The ACSL3-LPIAT1 signaling drives prostaglandin synthesis in non-small cell lung cancer

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Abstract
Enhanced prostaglandin production promotes the development and progression of cancer. Prostaglandins are generated from arachidonic acid (AA) by the action of cyclooxygenase (COX) isoenzymes. However, how cancer cells are able to maintain an elevated supply of AA for prostaglandin production remains unclear. Here, by using lung cancer cell lines and clinically relevant KrasG12D-driven mouse models, we show that the long-chain acyl-CoA synthetase (ACSL3) channels AA into phosphatidylinositols to provide the lysophosphatidylinositol-acyltransferase 1 (LPIAT1) with a pool of AA to sustain high prostaglandin synthesis. LPIAT1 knockdown suppresses proliferation and anchorage-independent growth of lung cancer cell lines, and hinders in vivo tumorigenesis. In primary human lung tumors, the expression of LPIAT1 is elevated compared with healthy tissue, and predicts poor patient survival. This study uncovers the ACSL3-LPIAT1 axis as a requirement for the sustained prostaglandin synthesis in lung cancer with potential therapeutic value.

Introduction
Arachidonic acid (AA) is a polyunsaturated fatty acid that, as arachidonate, is maintained at low concentrations, but it is highly abundant in its esterified form in membrane phospholipids. Therefore, the amount of AA is tightly controlled by the membrane phospholipid reacylation/deacylation cycle, known as the Lands cycle [1–3]. Depending on the cellular demand, AA can be released through phospholipid hydrolysis by phospholipase A2 (PLA2), phospholipase D, or phospholipase C pathways [4], and is then converted to prostaglandins by cyclooxygenases 1 and 2 (COX1 and COX2). Whereas COX1 is constitutively expressed, COX2 is induced by proinflammatory cytokines, and plays a central role in the insurgence of cancer inflammation and tumor progression [5, 6]. Notably, prostaglandins promote tumor growth both by directly activating signaling pathways, which control cancer cell proliferation, anchorage-independent growth, migration, and apoptosis, and by orchestrating interactions between tumor cells and the surrounding stromal cells, establishing an immunosuppressive tumor microenvironment [7–10].

Non-small cell lung cancer (NSCLC) constitutes about 85% of all lung malignancies, out of which 30% harbor KRAS mutations that are associated with aggressive, therapy-resistant tumors [11]. KRAS upregulates COX2, and the latter produces prostaglandins, including prostaglandin E2 (PGE2), in order to promote tumor growth and metastasis [12]. The transcriptional regulation of COX2 has been mainly attributed to MAPK signaling cascade, particularly to the ERK1/2, JNK/SAPK, and p38/RK/Mpk2 pathways [13–15]. However, it is unclear how KRAS is able to maintain a continuous supply of AA to feed COX2 and drive prostaglandin synthesis.

Mutant KRAS drives aberrant lipid metabolism in NSCLC by scavenging extracellular fatty acids [16, 17].
Indeed, we previously found that enhanced activity of the acyl-CoA synthetase long-chain 3 (ACSL3), an enzyme that catalyzes the activation of long-chain fatty acids to CoA thioesters, boosts extracellularly derived fatty acid activation in mutant KRAS NSCLC [17]. Knockdown of ACSL3 in NSCLC cell lines results in reduced cancer cell proliferation, while its deletion in mice suppresses KrasG12D-driven tumor initiation [17]. Thus, given the importance of ACSL3 in KRAS-driven tumorigenesis, and due to the fact that ACSL3 preferentially utilizes long-chain fatty acids, including arachidonate as substrates [18], we set out to investigate whether ACSL3 plays a role in mediating the KRAS-dependent prostaglandin production in lung cancer. To this aim, we combined mass spectrometry-based targeted lipidomics, in vivo cell-based assays, targeted genetic manipulations in cancer cells and in mouse models, as well as analysis of patient NSCLC samples.

**Results**

**ACSL3 promotes channeling of AA to phosphatidylinositol in NSCLC cells**

To understand whether ACSL3 is required to relay the KRAS-mediated AA cascade, we examined the lipid profile of the A549 NSCLC cell line upon ACSL3 knockdown by performing mass-spectrometry-based targeted lipidomics. We quantified the most abundant lipid species containing AA (20:4) in four phospholipid classes: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidyethanolamine (PE), and phosphatidylinositol (PI). We found that ACSL3 knockdown significantly altered the quantity of phospholipids containing AA in combination with 16:0 (palmitic), 16:1 (palmitoleate), 18:0 (stearate), and 18:1 (oleate) (Fig. 1a). Strikingly, the knockdown of ACSL3 resulted in a strong decrease in the AA content of PE and PI. Interestingly, we found a 40% decrease in PI containing C18:0–20:4 fatty acids (PI C18:0–20:4), the most abundant PI in mammalian cells [19–21] (Fig. 1a and Supplementary Fig. 1A). Of note, the knockdown of ACSL3 did not affect the protein levels of ACSL4, another ACSL with substrate preference for AA, underscoring the lack of compensatory effects (Supplementary Fig. 1B). These results strongly suggest that in mutant KRAS NSCLC cells, ACSL3 channels AA in glycerophospholipids.

PIs are reversibly phosphorylated at the inositol headgroup generating phosphoinositides, including phosphatidylinositol-4 phosphate (PI4P) and the plasma membrane-localized phosphatidylinositol-4, 5 bisphosphate, PI(4,5)P2 [22, 23]. We hypothesized that a decrease in the most abundant PI, C18:0–20:4 PI4P and C18:0–20:4 PI(4,5)P2. Indeed, upon ACSL3 knockdown, we observed a decrease in C38:4–PI4P and C38:4–PI(4,5)P2 lipids presumably due to the reduction of PI C18:0–C20:4 (Fig. 1b, c). This result additionally confirms a role of ACSL3 in esterifying AA into PI in mutant KRAS lung cancer cells.

**ACSL3 drives prostaglandin synthesis in NSCLC**

PI is the major source of AA, and in our lipidomic analysis, the AA-containing PI was consistently downregulated upon ACSL3 knockdown (Fig. 1a). Thus, we hypothesized that reduced AA-containing PIs would result in reduced prostaglandin synthesis. To this aim, we quantified PGE2, a prostaglandin produced by PGE2 synthase from COX-derived prostaglandin H2 [24]. We found that PGE2 was strongly reduced upon ACSL3 knockdown in a panel of NSCLC cell lines harboring KRAS mutations, namely A549, A427, H1264, and H358, and this coincided with decreased cell proliferation (Fig. 1d, e and Supplementary Fig. 1C). To investigate whether ACSL3 knockdown impairs PGE2 production in lung cancer cells carrying wild-type KRAS, we assessed four representative cancer cell lines with known differential sensitivity to ACSL3 knockdown, namely H596, H838, H125, and HCC95 (ref. [17] and Supplementary Table 1). The proliferation of H596 and H838 cell lines was unaffected by ACSL3 knockdown (hereafter these cells are mentioned as ACSL3-independent), while the proliferation of H125 and HCC95 is significantly reduced (hereafter these cells are mentioned as ACSL3-dependent) (Supplementary Figs. 1D and 1E). We measured PGE2 upon ACSL3 knockdown with two different shRNAs, and found that the loss of ACSL3 caused either a mild decrease (H596) or had no effect (H838) in PGE2 production in the ACSL3-independent cancer cell lines, while it highly suppressed PGE2 production in the ACSL3-dependent cell lines H125 and HCC95 (Supplementary Fig. 1F).

To confirm these results in vivo, we generated a mouse model bearing a Cre-activatable mutant Kras allele (LSL-KrasG12D/WT), homozygous for a Cre-conditional p53 knockout allele (p53fl/fl) [25, 26] and either wild type (LSL-KrasG12D/WT;p53fl/fl;Acsl3+/−) or knockout for Acsl3 (LSL-KrasG12D/WT;p53fl/fl;Acsl3−/−). In the LSL-KrasG12D/WT;p53fl/fl model, Cre-mediated loss of a stop cassette permits expression of the oncogenic KrasG12D allele from its endogenous promoter, and recapitulates key features of the human disease, including histologic features and response to conventional and targeted therapies [27]. Of note, we have previously shown that the LSL-KrasG12D/WT; Acsl3−/− mice display impaired lung tumor initiation and progression compared with their wild-type littermates [17]. Therefore, we employed this mouse model to assess...
prostaglandin production in mouse lungs 10 weeks after adenoviral-mediated Cre delivery to the lungs. In both genotypes, the level of different prostaglandins (PGE2, PGD2, and PGI2) was significantly increased in tumor lesions compared with healthy lungs (Fig. 1f). However, we found in both lung tumors and healthy lungs that Acsl3 knockout causes a striking reduction of the aforementioned prostaglandins (Fig. 1f). Thus, our results suggest that ACSS3 is important for prostaglandin synthesis regardless of the oncogenic mutational status. Nevertheless, our data indicate that ACSS3 significantly enhances prostaglandin synthesis in tumors compared with healthy lung, suggesting that this may fulfill the increased requirement for prostaglandin synthesis of lung cancer.
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that ACSL3 knockdown in A549 cells led to a reduction in –

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whether LPIAT1 knockdown suppresses PGE2 production

the enzyme responsible for the conversion of LPI–PIs with

and improved their overall survival (Fig. 3a–d). These

caused either a mild decrease (H596) or had no effect

improves the survival of mice bearing human lung cancer xenografts

Our results suggest that LPIAT1 may be essential for

In order to expand the significance of our finding in vivo, we injected the A549 (mutant KRAS) and HCC95 (wild-type KRAS, ACSL3-dependent) NSCLC cells into immunocompromised NOD scid gamma (NSG) mice, previously transduced with a control or shRNA against LPIAT1. In this model, we found that knockdown of LPIAT1 significantly suppressed tumorigenesis in mice and improved their overall survival (Fig. 3a–d). These results indicate that LPIAT1 plays a role in tumor progression.
The ACSL3-LPIAT1 signaling drives prostaglandin synthesis in non-small cell lung cancer.
LPIAT1 is upregulated in human NSCLC and positively correlates with poor patient survival

To explore the relevance of LPIAT1 in lung cancer, we investigated a lung adenocarcinoma cohort (subset LUAD) that includes information on KRAS mutational status from the The Cancer Genome Atlas (TCGA) database, to compare the gene expression of LPIAT1 between wild-type KRAS tumors, mutant KRAS tumors and healthy lung tissue [31]. Our analysis evidenced a higher expression of LPIAT1 in lung tumors compared with healthy lung tissue samples (Fig. 4a). However, the expression of LPIAT1 was higher in tumors with KRAS mutations compared with tumors carrying wild-type KRAS allele (Fig. 4a). Moreover, high LPIAT1 expression highly correlated with high Prostaglandin E Synthase expression, an enzyme that catalyzes the conversion of prostaglandin H2 to PGE2 (Fig. 4b). These data suggest that high LPIAT1 expression is not restricted to mutant KRAS tumors and underscores a broader relevance of LPIAT1 in NSCLC.

To validate the database analysis, we performed immunoblot using human patient-derived NSCLC biopsies with known KRAS mutations and the corresponding adjacent healthy tissue. Our results indicated that the protein levels of ACSL3, LPIAT1 and the cytosolic phospholipase A2 (cPLA2), an enzyme that catalyzes the hydrolysis of membrane phospholipids to release AA for prostaglandin and other eicosanoid production, are upregulated in patient-derived mutant KRAS tumors compared with adjacent healthy tissue (Fig. 4c). Thus, these data confirm that the ACSL3-LPIAT1 metabolic pathway is enhanced in NSCLC.

To assess the relationship between ACSL3 and LPIAT1 expression, we employed a NSCLC cohort that includes squamous lung carcinomas (LUSC) and lung adenocarcinomas (LUAD), stratified by ACSL3-high and ACSL3-low expression, and we found a direct correlation between the expression levels of LPIAT1 and ACSL3, suggesting a co-regulation of these enzymes in NSCLC (Fig. 4d).

Next, we examined the relationship between patient survival and ACSL3 or LPIAT1 expression. Kaplan–Meier analysis of LUSC and LUAD patient cohorts stratified by high versus low ACSL3 or LPIAT1, evidenced that patients with either high ACSL3 or high LPIAT1 expression had lower overall survival (Fig. 4e, f). These results suggest that both ACSL3 and LPIAT1 overexpression are clinically relevant and may have prognostic value for survival outcomes in NSCLC patients.

Discussion

Elevated prostaglandin levels have been extensively associated with enhancement of cancer cell survival and tumor growth, migration, invasion, and immunosuppression [3]. In several types of cancer, including mutant KRAS lung tumors, an important part of this effect has been attributed to the enhanced activity of COX1 and 2, the enzymes responsible for the production of prostaglandins from AA [32–34]. However, how the metabolism of AA is remodeled in cancer cells to cope with the high demand for prostaglandin synthesis remains elusive. Here, we found that, in mutant KRAS and in a subset of wild-type KRAS lung cancer cells, high prostaglandin levels are sustained by LPIAT1 activity and depend on the ACSL3-activated AA substrate availability (Fig. 2 and Supplementary Fig. 2). Importantly, the ACSL3-LPIAT1 metabolic axis drives prostaglandin synthesis to promote tumorigenesis in NSCLC (Fig. 3). We found that a subset of wild-type KRAS cancer cells show virtually no effect in PGE2 suppression and cell proliferation upon ACSL3 or LPIAT1 knockdown. These data suggest that alternative signaling pathways may confer resistance to ACSL3 or
LPIAT1 inhibition and future studies will be necessary to identify these mechanisms. For instance, since the production of free AA is highly regulated by a PLA2-dependent deacylation reaction and a LPIAT1-dependent reacylation/transfer into PI pools, PLA2 overexpression may result in increased release of free AA available for prostaglandin synthesis leading to resistance to LPIAT1 inhibition. Indeed, cPLA2 overexpression is common in NSCLC [35]. Moreover, PLA2 enzymes can also liberate free AA from other phospholipids such as PC and PS [36]. Importantly, we found an upregulation of some PC and PS species bound to AA (C14:0–C20:4 PC, C16:0–C20:4 PC, and C16:1–C20:4 PS) upon ACSL3 knockdown, suggesting that ACSL3 suppression results in a remodeling of PC and PS membrane phospholipids that PLA2 could, in principle, deacylate to provide free AA for prostaglandin synthesis (Fig. 1a).

Our data show that cancer cell lines that are sensitive to ACSL3 knockdown are also sensitive to LPIAT1 knockdown, while others that are resistant to ACSL3 knockdown are also resistant to LPIAT1 knockdown (Supplementary Fig. 1D-1F and Supplementary Fig. 2B–2D). We also found that overexpression of LPIAT1 causes enhanced cancer cell proliferation and increased colony formation, an effect that was rescued by knocking down ACSL3 (Fig. 2e, h). In this context, knockdown of ACSL3 reduced the availability of arachidonoyl-CoA, the substrate of LPIAT1, hence reducing the most abundant PI species (C18:0–20:4 PI) and prostaglandin synthesis (Figs. 1a, d and 2f). Thus, our data indicate that ACSL3 and LPIAT1 regulate cancer cell proliferation, at least in part, by acting on the same metabolic axis. Whether the overexpression of ACSL3 is sufficient to increase synthesis of prostaglandins and promote cancer proliferation remains to be investigated.

We previously found that ACSL3 is required for fatty acid β-oxidation in NSCLC cells [17]. Therefore, it is plausible that LPIAT1 and ACSL3 can sustain lung cancer proliferation by also serving other pathways. In this regard, it has been recently shown that during human platelet activation, prostaglandins and other eicosanoids are fed at high rates into β-oxidation in a cPLA2-dependent manner. Accordingly, cPLA2 blockade decreased β-oxidation and impaired mitochondrial respiration [37]. Thus, it would be of interest to investigate whether lung cancer cells rely on prostaglandins as substrates to support β-oxidation.

Although nonsteroidal anti-inflammatory drugs, which target COX enzymes and specific COX2 inhibitors, are among the most promising drugs against cancer, the serious cardiovascular and gastrointestinal side effects have reduced the enthusiasm for their use [38]. Therefore, the identification of novel tumor-specific targets related to AA...
remodeling may help develop therapies with a greater benefit and fewer side effects. Our results suggested that prostaglandin synthesis is higher in lung tumors compared with healthy tissue (Fig. 1f). Of note, mice with germline deletion of \textit{Acsl3} are viable and do not show any overt dysfunctions [17]. Thus, in principle, pharmacologic targeting of the ACSL3-LPIAT1 axis may benefit patients to selectively target tumor-derived prostaglandin synthesis while sparing normal cellular functions. However, germline deletion of LPIAT1 in mice results in defective brain development and mortality [39]. It would therefore be of interest to determine whether post-developmental knockout or inhibition of LPIAT1 would lead to a similar or other body dysfunctions, and whether it could be used for cancer treatment in the future. Notably, we found that high \textit{LPIAT1} expression predicts poor survival in mouse xenografts and human NSCLC patients (Figs. 3, 4). Hence, the status of ACSL3-LPIAT1 axis in human lung tumors may serve as a biomarker for personalized anti-cancer treatment.

Increased synthesis of prostaglandins is a negative prognostic marker in lung cancer and several other malignancies (e.g., gastric, colorectal, breast, hepatic, bladder, and renal cancers) [10, 40, 41]. Thus, future experiments aimed at assessing the role of LPIAT1 in prostaglandin synthesis and tumorigenesis in other types of cancer are warranted.

In conclusion, we have unraveled the ACSL3-LPIAT1 metabolic axis as a requirement for prostaglandin production and tumorigenesis in NSCLC that could be exploited for therapeutic intervention.

**Materials and methods**

**Resource sharing**

Further information and requests for resources and reagents should be directed to, and will be fulfilled by the Lead Contact, Georgia Konstantinidou, Institute of Pharmacology,
University of Bern, 3010 Bern, Switzerland (georgia.konstantinidou@pki.unibe.ch). Requests will be handled according to the University of Bern policies regarding MTA and related matters.

Reagents and plasmids

pLKO.1 puro (Addgene plasmid #8453; http://n2t.net/addgene:8453; RRID:Addgene_8453), pLKO.1 hygro (Addgene plasmid #24150; http://n2t.net/addgene:24150; RRID:Addgene_24150), pCMV-VSV-G (Addgene plasmid #8454; http://n2t.net/addgene:8454; RRID:Addgene_8454), and pCMV-dR8.2 dvpr (Addgene plasmid #8455; http://n2t.net/addgene:8455; RRID:Addgene_8455) were a gift from Prof. Bob Weinberg [42]. The human LPIAT1-containing net/addgene:8454; http://n2t.net/addgene:8454; RRID:Addgene_8454), pLKO.1 hygro (for the combination studies with LPIAT1 over-expression) plasmids after digestion with AgeI/EcoRI. The ACSL3 shRNAs were obtained as bacterial glycerol stock from Sigma-Aldrich and the sequence of interest was subcloned into the pLKO-puro backbone or into pLKO-hygro (for the combination studies with LPIAT1 over-expression) plasmids after digestion with AgeI/EcoRI. The final shRNA constructs were confirmed with sequencing.

Cell lines

All human NSCLC cell lines used in this study (A549, H358, H1264, A427, H838, H596, H125, and HCC95) were derived from male patients, and were provided by Dr. John Minna (UT Southwestern Medical Center) [43]. All cell lines were DNA fingerprinted for provenance. Cell lines were screened free for mycoplasma, and cultured in an incubator at 37 °C and 5% CO2 in RPMI-1640 medium.

Animal studies

Mice were maintained under controlled humidity and temperature conditions, with a standard 12-h light/dark cycle and were fed ad libitum. Mixed background LSL-Kras<sup>G12D/WT;</sup> p53<sup>lox/lox</sup> mice were generated by crossing B6.129SS4-Kras<sup>tm1Tyj/J</sup> (from JaxLab, Stock number 008179) [25], with B6.129P2-Tp53<sup>tm1Ben/J</sup> (from JaxLab, Stock number 008462) mouse [26]. The mixed background Cre-inducible LSL-Kras<sup>G12D/WT;</sup> p53<sup>lox/lox</sup> Acs3<sup>–/–</sup> mouse model was obtained by crossing LSL-Kras<sup>G12D/WT;</sup> p53<sup>lox/lox</sup> mice with B6.129S5-Acs3<sup>Gt(OST148301)Lex/Orl</sup> [44]. LSL-Kras<sup>G12D/WT;</sup> p53<sup>lox/lox</sup> Acs3<sup>–/–</sup> mice were backcrossed for eight generations, before creating the experimental groups. The NOD.Cg-Prkd<sup>ox</sup>/I2rg<sup>tm1Wij</sup>/SzJ NSG mice were from Jackson labs (stock number: 008179) and the sequence of interest was subcloned into the pLKO-puro backbone or into pLKO-hygro (for the combination studies with LPIAT1 over-expression) plasmids after digestion with AgeI/EcoRI. The final shRNA constructs were confirmed with sequencing.

Human studies

The patient-derived frozen lung adenocarcinoma samples used for Fig. 4 were provided by the institute of pathology, translational research unit. The sex of the patients is the following: Patient #1: female, #2: female, #3: male, and #4: female. The use of human samples was approved by the ethics commission (swissethics), ID: 2017-01322. All samples were provided upon patients’ consent.

TCGA data analysis

TCGA LUSC and lung adenocarcinoma (datasets LUSC and LUAD, respectively) datasets were retrieved from http://cancergenome.nih.gov. The data were downloaded with the help of the web graphic user interface Xenabrowser (xenabrowser.net) [45] and analyzed with GraphPad Prism version 7.0. Incomplete data, missing expression values and/or survival were eliminated from the analysis and only primary tumors were considered. RSEM gene quantifications as provided by TCGA were taken. The stratification in high-/
low-expressing groups in Fig. 4b, d was performed by median separation as indicated in the related figure legends. For the patient survival analysis (Fig. 4e, f), patients were classified into two groups, and association between prognosis (survival) and gene expression (FPKM) was examined. The best expression cut-off refers the FPKM value that yields maximal difference with regards to survival between the two groups at the lowest log-rank P value.

shRNAs, virus production, and transduction

Recombinant lentiviruses were produced by transfecting HEK 293 T cells, using the TransIT®-293 Transfection Reagent (Mirus; MIR2705), with pCMV-VSV-G (VSV-G protein), pCMV-dR8.2 (lentivirus packaging vector), and lentiviral constructs, according to the manufacturer’s instructions.

Mass spectrometry-based shotgun lipidomics

Lipid extraction

Mass spectrometry-based lipid analysis was performed by Lipotype GmbH (Dresden, Germany) as previously described [46]. Lipids were extracted using a two-step chloroform/methanol procedure [47]. Data analysis and post-processing. Data were analyzed with in-house developed lipid identification software based on LipidXplorer [48, 49]. Data post-processing and normalization were performed using an in-house developed data management system. Only lipid identifications with a signal-to-noise ratio >5, and a signal intensity fivefold higher than that in the corresponding blank samples, were considered for further data analysis. Experimenters were blinded during data analysis.

Ultra-performance liquid chromatography-tandem mass spectrometry

Reagents

Methanol, chloroform, dichloromethane, and acetonitrile (Fisher) were all of mass spectrometry grade. Sodium formate and HCl were from Sigma, and TMS-diazomethane (TMS-DM, 2.0 M in hexanes) from Sigma-Aldrich and Acros. Lipid standards were ammonium salts of 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1′,4′,5′-trisphosphate) [17:0-20:4 PI(3,4,5)P3] Avanti Polar Lipids, LM1906, Trimyristin (14:0, 14:0, 14:0), Tripalmitin (16:0, 16:0, 16:0), Triolein (18:1, 18:1, 18:1) Trilinolein (18:2,18:2,18:2), Tristearin (18:0,18:0,18:0), Triarachidonin (20:4,20:4,20:4) (NuChek-Prep, Inc. Elysian, MN).

Sample processing

Cells (2 × 10⁶) were washed twice with PBS and incubated with 0.5 M trichloroacetic acid (TCA) for 5 min on ice. Cells were then scraped from the dish, vortexed for 30 s and further incubated on ice for 5 min. The TCA-treated samples were centrifuged at 20,000 × g for 3 min at 4 °C. After discarding the supernatant, the pellet was resuspended in 1 mL of 5% (w/v) TCA + 10 mM EDTA and centrifuged at 20,000 × g for 3 min at 4 °C. After repeating the same step once, the pellet was used for the lipid extraction.

Lipid extraction

Prior to lipid extraction the following lipid analytical internal standards were added to the TCA precipitates: 17:0–20:4 PI(4,5)P2, 17:0–20:4 PI(3,4,5)P3, 17:0–20:4 PIP. Lipids were extracted using a modification of an acidified chloroform-methanol extraction protocol [50, 51]. It was initiated by adding 670 µL of chloroform:methanol:12 N HCl (40:80:1) to the TCA precipitate followed by vigorous vortexing for 5 min and incubation for 10 min at 4 °C. Then 650 µL of ice-cold chloroform was added and the samples were vortexed for another 2 min and allowed to sit for 5 min at 4 °C after which 300 µL of ice cold 1 M HCl was added. The samples were vortexed for 2 min, centrifuged at 10,000 × g for 2 min, and the lower phase was then collected in a fresh 2-mL microcentrifuge tube. Ice cold theoretical lower phase (900 µL) generated by combining chloroform:methanol:1.74 M HCl mixture (86:14:1,v/v/v) was added to the upper phase and the mixture was vortexed and centrifuged. The lower phase was then combined with the previously collected lower phase and dried under a stream of N₂ and subsequently methylated as previously described [52].

Mass spectroscopy

LC/MS was carried out essentially as previously described [52]. Aliquots of sample resuspended in 20–100 µL of 100% mass spectroscopy grade methanol were injected with a Waters Acquity FTN autosampler into the UPLC/MS. Chromatography over a Waters Acquity UPLC C4 column (Waters Acquity UPLC Protein BEH C4, 1.7 μm 1.1 × 100; 300 A) was carried out with an acetonitrile formic acid gradient monitored by a Waters XEVO TQ-S MS/MS in multiple reaction monitoring mode using electrospray and
positive ion mode. The gradient was initiated with 10 mM formic acid in water/10 mM formic acid in acetonitrile (33:67 v/v), held for 1 min, then increased to 15:85, v/v in 9 min following injection, held at 85% for 1 min, then increased to 100% for an additional 3 min, and then re-equilibrated to starting conditions for 3 min.

Quantification of PGE2, PGD2, and PGI2 by LC–MS/MS

Sample preparation

Lung tissue samples were weighted while still frozen and transferred into 2 mL XXTuff reinforced microvials (Bio Spec Products Inc., OK, USA) with three chrome-steel beads (diameter, 2.3 mm; Bio Spec Products Inc., OK, USA) and the corresponding volume of 0.1 M formic acid to reach 100 mg/mL. Samples were homogenized using a Mini-Beadbeater-24 (Bio Spec Products Inc., OK, USA) and the extraction of AA, PGE2, PGD2, and PGI2 (since PGI2 is not metabolically stable, this analyte was quantified using its stable hydrolysis product 6k-PGF1α) as previously described [53]. LC–MS/MS conditions. LC–MS/MS analysis was performed using our previously described protocol [53] with some changes. We used a Shimadzu UFLC coupled to a TripleQuad 5500 QTRAP mass spectrometer (AB Sciex, Canada). The LC column was a Reprosil-PUR C18 column (3 μm particle size; 2 × 50 mm; Dr. A. Maisch HPLC GmbH, Germany) maintained at 40 °C with a mobile phase flow rate of 0.3 mL/min. The mobile phase composition was a mixture of (A) 2 mM ammonium acetate plus 0.1% formic acid and (B) acetonitrile plus 0.1% formic acid. A gradient elution was used, starting with 95% of phase A and linear increase of phase B reaching 40% at minute 3; then the linear increase rate was decreased to reach 65% B at minute 9. Finally, to flush the column, phase B was increased by 95% at minute 10 and kept for 4 min with a subsequent re-equilibration by decreasing phase B down to 5%. The total analysis time was 17 min. For quantification, an 11-point calibration curve was analyzed, determining the slope, intercept, and regression coefficient, and analytes concentration in the samples was calculated applying the model previously described [53]. The values were normalized to total proteins. Protein quantification was done with a BCA kit.

PGE2 ELISA assay

PGE2 levels were measured in culture medium of A549, A427, H1264, H358, H596, H838, H125, and HCC95 human NSCLC cell lines using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, for every cell line 3–3.5 × 10^5 cells were plated in six-well plates in a standard volume of culture medium. The supernatant was collected at 24 h, and the assay was performed according to the manufacturer’s protocol (Cayman Chemical, 514010). The assay had a range from 7.8 to 1000 pg/ml and its sensitivity was ~15 pg/ml. Proteins from the adherent cells were then extracted in a standard volume of RIPA buffer and the total protein content was used as normalization factor for PGE2.

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing complete EDTA-free protease inhibitors (Roche) and 1 mM PMSF. Samples were resolved by SDS-PAGE in Bio-Rad blotting chamber, transferred to nitrocellulose membrane using a semi-dry chamber (Bio Rad) and blocked in 5% BSA. Membranes were then incubated overnight at 4 °C with primary antibody diluted in 5% BSA in TBS containing 0.1% Tween. Secondary fluorescent-tagged antibodies were from Li-Cor biosciences, and development was done in Li-Cor fluorescence–chemiluminescence detector. All antibodies and their dilutions are listed in Supplementary Table 2.

RT-PCR

RNA was extracted using the RNAeasy kit (QIAGEN, 74104), and cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622). RT-PCR was performed in 96-well plates (TrefLab) with FastSybr green (Thermo Scientific, 4367659). The normalization was performed with the ΔΔCT method. The full list of the oligonucleotides used can be found in Supplementary Table 2.

Cell proliferation assay

Cells were plated at low confluency in 24-well plates (8000 cells/well for A549, 9000 cells/well for H358, H1264, and A427) and allowed to proliferate for 48 or 96 h. Cell viability was measured by crystal violet (Sigma-Aldrich) staining (0.1% in 20% methanol) of adherent cells after 10 min fixation in 4% paraformaldehyde (Sigma-Aldrich). After washing twice and air-drying, stained cells were decolored with 5% acetic acid, and OD_{600} was measured with a spectrophotometer.

Soft agar colony formation assay

Cells (3 × 10^5/well) were seeded on semi-solid agar medium (bottom layer 0.6% and top layer 0.4% mixed with cells) in
a six-well plate. After 14–21 days cells were formalin-fixed and stained with 0.005% Iodonitrotetrazolium. The colonies were counted using a microscope.

**Statistical analysis**

All data sets were organized and analyzed in Microsoft excel 2016 and GraphPad Prism version 7.0.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All data presented are expressed as mean ± SEM or ±SD of three or more biological replicates/group (except for the in vivo experiments and the human data analysis, the number is indicated in the related figure legends). The mass spectrometry-based lipidomics analyses and PGE2 measurements were repeated twice. The significance of the results was determined by employing two-tailed unpaired Student’s t test and one- or two-way ANOVA (Tukey’s post test) when more than two groups were compared, and significance is indicated in the related figure legends. No outliers were found in any data set and no animals or data were excluded from statistical analysis.

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**Author contributions**

Experiments were conceived and designed by GK and MS, performed by MS, CP, MRS, and IR, JG and IR performed lipid measurement quantification and statistical analysis. This study was supported by the Swiss National Science Foundation (SNSF) professorship PP00P3_163929, Novartis Foundation for medical–biological research #16C190, UniBern Forschungsstiftung (to GK), and SNSF research grant #163359 (to JG).

**Compliance with ethical standards**

**Conflict of interest**

The authors declare that they have no conflict of interest.

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