

Myc expression drives aberrant lipid metabolism in lung cancer

Running title: Role of lipids in high Myc lung tumours

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Abstract

MYC mediated pathogenesis in lung cancer continues to attract interest for new therapeutic strategies. In this study, we describe a transgenic mouse model of KRAS-driven lung adenocarcinoma that affords reversible activation of MYC, used here as a tool for lipidomic profiling of MYC-dependent lung tumors formed in this model. Advanced mass spectrometric imaging and surface analysis techniques were employed to characterize the spatial and temporal changes in lipid composition in lung tissue. We found that normal lung tissue was characterized predominantly by saturated phosphatidylcholines and phosphatidylglycerols, which are major lipid components of pulmonary surfactant. In contrast, tumor tissues displayed an increase in phosphatidylinositol and arachidonate-containing phospholipids that can serve as signalling precursors. Deactivating MYC resulted in a rapid and dramatic decrease in arachidonic acid and its eicosanoid metabolites. In tumors with high levels of MYC, we found an increase in cytosolic phospholipase A2 (cPLA2) activity with a preferential release of membrane-bound arachidonic acid, stimulating the lipoxygenase (LOX) and cyclooxygenase (COX) pathways also amplified by MYC at the level of gene expression. Deactivating MYC lowered cPLA2 activity along with COX2 and 5-LOX mRNA levels. Notably, inhibiting the COX/5-LOX pathways in vivo reduced tumor burden in manner associated with reduced cell proliferation. Taken together, our results show how MYC drives the production of specific eicosanoids critical for lung cancer cell survival and proliferation, with possible implications for the use of COX and LOX pathway inhibitors for lung cancer therapy.

Introduction

Non-small cell lung cancer is a leading cause of cancer death. The single name encompasses several genetically distinct pathologies, and increasingly efforts to identify therapies for this disease have attempted to target specific mutations. In adenocarcinoma, more than a third of tumours have an

activating mutation in KRAS, and MYC has been shown to be frequently overexpressed [1]. Whilst the oncogenic activation of MYC results in uncontrolled cell proliferation [2], MYC inhibition leads to tumour regression and re-differentiation of cells [3, 4]. As such MYC is an attractive candidate for cancer drug therapies; however all its functions and interactions are not fully understood.

Cancer cell proliferation is typically accompanied by metabolic reprogramming, with increased glucose uptake and aerobic glycolysis, as part of the Warburg effect [5]. In addition, cancer cells synthesise lipids through *de novo* lipogenesis [5]. These lipids play a structural role in new cell membranes and are likely to be further involved in signalling events important for cancer development [5, 6]. Whilst the precise role of aberrant lipid biosynthesis is unknown, several studies have shown that the lipid profile has been linked to survival, ER status and tumour grade in human breast cancer [7, 8]. The lipid composition of tumours is also affected by dynamic processes, having been observed to vary with cell proliferation, apoptosis and necrosis [9]. Furthermore, a recent study has revealed distinct lipid signatures in MYC- or RAS-induced lymphoma suggesting that the observable lipid profile can be related to specific oncogene expression [10]. In addition to genotype specificity, evidence suggests that metabolic profiles are highly tissue specific [11].

Here we use a novel transgenic mouse model of KRAS^{G12D}-driven lung adenocarcinoma with reversible activation of MYC, to study changes to the lung lipid profile following tumorigenesis with high MYC activity, and subsequent deactivation of MYC over time. We characterised the changes in lipid composition and abundance using matrix assisted laser desorption ionisation-mass spectrometry imaging (MALDI-MSI) and liquid extraction surface analysis-mass spectrometry (LESA-MS). MALDI-MSI is ideally placed for *in situ* molecular mapping since lipids are directly ablated and ionized from tissue by a moving laser pulse, retaining critical spatial information [12-15]. In LESA-MS lipids are extracted directly from the surface of tissue with solvent, providing an orthogonal

approach to the direct analysis of lipids from tissues [16-18]. This allowed us to follow lipidome changes and to map these effects across the tissue.

Overall we established the lipid signatures for normal and tumour lung tissue in mice with increased MYC activity and endogenous levels of oncogenic KRAS. We found that normal tissue was predominantly characterised by pulmonary surfactant lipids, whereas tumour tissue had increased signalling precursor phospholipids, such as the phosphatidylinositols and arachidonate-containing phospholipids. We observed an increase in free arachidonic acid, which is released from membrane phospholipids by cytosolic phospholipase A2 (cPLA2), in tumours with high MYC activity and linked this to increased cPLA2 activity. Moreover, we report evidence at the metabolite and gene expression level, linking MYC to increased synthesis of arachidonic acid-derived eicosanoids via the lipoxygenase (LOX) and cyclooxygenase (COX) pathways. Following MYC deactivation, specific metabolites and mRNAs associated with these pathways, and cPLA2 activity, decreased with time. By inhibiting the COX and 5-LOX pathways in high MYC mice, a reduction in tumour proliferation and increase in apoptosis was observed, highlighting these pathways as potential drug targets for lung cancer.

Materials and Methods

Tumour models

Lung tumours were generated by intranasal delivery of adeno-Cre (7×10^6) virus particles to either *Kras^{tm1Tyj}* (*LSL-Kras^{G12D}*) mice, which express oncogenic *Kras^{G12D}* upon Cre-mediated deletion, or *LSL-*

Kras^{G12D}; *R26*^{LSL-CAG-c-MycER/ LSL-CAG-c-MycER} (*R26*^{LSL-CMER}) mice, which in addition to oncogenic KRAS express a high level of deregulated c-MYCER^{T2} (driven by a CAGGs (chicken beta actin/CMV) enhancer) from the *Rosa26* locus. The *Rosa26* model of c-MYCER^{T2} is regulated at two levels; expression of cre-recombinase is necessary to remove the stop element and addition of tamoxifen to the diet is necessary to activate the c-MYCER^{T2} protein, providing a means to activate or deactivate MYC [19]. Detailed characterisation of this novel mouse model will be described elsewhere (manuscript in preparation).

Ten adeno-Cre injected *LSL-Kras*^{G12D}; *R26*^{LSL-CMER} mice were fed a tamoxifen-containing diet for 4 weeks. Tamoxifen was removed from the diet for 24 hours (n=3) or 72 hours (n=3), in order to deactivate MYC (referred to as “MYC inactive”) before culling. The remaining mice (n=4, referred to as “MYC activated”) were maintained on the tamoxifen-containing diet continuously. As a further control and to ensure the overall lipid content was not altered by the addition/removal of tamoxifen, *LSL-Kras*^{G12D} mice, where MYC expression was not controlled (referred to as “KRAS only”), were maintained either on a tamoxifen-containing (n=2) or normal diet (n=2). Lung samples were collected, snap-frozen with liquid nitrogen and stored at -80 °C. Samples were embedded in Tissue-Tek® OCT™ and 12 µm frozen sections on glass microscope slides were prepared using a cryostat. Adjacent sections were stained with haematoxylin and eosin (H&E). All animal procedures were approved by the UK Home Office and the University of Cambridge.

MALDI Imaging MS

Matrix solutions of 10 mg/mL [2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich, St Louis, MO) or 2, 4, 6-trihydroxyacetophenone monohydrate (THAP; Sigma-Aldrich)] were administered to the tissue surface using a nebulised sprayer (Suncollect MALDI spotter, KR Analytical Ltd, Cheshire, UK).

Imaging experiments were carried out using a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Hemel Hempstead, UK) at 50 µm step increments across the tissue. The chemical composition of the lipid head group determines whether it has a greater propensity to form positive or negative ions. To detect as many different lipid species as possible, spectra were acquired in positive (DHB) and negative (THAP) ion mode, from 250-1000 *m/z* at 60,000 resolution. Mass accuracy in positive and negative ion mode was approximately 1 and 10 ppm, respectively. Lipid identity was performed by accurate mass using the Lipid Maps database (accessed December 2015) [20]. The predominant fatty acid composition of lipids was confirmed where possible by collision-induced dissociation (CID). Na⁺ and K⁺ lipid adducts commonly observed in positive ion mode using MALDI have a low yield of stable charged fragments. Therefore, in order to improve fragmentation efficiency in positive ion mode, the formation of lithium adducts more prone to fragmentation [21] was induced by a 5 s washing step in lithium nitrate (100 mM) prior to deposition of matrix, and the corresponding lithiated ions subjected to fragmentation by CID.

LESA-MS

Lipids were extracted using LESA at multiple user-defined points (1 mm²) across the tissue surface by dispensing 0.8 µL 1:2:4 chloroform: methanol: isopropanol with 10 mM ammonium formate, and incubating for 2.5 s. Analytes extracted from the surface were infused using a Triversa Nanomate® (Advion BioSciences, Ithaca, NY), with capillary voltage 1.4 kV, 0.3 gas flow for 1 min into an LTQ Orbitrap Elite™ (Thermo Fisher Scientific). Spectra were acquired in positive and negative mode, from 200 – 1000 *m/z* at 60,000 resolution.

Data processing

Data from MALDI-MSI were converted to imzML format for processing [22]. Using an in-house R script ions were retained above a threshold intensity and the *m/z* ratio of ions detected were summed across integral regions of 10 ppm to allow for alterations in *m/z* mass accuracy across the experiment (“binning”). Only features detected above a threshold proportion of pixels were retained. Ion intensities were normalised to the total ion count, prior to creating two-dimensional ion images. Clustering of pixels was performed using *k*-means clustering [23], in order to define tumour and normal tissue regions, and to establish the average spectra in each region from the cluster centers (**Figure S1**).

Data from LESA-MS were converted to mzML format for subsequent data processing, and features were picked using an in-house R script. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) models [24] were constructed using SIMCA 14 (Umetrics, Umea, Sweden), following normalisation to the total ion count, and Pareto scaling [25]. For univariate statistics, *P* values were determined using Student’s *t*-tests ($\alpha = 0.05$) and are adjusted for multiple testing using the Benjamini-Hochberg procedure.

Eicosanoid assay

Ten microliters of butylated hydroxytoluene (0.2 mg/mL, Sigma-Aldrich), 40 μ L of an internal standard mix (containing 100 nM each of 12-HETE-D8, PGE₂-D4, and 8-iso-PGF_{2 α} -D4, Cayman Chemical Company, Ann Arbor, MI), and 1 mL of methanol were added to approximately 100 mg of frozen tissue sample which was homogenised using a TissueLyser (Qiagen Ltd., Manchester, UK) for 10 min at 30 Hz. Samples were centrifuged (17,000 $\times g$, 5 min) and the supernatants dried down under nitrogen and reconstituted in 40 μ L 1:1 (v/v) methanol: water. Samples were analysed on QTRAP 5500 (ABSciex UK Limited, Warrington, UK) coupled to an ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters Ltd., Wilmslow, UK). The mobile phase gradient (0.5 mL/min) is detailed in **Table S1**; mobile phase A was 0.1 % acetic acid in water, mobile phase B was

0.1 % acetic acid in acetonitrile: methanol 80:20. Sample was injected (10 µL) into a Kinetex 2.6 µm XB-C18 100 Å column (100 x 2.1 mm, Phenomenex, Macclesfield, UK) at 30 °C. The electrospray source was operated in negative ion mode (4.5 kV and 650 °C). Mass transitions of 51 analytes and 3 isotopically labelled standards (**Table S2**) were acquired in scheduled MRM mode. Peaks were integrated by the Quantitation Wizard within Analyst™ version 1.6 and normalised against wet tissue weight and to the intensity of the internal standards.

Immunohistochemistry

Staining was performed on 4 µm paraffin-embedded sections. Sections were deparaffinised, treated with citrate buffer for epitope retrieval and endogenous peroxidases blocked in 3% H₂O₂. Immunohistochemical staining was performed using the Rabbit VECTASTAIN ELITE ABC horseradish peroxidase kit (Vector Laboratories) following the manufacturer's protocol. Primary antibodies were anti-Ki67 (*Thermo Scientific*; clone: SP6; 1:200), anti-caspase 3 antibody (Cell Signalling Technology; clone; 5A1E 1:2000), anti-c-MYC antibody [Y69] (Abcam; ab32072; 1:1000), anti-phospholipase A2 (Abcam; ab58375; 1:1000) and anti-phospholipase A2 (phospho Ser505) (Abcam; ab53105; 1:200). Staining was imaged on a Zeiss Axio Imager using the Zeiss AxioVision 4.8 software. Quantification was performed by counting number of positive cells per tumour area for 5 images; the mean of 5 raw counts was calculated and represents one data point per graph.

Phospholipase A2 activity assay

Enzymatic activity was assessed using the cytosolic phospholipase A2 assay kit (Abcam; ab133909) and following the manufacturer's instructions. Calcium independent PLA2 activity was inhibited by the addition of bromoenol lactone, and secretory PLA2 isoforms removed using a membrane filter with molecular weight cut-off 30 kDa. Activity was normalised to total protein content.

Transcript quantification by qPCR

Total RNA was extracted and purified from homogenised frozen lung tissue (20 mg) using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. Purified RNA concentration was quantified at 260 nm using a NanoDrop 100 (Thermo Fisher Scientific). Genomic DNA contamination was eliminated and complimentary DNA produced using the RT² First Strand Kit (Qiagen). Relative abundance of transcripts of interest was assessed by quantitative-PCR in RT² SYBRgreen Mastermix (Qiagen) with a StepOnePlus detection system (Applied Biosciences). RT² primer assays for mouse *Rn18s* (endogenous control), *Pla2g4a* (*cPla2*), *Ptgs2* (*Cox2*), *Alox5*, *Alox15*, *Alox12*, *Tnf-α*, *Il-1β* were obtained from Qiagen. Expression levels were normalised to endogenous controls using the ΔΔC_T method and fold changes reported were relative to the "KRAS only" group (tamoxifen diet). Additional mouse lung samples were prepared, such that 4-6 replicates were used per group. Statistical significance was determined using one way analysis of variance ($\alpha = 0.05$) and adjusted for multiple comparisons using the Holm-Sidak method.

In vivo inhibitor study

Eight adeno-Cre infected *LSL-Kras^{G12D};R26^{LSL-CMER}* mice were fed a tamoxifen-containing diet as described above. Two weeks following the administration of tamoxifen diet, mice were treated with licofelone (n=4) or vehicle (n=4) once daily for 4 days, and culled two hours following the final treatment. Licofelone (Calbiochem; #435801), was freshly suspended in sunflower oil at a concentration of 20 mg/mL and given by oral gavage at a dose of 100 mg/kg.

Results

Tumours have distinct lipid signatures to normal tissue

First we sought to characterise the lipid signature in KRAS-driven lung tumours with increased MYC activity, and compare this to the lipid profile of adjacent normal tissue using MALDI-MSI. In order to distinguish between normal and tumour tissue, pixels of each image were clustered according to their spectral similarity (**Figure 1A**). This was validated by comparing to histological features in adjacent H&E stained sections (**Figure 1**), and the average spectra of the tumour and normal tissue in both positive and negative mode compared (**Figure 1B-C**). Using their accurate masses, and database searching (**Table S3**) the majority of ions observed in positive ion mode were found to be sodiated (Na^+) and potassiated (K^+) adducts of phosphatidylcholines (PC) and their corresponding fragment ions (partial loss of head group, $\Delta m/z$ 59.074), whereas in negative ion mode spectra were predominantly characterised by deprotonated phosphatidylinisotols (PI), phosphatidylglycerols (PG), and free fatty acids (FFA). Characteristic product ion spectra were used to confirm lipid class and fatty acid composition (**Table S4**).

Notable differences were observed in the lipid profiles of normal and tumour tissue (**Figure 1B, 2A**). We found that the lipid profile of normal tissue was predominantly characterised by PC(32:0) (m/z 756.550 $[\text{M}+\text{Na}]^+$ and m/z 772.526 $[\text{M}+\text{K}]^+$; composed of fatty acids 16:0/16:0 according to fragmentation data, **Table S4**) and PC(32:1) (m/z 754.535 $[\text{M}+\text{Na}]^+$ and m/z 770.510 $[\text{M}+\text{K}]^+$; 16:0/16:1). In contrast, tumour tissue contained a wider distribution of lipids, including PC(34:1) (m/z 782.568 $[\text{M}+\text{Na}]^+$ and m/z 798.542 $[\text{M}+\text{K}]^+$; 16:0/18:1) and PC(34:2) (m/z 780.552 $[\text{M}+\text{Na}]^+$ and m/z 796.526 $[\text{M}+\text{K}]^+$; 16:0/18:2). In particular, PC(36:4) (m/z 804.552 $[\text{M}+\text{Na}]^+$ and m/z 820.526 $[\text{M}+\text{K}]^+$; 16:0/20:4) had a significantly increased relative abundance in tumours compared to normal tissue. Ion images for the two most distinguishing ions, PC(32:0) $[\text{M}+\text{Na}]^+$ for normal tissue and PC(36:4) $[\text{M}+\text{K}]^+$ for tumours, were created highlighting their regio-specificity (**Figure 2B-2C**). Interestingly, differences were observed for the distribution of salt adducts, with a higher proportion of sodiated lipids present in normal tissue, and potassiated lipids in tumour regions (**Figure 2D-2F**).

Similarly the lipid profiles in negative ion mode were examined (**Figure 1C, S2**). The most abundant species in both tissue types was PI(38:4) (m/z 885.539 [M-H] $^-$; 18:0/20:4, **Table S3**), whereas the second most abundant species was PG(34:1) (m/z 747.509 [M-H] $^-$; 16:0/18:1) in normal tissue and PI(36:4) (m/z 857.508 [M-H] $^-$; 16:0/20:4) in tumour tissue (**Figure 1C, Table S3**). Overall tumour tissue had an increased relative abundance of PIs, whereas normal tissue had an increased relative PG content (**Figure S2B-S2C**). Free fatty acids revealed a relative increase in palmitic acid, FFA(16:0) [M-H] $^-$, in normal tissue and in arachidonic acid, FFA(20:4) [M-H] $^-$, in tumour tissue, in line with observations for the PCs (**Figure S2A**).

We complemented our results from MALDI-MSI with LESA-MS, which was carried out on different sections of the same samples (**Figure S3**). Lipids were extracted at multiple sites in both tumour and adjacent normal tissue and directly infused to the MS. Lipids identified in positive and negative ion mode were combined, and an OPLS-DA model built to identify which lipid species were important for classifying samples as either normal or tumour (**Figure S3**). Analysis supported the results from MALDI-MSI, and suggest that PC(32:0) [M+H] $^+$ was the most distinguishing ion for normal tissue, whereas free arachidonic acid and related lipids, including PC(36:4) [M+H] $^+$, were the most important lipid species differentiating tumour tissue.

Imaging MS distinguishes tumour models with different MYC activities

Having established the major differences in lipid profiles between normal and tumour tissue, we sought to identify the lipid profile differences attributable to high MYC activity. Removing tamoxifen from the diet results in rapid MYCER-fusion protein deactivation over the course of 24 hours [26]. As a consequence of MYC deactivation, reduced tumour proliferation and apoptosis were observed

(Figure S4A-S4B). Using MALDI-MSI we therefore compared lung sections from *LSL-Kras*^{G12D};R26^{LSL-CMER} mice with high MYC activity (“MYC activated”) and those after 72 hours MYC deactivation (“MYC inactive”). As a control to ensure the overall lipid content was not altered by the addition of tamoxifen to the diet, we also examined lung tumours from *LSL-Kras*^{G12D} mice on a tamoxifen-containing diet, where MYC expression was low and unmodified (“KRAS only”, Figure S4C).

We examined the spatial distributions for PC(36:4) [M+K]⁺ and PC(32:0) [M+Na]⁺ – the most distinguishing lipid species for tumour and normal tissue, respectively (Figure 3A-3C). A striking difference in the relative abundance of PC(36:4) was noted between the three tumour models (Figure 3D). PC(36:4) accounts for a large proportion of the overall signal intensity in “MYC activated” tumours (Figure 3B). On the other hand, after deactivation of MYC the relative abundance of PC(36:4) in tumours was dramatically reduced (Figure 3C). “KRAS only” tumours had lower relative PC(36:4) content than “MYC activated”, but higher than “MYC inactive”, highlighting the effect that specific oncogenes or combinations thereof can have on the lipid profile. In-line with previous observations [27], PC(36:4) was also observed to localise prominently in the airway linings – this was more notable in the “MYC inactive” and “KRAS only” samples.

MS suggests a role for arachidonic acid in MYC-dependent tumour models

We next focussed on LESA-MS in order to further probe differences in the lipid profile for the three tumour types (“MYC activated”, “MYC inactive” and “KRAS only”). Mass spectra were obtained in positive and negative ion modes. In positive ion mode, the most prominent lipids were protonated PCs (Figure 4A). The lipid profiles for “MYC inactive” and “KRAS only” tumour samples looked broadly similar, with a high relative content of PC(32:0) (*m/z* 734.569; [M+H]⁺) and PC(32:1) (*m/z* 732.555; [M+H]⁺). However, the lipid profile for “MYC activated” tumour samples was strikingly different, with a notable relative increase in longer chain PCs, including PC(36:4) (*m/z* 782.569;

$[M+H]^+$) (**Figure 4A**). In negative ion mode, a wider distribution of lipids was observed including formate adducts of PCs, and deprotonated PIs, PGs, phosphatidylethanolamines (PE), plasmalogens and free fatty acids (**Figure 4B**). Of particular note was a marked increase in the relative abundance of free arachidonic acid (m/z 303.234; $[M-H]^-$) in “MYC activated” tumours compared to “MYC inactive” and “KRAS only” tumours (**Figure 4B**).

Identified features in positive and negative mode were combined and used to construct an OPLS-DA (**Figure 4C**) model. There was clear grouping of the three tumour classes ($R^2 = 0.98$, $Q^2 = 0.91$). Examination of the corresponding loadings plots (**Figure 4D**) revealed that the most important lipid species for distinguishing “MYC activated” tumours was arachidonic acid, which was increased relative to the other two groups.

To further establish the changes in arachidonate, we examined the relative abundance of free arachidonic acid and related lipids, including its precursor linoleic acid (FFA(18:2)) and arachidonate-containing lipids PC(36:4) (16:0/20:4) and PC(38:4) (18:0/20:4). In all cases a decrease over time was observed following deactivation of MYC: this was particularly notable for arachidonic acid itself. This can be attributed to the lower MYC level and not to diet, since lipid abundances in “KRAS only” controls on normal and tamoxifen-containing diets were not significantly different (**Figure 4E**).

Increased eicosanoids implicated in tumours with high MYC activity

Arachidonic acid is the major precursor for eicosanoids, important signalling molecules responsible for regulation of the inflammatory response, and implicated in facilitating cancer progression [28, 29]. Due to their low abundance, eicosanoids and related lipid mediators are not easily detected by imaging techniques, and were therefore extracted from lung tissue and analysed using liquid

chromatography-tandem mass spectrometry (LC-MS/MS). Based on chromatographic retention times and characteristic fragmentation pathways (**Figure S5, Table S2**), we detected 44 different eicosanoids and lipid mediators, 28 of which were derived from arachidonic acid. Identified species included 10 prostaglandins, 2 leukotrienes, 6 hydroxyeicosatetraenoic acids (HETEs), 4 epoxyeicosatrienoic acids (EETs), 4 dihydroxyeicosatrienoic acids (DHETs), 3 hydroxyeicosapentaenoic acids (HEPEs), 2 dihydroxyoctadecanoic acids (DiHOMEs) and 2 hydroxyoctadecadienoic acids (HODEs). Interestingly, many of the identified species were found to be increased in “MYC activated” samples, and the top ten most increased eicosanoids were all arachidonic acid-derived (**Table S5**). Using multivariate statistics (**Figure S6A-S6B**), we determined that the most important eicosanoids for distinguishing “MYC activated” samples from the other groups were the HETEs, which were present at higher concentrations in “MYC activated” samples (**Figure 4F**). In addition to the HETEs, levels of prostaglandins (including PGE2, PGF2 α), thromboxanes (TxB2) and leukotrienes (LTB4), amongst others, were increased in “MYC activated” lung tissue (**Figure 4F, Table S5**).

MYC linked to cPLA2 activity and COX/LOX expression

The observed increase in arachidonic acid-derived eicosanoids in “MYC activated” tumours could arise from enhanced substrate availability through increased cPLA2 expression or activity, from increased expression of LOX/COX, or a combination of the above. To test this we performed immunostaining on *LSL-Kras*^{G12D};R26^{LSL-CMER} tumours before and after MYC deactivation. We stained specifically for the active phosphorylated form (**Figure 5A**), finding a higher number of positively-stained cells for the “MYC activated” tumours, with a significant decrease over time following MYC deactivation (**Figure 5A-5B**). We then verified cPLA2 activity using an enzyme activity assay (**Figure 5C**), which confirmed the significantly higher phospholipase activity in “MYC activated” tissue.

Interestingly, when examining the staining, we noticed that in many instances positively-stained cells were visibly undergoing cell division, suggesting a proliferative role for cPLA2 (**Figure 5D**).

Next we examined the gene expression in lung tissue from *LSL-Kras^{G12D};R26^{LSL-CMER}* mice, before and after MYC deactivation. We quantified transcripts for cPLA2 (*cPla2*), COX-2 (*Cox2*), 5-LOX (*Alox5*), 12/15-LOX (*Alox15*) and platelet-type 12-LOX (*Alox12*). As a reference group, we used lung tissue from *LSL-Kras^{G12D}* mice. No difference in *cPla2* mRNA levels was observed between whole lung tissue containing “KRAS only” and “MYC activated” tumours (**Figure 5E**). However, upon MYC deactivation there was a significant increase in mRNA levels after 72 hours, which may be a compensatory effect to the overall reduction in cPLA2 activity. This suggests the changes observed in cPLA2 activity with MYC activation/deactivation are not governed at the transcriptional level. In contrast, transcript levels for *Cox2* and *Alox5* were significantly increased in “MYC activated” compared to “KRAS only” lung tissue (**Figure 5E**). Following MYC deactivation, these transcripts decreased with time, suggesting their amplification at the gene expression level is linked to MYC. Finally, we examined the gene expression for pro-inflammatory cytokines TNF- α and IL-1 β , that may play a role in regulating cPLA2 and eicosanoid synthesis [30-33]. We found a modest increase in *Tnf- α* with MYC activation, whilst *IL-1 β* gene expression was closely correlated to MYC activity (**Figure 5F**).

In vivo inhibition of COX/5-LOX pathways reduces proliferation in lung tumours

We have shown that COX-2 and 5-LOX transcripts and metabolites are linked to MYC activity in KRAS-driven adenocarcinoma. In order to confirm the importance and assess the feasibility of targeting these pathways for the treatment of such lung cancers, we designed an *in vivo* inhibitor study using the novel *LSL-Kras^{G12D};R26^{LSL-CMER}* mouse model, with MYC activated. Mice were infected with adeno-Cre and transferred to a tamoxifen-containing diet, as in the earlier experiments. Two weeks following the addition of tamoxifen, mice were treated with either vehicle or licoferone – a

dual COX/5-LOX pathway inhibitor, which has shown promising chemopreventive efficacy in lung and colon cancers [34, 35]. Remarkably, after only four doses we found substantially reduced tumour load when compared to vehicle-treated controls (**Figure 6A**). In order to investigate this further, we performed staining for proliferation (Ki67; **Figure 6B-6C**) and apoptosis (cleaved caspase 3, **Figure 6D**). We found increased apoptosis (although this did not reach significance) and significantly decreased proliferation (**Figure 6C**) in tumours. Using mass spectrometry, we confirmed that the COX-2/5-LOX metabolites had been reduced, as anticipated (**Figure 6E**).

Discussion

Here we have probed the lipid profiles of mouse lung tumours with oncogenic KRAS and increased MYC activity, comparing normal and tumour tissue, and monitoring changes following the deactivation of MYC. The most intriguing discovery was a striking increase in arachidonic acid and arachidonate-containing phospholipids in “MYC activated” tumours compared to normal tissue. These levels dramatically decreased with time in tumours following MYC deactivation. Arachidonic acid is an omega-6 polyunsaturated fatty acid, and is liberated from membrane phospholipids by cPLA2 [28]. The metabolism of arachidonic acid by COX, LOX and cytochrome P450 generates eicosanoids, including prostaglandins, leukotrienes and HETEs (**Figure 6F**). Whilst the functional role of many eicosanoids remains unknown, a number act as potent mediators of inflammation, and have recently been implicated in cancers through effecting cell proliferation, survival, angiogenesis, migration and invasion [36-38]. In particular, arachidonic acid and its eicosanoid metabolites have been shown to activate peroxisome proliferator-activated receptors (PPARs), induce nuclear translocation of NF- κ B and to activate the phosphoinositide-3-kinase signalling cascade (PI3K/AKT) - a major driver for proliferation in cancer [33, 39].

In this study, we uncovered an increase in arachidonic acid-derived eicosanoids in cancerous lung tissue from “MYC activated” mice, notably metabolites of the COX and LOX pathways (**Figure 6F**). We further found increased cPLA2 activity, which decreased with time following MYC deactivation, concomitant with decreased downstream metabolite levels. High cPLA2 staining was found in mitotic cells, suggesting a role for cPLA2 or its metabolites in proliferation. cPLA2 is activated by phosphorylation via the mitogen-activated protein kinase (MAPK) pathway, and effectors may include TNF- α and IL-1 β , amongst many others [33, 40]. Here, we found that gene expression for IL-1 β in particular was linked to MYC activity. We additionally uncovered a link between upregulated COX-2 and 5-LOX gene expression and MYC activity. Interestingly, both genes contain an E-box sequence in their promoter region, so could be directly regulated by MYC abundance [41, 42]. Alternatively, their response to MYC may be a result of complex interdependencies, occurring downstream of primary MYC targets. Inhibiting these two pathways *in vivo* resulted in marked reduction in tumour load and significantly decreased proliferation, highlighting their potential as targets for lung adenocarcinoma treatment, particularly with the involvement of MYC [43, 44].

Upregulation of lipid synthesis is a hallmark of cancers as a strategy to generate new cell membranes and signalling molecules, thus enabling the extensive proliferation of tumour cells [45]. When comparing tumour and normal lung tissue, we observed a relative increase in PIs in tumours. PIs are precursors of the PI polyphosphates (PIP_n), an important class of signalling molecules involved in the PI3K signalling cascade. PIs are not considered to be rate-limiting for the formation of PIP_n, and their roles in many cancers are yet to be defined. However, specific PIs have been proposed as biomarkers for prostate cancer [46], and have been shown to be elevated in human lung tumours [47]. One possibility here is that PI may provide an alternative source of arachidonic acid, since the most abundant PI identified was arachidonate-containing PI(38:4).

Whilst lipids serve as signalling precursors and structural components of the cell membrane, they also make up ~ 90 % of pulmonary surfactant, which lines the alveolar surface of healthy lung. This fluid is responsible for reducing surface tension at the air-water interface, allowing efficient exchange of gases [48, 49]. The lipid content of this surfactant is predominantly PC and PG, the most abundant of which are PC(32:0) (~ 50 %) and PC(32:1) (~ 10 %) [27, 49]. During our analysis, we found that PC(32:0), PC(32:1) and PGs were substantially increased in normal mouse lung tissue compared to tumour tissue. This may be as a result of the breakdown of the fine alveolar structure with tumour progression. An alternate explanation is the composition of surfactant in lung tumours may be altered by catabolic phospholipase activity or acyl chain elongation [50].

Finally, differences were observed for the distribution of salt adducts, with a higher proportion of sodiated phospholipids present in normal tissue, and potassiated lipids in tumour regions. In healthy cells, there is typically higher intracellular K⁺ whereas extracellular fluid such as lung surfactant contains a higher proportion of Na⁺. This balance can be disrupted by the upregulation of Na⁺/H⁺ exchangers in cancerous cells with high aerobic glycolytic activity. Furthermore, dysregulation of potassium channels, Na⁺/K⁺ ATPase or Na⁺/Ca²⁺ exchangers may also be important, particularly given the role of Ca²⁺ in cPLA2 activation [51, 52]. All these could result in altered Na⁺ permeability into the cell and/or export of K⁺. An increased extracellular K⁺ associated with tumours may account for the preference of K⁺ adduct formation during ionisation of phospholipids by MALDI-MSI, and offers unique insights into the tumour micro-environment.

Conclusion

Using a transgenic mouse model of KRAS-driven lung adenocarcinoma with reversible activation of MYC, we compared the lipid profiles of normal and tumour tissue, finding increased signalling-precursor lipids and eicosanoids, and decreased surfactant lipids in tumours. When MYC was

deactivated, the tumour lipid signature markedly changed, with a large relative decrease in arachidonic acid and related lipids. This coincided with decreased cPLA2 activity and downstream metabolites, and decreased transcript levels for COX-2 and 5-LOX in lung tissue. We hypothesise that tumours with high MYC activity have increased levels of arachidonic acid as a consequence of increased cPLA2 activity. Free arachidonic acid serves as a substrate pool for upregulated eicosanoid synthesis, which can influence cancer progression in multiple ways. Dual inhibition of COX/5-LOX pathways *in vivo* resulted in reduced proliferation and a marked decrease in tumour load, and suggests these druggable pathways warrant further investigation as lung cancer therapies. MS imaging and associated surface analysis techniques have been shown here as powerful analytical tools, capable of obtaining detailed and spatially precise molecular signatures of tumour sub-environments, and paving the way for further studies in the exciting and rapidly developing field of lipidomics.

Acknowledgements

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References

1. The Cancer Genome Atlas Research Network: **Comprehensive molecular profiling of lung adenocarcinoma.** *Nature* 2014, **511**:543-550.
2. Robson SC, Ward L, Brown H, Turner H, Hunter E, Pelengaris S, Khan M: **Deciphering c-MYC-regulated genes in two distinct tissues.** *BMC Genomics* 2011, **12**:476.
3. Soucek L, Whitfield JR, Sodir NM, Masso-Valles D, Serrano E, Karnezis AN, Swigart LB, Evan GI: **Inhibition of Myc family proteins eradicates KRas-driven lung cancer in mice.** *Genes Dev* 2013, **27**:504-513.

4. Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, Bachmann MH, Borowsky AD, Ruebner B, Cardiff RD, et al: **MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer.** *Nature* 2004, **431**:1112-1117.
5. Baenke F, Peck B, Miess H, Schulze A: **Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development.** *Dis Model Mech* 2013, **6**:1353-1363.
6. Louie SM, Roberts LS, Mulvihill MM, Luo K, Nomura DK: **Cancer cells incorporate and remodel exogenous palmitate into structural and oncogenic signaling lipids.** *Biochim Biophys Acta* 2013, **1831**:1566-1572.
7. Hilvo M, Denkert C, Lehtinen L, Muller B, Brockmoller S, Seppanen-Laakso T, Budczies J, Bucher E, Yetukuri L, Castillo S, et al: **Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression.** *Cancer Res* 2011, **71**:3236-3245.
8. Brockmoller SF, Bucher E, Muller BM, Budczies J, Hilvo M, Griffin JL, Oresic M, Kallioniemi O, Iljin K, Loibl S, et al: **Integration of metabolomics and expression of glycerol-3-phosphate acyltransferase (GPAM) in breast cancer-link to patient survival, hormone receptor status, and metabolic profiling.** *J Proteome Res* 2012, **11**:850-860.
9. Griffin JL, Lehtimäki KK, Valonen PK, Gröhn OHJ, Kettunen MI, Ylä-Herttuala S, Pitkänen A, Nicholson JK, Kauppinen RA: **Assignment of ¹H Nuclear Magnetic Resonance Visible Polyunsaturated Fatty Acids in BT4C Gliomas Undergoing Ganciclovir-Thymidine Kinase Gene Therapy-induced Programmed Cell Death.** *Cancer Res* 2003, **63**:3195-3201.
10. Eberlin LS, Gabay M, Fan AC, Gouw AM, Tibshirani RJ, Felsher DW, Zare RN: **Alteration of the lipid profile in lymphomas induced by MYC overexpression.** *Proc Natl Acad Sci U S A* 2014, **111**:10450-10455.
11. Yuneva MO, Fan TW, Allen TD, Higashi RM, Ferraris DV, Tsukamoto T, Mates JM, Alonso FJ, Wang C, Seo Y, et al: **The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type.** *Cell Metab* 2012, **15**:157-170.
12. Angel PM, Caprioli RM: **Matrix-assisted laser desorption ionization imaging mass spectrometry: in situ molecular mapping.** *Biochemistry* 2013, **52**:3818-3828.
13. Berry KA, Hankin JA, Barkley RM, Spraggins JM, Caprioli RM, Murphy RC: **MALDI imaging of lipid biochemistry in tissues by mass spectrometry.** *Chem Rev* 2011, **111**:6491-6512.
14. Goto-Inoue N, Hayasaka T, Zaima N, Setou M: **Imaging mass spectrometry for lipidomics.** *Biochim Biophys Acta* 2011, **1811**:961-969.
15. Addie RD, Balluff B, Bovee JV, Morreau H, McDonnell LA: **Current State and Future Challenges of Mass Spectrometry Imaging for Clinical Research.** *Anal Chem* 2015, **87**:6426-6433.
16. Eikel D, Vavrek M, Smith S, Bason C, Yeh S, Korfmacher WA, Henion JD: **Liquid extraction surface analysis mass spectrometry (LESA-MS) as a novel profiling tool for drug distribution and metabolism analysis: the terfenadine example.** *Rapid Commun Mass Spectrom* 2011, **25**:3587-3596.
17. Griffiths RL, Dexter A, Creese AJ, Cooper HJ: **Liquid extraction surface analysis field asymmetric waveform ion mobility spectrometry mass spectrometry for the analysis of dried blood spots.** *Analyst* 2015.
18. Swales JG, Tucker JW, Spreadborough MJ, Iverson SL, Clench MR, Webborn PJ, Goodwin RJ: **Mapping Drug Distribution in Brain Tissue Using Liquid Extraction Surface Analysis Mass Spectrometry Imaging.** *Anal Chem* 2015.
19. Murphy DJ, Junntila MR, Pouyet L, Karnezis A, Shchors K, Bui DA, Brown-Swigart L, Johnson L, Evan GI: **Distinct Thresholds Govern Myc's Biological Output In Vivo.** *Cancer Cell* 2008, **14**:447-457.

20. Sud M, Fahy E, Cotter D, Brown A, Dennis EA, Glass CK, Merrill AH, Murphy RC, Raetz CRH, Russell DW, Subramaniam S: **LMSD: LIPID MAPS structure database.** *Nucleic Acids Research* 2007, **35**:D527-D532.
21. Hsu F-F, Bohrer A, Turk J: **Formation of Lithiated Adducts of Glycerophosphocholine Lipids Facilitates their Identification by Electrospray Ionization Tandem Mass Spectrometry.** *J Am Soc Mass Spectrom* 1998, **9**:516-526.
22. Rompp A, Schramm T, Hester A, Klinkert I, Both JP, Heeren RM, Stockli M, Spengler B: **imzML: Imaging Mass Spectrometry Markup Language: A common data format for mass spectrometry imaging.** *Methods Mol Biol* 2011, **696**:205-224.
23. Hartigan JA, Wong MA: **Algorithm AS 136: A K-Means Clustering Algorithm.** *J Royal Stat Soc C* 1979, **28**:100-108.
24. Trygg J, Wold S: **Orthogonal projections to latent structures (o-PLS).** *J Chemometrics* 2002, **16**:119-128.
25. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ: **Centering, scaling, and transformations: improving the biological information content of metabolomics data.** *BMC Genomics* 2006, **7**:142.
26. Wilson CH, Gamper I, Perfetto A, Auw J, Littlewood TD, Evan GI: **The kinetics of ER fusion protein activation in vivo.** *Oncogene* 2014, **33**:4877-4880.
27. Berry KA, Li B, Reynolds SD, Barkley RM, Gijon MA, Hankin JA, Henson PM, Murphy RC: **MALDI imaging MS of phospholipids in the mouse lung.** *J Lipid Res* 2011, **52**:1551-1560.
28. Dennis EA, Norris PC: **Eicosanoid storm in infection and inflammation.** *Nat Rev Immunol* 2015, **15**:511-523.
29. Astarita G, Kendall AC, Dennis EA, Nicolaou A: **Targeted lipidomic strategies for oxygenated metabolites of polyunsaturated fatty acids.** *Biochim Biophys Acta* 2015, **1851**:456-468.
30. Homaidan RF, Chakroun I, Dbaibo SG, El-Assaad W, El-Sabban EM: **IL-1 activates two phospholipid signaling pathways in intestinal epithelial cells.** *Inflammation Res* 2001, **50**:375-381.
31. Yang C-M, Lee I-T, Chi P-L, Cheng S-E, Hsiao L-D, Hsu C-K: **TNF- α induces cytosolic phospholipase A2 expression via Jak2/PDGFR-dependent Elk-1/p300 activation in human lung epithelial cells.** *American Journal of Physiology - Lung Cellular and Molecular Physiology* 2014, **306**:L543-L551.
32. McLeish KR, Knall C, Ward RA, Gerwins P, Coxon PY, Klein JB, Johnson GL: **Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF.** *J Leukocyte Biol* 1998, **64**:537-545.
33. Hyde CA, Missailidis S: **Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis.** *Int Immunopharmacol* 2009, **9**:701-715.
34. Sharma S, Lee J, Zhou J, Steele VE: **Chemopreventive efficacy and mechanism of licoferone in a mouse lung tumor model via aspiration.** *Cancer Prev Res (Phila)* 2011, **4**:1233-1242.
35. Mohammed A, Janakiram NB, Li Q, Choi CI, Zhang Y, Steele VE, Rao CV: **Chemoprevention of colon and small intestinal tumorigenesis in APC(Min $^{+/-}$) mice by licoferone, a novel dual 5-LOX/COX inhibitor: potential implications for human colon cancer prevention.** *Cancer Prev Res (Phila)* 2011, **4**:2015-2026.
36. Wang D, Dubois RN: **Eicosanoids and cancer.** *Nat Rev Cancer* 2010, **10**:181-193.
37. Greene ER, Huang S, Serhan CN, Panigrahy D: **Regulation of inflammation in cancer by eicosanoids.** *Prostaglandins Other Lipid Mediat* 2011, **96**:27-36.
38. Nie D, Honn KV: **Eicosanoid regulation of angiogenesis in tumors.** *Semin Thromb Hemost* 2004, **30**:119-125.
39. Hughes-Fulford M, Li CF, Boonyaratanaornkit J, Sayyah S: **Arachidonic acid activates phosphatidylinositol 3-kinase signaling and induces gene expression in prostate cancer.** *Cancer Res* 2006, **66**:1427-1433.

40. Das S, Rafter JD, Kim KP, Gygi SP, Cho W: **Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation.** *J Biol Chem* 2003, **278**:41431-41442.
41. Mestre JR, Rivadeneira DE, Mackrell PJ, Duff M, Stapleton PP, Mack-Strong V, Maddali S, Smyth GP, Tanabe T, Daly JM: **Overlapping CRE and E-box promoter elements can independently regulate COX-2 gene transcription in macrophages.** *FEBS Lett* 2001, **496**:147-151.
42. Zeller KI, Zhao X, Lee CWH, Chiu KP, Yao F, Yustein JT, Ooi HS, Orlov YL, Shahab A, Yong HC, et al: **Global mapping of c-Myc binding sites and target gene networks in human B cells.** *Proc Natl Acad Sci U S A* 2006, **103**:17834-17839.
43. Sarveswaran S, Chakraborty D, Chitale D, Sears R, Ghosh J: **Inhibition of 5-lipoxygenase selectively triggers disruption of c-Myc signaling in prostate cancer cells.** *J Biol Chem* 2015, **290**:4994-5006.
44. Meyer AM, Dwyer-Nield LD, Hurteau GJ, Keith RL, O'Leary E, You M, Bonventre JV, Nemenoff RA, Malkinson AM: **Decreased lung tumorigenesis in mice genetically deficient in cytosolic phospholipase A2.** *Carcinogenesis* 2004, **25**:1517-1524.
45. Santos CR, Schulze A: **Lipid metabolism in cancer.** *FEBS J* 2012, **279**:2610-2623.
46. Goto T, Terada N, Inoue T, Nakayama K, Okada Y, Yoshikawa T, Miyazaki Y, Uegaki M, Sumiyoshi S, Kobayashi T, et al: **The expression profile of phosphatidylinositol in high spatial resolution imaging mass spectrometry as a potential biomarker for prostate cancer.** *PLoS One* 2014, **9**:e90242.
47. Marien E, Meister M, Muley T, Fieuws S, Bordel S, Derua R, Spraggins J, Van de Plassche R, Dehairs J, Wouters J, et al: **Non-small cell lung cancer is characterized by dramatic changes in phospholipid profiles.** *Int J Cancer* 2015, **137**:1539-1548.
48. Akella A, Deshpande SB: **Pulmonary surfactants and their role in pathophysiology of lung disorders.** *Indian J Exp Biol* 2013, **51**:5-22.
49. Veldhuizen R, Nag K, Orgeig S, Possmayer F: **The role of lipids in pulmonary surfactant.** *Biochim Biophys Acta* 1998, **1408**:90-108.
50. Marien E, Meister M, Muley T, Gomez Del Pulgar T, Derua R, Spraggins JM, Van de Plassche R, Vanderhoydonc F, Machiels J, Binda MM, et al: **Phospholipid profiling identifies acyl chain elongation as a ubiquitous trait and potential target for the treatment of lung squamous cell carcinoma.** *Oncotarget* 2016.
51. Haltiwanger S: **Electroporation-based therapies for cancer: From basics to clinical applications, Chapter 5: Why electroporation is a useful technique for cancer treatments?** Edited by Raji Sundararajan, Woodhead Publishing Series in Biomedicine, Cambridge, UK 2014:103-125.
52. Amstalden van Hove ER, Blackwell TR, Klinkert I, Eijkel GB, Heeren RM, Glunde K: **Multimodal mass spectrometric imaging of small molecules reveals distinct spatio-molecular signatures in differentially metastatic breast tumor models.** *Cancer Res* 2010, **70**:9012-9021.

Figure legends

Figure 1. Lipid signatures in normal and high MYC tumour tissue in lung. Using MALDI-MSI and clustering, normal (green) and tumour (red) lung tissue were differentiated by their mass spectra (A). The average spectra in positive (B) and negative (C) ion modes for normal and tumour tissue

clusters are shown. The inset shows an adjacent H&E stained section of lung. Tumours are depicted by densely packed cells and stained dark pink, whereas normal tissue is thin and filled with voids. A blood vessel can be observed in the top center of the section (orange).

Figure 2. Characterising the lipidome in KRAS-driven lung tumours, with high MYC activity. Relative abundances of major phosphatidylcholines (PC) in tumour compared to normal lung tissue determined by MALDI-MSI. The intensities for multiple adducts were summed for each lipid species (A). Two-dimensional ion images for PC(32:0) ($[M+Na^+]$; m/z 756.550, B) and PC(36:4) ($[M+K^+]$; m/z 829.526, C) show a predominantly normal or tumour tissue distribution, respectively. Relative proportion of sodiated or potassiated PC adducts in tumour and normal tissue (D). MALDI-MSI images for sodiated (m/z 754.535, E) and potassiated (m/z 770.510, F) PC(32:1) show different spatial distributions. Data is mean ($n=3$) \pm standard error of mean (SEM) (* $p < 0.05$).

Figure 3. MS imaging differentiates between levels of MYC activity. MALDI-MSI was carried out on lung tissue containing tumours driven by oncogenic KRAS only (A), with MYC activated (B), and with MYC deactivated for 72 hours (C). The spatial distributions of PC(36:4) [$M+K^+$] is shown in red (top) and overlaid with that of PC(32:0) [$M+Na^+$] in green (middle). Brighter intensity of colour indicates higher ion abundance. Adjacent sections stained with H&E are used as a reference (bottom). The relative proportion of signal for PC(36:4) was compared for the three different tumours, and found to be substantially higher in MYC-activated tumours (D). Data is mean ($n=2$ or 3) \pm SEM.

Figure 4. Arachidonic acid-related lipids differentiate lung tumours with varying MYC activities. Typical LESA mass spectra for lung tumours in positive (A) and negative ion mode (B). Asterisks indicate PC(36:4) (m/z 782.569; $[M+H^+]$), and arachidonic acid (FFA(20:4), m/z 303.234; $[M-H^-]$). LESA-MS spectra at three sites across lung tumours (“MYC activated” - $n=4$, “MYC inactive” - $n=6$, “KRAS only” - $n=4$) were used to construct an OPLS-DA model (C) and related loadings plot (D). A significant decrease over time was observed for arachidonic acid (FFA(20:4)) and related lipids following deactivation of MYC. MYC deactivation was achieved by the removal of tamoxifen from the

diet; as an additional control “KRAS only” lung tumours from mice on a normal diet and a tamoxifen-containing diet were compared, with no significant (*n.s.*) differences in lipid content noted (**E**). Higher levels of eicosanoids, including HETEs, prostaglandins and thromboxanes were measured by LC-MS/MS in “MYC activated” compared to “MYC inactive” samples (**F**). Data is mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5. Cytosolic phospholipase A2 activity linked to MYC. Immunostaining of lung tumours for the active phosphorylated cPLA2 revealed a decrease in positively-stained cells with MYC deactivation (**A**). Quantification of staining revealed a significant decrease in phosphorylated cPLA2 levels with time after MYC deactivation (**B**). cPLA2 activity was determined using an enzyme assay and found to decrease with MYC deactivation (**C**). Closer inspection of staining for phosphorylated (left) or total (right) cPLA2 revealed that many of the positively-stained cells were visibly undergoing mitosis (black arrows; **D**). Transcript quantification in lung revealed that only *Cox2* and *Alox5* mRNAs were increased in “MYC activated” compared to “KRAS only” lung tissue (**E**). These levels decreased following MYC deactivation. Similarly *Tnf- α* and *IL-1 β* mRNAs increased with MYC activation (**F**). Data is mean ± SEM (* $p < 0.05$, ** $p < 0.01$).

Figure 6. In vivo inhibition of COX/5-LOX pathways. Licofelone treatment in mice with KRAS-driven adenocarcinoma and high MYC resulted in reduced tumour load (H&E; x40 magnification; **A**) and proliferation staining (Ki67; x200; **B**) compared to vehicle. Quantification of proliferation (**C**) and apoptosis (**D**) by Ki67 and cleaved caspase 3 (cc3) immunostaining, respectively, was performed. LC-MS/MS confirmed reduction of 5-LOX and COX-2 metabolites (**E**). Data is mean ± SEM ($n = 4$); * $p < 0.01$ (Mann-Whitney U test). cPLA2 is activated by phosphorylation through the mitogen-activated protein kinase (MAPK) pathway. Phosphorylated cPLA2 liberates arachidonic acid from intact phospholipids, which is metabolised by the COX, LOX and cytochrome P450 pathways to form eicosanoids. Complex feedback mechanisms exist between LOX/COX, cPLA2 pathways, and pro-inflammatory cytokines such as TNF- α and IL-1 β . Eicosanoids have a wide range of effects which can

result in increased proliferation, cell survival and angiogenesis. Red arrows indicate a positive correlation to MYC activity level, as determined in this study (**F**).

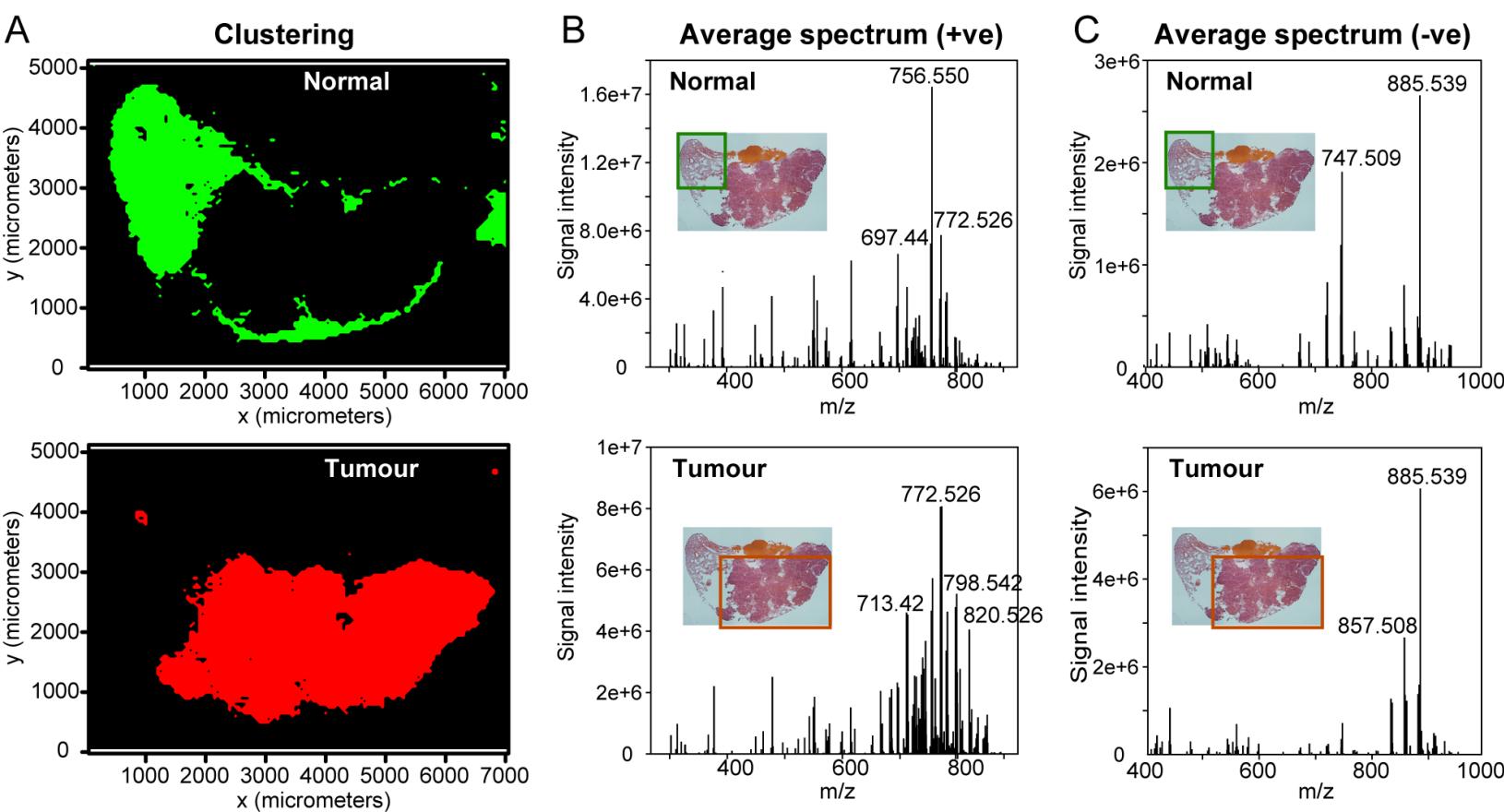


Figure 1

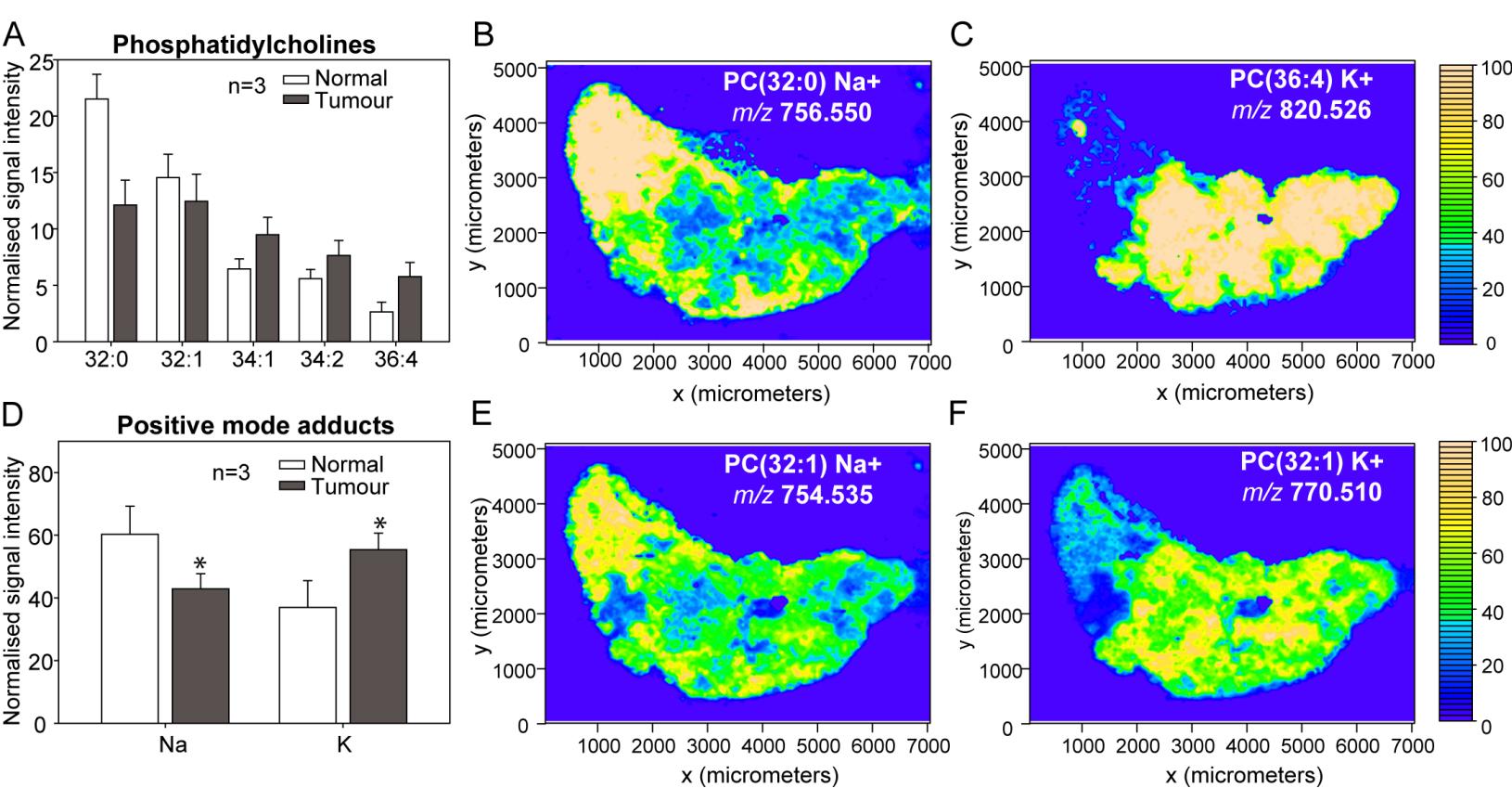


Figure 2

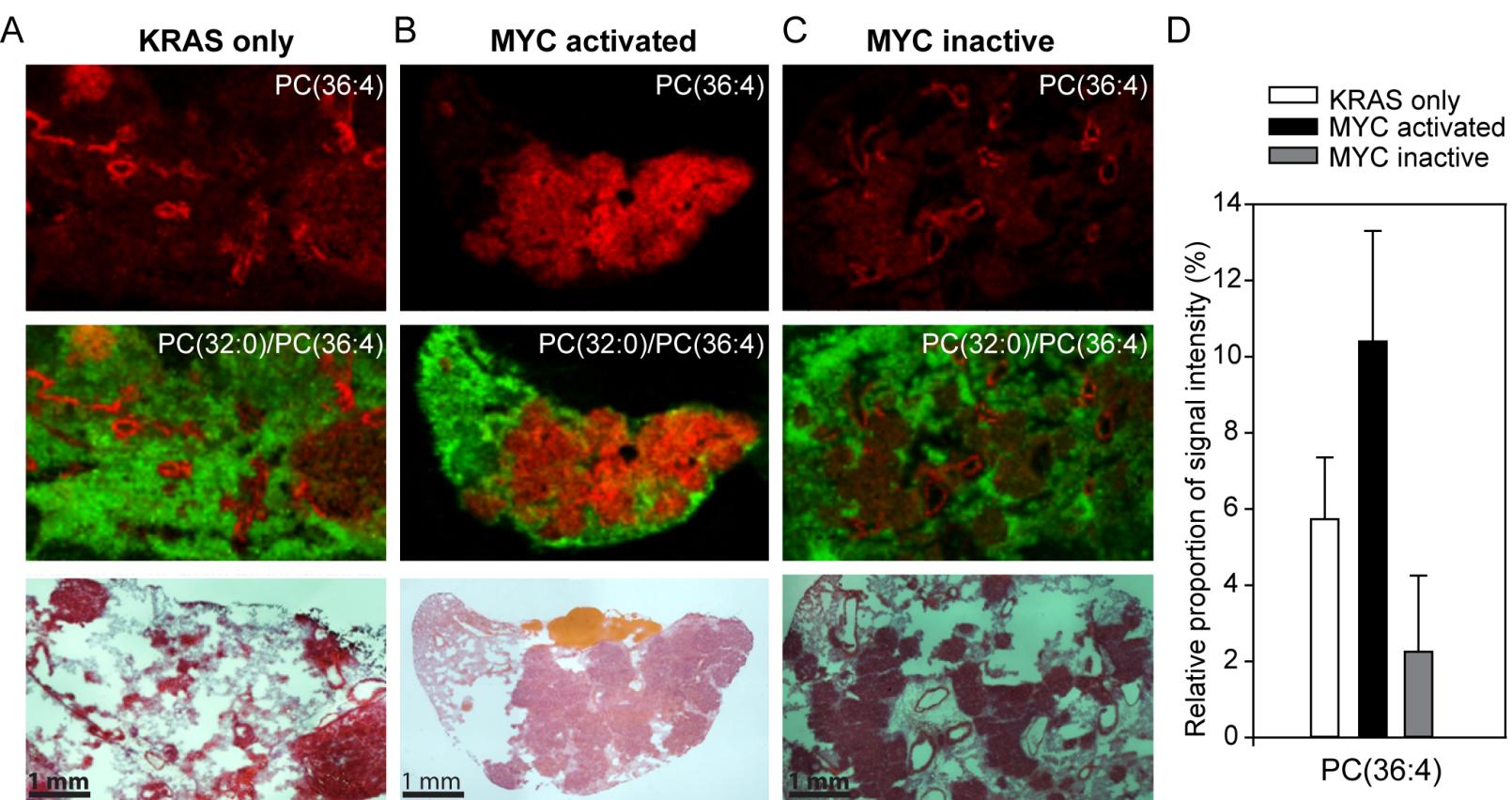


Figure 3

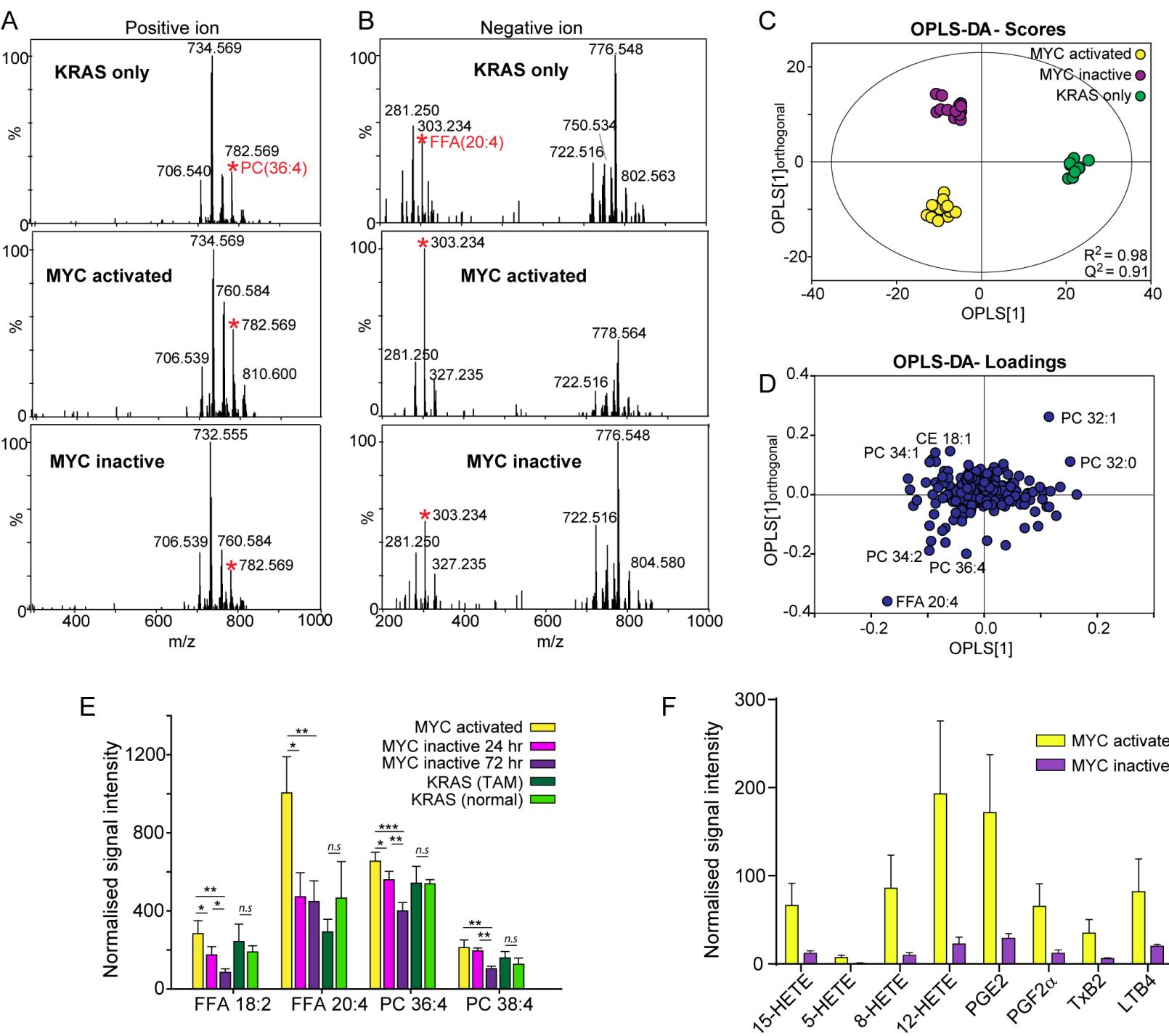


Figure 4

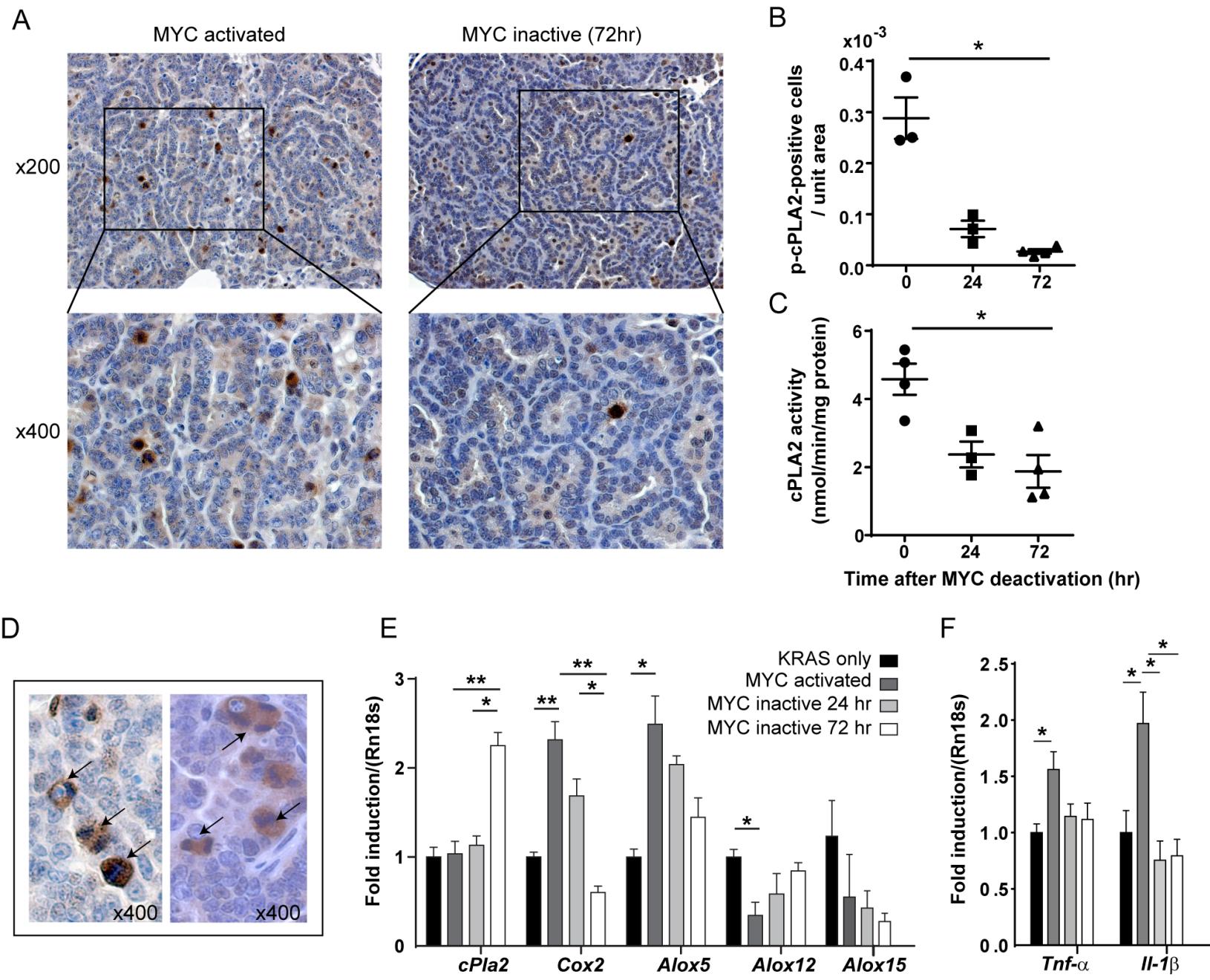


Figure 5

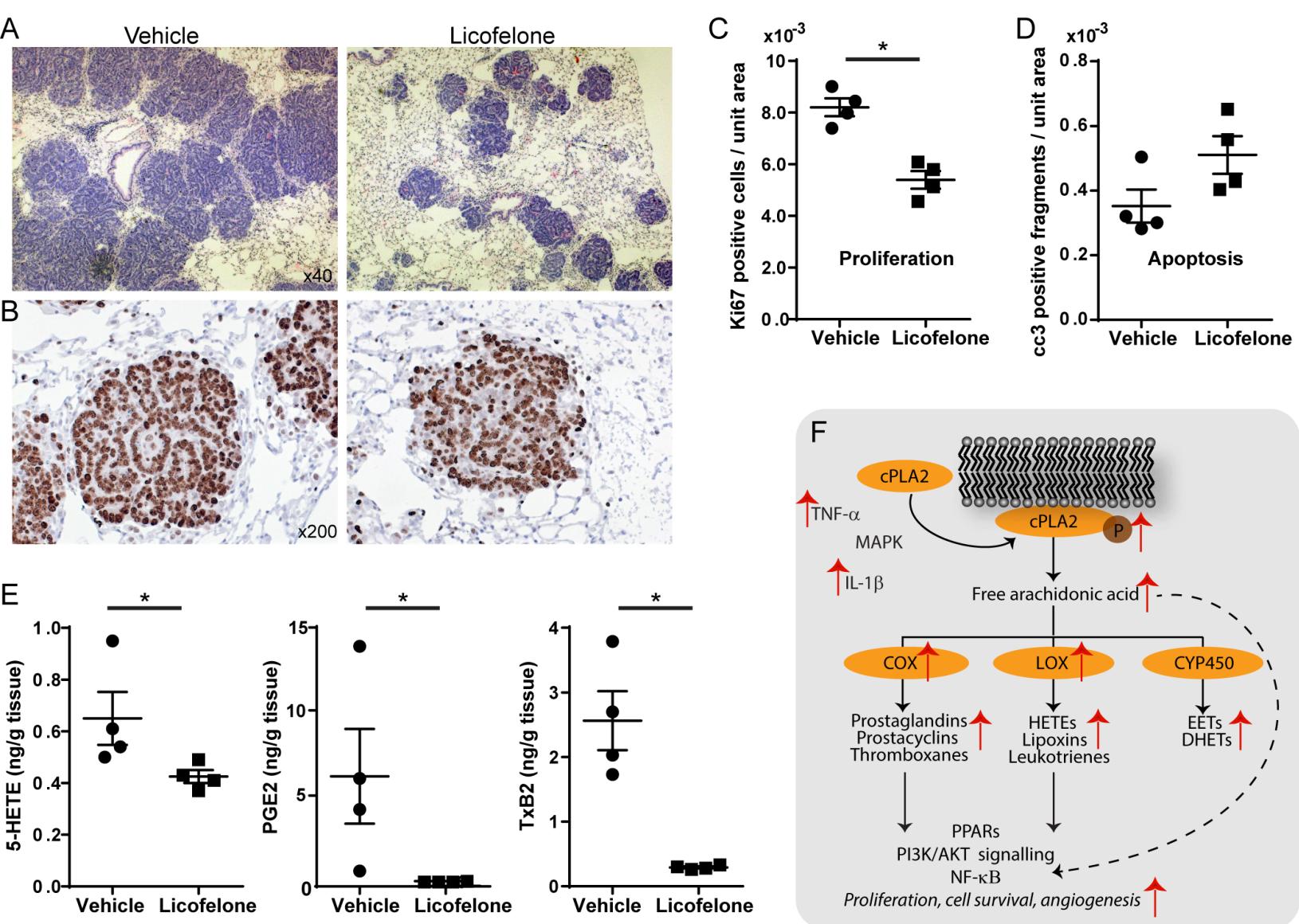


Figure 6