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Activity-Based Protein Profiling of Oncogene-Driven Changes in Metabolism Reveals Broad Dysregulation of PAFAH1B2 and 1B3 in Cancer

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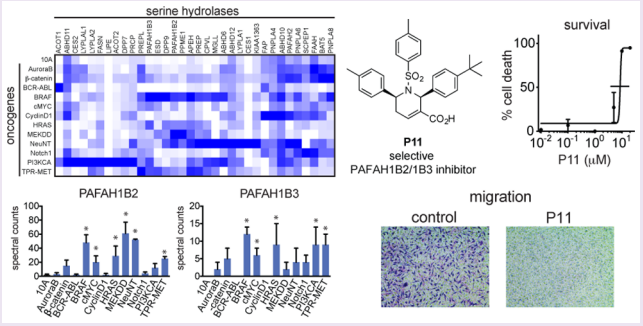
Supporting Information

ABSTRACT: Targeting dysregulated metabolic pathways is a promising therapeutic strategy for eradicating cancer. Understanding how frequently altered oncogenes regulate metabolic enzyme targets would be useful in identifying both broad-spectrum and targeted metabolic therapies for cancer. Here, we used activity-based protein profiling to identify serine hydrolase activities that were consistently upregulated by various human oncogenes. Through this profiling effort, we found oncogenic regulatory mechanisms for several cancer-relevant serine hydrolases and discovered that platelet activating factor acetylhydrolase 1B2 and 1B3 (PAFAH1B2 and PAFAH1B3) activities were consistently upregulated by several oncogenes, alongside previously discovered cancer-relevant hydrolases fatty acid synthase and monoacylglycerol lipase. While we previously showed that PAFAH1B2 and 1B3 were important in breast cancer, our most recent profiling studies have revealed that these enzymes may be dysregulated broadly across many types of cancers. Here, we find that pharmacological blockade of both enzymes impairs cancer pathogenicity across multiple different types of cancer cells, including breast, ovarian, melanoma, and prostate cancer. We also show that pharmacological blockade of PAFAH1B2 and 1B3 causes unique changes in lipid metabolism, including heightened levels of tumor-suppressing lipids. Our results reveal oncogenic regulatory mechanisms of several cancer-relevant serine hydrolases using activity-based protein profiling, and we show that PAFAH1B2 and 1B3 are important in maintaining cancer pathogenicity across a wide spectrum of cancer types.



Cancer cells frequently possess rewired metabolic pathways that include glycolytic addiction, heightened *de novo* lipogenesis, and glutamine-dependent anaplerosis that support cancer pathogenicity.[1,2] As such, targeting metabolic drivers of cancer has become an attractive strategy for combatting cancer. These therapeutic strategies include the classical antifolate and antinucleotide chemotherapy agents, pharmacological activators of the glycolytic enzyme pyruvate kinase M2, inhibitors of the mutant isocitrate dehydrogenases disrupting generation of the oncometabolite 2-hydroxyglutarate, and inhibitors of the *de novo* lipogenic enzyme fatty acid synthase (FASN).[1,2] However, efforts to target metabolic drivers of cancer have largely focused on major pathways in central carbon metabolism despite emerging evidence for the importance of other enzymes involved in other facets of metabolism.

Indeed, more recent glimpses into understudied aspects of metabolism have revealed several novel and important metabolic drivers of cancer, frequently found through profiling efforts of metabolic enzyme targets in tumor cell line panels or in primary tumors.[3−8] These metabolic targets are likely regulated through mutational activation, amplification, or

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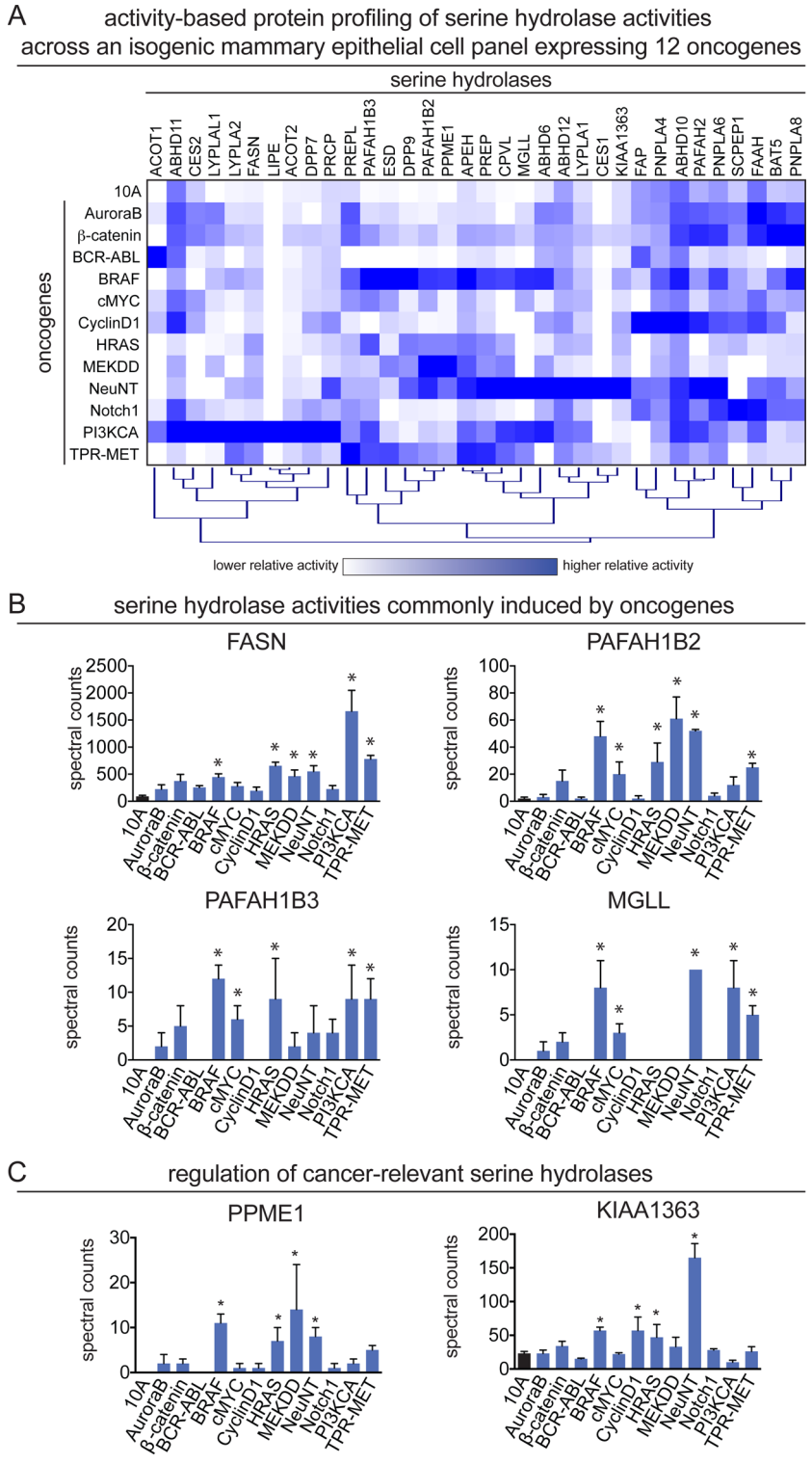

Figure 1. ABPP-MudPIT profiling of serine hydrolase activities in an isogenic MCF10A panel expressing 12 human oncogenes. (A) Heatmap of serine hydrolase activities detected and quantified by ABPP-MudPIT profiling in vector-transfected control (10A) cells and oncogene-induced MCF10A cells (listed by name of oncogene that was induced). Each column of serine hydrolase activities was normalized to the line showing the highest spectral counts. Dark blue and light blue denote high and low relative activity, respectively. Cell lysates were labeled with the serine hydrolase activity-based probe FP-biotin, after which, labeled proteins were avidin-enriched, and tryptic digests were analyzed by MudPIT analysis. (B) Shown are the serine hydrolase activities commonly induced by five or more oncogenes. (C) Shown are serine hydrolase activities for PPME1 and KIAA1363, enzymes that have been previously shown to be important in cancer. Data in B and C are presented as mean ± SEM, n = 3−10. Significance is presented as *p < 0.05 compared to MCF10A control cells using a one-way ANOVA for multiple comparisons against MCF10A control cells. Detailed data from the ABPP-MudPIT study can be found in Supporting Information Table 1.

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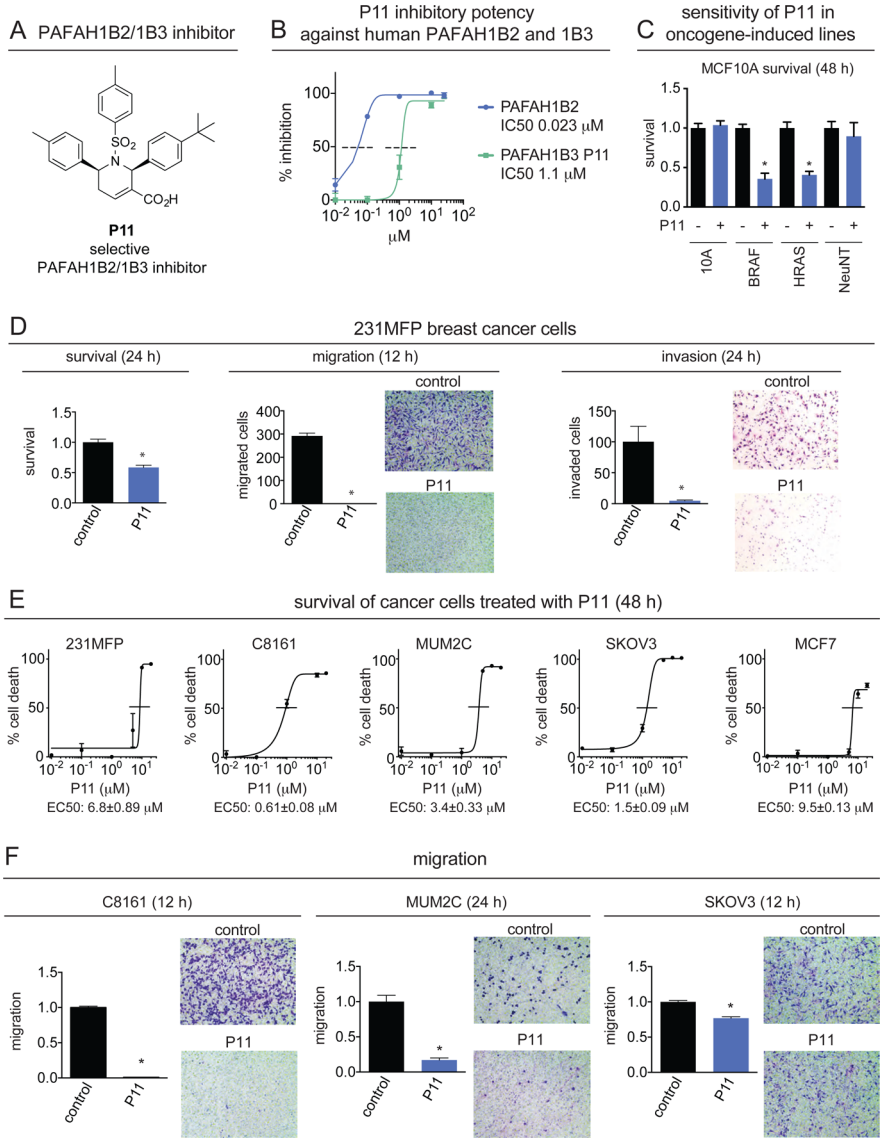

Figure 2. PAFAH1B2 and 1B3 dual blockade impairs cancer cell pathogenicity of multiple cancer cell types. (A) Structure of selective PAFAH1B2 and 1B3 dual inhibitor P11. (B) Concentration−response curves of human PAFAH1B2 and PAFAH1B3 inhibition by P11. P11 inhibitory potency was determined by PAF hydrolytic activity assays measuring the formation of lyso-PAF by LC-MS/MS. (C) P11 (1 μM, 48 h) treatment impairs serum-free cell survival in MCF10A cells expressing BRAF and HRAS but not in NeuNT-expressing or control MCF10A cells. (D) P11 (10 μM) impairs 231MFP breast cancer cell serum-free survival (24 h), migration (12 h), and invasion (24 h). (E) P11 impairs serum-free cell survival (48 h) in a concentration-dependent manner in 231MFP breast, C8161 melanoma, MUM2C melanoma, SKOV3 ovarian, and MCF7 breast cancer cells with 50% effective concentration (EC50) values of 6.8, 0.61, 3.4, 1.5, and 9.5 μM, respectively. (F) P11 (10 μM) significantly impairs cellular migration in C8161 (12 h), MUM2C (24 h), and SKOV3 (12 h) cells. MCF7 migration was not measured since MCF7 cells do not migrate. For C−F, cells were treated with either DMSO (0.1% final concentration) or P11 in DMSO during the seeding of cells into migration, invasion, and survival assays. Data are presented as mean ± SEM, n = 4−5. Significance is presented as *p < 0.05 compared to vehicle (DMSO)-treated control cells.

upregulation of oncogenes or inactivation of tumor suppressors to promote cellular transformation and cancer progression. Thus, understanding the metabolic enzymes that are consistently dysregulated by oncogenic stimuli could enable the identification of important metabolic drivers of cancer, which may also serve as therapeutic targets that have broad applicability toward a wide range of cancer types. Additionally, understanding oncogene-specific alterations in metabolic pathways may potentially be used to define a responsive patient population for future metabolic cancer therapies.

We have used a functional proteomic platform termed activity-based protein profiling (ABPP),[9,10] which employs active-site directed chemical probes to assess the activities of enzymes *en masse* within native proteomes. Using ABPP, we uncover here commonly dysregulated enzyme activities induced by 12 distinct human oncogenes expressed in an isogenic mammary epithelial MCF10A cell line background.[11] We focused this study on mapping the oncogenic regulation of the serine hydrolase superfamily of enzymes, as this is one of the largest metabolic enzyme classes encompassing hydrolases, lipases, esterases, thioesterases, peptidases, and proteases. We, and others, have previously uncovered several serine hydrolases that are important in cancer.[5,8,12−14] Furthermore, many pharmacological tools have been generated for interrogating serine hydrolases in cancer cells.[5,8,12−14] We use here the serine hydrolase-directed activity-based probe, fluorophosphonate

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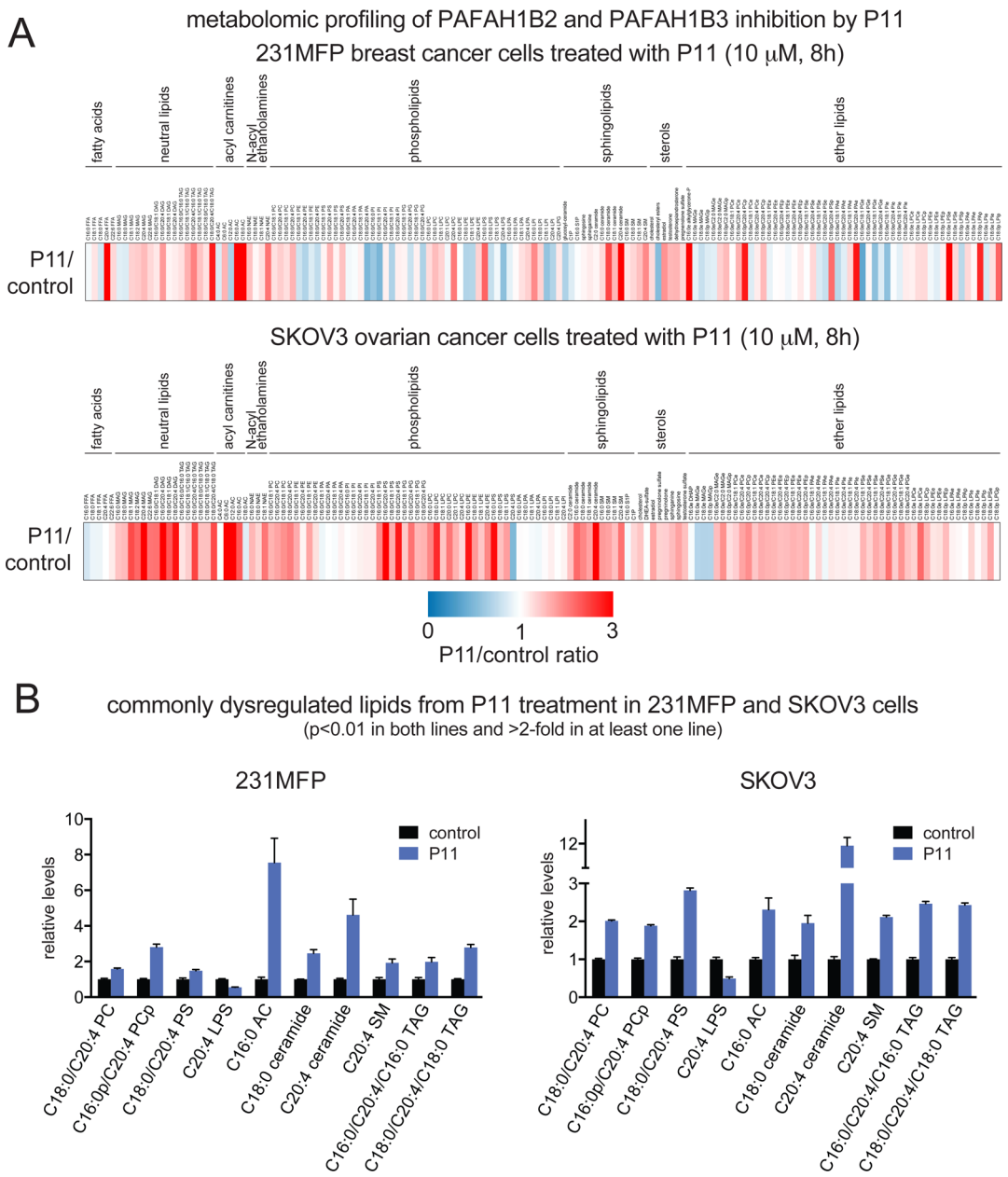

Figure 3. Metabolomic profiling of PAFAH1B2 and 1B3 inhibition by P11 in 231MFP breast cancer and SKOV3 ovarian cancer cells. (A) Heatmap showing metabolomic profile of lipid species identified through targeted single reaction monitoring (SRM)-based-LC-MS/MS lipidomic analysis. Heatmap shows relative metabolite levels normalized to 231MFP or SKOV3 DMSO-treated control cells. Red shading on the heatmap corresponds to P11/control ratios of >1. White corresponds to a P11/control ratio of 1, and blue shading corresponds to P11/control ratios < 1. (B) Bar graph showing lipid species that were significantly ($p < 0.01$) changing with P11 treatment in both 231MFP and SKOV3 cells compared to DMSO-treated controls by >2-fold in at least one line. Data are presented as mean ± SEM, $n = 5$. Detailed data for the metabolomics analyses can be found in Supporting Information Table 2.

(FP)-biotin, to label active serine hydrolases in the MCF10A or oncogenically altered MCF10A proteomes, followed by subsequent enrichment of probe-labeled targets and mass spectrometry analysis (Multidimensional Protein Identification Technology (MudPIT)). Using this ABPP-MudPIT platform, we identified 34 serine hydrolases, of which only four of these enzyme activities were consistently upregulated in five or more of the 12 oncogene-induced MCF10A cells (Figure 1A,B, Supporting Information Table 1). These included fatty acid synthase (FASN), platelet activating factor acetylhydrolase 1B2 and 1B3 (PAFAH1B2 and PAFAH1B3), and monoacylglycerol lipase (MGLL), which were each regulated by a different subset of oncogenic drivers (Figure 1B). Heightened *de novo* lipogenesis through the upregulation of FASN is a well-established hallmark of cancer to generate fatty acids for cell membrane biosynthesis, lipid raft maintenance, and signaling molecules.[15] We have also previously discovered that MGLL fuels cancer aggressiveness through regulating a fatty acid network enriched in protumorigenic signaling lipids.[8,16] More recently, we have also shown that PAFAH1B2 and PAFAH1B3 are critical metabolic drivers of breast cancer.[14] Thus, we show here the oncogenic regulatory mechanisms of several serine hydrolases previously shown to be critical in driving cancer

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pathogenicity using ABPP coupled with the profiling of a series of isogenic oncogene-driven MCF10A lines.

Previous studies have also demonstrated the importance of other serine hydrolases in cancer, including protein methylesterase 1 (PPME1) that demethylates and inactivates the tumor suppressor PP2A and KIAA1363 that regulates oncogenic ether lipid signaling pathways.[5,17−20] Our data found that PPME1 (BRAF, HRAS, MEKDD, NeuNT) and KIAA1363 (BRAF, CyclinD1, HRAS, NeuNT) were each differentially regulated by a select subset of oncogenes (Figure 1C), indicating that these serine hydrolases may be more narrowly regulated by specific oncogenes rather than more globally altered by multiple oncogenic stimuli.

While previous studies have already shown that pharmacological blockades of FASN and MGLL are promising therapeutic strategies for treating cancer, the potential anticancer effects of PAFAH1B2/1B3 inhibitors are less well understood. We recently showed that RNA interference (RNAi)-mediated reduction of PAFAH1B2 or PAFAH1B3 results in substantially impaired cellular survival, motility, invasiveness, and *in vivo* tumor xenograft growth of 231MFP triple-negative breast cancer cells.[14] A selective dual PAFAH1B2 and PAFAH1B3 inhibitor P11 has also been recently generated (Figure 2A),[21] showing the ability of P11 to selectively block both PAFAH1B2 and 1B3 activity *in situ* and impair cell survival in Neuro2a neuroblastoma and PC3 prostate cancer cells. Here, we substantially expand upon these initial findings to characterize in-depth the pharmacological blockade of PAFAH1B2 and 1B3 enzymes. We show that P11 inhibits recombinant purified human PAFAH1B2 and PAFAH1B3 PAF hydrolytic activity with respective 50% inhibitory concentration (IC50) values of 0.023 and 1.1 μM (Figure 2B).

We next wanted to investigate whether certain oncogenes may confer sensitivity to P11. We show that P11 impairs serum-free cell survival in BRAF and HRAS-expressing MCF10A cells, but not in the 10A control and NeuNT-expressing 10A cells (Figure 2C). Interestingly, HRAS and BRAF-expressing 10A cells possess upregulated PAFAH1B2 and 1B3, whereas the less sensitive control MCF10A and NeuNT-expressing 10A cells have undetectable activity or only show an upregulation in PAFAH1B2, respectively (Figure 1B). We have also previously shown that 10A cells transformed by the Hippo transducer TAZ have increased PAFAH1B2 and 1B3 activity and are sensitive to knockdown of either enzyme.[14] When taken together, our studies suggest that perhaps tumors dependent on HRAS, BRAF, and TAZ may be more sensitive to PAFAH1B2/1B3 inhibitors compared to NEU-driven tumors or normal mammary epithelial cells. Further studies are required to understand the mechanisms underlying sensitivity versus resistance to PAFAH1B2/1B3 inhibitors.

We previously showed that genetic inactivation of PAFAH1B2 or 1B3 in triple-negative breast cancer cells impaired cancer cell pathogenicity.[14] We show here that pharmacological blockade of both PAFAH1B2 and 1B3 with P11 also significantly impairs 231MFP breast cancer survival, motility, and invasiveness (Figure 2D). While these data look quite striking, we note that the motility and invasiveness impairments conferred by P11 may be in part due to the survival impairments observed with P11, although the 50% cell survival impairments after 24 h in 231MFP cells do not account for the >90% impairments in motility and invasion in 231MFP cells at 12 and 24 h, respectively.

Nilsson et al. recently showed that PAFAH1B3 was among the 50 most commonly upregulated metabolic enzymes across >1000 primary human tumors across 19 cancer types, indicating that PAFAH1B enzymes may be important to additional cancers.[22] Indeed, we have also observed that PAFAH1B2 and 1B3 are also highly expressed in multiple other human cancer cell lines including C8161 and MUM2C melanoma, SKOV3 ovarian, and MCF7 breast cancer cells.[8] We show here that P11 also impairs 48 h serum-free cell survival in a concentration-dependent manner and significantly reduces cellular motility across these cell lines (Figure 2E,F). Again, we cannot rule out that the motility impairments may be due to the survival impairments observed with P11. Nonetheless, we show here that dual blockade of PAFAH1B2 and 1B3 enzymes dramatically impairs cancer cell pathogenicity across a broad spectrum of human cancer cell types.

While previous studies have shown that PAFAH1B2 and 1B3 are capable of hydrolyzing the lipid species known as platelet activating factor (PAF), we recently demonstrated that RNAi-mediated knockdown of PAFAH1B2 or 1B3 does not alter PAF levels or PAF hydrolytic activity, indicating that these enzymes may possess alternate endogenous substrates.[14] Surprisingly, we observed that PAFAH1B2 and 1B3 inactivation led to far wider alterations in lipid metabolism including increases in several tumor-suppressing lipids.[14] We next determined whether P11 produced similar metabolic changes to those observed with knockdown of these enzymes in 231MFP breast cancer cells and whether these changes were also seen in other cancer cell types. Thus, we performed targeted lipidomic analysis on P11-treated 231MFP breast and SKOV3 ovarian cells (Figure 3A,B, Supporting Information Table 2). Out of 148 lipid species measured, we show that the levels of 10 lipids are significantly ($p < 0.01$) and commonly altered upon P11 treatment in both 231MFP and SKOV3 cancer cells by >2-fold in at least one cell line. These common changes include elevations in phosphatidylcholine (PC) and phosphatidylcholine-plasmalogens (PCp), phosphatidylserine (PS), acyl carnitines (AC), ceramides, sphingomyelins (SM), and triacylglycerols (TAG) levels and a decrease in lysophosphatidylserine (LPS) levels. Importantly, many of these changes in lipid species—namely, elevations in PC, PS, ceramides, SM, and TAGs—are common with our previously described metabolomic signature of PAFAH1B3 genetic knockdown (Figure 3B).[14] Among these lipid changes, ceramides are known tumor-suppressing pro-apoptotic lipids, PC species have been shown to stimulate the antitumorigenic PPARα, and PS is also associated with pro-apoptotic pathways.[23−27] The other changes in lipid species such as AC elevations and LPS decreases may potentially be lipidomic changes resulting from PAFAH1B2 inhibition or dual PAFAH1B2/1B3 inhibition. Our results thus indicate that dual PAFAH1B2 and 1B3 inhibition results in broad-based changes in the lipidome, which include elevations in key tumor-suppressing signaling lipids such as ceramides, PCs, and PSs. Intrigued by the large-fold changes in AC and ceramide levels, we tested whether purified recombinant PAFAH1B2 or PAFAH1B3 was capable of *in vitro* hydrolysis by LC/MS, but we did not detect formation of hydrolysis products for either lipid species (data not shown). Thus, while we observe striking changes in the lipidome we still do not understand the identity of endogenous PAFAH1B2 or 1B3 substrates. Further studies need to be undertaken to identify the substrates of these two enzymes and determine if those substrates contribute to the lipidomic and pathogenic effects we observe here.

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In summary, we show the ability of oncogenic stimulus to radically alter serine hydrolase activities using ABPP and show that four of these enzymes, FASN, PAFAH1B2, PAFAH1B3, and MGLL, show consistent upregulated activity upon induction by different oncogenes. We show that targeted and selective pharmacological blockade of the targets PAFAH1B2 and 1B3 leads to dramatic impairment in cancer cell survival and aggressiveness *in vitro* using multiple different cancer cell types. This inhibition produces broad metabolic alterations in lipid metabolism and leads to elevated levels of key tumor-suppressing signaling lipids, potentially mediating the observed reduction in cancer cell pathogenicity. While we have focused here on serine hydrolases that were broadly regulated by multiple oncogenes, it will also be of future interest to delve into serine hydrolase activities that are tightly regulated by unique oncogenic drivers, as these may represent nodal points of control for specific types of tumors. Taken together, our study highlights the utility of functional proteomic profiling of oncogenic changes to uncover metabolic pathways that are commonly dysregulated in cancer and may constitute targets for therapeutic intervention.

METHODS

Chemicals. P11 was obtained from Professor Benjamin Cravatt at The Scripps Research Institute.

Cell Culture. MCF10A and derived lines were obtained from Sourav Bandyopadhyay and Andrei Goga's groups.[11] The generation and validation of these lines are described in a recent publication by the Bandyopadhyay group.[11] These lines were cultured in DMEM/F12K media with 5% horse serum, glutamine (4.5 mM), 500 ng/mL hydrocortisone, cholera toxin (100 ng/mL), epidermal growth factor (20 ng/mL), and insulin (10 ng/mL) at 37 °C and 5% CO_2. 231MFP and SKOV3 cells were obtained Dr. Benjamin Cravatt and are *in vivo* passaged lines derived from MDA-MB-231 and parental SKOV3 cells. C8161 cells were obtained from Mary Hendrix. MCF7 and MUM2C cells were purchased from ATCC. 231MFPs were grown in L15 media with 10% FBS and supplemented glutamine (4 mM) at 37 °C and 0% CO_2. C8161, MCF7, MUM2C, and SKOV3 were grown in RPMI media with 10% FBS and supplemented glutamine (4 mM) at 37 °C and 5% CO_2.

ABPP-MudPIT Analysis of Serine Hydrolase Activities. Identification and comparative quantitation of serine hydrolase activities by ABPP-MudPIT from MCF10A and oncogene-induced MCF10A lines were conducted as previously described using FP-biotin (5 μM) and analyzed using the Integrated Proteomics Platform (IP2).[8]

PAF, Acyl Carnitine, and Ceramide Hydrolytic Activity Assay. Hydrolytic activity assays were performed as previously described.[14,28] Briefly, recombinant human PAFAH1B2 and PAFAH1B3 were preincubated with either DMSO or P11 for 30 min at 37 °C followed by the addition of C16:0e PAF, C16:0 acyl carnitine, or C16:0 ceramide (100 μM) for 30 min at 25 °C in PBS (200 μL final volume). Reactions were quenched by the addition of 3 × 2:1 chloroform/methanol. A total of 10 nmol of dodecylglycerol was added as an internal standard, vortexed, and centrifuged, and the bottom organic layer was removed for LC/MS analysis of product formation (C16:0e LPCe (lyso-PAF) for PAF and C16:0 FFA for AC and ceramide). IC50 values were calculated using Prism Software.

Cellular Phenotype Studies. Migration, cell survival, and invasion assays were performed as previously described.[3,8] Migration assays were performed in Transwell chambers (Corning) coated with collagen. Invasion assays were performed using the BD Matrigel Invasion Chambers. Cell survival assays were performed using Hoechst 33342 dye (Invitrogen).

Metabolomic Profiling of P11-Treated 231MFP Breast Cancer Cells. Targeted lipidomic analyses were conducted using previously described methods.[3,14] Briefly, 2 million cells were plated overnight, after which cells were washed with PBS, and then treated with DMSO or P11 for 8 h in serum-free media. Cells were washed with PBS, harvested by scraping, and flash frozen. Flash frozen cell pellets were then extracted in 4 mL of 2:1:1 chloroform/methanol/PBS with internal standards dodecylglycerol (10 nmoles) and pentadecanoic acid (10 nmoles). Organic and aqueous layers were separated by centrifugation, and the organic layer was removed. The aqueous layer was acidified with 0.1% formic acid followed by reextraction with chloroform (2 mL). The second organic layer was combined with the first organic extract and dried under nitrogen, after which lipids were resuspended in chloroform (120 μL), of which an aliquot (10 μL) was analyzed by targeted single-reaction monitoring LC-MS/MS. Quantification of relative levels of metabolites were performed by calculating the area under the peak, normalizing to the internal standard and external standard curves, and the final value is expressed in relation to normalized DMSO-treated controls.

ASSOCIATED CONTENT

Supporting Information

Supporting Tables showing detailed data for both ABPP and metabolomic profiling. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00053.

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Author Contributions

R.A.K. designed research, performed research, analyzed data, and wrote the paper. M.M.M. designed research, performed research, and analyzed data. D.K.N. designed research, performed research, analyzed data, and wrote the paper. J.W.C., K.-L.H., and B.F.C. provided reagents. A.S., S.B., and A.G. provided cell lines.

Notes

The authors declare no competing financial interest.

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