Upregulated phospholipase D2 expression and activity is related to the metastatic properties of melanoma

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Received September 30, 2021; Accepted February 1, 2022

DOI: 10.3892/ol.2022.13260

Abstract. The incidence rates of melanoma have increased steadily in recent decades and nearly 25% of the patients diagnosed with early-stage melanoma will eventually develop metastasis, for which there is currently no fully effective treatment. The link between phospholipases and tumors has been studied extensively, particularly in breast and colon cancers. With the aim of finding new biomarkers and therapeutic options for melanoma, the expression of different phospholipases was assessed in 17 distinct cell lines in the present study, demonstrating that phospholipase D2 (PLD2) is upregulated in metastatic melanoma as compared to normal skin melanocytes. These results were corroborated by immunofluorescence and lipase activity assays. Upregulation of PLD2 expression and increased lipase activity were observed in metastatic melanoma relative to normal skin melanocytes. So far, the implication of PLD2 activity in melanoma malignancies has remained elusive. To the best of our knowledge, the present study was the first to demonstrate that the overexpression of PLD2 enhances lipase activity, and its effect to increase the proliferation, migration and invasion capacity of melanoma cells was assessed with XTT and Transwell assays. In addition, silencing of PLD2 in melanoma cells reduced the metastatic potential of these cells. The present study provided evidence that PLD2 is involved in melanoma malignancy and in particular, in its metastatic potential, and established a basis for future studies evaluating PLD2 blockade as a therapeutic strategy to manage this condition.

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Abbreviations: Cho, choline; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; PA, phosphatidic acid; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PLD, phospholipase D

Key words: melanoma, metastasis, phospholipases, phospholipase D2, enzyme activity, phosphatidylcholine, lipidomics, cell migration, cell invasion, cell proliferation

Introduction

Phospholipase D (PLD) is a phospholipase that participates in the catabolism of glycerol-based phospholipids. There are six different isoforms of the phospholipase D subclass (PLD1-6) and PLD1 and PLD2 are the best-studied isoforms. The lipase activity of both these enzymes involves the preferential hydrolysis of phosphatidylcholines (PCs), to release phosphatidic acid (PA) and choline (Cho), crucial lipid mediators of cell signaling pathways involved in cell proliferation, growth and survival (1).

Cho is an essential nutrient for humans and it has a wide range of vital physiological roles (2,3), although higher levels of Cho have been associated with an increased risk of melanoma and other cancer types (3-5). However, as only small amounts of this molecule may be synthesized *de novo* in humans, it must be obtained from other sources, such as through the hydrolysis of the most abundant phospholipid, PC, by the lipase activity of PLDs (3). PA is the other lipid mediator produced by the lipase activity of PLDs, which may subsequently be converted into essential second messengers such as lyso-PA by phospholipase A_2 (PLA_2) or diacylglycerol (DAG) by PA-phosphohydrolase. These products significantly expand the range of the effects in which these enzymes may participate.

Aside from the lipase activity of PLD2, a unique feature of this isoform is that it also acts as a guanine nucleotide exchange factor (GEF) (6,7). Certain small GTPases regulate PLD2 activity through a complex regulatory program of phosphorylation and dephosphorylation switching involving S6K, Grb2, SoS, WASp and Rac2 (7). The GEF activity of PLD2 has been associated with the migration of cancer cells, hypothesizing that PLD2 constitutively activates Rac2, a GTPase known to participate

in cancer cell metastasis (8). Furthermore, the GEF activity of PLD2 regulates the PA released by hydrolysis of PC, adding a further layer of complexity to these regulatory circuits (6,9,10).

It is known that both the expression and activity of PLD1 and PLD2 are upregulated in several cancer types (1,10-18), altering numerous signaling pathways involved in tumor progression and metastasis. Indeed, it has been suggested that PLD2 has a pivotal role in tumor cells, primarily in metastatic dissemination (16,19). In fact, PLD2 activity promotes proliferation and invasiveness of lymphomas (20) and breast cancer cells (12,21), as well as representing a marker of poor prognosis in colorectal and renal neoplasms (14-16). PLD2 has also been observed to be overexpressed in melanoma along with enhanced lipase activity (22,23), although there is no clear evidence of its specific role in this pathology.

Melanoma is a cancer that arises from the transformation of melanocytes and it mainly occurs in the skin. It is the seventh most common tumor type in Europe and although the incidence of most cancers is declining, the number of melanoma cases is expected to continue to rise in western populations. While early diagnosis guarantees a 5-year overall survival of 99% for most melanomas, this rate falls to 25% in melanomas with distant metastases (24). Although numerous treatment options have been approved for metastatic tumors in the past decade (25), there is still no effective therapy for advanced melanoma. Thus, the aim of the present study was to obtain novel insight into the contribution of PLD2 in the development and progression of melanoma, assessing its value as a potential novel biomarker or therapeutic target for melanoma. It was indicated that an increase in the expression of PLD2 and its lipase activity contribute to increased proliferation, migration and invasion of melanoma cells.

Materials and methods

Commercial cell lines. The HEMn-LP, HEMn-MP and HEMn-DP melanocyte cell lines were purchased from Thermo Fisher Scientific, Inc., while the A375, G361, Sk-Mel-28, HT144, Hs294t, A2058, Sk-Mel-2, Sk-Mel-3, WM-266-4 and VMM1 melanoma cell lines were obtained from the American Type Culture Collection (ATCC). The Mel-Ho, Colo-800 and RPMI-7951 melanoma cell lines were acquired from Innoprot S.L.

The cell lines used in the present study may be classified into three different groups according to their origin: Normal melanocytes (HEMn-LP, HEMn-MP and HEMn-DP), primary melanomas (A375, G361, Sk-Mel-28, ME4405 and Mel-Ho) and metastatic melanomas (HT144, Hs294t, A2058, Sk-Mel-2, Sk-Mel-3, WM-266-4, VMM1, RPMI-7951 and Colo-800). Table SI provides a brief description of these cell lines.

The Mel-Ho cell line was authenticated by short-tandem repeat DNA profiling analysis (TH01: 7; D21S11: 29; D5S818: 12; D13S317: 11; D7S820: 10,11; D16S539: 11; CSF1P0: 12; AMEL: X; VWA: 14,18; TPOX: 8,10. The numbers after the loci name indicate the number of repeats of the repeat unit for that locus in the analysed cells' genome).

Cell culture. Melanocyte cell lines were cultured in medium 254 supplemented with human melanocyte growth supplement (Thermo Fisher Scientific, Inc.). The A375, ME4405, Hs294t, RPMI-7951, A2058 and HT144 lines were maintained in DMEM culture medium (MilliporeSigma), while the Sk-Mel-28, WM-266-4 and Sk-Mel-2 lines were cultured in Eagle's Minimum Essential Medium (ATCC). RPMI 1640-GlutaMAX™ (Thermo Fisher Scientific, Inc.) was used to culture the Mel-Ho, Colo-800 and VMM1 cell lines, and McCoy's 5A was used for G361 and Sk-Mel-3 cells (Thermo Fisher Scientific, Inc.). All the culture media used for melanoma cell lines were supplemented with 10% FBS (v/v; Cytiva), 100 UI/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.). The cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. All of the cultured cell lines tested negative for mycoplasma (Venor® GeM One Step Test Kit; Minerva Biolabs).

Western blot analysis. Cell protein extracts were obtained from cells incubated for 15 min in RIPA buffer (Thermo Fisher Scientific, Inc.) containing protease inhibitor cocktail-3 and a phosphatase inhibitor (MilliporeSigma), and then centrifuged for 5 min at 15,000 x g at 4˚C. The proteins were quantified with a BCA assay (EMD Millipore) and equal amounts (40 μg) of each protein extract were denatured for 30 min at 37˚C (22) and separated by 7.5% SDS-PAGE electrophoresis under reducing conditions. The proteins were then wet-transferred to a nitrocellulose membrane (Cytiva) that was probed overnight at 4˚C with primary antibodies against PLA_2, PLC, PLD1, PLD2 and tubulin. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The details of the antibodies are provided in Table I. The bands were visualized by enhanced chemiluminescence and digital images of the bands were obtained with a charge-couple device camera-based imager, the Syngene G box imaging system (Syngene).

Immunofluorescence staining. Cells were seeded on round coverslips, incubated overnight, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated overnight at 4˚C with anti-human PLD2 primary antibody (1:200 dilution; Abgent), washed and then incubated for 2 h at room temperature in the dark with an Alexa-Fluor 594-conjugated secondary antibody (1:5,000 dilution; cat. no. ab150080; Abcam). Finally, the cells were incubated with Hoechst-33342 (Thermo Fisher Scientific, Inc.) for 5 min and the coverslips were mounted with Fluoromount-G (MilliporeSigma) on a microscope slide. The cells were visualized by generating 20 z-stacks on a Leica SP8 confocal fluorescence microscope (Leica Microsystems GmbH) at an original magnification of x63.

PLD lipase activity. PLD activity in melanoma cells was assessed using a protocol described previously (26). First, liposomes were prepared from a short chain PC (8:0/8:0) (3.5 mM; Avanti Polar Lipids) in HEPES (pH 7.8; 45 mM), phosphatidylinositol 4,5-biphosphate (1 μM; Avanti Polar Lipids, Inc.) and 1.0 μCi [^{3}H]-n-butanol (American Radiolabeled Chemicals Inc.). Pellets of the cell lines studied were resuspended in lysis buffer [5 mM HEPES (pH 7.8), 100 μM Na orthovanadate, 0.4% Triton X-100, 2 μg/ml aprotinin and 5 μg/ml leupeptin], sonicated on ice and the protein content of the sonicates was measured with a BCA assay. The liposomes were incubated for 20 min on a shaker with 50 μg of the cell sonicates at 30˚C to achieve transphosphatidylation of PLD. This reaction was

Table I. Antibodies for western blot analysis.

Antibody	Company (cat. no)	Source	Target species	Dilution
PLD2	Abgent (AO2358a)	Mouse	Human	1/1500
PLD1	Cell Signaling Technology, Inc. (3832)	Rabbit	Human	1/1,000
PLA_2	Santa Cruz Biotechnology, Inc. (Sc-376563)	Mouse	Human	1/1,000
PLC	Santa Cruz Biotechnology, Inc. (Sc-5291)	Mouse	Human	1/1,000
α-Tubulin	MilliporeSigma (T9026)	Mouse	Human	1/3,000
Mouse IgGκ-HRP	Santa Cruz Biotechnology, Inc. (Sc-516102)	Goat	Mouse	1/5,000
Mouse IgG-HRP	SouthernBiotech (1032-05)	Goat	Mouse	1/8,000
Rabbit IgG-HRP	Abcam (Ab102279)	Goat	Rabbit	1/10,000

PLD, phospholipase D; IgG, immunoglobulin G; HRP, horseradish peroxidase.

stopped by adding ice-cold chloroform/methanol (1:2) and the lipids in the samples were isolated and resolved by thin-layer chromatography. The amount of [^{3}H]-phosphatidylbutanol (PBut) that co-migrated with the PBut standards was measured in a scintillation counter (PerkinElmer, Inc.).

PLD2 overexpression. Melanoma cells were seeded in 6-well plates and grown until 60-70% confluence. They were then transfected by incubation for 48 h with a transfection mixture of 1 μg of plasmid DNA (cat. no. RC202042; Origene Technologies, Inc.), TransIT®-2020 transfection reagent (Mirus Bio, LLC) and Opti-MEM™ (Thermo Fisher Scientific, Inc.). Control cells were transfected with empty plasmid (pc-DNA3.1-myc; Origene Technologies, Inc.) following the same protocol and the overexpression efficiency was confirmed using western blot analysis.

PLD2 silencing. When the melanoma cells cultured in 6-well plates reached 60-70% confluence, the culture medium was replaced with silencing mixture containing 50 nM small interfering (si)RNA (Ambion; Thermo Fisher Scientific, Inc.), Opti-MEM™ and Lipofectamine RNAiMax (Thermo Fisher Scientific, Inc.), and the cells were incubated for 48 h prior to performing the experiments. For control transfections, a non-targeting siRNA molecule suitable for negative controls (cat. no. AM4611; Ambion; Thermo Fisher Scientific, Inc.) was used following the same procedure and the correct silencing of the cells was confirmed using western blot analysis.

Cell proliferation assay. The relative proliferation capacity of cells after PLD2 silencing and overexpression was measured as the number of viable cells in that condition after 24 h in culture, relative to their control. Viable cells were quantified by XTT, which is based on dehydrogenase enzymes that reduce the tetrazolium salt to a highly coloured formazan dye. The amount of the formazan produced is proportional to the number of viable cells. It was confirmed that the treatments did not affect dehydrogenase enzyme activity compared to the results obtained by XTT and those calculated by trypan blue, so XTT is valid for measuring cell viability in the present study. Melanoma cells were grown overnight in 96-well plates in complete culture medium and then the medium was discarded and low-FBS medium (0.5%) was added. After 2 h in the incubator at 37˚C, the medium was replaced with complete culture medium and 24 h later, XTT reagent (MilliporeSigma) was added to the cells. The cell absorbance in each experimental condition was measured at 450 nm in a Synergy HT spectrophotometer (BioTek Instruments, Inc.) and the proliferation rate of the cells was expressed as a ratio relative to their corresponding transfection controls.

Cell migration and invasion assay. Cell migration was assessed using Boyden chambers with 8-μm pores (Corning, Inc.). Melanoma cells were incubated for 2 h at 37˚C in low-FBS medium (0.5%) and 400 μl of $5x10^4$ cells/ml were seeded in the upper chambers in medium containing 0.5% FBS. For invasion, 400 μl of $7.5x10^5$ cells/ml in 0.5% FBS medium were seeded in the upper chambers of Matrigel®-coated Boyden chambers (Corning, Inc.). Complete culture medium was added to the bottom well and after 20 h of incubation at 37˚C, the non-migrated/invaded cells were removed by aspiration and wiping the upper side of the membrane with a cotton bud, while the migrated/invaded cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Inc.) and stained with 0.2% crystal violet at room temperature for 8 min. Finally, the filter was mounted and observed under a microscope at a magnification of x20. Images of six different fields were acquired and the cells in each field were counted. The migratory/invasive capacity of each cell line was calculated as a ratio relative to their corresponding transfection control.

Statistical analysis. Values are expressed as the mean ± standard error of the mean of at least three experiments and the statistical significance of the differences between the means was calculated using one-way ANOVA and Bonferroni's post-hoc correction for the comparison of more than two data sets, or Dunnett's multiple-comparison test post-hoc correction for comparison of multiple data sets with their control as indicated in each experiment (GraphPad Prism 5.01; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PLD2 protein expression is upregulated in melanoma cell lines. As several phospholipases are overexpressed in a variety

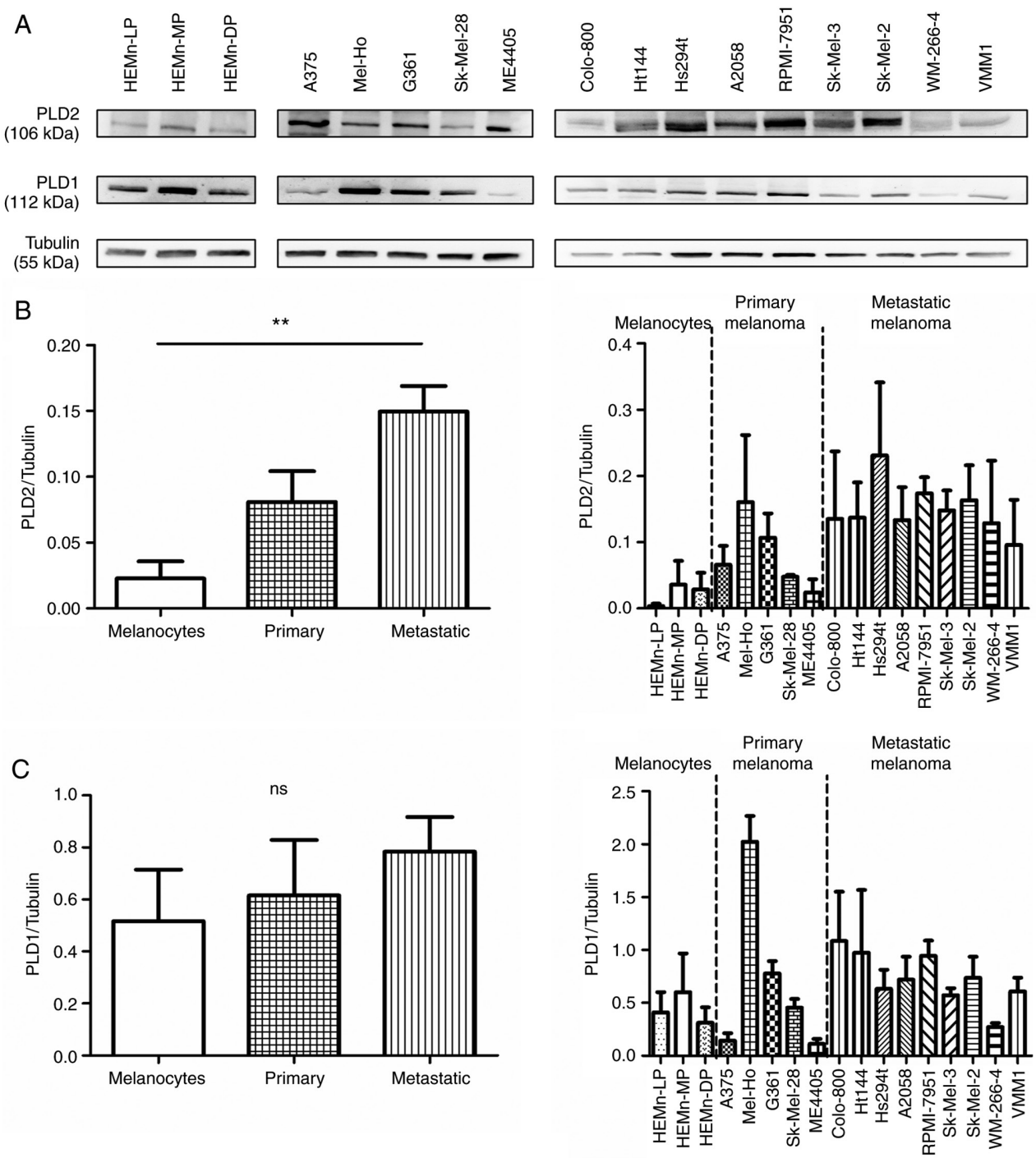


Figure 1. Phospholipase expression in skin melanocytes and melanoma cell lines. (A) Western blots of PLD2 and PLD1 expression, using α-tubulin as a loading control. Full-length western blot images are provided in Fig S1. For each experiment, two gels were made and processed in the same way using the same washing and developing times. Melanocytes and primary melanoma lines were loaded in one gel and metastatic melanomas in the other gel. The figure is representative of four independent experiments. Quantification of (B) PLD2 and (C) PLD1 expression relative to α-tubulin in each study group and cell line: Skin melanocytes, primary melanomas (squares) and metastatic melanomas (lines). Values are expressed as the mean ± standard error of the mean. **P<0.01 (one-way ANOVA, and post-hoc correction with Bonferroni's test). ns, no significance; PLD, phospholipase D.

of cancers (1,27) and they participate in their malignant transition, the presence of this family of enzymes was assessed in melanoma. The expression of PLD2, PLD1, PLA_2 and PLC was observed to vary in western blots of 17 cell lines, including skin melanocytes, as well as in primary and metastatic melanoma cell lines. In particular, while PLD2 was weakly expressed in skin melanocytes, it was expressed more strongly in melanoma cells (Figs. 1A and B and S1), particularly metastatic cells, although this increase in expression was cell line-specific. Conversely, no clear variation was observed in PLD1 protein expression between skin melanocytes and melanoma cells (Figs. 1A and C and S2). In fact, the expression of this protein in the HEMn-MP cell line was similar to that in all the primary melanomas except Mel-Ho. PLA_2 was expressed more strongly by skin melanocytes than by primary melanomas (Fig. S3), although HT144, Colo-800 and RPMI-7951 metastatic melanomas had the highest levels of

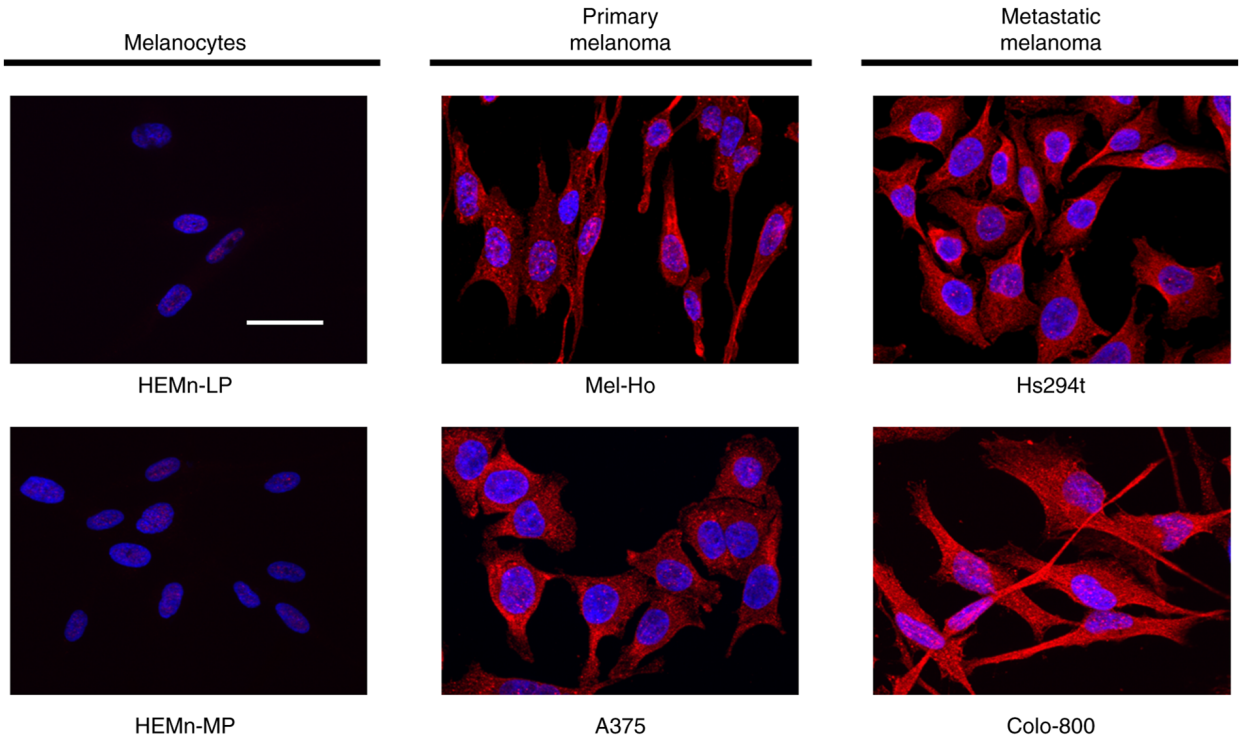


Figure 2. PLD2 expression in skin melanocytes, primary melanomas and metastatic melanoma cell lines as assessed by immunofluorescence. PLD2 is in red and the nuclei in blue (scale bar, 50 μm; original magnification, x63). The images are representative of three different experiments. PLD, phospholipase D.

PLA_2, a trait that was cell line-specific as it was not common to all the metastatic melanomas. PLC was expressed relatively homogeneously in the cell lines studied and no marked differences were observed (Fig. S3). The enhanced presence of PLD2 in melanoma cells was specific to this isoform, since the PLD1, PLA_2 and PLC proteins remained relatively constant in melanocytes and melanoma cells.

Immunofluorescence analyses confirmed that PLD2 expression was upregulated in the malignant cell lines studied (Fig. 2). PLD2 was expressed more intensely in primary melanomas (A375, Mel-Ho) and metastatic melanomas (Hs294t, Colo-800), while its expression was faint in skin melanocytes (HEMn-LP, HEMn-MP). By contrast, immunofluorescence analysis of the other phospholipases (PLA_2, PLC and PLD1) did not indicate any consistent differences between the cell lines studied (data not shown). Indeed, both the western blot and immunofluorescence data corroborated the enhanced expression of PLD2 in primary and metastatic melanomas relative to normal skin melanocytes.

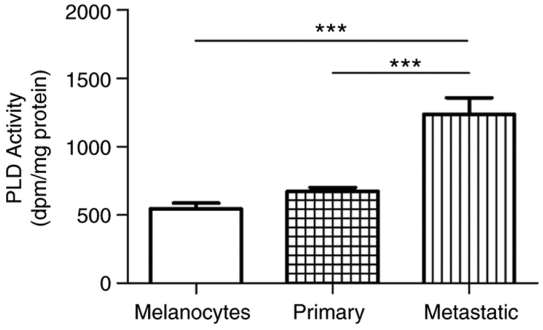
Enhanced PLD lipase activity in melanoma cells. A comparative analysis of PLD activity was performed in melanocytes, primary melanomas and metastatic melanomas (Fig. 3). The results indicated that melanoma cells exhibited enhanced PLD activity and that this was associated with melanoma progression. Indeed, metastatic melanoma cells had significantly more lipase activity, while primary melanoma cell lines also had higher activity than skin melanocytes. However, the activity in the HEMn-LP melanocyte cell line was comparable to that in primary melanomas and therefore, the differences were not statistically significant between both groups. These results confirmed that, overall, the lipase activity of PLD enzymes is increased in melanoma cells and raise the question of whether they have a role in melanoma, particularly in the metastatic process.

PLD2 overexpression and silencing in melanoma cell lines. After demonstrating that aggressive melanoma cells have stronger PLD2 expression and activity than skin melanocytes, modifications to the amounts of this enzyme were performed to shed light on the implication of PLD2 in specific carcinogenic events. The effects of transient overexpression and silencing of PLD2 were examined in primary melanoma cells (A375, Mel-Ho) or metastatic melanoma cells (Colo-800), as confirmed in western blots (Figs. 4A and S4). Furthermore, these molecular alterations were also manifested through enzymatic activity, as lipase activity was altered in accordance with protein expression in all cases, although only in the A375 line, the lipase activity exhibited statistically significant differences compared to the transfection controls. As indicated in Fig. 4B, PLD2 overexpression was associated with an increase in lipase activity when the ratio relative to the transfection control was considered: A375 cells, 1.87; Mel-Ho, 1.54; and Colo-800, 1.4. By contrast, PLD2-silenced cells exhibited weaker lipase activity: A375, 0.52; Mel-Ho, 0.82; and Colo-800, 0.68.

PLD2 enhances the proliferation, migration and invasion of melanoma cells. The functional implications of the modifications to PLD2 expression were analyzed by studying the differences in proliferation, migration and invasion of transformed cells. Although these changes were cell line-specific, there was a clear trend towards an increase in proliferation, migration and invasion of PLD2-overexpressing cells, while these processes were downregulated in PLD2-silenced

A

Group	dpm/mg protein
Melanocytes	543.50±42.97
Primary melanoma	673.33±26.38
Metastatic melanoma	1233.50±123.00



B

Cell line	dpm/mg protein
HEMn-LP	711.50±12.50
HEMn-MP	478.00±8.00
HEMn-DP	492.25±47.81
A375	693.00±51.07
Sk-Mel-28	657.75±66.45
Mel-Ho	669.25±20.42
Colo-800	1103.25±55.17
A2058	1363.75±237.23

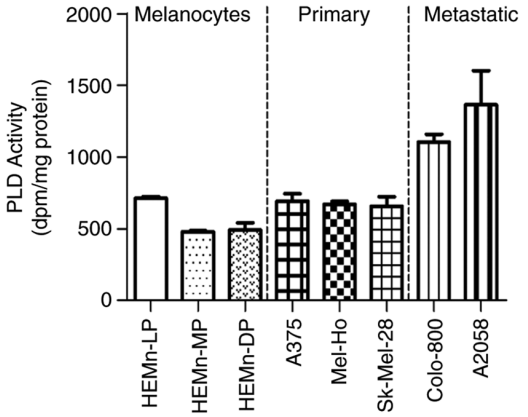


Figure 3. PLD activity in melanocytes and melanoma cells. Summary and graphic representation of the PLD activity in (A) the three study groups and (B) in the individual cell lines. PLD activity is expressed as dpm/mg of protein and values are expressed as the mean ± standard error of the mean from three different experiments. ***P<0.001 (one-way ANOVA and post-hoc correction with Bonferroni test). PLD, phospholipase D.

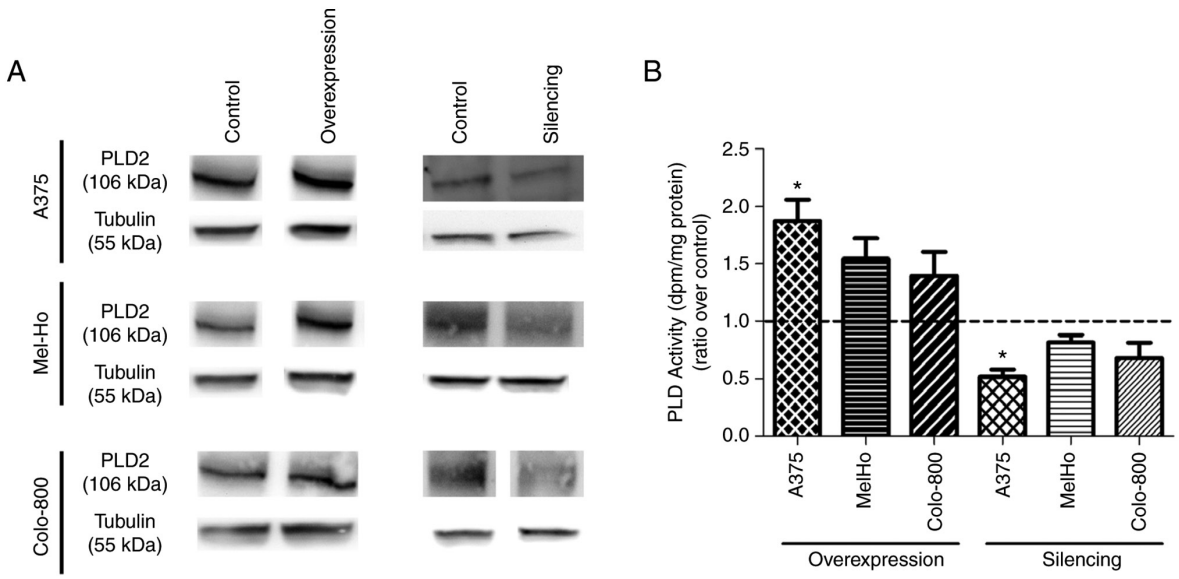


Figure 4. (A) PLD2 protein expression and (B) PLD lipase activity in PLD2-overexpressing and -silenced cells. (A) The images are representative of three different transfections and western blots. All bands presented together have been probed on the same membrane. In the case of A375 and Mel-Ho PLD-overexpressing cells and Colo-800 PLD-silenced cells, bands from the same gel have been cropped and grouped to maintain the same order in the figures. Full-length western blot images are provided in Fig. S4. (B) PLD enzymatic activity is expressed as the ratio of dpm/mg of protein in each sample relative to the transfection controls. The results are representative of three independent experiments and values are expressed as the mean ± standard error of the mean. *P<0.05 (one-way ANOVA and post-hoc correction with Dunnett's multi-comparison test). PLD, phospholipase D.

A

		A375	Mel-Ho	Colo-800
Overexpression	Mean	1.511	1.070	1.678
	SEM	0.133	0.005	0.128
	p-value	0.0003	0.9262	0.0001
Silencing	Mean	0.571	0.796	0.785
	SEM	0.049	0.019	0.060
	p-value	0.0007	0.1835	0.1506

B

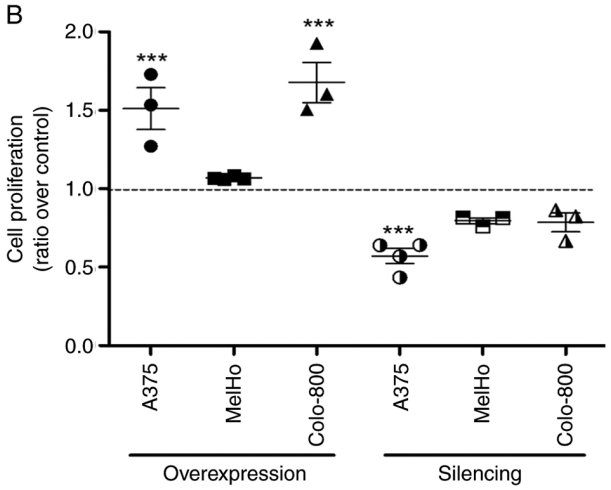


Figure 5. Cell proliferation in PLD2-overexpressing and -silenced melanoma cells. (A) Proliferation relative to the corresponding transfection controls. (B) Graphs presenting the proliferation of the representative cell lines. No substantial differences were observed in the basal proliferation rates of the three cell lines. Values are expressed as the mean ± SEM of at least three different transfections. ***P<0.001 (one-way ANOVA and post-hoc correction with Dunnett's multi-comparison test). PLD, phospholipase D; SEM, standard error of the mean.

cells (Figs. 5 and 6). Indeed, cell proliferation increased markedly in A375 and Colo-800 cells overexpressing PLD2 but only slightly in MelHo cells. Conversely, in cells that downregulated PLD2, there was a consistent reduction in proliferation: A375, 0.55 (P<0,001); Mel-Ho, 0.8; and Colo-800, 0.79 (Fig. 5).

Melanoma cell migration and invasion were then assessed and PLD2-overexpressing primary melanoma cells exhibited moderate increments in cell migration: A375, 1.39; and Mel-Ho, 1.31. This effect was markedly enhanced in metastatic cells (Colo-800, 2.13), whereas the results were more similar among the silenced cell lines: A375, 0.66; Mel-Ho, 0.53; and Colo-800, 0.54 (Fig. 6A and B; images are provided in Fig. S5). Finally, increases in cell invasion in association with PLD2 overexpression were observed in primary melanoma cells (A375, 1.68; Mel-Ho, 1.71) relative to metastatic cells (Colo-800, 1.43). However, in the silenced cells, the results were more similar among the three cell lines, although primary melanoma cells were still more strongly affected: A375, 0.48; Mel-Ho, 0.53; and Colo-800, 0.60 (Fig. 6C and D; images are provided in Fig. S6). In conclusion, the present results suggested that higher levels of PLD2 increased the proliferation, migration and invasion of melanoma cells, while silencing of this enzyme decreased those activities.

Discussion

Phospholipases metabolize phospholipids, generating bioactive molecules that influence cell fate by regulating distinct cell activities, including events that favor tumorigenesis and metastasis (11-13,28). Deregulation of the expression and activity of phospholipases has been implicated in different diseases, including cancer. Indeed, the overexpression and enhanced activity of PLA2 serves as a diagnostic marker of breast cancer and is correlated with the tumor stage (29). Similarly, the present analysis of the PLD2 protein demonstrated that it accumulates at higher levels in metastatic melanoma than in normal skin melanocytes, as indicated by the PLD2 expression analyzed by western blot and immunofluorescence. However, the immunohistochemical analysis performed was limited, as neither the subcellular localization of PLD2 nor the changes in PLD2 expression in the transfected cells was examined. The results prompted us to analyze the activity of PLD, based on the notion that not only is PLD expression enhanced in cancer tissues but its activity is also increased (10,30). PLD activity was enhanced particularly in murine melanoma cells, in association with an acidic pH of the tumor microenvironment (31), consistent with the enhanced PLD activity observed previously in melanoma cells relative to skin melanocytes (22). This trait was verified in the present study, as significantly stronger PLD activity was observed in malignant cells. Furthermore, the present study was the first, to the best of our knowledge, to indicate that PLD activity in metastatic melanoma cells was substantially higher than in primary melanoma cells.

One role of PLD2 is the hydrolysis of PC present in cell membranes to release free Cho and a PA molecule. The molecules liberated are known precursors and second messengers that support a tumorigenic phenotype. Indeed, increased Cho levels have been identified in cancer cells (3-5). Cho is essential for the synthesis of PC and sphingomyelin lipids, important structural and signaling lipids involved in generating new cell membranes and reprogramming the metabolism of cancer cells, thereby supporting their malignant transformation. However, the effect of PLD activation is principally related to PA, a ubiquitous second messenger that interacts with important signaling molecules such as Akt and mTOR (13,23,32), both in physiological and pathological processes (1,33). These signaling pathways are constitutively activated in melanoma, promoting cell proliferation, migration, survival, angiogenesis, metabolism and chemoresistance. PLD-produced PA favors the phosphorylation of these interactors in melanoma cells with downregulated liver kinase B1 (23), thereby producing pro-tumorigenic effects (1,33-40). In addition, PA may also be dephosphorylated to yield DAG or hydrolyzed to yield Lyso-PA, both of which are potent signaling molecules known to favor carcinogenesis (32,41). In particular, Lyso-PA supports the migration of melanoma cells (42).

For the first time, to the best of our knowledge, the present study demonstrated the influence of PLD2 expression and activity on distinct carcinogenic events by overexpressing and silencing this enzyme in primary and metastatic melanoma cells. The results demonstrated that augmenting the activity and expression of PLD2 promoted a pro-tumorigenic phenotype in melanoma, driving the proliferation, invasion and migration of these cells, particularly in metastatic cells. Conversely, downregulation

A

		A375	Mel-Ho	Colo-800
Overexpression	Mean	1.388	1.310	2.130
	SEM	0.046	0.039	0.137
	p-value	0.0003	0.0023	<0.0001
Silencing	Mean	0.668	0.529	0.536
	SEM	0.024	0.046	0.019
	p-value	0.0026	<0.0001	<0.0001

B

		A375	Mel-Ho	Colo-800
Overexpression	Mean	1.681	1.715	1.437
	SEM	0.050	0.070	0.075
	p-value	<0.0001	<0.0001	<0.0001
Silencing	Mean	0.483	0.526	0.609
	SEM	0.093	0.042	0.040
	p-value	<0.0001	<0.0001	0.0003

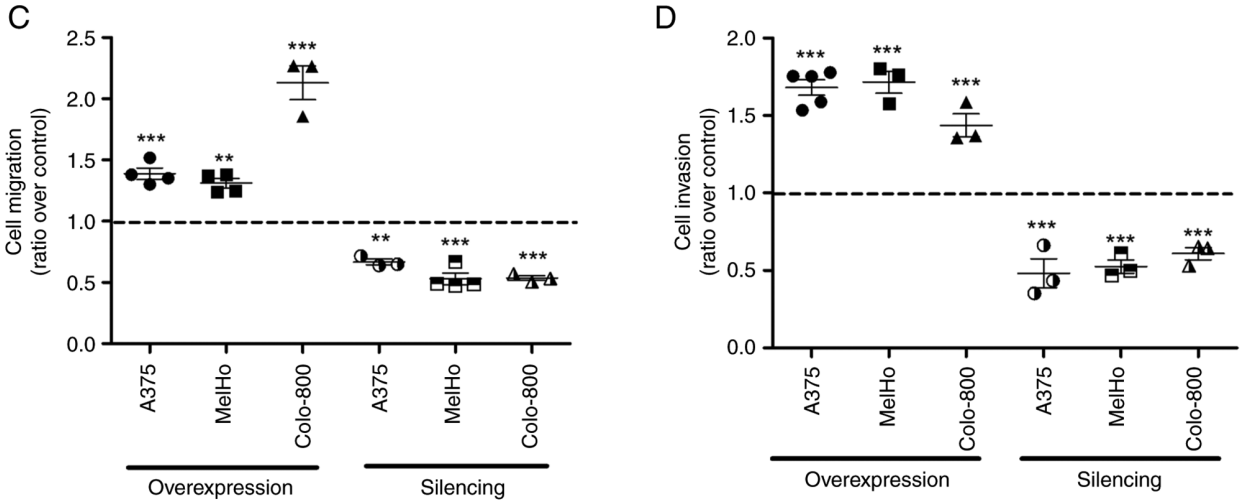


Figure 6. Cell migration and invasion of PLD2-overexpressing and -silenced melanoma cells. (A) Summary of the migration relative to the corresponding transfection controls. (B) Summary of the invasion data represented as a ratio relative to the corresponding transfection controls. (C and D) Graphical representation of the results from (C) the migration and (D) invasion assay. The three cell lines had a similar basal migration capacity, while the basal invasion capacity of Colo-800 was significantly lower than that of the primary melanomas. Representative images for the migration and invasion assays for the three lines and all the conditions tested are provided in Figs. S5 and S6, respectively. Values are expressed as the mean ± SEM of at least three different transfections. **P<0.01; ***P<0.001 (one-way ANOVA and post-hoc correction with Dunnett's multi-comparison test). PLD, phospholipase D; SEM, standard error of the mean.

of PLD2 inhibited these activities. Similar results have been described in lymphoma (20) and breast cancer cells (12,19), and it was determined that PLD2 promoted metastatic activity by phosphorylating FAK and PAK, and by activating mTOR, JAK3 and Akt (20,43). Furthermore, PLD2 is present in the exosomes secreted by cancer cells, favoring the metastatic spread of prostate cells (44) and the crosstalk of colon cancer cells with their microenvironment (45). Thus, PLD has been associated with cancer progression and metastasis in several tumor types (10-12,17,21,28,44-48), to which melanoma may now be added. Concerning this point, the survival of melanoma patients with high and low PLD2 expression from The Cancer Genome Atlas PanCancer panel data were analysed using the cBioPortal platform (49,50). Although the results of this analysis should be interpreted with caution, they indicate a significant difference in overall survival between the two groups, where higher PLD2 expression (z score >1) is associated with shorter survival (Fig. S7). These data support the experimental results of the present study and reinforce the idea that, in melanoma, high PLD2 expression may be associated with malignant progression.

The association between PLD activity detected and the functional processes studied in transfected cells is not perfect, since the magnitude of this relationship differs between cell lines and the type of process. For instance, in Mel-Ho and Colo-800 cells overexpressing PLD2, similar levels of PLD activity result in quite different rates of proliferation and migration. The variability observed among them may be due to an activity of PLD2 other than its lipase activity. In fact, the GEF activity of PLD2 is important for cell motility and other functions (1,9,10). Indeed, PLD2 is a GEF for the small GTPases Rho, Rac-2 and Arf6, which fulfills an essential role in the actin rearrangements and filopodia formation required for cell motility and migration (6,16,51-53). These activities are consistent with the present results, as it was observed that the migratory and invasive capacities of melanoma cells increase when PLD2 is overexpressed, particularly in metastatic melanoma. In addition, the PLD activity determined indicated the presence of enzymatic activity of both PLD1 and PLD2, while in the functional study, only the protein levels of PLD2 were modified. On the other hand, the fact that the products of PLD2 activity may be converted into

essential second messengers means that the range of effects of the enzyme is broad and its effect on biological activities such as those studied may be cell line-dependent.

Pharmacological inhibition or genetic downregulation of PLD2 has been proposed as a potential treatment option for certain cancer types. In breast cancer in particular, previous studies have indicated that the use of a PLD2-specific inhibitor markedly reduced the invasive capacity of cultured cell lines (21) and it promoted cancer cell apoptosis, while overexpression of PLD2 provided resistance to chemotherapeutic agents (54). Silencing of PLD2 or its pharmacological inhibition in a mouse model repressed tumor progression, whereas overexpression of this enzyme translated into an increase in tumorigenic potential (12). In lymphoma, catalytic inactivation of PLD2 reduced the formation of lung metastases (20) and in colorectal cancer cells, the pharmacological inhibition and silencing of PLD2 resulted in cancer cell autophagy (55).

In conclusion, PLD2 contributes to different aspects of cancer development including the production of survival signals (10,46,56-59), proliferation (60-63), resistance to apoptosis (10,64), angiogenesis (10,65), deregulated cellular energetics (10), as well as matrix substrate degradation, invadopodia formation, tumor-cell migration, invasion and metastatic dissemination (10,19,31,43,46,47,66,67). However, in melanoma, a specific role for PLD2 has yet to be described in tumorigenesis. The present study was the first to report increased PLD activity in metastatic melanoma cells relative to primary melanoma cells, as well as the influence of changes in PLD2 levels and activity in several properties of metastatic melanoma. In the light of the data on PLD2 from other cancers and the present *in vitro* results in melanoma, it is suggested that PLD2 may be a promising therapeutic target and biomarker for melanoma tumors, which merits further study.

Acknowledgements

The authors would like to thank Dr Julian Gomez-Cambronero from the Department of Biochemistry and Molecular Biology of Wright State University (Dayton, OH, USA) for his support with the PLD lipase activity measurements and PLD2 transfection in cultured cells.

Funding

This study was supported by grants from the University of the Basque Country/EHU (grant no. GIU17/066) and Ministerio de Economía y Competividad MINECO-ONCOFINDER of the Spanish Government (grant no. RTC.2015-3693-1).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contribution

APV performed the experiments described in the study, collected the data and drafted the manuscript. MDB and AA guided the design and execution of the study and revised the manuscript. BO provided advice on lipid biology and function and analyzed the results of certain experiments. KNS participated in the execution of PLD activity assays and PLD2 transfection assays. EA and GBG obtained the financial support and performed parts of the statistical analysis. APV and AA confirm the authenticity of all the raw data. All authors read, reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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