signalling. *Semin Cell Dev Biol* **23**, 145-153

608 61. Matallanas, D., Sanz-Moreno, V., Arozarena, I., Calvo, F., Agudo-Ibanez, L., Santos, E.,
609 Berciano, M. T., and Crespo, P. (2006) Distinct utilization of effectors and biological
610 outcomes resulting from site-specific Ras activation: Ras functions in lipid rafts and Golgi
611 complex are dispensable for proliferation and transformation. *Mol. Cell. Biol.* **26**, 100-116
612 62. Cheng, C. M., Li, H., Gasman, S., Huang, J., Schiff, R., and Chang, E. C. (2011)
613 Compartmentalized Ras proteins transform NIH 3T3 cells with different efficiencies. *Mol.*
614 *Cell. Biol.* **31**, 983-997
615 63. Torii, S., Kusakabe, M., Yamamoto, T., Maekawa, M., and Nishida, E. (2004) Sef is a
616 spatial regulator for Ras/MAP kinase signaling. *Dev Cell* **7**, 33-44
617 64. Kolch, W. (2005) Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat*
618 *Rev Mol Cell Biol* **6**, 827-837
619 65. Kraft, C. A., Garrido, J. L., Fluharty, E., Leiva-Vega, L., and Romero, G. (2008) Role of
620 phosphatidic acid in the coupling of the ERK cascade. *J. Biol. Chem.* **283**, 36636-36645
621 66. Noh, D., Ahn, S., Lee, R., Park, I., Kim, J., Suh, P., Ryu, S., Lee, K., and Han, J. (2000)
622 Overexpression of phospholipase D1 in human breast cancer tissues. *Cancer Lett* **161**, 207-
623 214
624 67. Uchida, N., Okamura, S., and Kuwano, H. (1999) Phospholipase D activity in human gastric
625 carcinoma. *Anticancer Res* **19**, 671-675
626 68. Yamada, Y., Hamajima, N., Kato, T., Iwata, H., Yamamura, Y., Shinoda, M., Suyama, M.,
627 Mitsudomi, T., Tajima, K., Kusakabe, S., Yoshida, H., Banno, Y., Akao, Y., Tanaka, M.,
628 and Nozawa, Y. (2003) Association of a polymorphism of the phospholipase D2 gene with
629 the prevalence of colorectal cancer. *J Mol Med* **81**, 126-131

69. Zhao, Y., Ehara, H., Akao, Y., Shamoto, M., Nakagawa, Y., Banno, Y., Deguchi, T., Ohishi, N., Yagi, K., and Nozawa, Y. (2000) Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. *Biochem. Biophys. Res. Comm.* **278**, 140-143

70. Buchanan, F. G., McReynolds, M., Couvillon, A., Kam, Y., Holla, V. R., Dubois, R. N., and Exton, J. H. (2005) Requirement of phospholipase D1 activity in H-RasV12-induced transformation. *Proc. Nat. Acad. Sci. USA* **102**, 1638-1642

71. Saito, M., Iwadate, M., Higashimoto, M., Ono, K., Takebayashi, Y., and Takenoshita, S. (2007) Expression of phospholipase D2 in human colorectal carcinoma. *Oncol Rep* **18**, 1329-1334

72. Zheng, Y., Rodrik, V., Toschi, A., Shi, M., Hui, L., Shen, Y., and Foster, D. A. (2006) Phospholipase D couples survival and migration signals in stress response of human cancer cells. *J. Biol. Chem.* **281**, 15862-15868

73. Iorio, E., Mezzanzanica, D., Alberti, P., Spadaro, F., Ramoni, C., D'Ascenzo, S., Millimaggi, D., Pavan, A., Dolo, V., Canevari, S., and Podo, F. (2005) Alterations of choline phospholipid metabolism in ovarian tumor progression. *Cancer Res.* **65**, 9369-9376

74. Gadir, N., Lee, E., Garcia, A., Toschi, A., and Foster, D. A. (2007) Suppression of TGF-beta signaling by phospholipase D. *Cell Cycle* **6**, 2840-2845

75. Shi, M., Zheng, Y., Garcia, A., Xu, L., and Foster, D. A. (2007) Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras. *Cancer Lett* **258**, 268-275

76. Chen, Y., Zheng, Y., and Foster, D. A. (2003) Phospholipase D confers rapamycin resistance in human breast cancer cells. *Oncogene* **22**, 3937-3942

652 77. Fiucci, G., Czarny, M., Lavie, Y., Zhao, D., Berse, B., Blusztajn, J. K., and Liscovitch, M.
653 (2000) Changes in phospholipase D isoform activity and expression in multidrug-resistant
654 human cancer cells. *Int J Cancer* **85**, 882-888
655 78. Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjoblom, T., Leary, R. J., Shen, D., Boca, S.
656 M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T.,
657 Nikolsky, Y., Karchin, R., Wilson, P. A., Kaminker, J. S., Zhang, Z., Croshaw, R., Willis, J.,
658 Dawson, D., Shipitsin, M., Willson, J. K., Sukumar, S., Polyak, K., Park, B. H.,
659 Pethiyagoda, C. L., Pant, P. V., Ballinger, D. G., Sparks, A. B., Hartigan, J., Smith, D. R.,
660 Suh, E., Papadopoulos, N., Buckhaults, P., Markowitz, S. D., Parmigiani, G., Kinzler, K.
661 W., Velculescu, V. E., and Vogelstein, B. (2007) The genomic landscapes of human breast
662 and colorectal cancers. *Science* **318**, 1108-1113
663 79. Foster, D. A. (2009) Phosphatidic acid signaling to mTOR: signals for the survival of human
664 cancer cells. *Biochim Biophys Acta* **1791**, 949-955
665 80. Rudge, S. A., and Wakelam, M. J. (2009) Inter-regulatory dynamics of phospholipase D and
666 the actin cytoskeleton. *Biochim Biophys Acta* **1791**, 856-861
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668

669 **Figure Legends**

670 Fig 1. Design of a new phosphatidic acid (PA) biosensor. (A) Schematic of the design of new PA
671 biosensors. To improve the PA accessibility, a nuclear export sequence (NES) derived from protein
672 kinase A inhibitor was added to either the N-terminus of Spo20-PABD, which was derived from
673 yeast t-SNEAR Spo20 protein. The new NES-Spo20-PABD is named PASS (Phosphatidic Acid
674 biosensor with Superior Sensitivity). PASS-4E is a mutant that four lysine residues were mutated to
675 glutamic acid (K66E, K68E, R71E, and K73E), which disrupted the PA binding of the original
676 Spo20-PABD (38). (B) PASS specifically binds to PA. Purified GST-PASS-His was mixed with
677 sucrose-loaded liposomes containing PA, phosphatidylinositol *4*,*5*-bisphosphate ($PI(4,5)P_2$), and
678 phosphatidylinositol 3,*4*,*5*-bisphosphate ($PI(3,4,5)P_3$). After centrifugation, the pellets bound to
679 liposomes were analyzed by Western blot using a GST antibody.
680
681 Fig 2. PASS specifically monitors the changes in cellular PA levels. (A) Minimal membrane
682 localization of previous PA biosensors (GFP-Spo20-PABD, GFP-Sos1-PH and Raf1-PABD) in
683 PMA-stimulated CHO cells. The original GFP-Spo20-PABD was dominantly localized in the
684 nucleus, and its plasma membrane localization was only observed in PMA-stimulated cells when
685 the images were overexposed. GFP-Raf1-PABD aggregates in some cells. (B) The majority of GFP-
686 and RFP-PASS is translocated to the plasma membrane upon PMA stimulation, which can be
687 inhibited by the PLD inhibitor FIPI (PLDi, 0.75μM). (C) PMA stimulation recruited GFP-PASS but
688 not RFP-PASS-4E to the plasma membrane. (D) Increased PLD activity recruited PASS to
689 membranes. Overexpression of PLD2 recruited GFP-PASS but not RFP-PASS-4E to the plasma
690 membrane in the same cells. Overexpression of PLD1 increased the localization of PASS on
691 intracellular vesicles upon PMA stimulation (compared to B and C). (E) Delivery of synthetic PA
692 (100 μM), but not PS (100 μM), recruited PASS to the plasma membrane. (F) Quantification of the

693 fluorescent intensity of PASS at the plasma membrane relative to that of the cytoplasm in the
694 experiment in B-E. N=3. *, $p < 0.001$ versus GFP-PASS.
695
696 Fig 3. Temporal production of PA by PLD and DGK in EGF-stimulated HCC1806 breast cancer
697 cells. (A) Different effects of PLD and DGK inhibition on EGF-promoted plasma membrane
698 translocation of GFP-PASS. Cells were pre-incubated with either DMSO, PLD inhibitor FIPI
699 (PLDi, 0.75 μM) or DGK inhibitor (DGKi, 10 μM) for 1 hr, and then stimulated with EGF for the
700 indicated time. (B) Quantification of the fluorescent intensity of GFP-PASS at the plasma
701 membrane relative to that of the cytoplasm in the same experiment in A. N=3. *, $p < 0.01$; **, $p <$
702 0.001 versus DMSO treatment at the same time points. (C) PLD2 is responsible for the generation
703 of PA on the plasma membrane. HCC1806 cells expressing GFP-PASS were infected with
704 lentiviruses carrying luciferase (Luc), PLD1 and PLD2 shRNAs, and stimulated with EGF for 3
705 min. (D) Western blot analysis of PLD1 and PLD2 knockdown. (E) Quantification of the
706 fluorescent intensity of GFP-PASS at the plasma membrane relative to that of the cytoplasm in the
707 same experiment in C and those treated with PLD1 and PLD2 inhibitor (PLD1i, 5 μM; PLD2i, 5
708 μM). N=3. **, $p < 0.001$ versus Luc shRNA or DMSO treatment. (F) PLD1 and PLD2 subcellular
709 localization in HCC1806 cells. Cells expressing Flag-PLD1 or Flag-PLD2 stimulated with or
710 without EGF were stained with an anti-Flag antibody.
711
712 Fig 4. PLD2 modulates the nuclear activity of ERK in EGF-stimulated cancer cells. (A) Inhibition
713 of either PLD (PLDi, 0.75 μM) or DGK (DGKi, 10 μM) has no effect on the global phosphorylation
714 of ERK in HCC1806 cells. Serum-starved cells were pre-incubated with inhibitors, and then
715 stimulated with EGF for the indicated time. Phospho-ERK1/2 (pERK) and total ERK1/2 were
716 detected on the same membranes by LI-COR Odyssey infrared imaging system using a mouse

717 monoclonal antibody and a rabbit polyclonal antibody, respectively. (B) Quantification of the
718 relative pixel intensity of pERK levels in the same experiment in A. pERK levels were normalized
719 by total ERK of the same samples. N=3. (C) Downregulation of PLD1 and PLD2 by shRNAs does
720 not affect the global activation of ERK. Serum-starved cells expressing luciferase (Luc), PLD1 or
721 PLD2, were stimulated with EGF for the indicated time. Phospho-ERK1/2 (pERK) and total
722 ERK1/2 were detected as described in A. (D) Quantification of the relative pixel intensity of pERK
723 levels in the same experiment in C. pERK levels were normalized by total ERK of the same
724 samples. N=3. (E) PLD inhibitor decreases the nuclear translocation of pERK. Cells grown on
725 coverslips were treated, stimulated for 0, 3, 6, 15, 30 and 60 min, as described in A, and stained for
726 a pERK antibody (only the images at 6 min of EGF stimulation are shown). (F) Quantification of
727 the fluorescent intensity of pERK in the nucleus relative to that of the cytoplasm in C. N=3. *, p
728 <0.001 versus DMSO treatment at the same time points. (G) PLD2 knockdown decreases the
729 nuclear translocation of pERK. Cells expressing shRNAs were grown on coverslips, stimulated with
730 EGF for 6 min, and stained for a pERK antibody. (H) Quantification of the fluorescent intensity of
731 pERK in the nucleus relative to that of the cytoplasm in E. N=3. *, p <0.001 versus Luc shRNA. (I)
732 Exogenous PA rescues reduced nuclear pERK staining caused by PLD2 inhibitor (5μM) in a dose-
733 dependent manner. Serum-starved cells were pre-treated by exogenous PA in indicated
734 concentrations 20 min before EGF stimulation. (J) Quantification of the fluorescent intensity of
735 pERK in the nucleus relative to that of the cytoplasm in I. N=3. *, p <0.01, **, p <0.001 versus cells
736 without PA addition. (K) PLD2 inhibition does not affect EGFR internalization. 1806 cells pre-
737 treated with DMSO or PLD2 inhibitor (5μM) were stimulated with EGF for 0, 3, 6, 15, and 30 min,
738 and then stained for EGFR antibody. There is no difference in the localization of EGFR on the
739 plasma membrane and endosomes. Only results from 0 and 15 min are shown.
740

741 Fig 5. PLD2 regulation of the nuclear ERK activity is a common mechanism of modulating EGF
742 signaling pathway in cancer cells. (A) Serum-starved cancer cells were pre-incubated with either
743 DMSO or PLD2 inhibitor (5μM), and then stimulated with EGF. The images shown were from the
744 time points of EGF stimulation at which pERK reached to the maximal levels, which varied in
745 different cancer cell lines, *e.g.,* 6 min for HCC827, 6 min for MDA-MB-468, 3 min for A-431, and
746 3 min for BT-20. (C) Cancer cells expressing luciferase (Luc) control and PLD2 shRNAs were
747 stimulated with EGF as those in A. (B, D) Quantification of the fluorescent intensity of pERK in the
748 nucleus relative to that of the cytoplasm in A and C. N=3. *, $p < 0.001$ versus DMSO or Luc shRNA
749 control in the same cell lines.
750
751 Fig 6. Inhibition of PLD2 decreases the phosphorylation and protein levels of ERK nuclear
752 substrates. Serum-starved HCC1806 cells pre-treated with inhibitors (A, E) or expressing shRNAs
753 (C) were stimulated with EGF for the indicated time. Total cell lysates were collected for Western
754 blot analyses of indicated proteins. (B, D, F) Quantification of the relative pixel intensity of
755 phosphorylated and total proteins in the same experiment in A, C and E. pElk1 levels were
756 normalized by total Elk of the same samples. Phosphorylated and total levels of other proteins were
757 normalized by α-tubulin of the same samples. PLDi, 0.75 μM; DGKi, 10 μM. N=3. *, $p<0.01$ versus
758 DMSO or Luc shRNA treatment at the same time points.
759
760 Fig 7. Inhibition of PLD2 decreases the proliferation of HCC1806 cells. (A) Inhibitor treatment.
761 (B) PLD1 and PLD2 knockdown by shRNA expression. Cells were seeded in 6-well plates in
762 RPMI-1640 media containing 0.1 % FBS and 5 ng/ml EGF in the presence of DMSO or inhibitors
763 (PLD1i, 5 μM; PLD2i, 5 μM; DGKi, 10 μM), or in the presence of control and PLD shRNAs.

764 Viable cells were measured by trypan blue exclusion. N=3. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$;

765 versus DMSO treatment or Luc shRNA at the same time points.