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Research article

ACLY is the novel signaling target of PIP_2/PIP_3 and Lyn in acute myeloid leukemia

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ABSTRACT

A fundamental feature of tumor progression is reprogramming of metabolic pathways. ATP citrate lyase (ACLY) is a key metabolic enzyme that catalyzes the generation of Acetyl-CoA and is upregulated in cancer cells and required for their growth. The phosphoinositide 3-kinase (PI3K) and Src-family kinase (SFK) Lyn are constitutively activate in many cancers. We show here, for the first time, that both the substrate and product of PI3K, phosphatidylinositol-(4,5)-bisphosphate (PIP_2) and phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), respectively, bind to ACLY in Acute Myeloid Leukemia (AML) patient-derived, but not normal donor-derived cells. We demonstrate the binding of PIP_2 to the CoA-binding domain of ACLY and identify the six tyrosine residues of ACLY that are phosphorylated by Lyn. Three of them (Y682, Y252, Y227) can be also phosphorylated by Src and they are located in catalytic, citrate binding and ATP binding domains, respectively. PI3K and Lyn inhibitors reduce the ACLY enzyme activity, ACLY-mediated Acetyl-CoA synthesis, phospholipid synthesis, histone acetylation and cell growth. Thus, PIP_2/PIP_3 binding and Src tyrosine kinases-mediated stimulation of ACLY links oncogenic pathways to Acetyl-CoA-dependent pro-growth and survival metabolic pathways in cancer cells. These results indicate a novel function for Lyn, as a regulator of Acetyl-CoA-mediated metabolic pathways.

1. Introduction

The most frequently activated signaling pathway in cancer is the phosphoinositide 3-kinase (PI3K) pathway (Traynor-Kaplan et al., 1988; Whitman et al., 1988; Goncalves et al., 2018). This is principally due to at least one, but more often multiple, genetic modifications in PI3K/PTEN and/or upstream activators such as *RAS* subfamily proteins, receptor tyrosine kinases, and non-receptor tyrosine kinases including Src family kinases (SFK) that are common in all types of cancer (Goncalves et al., 2018). Two key signaling molecules common to these pathways are the phospholipids, PI(4,5)P_2 and PI(3,4,5)P_3, whose alterations trigger cascades of pro-cancer responses such as cell proliferation, survival, adhesion and chemotaxis ((Traynor-Kaplan et al., 1988; Whitman et al., 1988; Goncalves et al., 2018). PI(4,5)P_2 and PI(3,4,5)P_3 couple to metabolic pathways through both AKT-dependent and AKT-independent mechanisms that can lead to tumor progression (Mahajan and Mahajan, 2012; Sivanand et al., 2017). Src was the first transforming protein (Rous, 1911) and protein tyrosine kinase (Hunter and Sefton., 1980) discovered. While the SFKs, particularly Lyn, are functionally and physically associated with PI3K (Ptasznik et al., 2002), and constitutively activated in acute myeloid leukemia (Dos Santos et al., 2008), chronic myeloid leukemia-blast crisis (Ptasznik et al., 2002, 2004), chronic lymphocytic leukemia (Contri et al., 2005), breast cancer (Tornillo et al., 2018), pancreatic cancer and fibrosis (Fu et al., 2006; Pham et al., 2016), glioblastoma (Stettner et al., 2005) and malignant melanoma (Zhang et al.,. 2019), Lyn's peculiar role in cancer cell metabolism remains to be elucidated.

A fundamental feature of tumor progression is reprogramming of metabolic pathways and gene regulation. ATP citrate lyase (ACLY) is a key enzyme for the synthesis of Acetyl-CoA, a critical precursor

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delivering acetyl groups for fatty acid/lipid/phospholipid synthesis and histone acetylation/gene regulation (Wellen et al., 2009; Cai et al., 2011; Zaidi et al., 2012; Sivanand et al., 2017). ACLY, and resulting lipid production and histone acetylation (Wellen et al., 2009), are upregulated in cancer (Cai et al., 2011; Zaidi et al., 2012). To examine the signaling and metabolic consequences of multiple pathogenic chromosomal aberrations and genetic mutations (SupplementalInformation), we measured the direct binding of PIP_2 and PIP_3 to ACLY in AML patient- and normal donor-derived living marrow cells by the tri-functional PIP_2 and PIP_3 derivatives. We also performed several ACLY/PIP specificity binding assays with the ACLY purified peptides. To identify phosphorylated by Lyn/Src tyrosine sites of ACLY we used the phosphoproteomics analysis. We evaluated the effects of PI3K and Lyn inhibition on the ACLY-mediated Acetyl-CoA and phospholipid synthesis, histone acetylation and growth of HL-60 AML cells. We report here a molecular mechanism in which both the substrate and product of PI3K, PIP_2 and PIP_3, respectively, directly bind to the Lyn tyrosine kinase-phosphorylated ACLY. This couples oncogenic signaling events, through a tyrosine kinase-mediated mechanism, with the Acetyl-CoA synthesis, phospholipid metabolism, histone acetylation and cell proliferation in cancer.

2. Results

2.1. ACLY interacts with PIP_2/PIP_3 in patient-derived AML cells

Because AML patient-derived blasts, in contrast to non-malignant myeloid cells, express multiple mutated proteins that can alter PI3K signaling (Table S1), we examined whether the substrate and product of PI3K, PIP_2 and PIP_3, respectively, could bind to ACLY in these cells. Investigations of PIP_2/PIP_3 actions are often hampered by a lack of tools that can be used in living cells. However, it has recently been demonstrated that the novel tri-functional lipid probes (Höglinger et al., 2017; Laguerre and Schultz, 2018), including the phosphatidylinositol probes

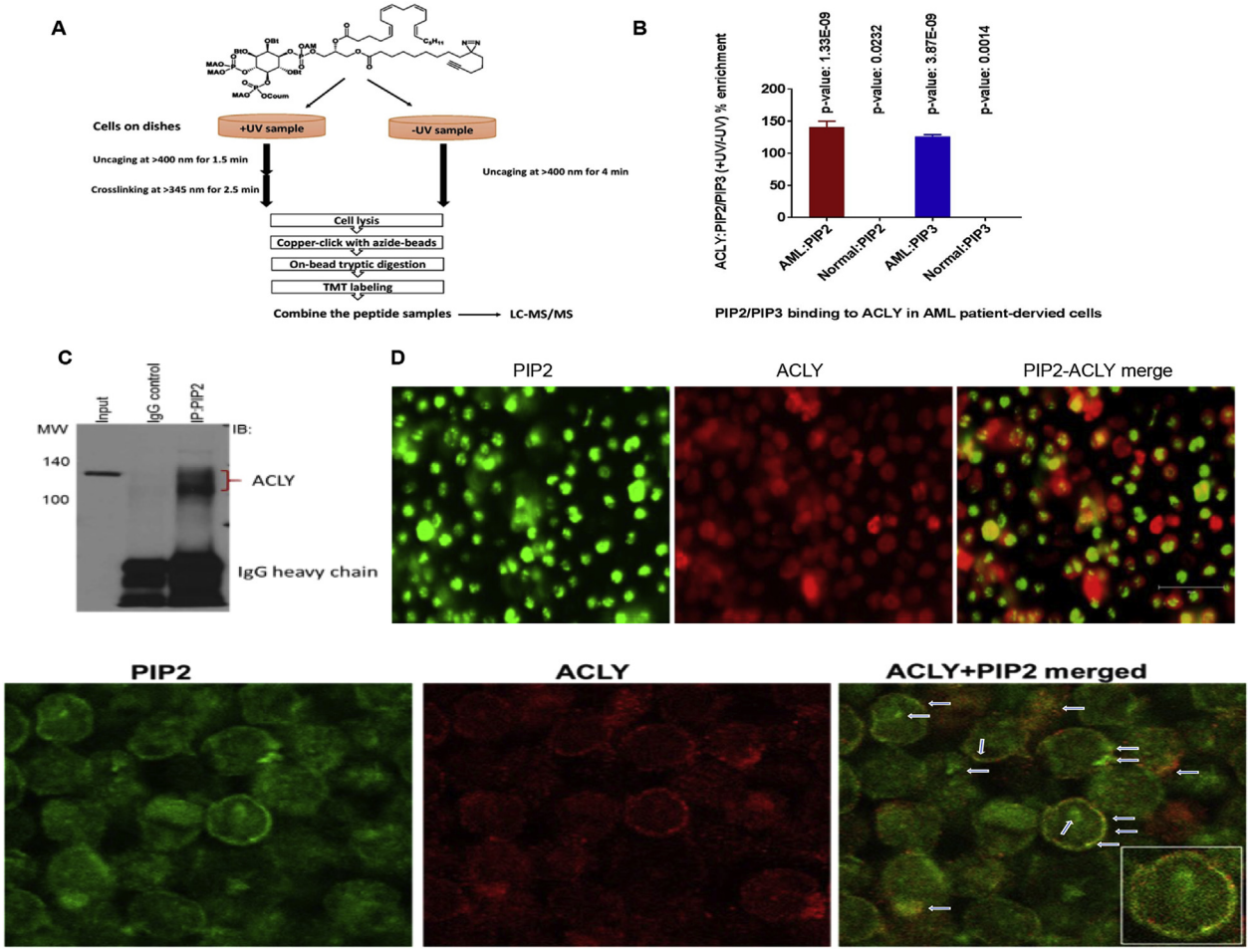


Figure 1. $PI(4,5)P_2$ and $PI(3,4,5)P_3$ directly interact with AC LY in acute myeloid leukemia (AML) patient-derived marrow blasts, but not non-malignant marrow CD34+ cells (A) Treatment of AML and normal cells with the tri-functional membrane-permeant PIP_2 or PIP_3 derivatives (Methods). The chemical structure of tri-functional $PI(4,5)P_2$ is shown (B) Normalized reporter ion intensities from +UV samples were divided by the respective -UV control values to yield the final enrichment factor. The bars show the average enrichment ratio (+UV/-UV) for each condition, the p values show the relation between + UV and –UV samples in each condition and they are highly statistically significant. ACLY was enriched with PIP_2 or PIP_3 more than 100% in AML patient cells while no enrichment was obtained in illuminated normal cells (normalization and statistics are described in Methods in detail, Figures S2, S3, S4) (C) ACLY is present in $PI(4,5)P_2$ precipitates from acute myeloid leukemia HL-60 cells. Cells were lysed and immunoprecipitated with anti-PIP_2 or IgG control and blotted for ACLY, as described under Methods. The input (5%) lysate was also analyzed. The position of ACLY is indicated. We have noticed that binding of negatively charged PIP_2 to positively charged amino acids on ACLY (co-immunoprecipitate) can cause slight change in electrophoretic mobilities in SDS-PAGE gel, as compared to input (the full, non-adjusted images of western blots are in Figure S7) (D) Colocalization of ACLY and $PI(4,5)P_2$ in HL-60 cells. Immunoreactivity for ACLY is shown in red and PIP_2 in green. When the two fluorescence spectra are merged (right panels) ACLY and PIP2 colocalization in the cell is shown by the yellow color. Confocal imaging with 60x magnification (lower panel) indicates PIP2 and ACLY colocalization throughout the cell, particularly in the cell membranes. The secondary Ab controls are shown in Figure S5. The colocalization of ACLY and $PI(3,4,5)P_3$ was also measured, but levels of endogenous PIP_3 were too low in these cells to be analyzed by this method (see explanation in Methods).

(Müller et al., 2020) well represent the endogenous lipid and phosphatidyinositol pool in living cells. Thus, we probed the association of PIP_2/PIP_3 with ACLY by incubating AML and control cells with the tri-functional derivatives of PIP_2 and PIP_3 (Figure 1A), and applying the properly normalized ACLY enrichment procedures and mass spectrometry analysis (Figure 1B) (Höglinger et al., 2017; Laguerre and Schultz, 2018; Müller et al., 2020). We observed that ACLY was enriched by PIP_2 and PIP_3 more than 100% in AML patient blasts, while no enrichment was observed in illuminated non-malignant myeloid cells (Figure 1B). These data show the direct association of PIP_2/PIP_3 with ACLY in living primary AML blasts. We confirmed the association of PIP_2 with ACLY in the HL-60 AML cell line by looking for ACLY in PIP_2 immunoprecipitates by Western blotting (Figure 1C) and colocalization of ACLY with PIP_2 by immunofluorescence (Figure 1D). PIP_3 was also measured, but the basal endogenous PIP3 levels were too low in these cells to be analyzed by immunofluorescence or blotting. It is well established that the abundance of PIP_3 in living cells is several orders of magnitude lower than PIP_2 (Methods). Therefore, we also probed the association of $PI(3,4,5)P_3$ with ACLY by binding the ACLY full length protein to membrane lipid strips (the membranes were spotted with 100pmol of fifteen biologically important lipids, see Methods) (Figure 2A). ACLY bound selectively to PIP, PIP_2 and PIP_3 in the membrane lipid strips binding assay (Figure 2B, C). In contrast, we detected no binding of ACLY to phosphatidylinositol (PI) and several other lipids, under the same conditions (Figure 2B,C). These data indicate that phosphorylated forms of phosphatidylinositol (PIP, PIP_2 and PIP_3), which are known to play important roles in cell signaling, can selectively interact with ACLY, in contrast to phosphatidylinositol (PI), which is their precursor and thus structurally very similar. It is consistent with our data obtained with the trifunctional PIP_2 and PIP_3 derivatives in living cancer cells (Figure 1B).

2.2. *Identification of the PIP_2 binding region of ACLY*

Based on the PIP_2 binding motif analysis and using the full length ACLY protein sequence, we predicted two potential PIP_2 binding regions: the ATP-binding domain and the CoA-binding domain of ACLY. Therefore, we synthesized two different ACLY peptides containing either the ATP-binding domain or the CoA-binding domain sequences (Figure 6A and Table S3). The binding of these ACLY peptides to phospholipids on the Cova PIP Specificity Plates (Figure 2D,E) and the ACLY mutant

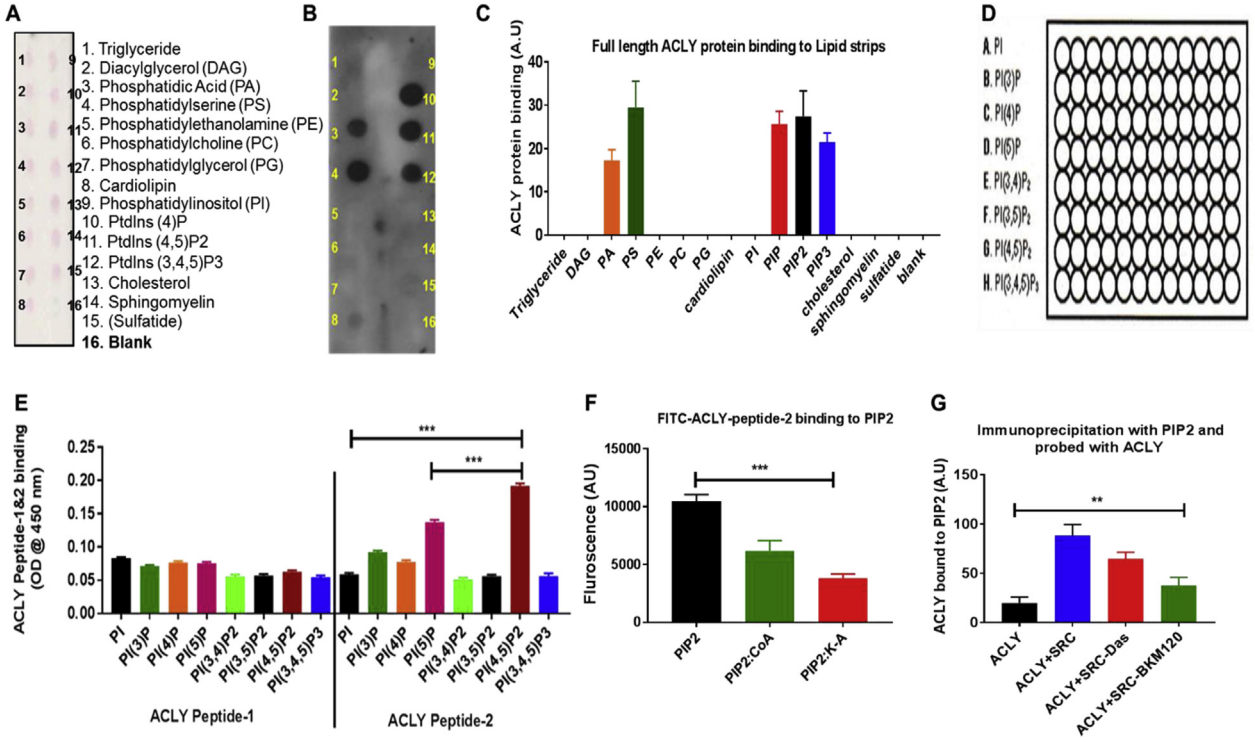
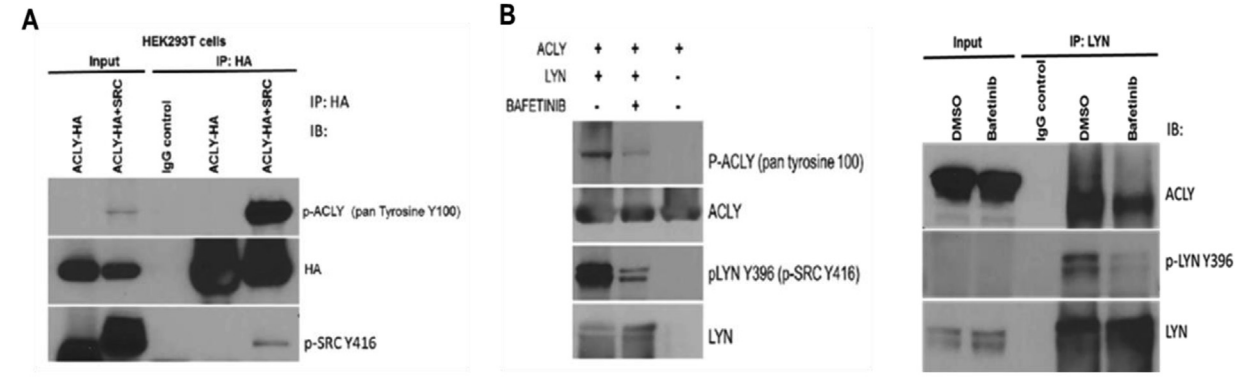


Figure 2. $PI(4,5)P_2$ and $PI(3,4,5)P_3$ directly interact with the purified ACLY peptides and the ACLY full length protein (A-C) The ACLY full length protein binds to $PI(4,5)P_2$ and $PI(3,4,5)P_3$ in the membrane lipid strips assay. ACLY-HA tagged full length protein binding to membrane lipid strip (Echelon Biosciences) (Methods). The membranes have been spotted with 100 pmol of fifteen biologically important lipids (A). This assay shows that ACLY selectively interacts with the phosphorylated phosphoinositides (PIP, PIP_2, PIP_3), Phosphatidic Acid and Phosphatidylserine, but not with phosphatidylinositol (PI) and nine other lipids (B). The membrane dots densitometry values were measured and used for the graph (C). The positive control is in Figure S6 (D-E) The ACLY peptide-2 (Co-A-binding domain), but not the ACLY peptide-1 (ATP-binding domain), binds to $PI(4,5)P_2$. Schematic representation of a PIP specificity plate (D). 96-well polystyrene microplate where each row has an individual phosphoinositide coated at 20pmols per well. The two ACLY peptides were designed, synthetized and used to detect phospholipids binding on the PIP specificity plates (E) (Methods) (the relevant peptide sequences are shown in Figure 6A and Table S3). For statistical analysis Graphpad prism software was used. Error bars, S.E.; n = 3, one-way ANOVA or unpaired t-test. Asterisks indicate significant difference between $PI(4,5)P_2$ and other phospholipids. ***, $p < 0.0001$ (F) The binding of the ACLY peptide-2 to PIP2 is decreased in the presence of Coenzyme A (PIP2:CoA) or with mutant ACLY peptide-2 with replacement of basic amino acid lysine-K to alanine-A (PIP2:K-A). For ACLY and PIP2 specificity binding assay was performed using the N-terminally FITC labelled wild type ACLY peptide-2 (CoA-binding domain sequence - peptide-2: ALTRKLIKKADQKGV), or in ACLY peptide-2 two basic amino acids lysine (K) were replaced with alanine (Kpeptide-2: ALTRKLIAAADQKGV), with or without 50 uM CoA in binding conditions. For statistical analysis Graphpad prism software was used. Error bars, S.E.; n = 3, one-way ANOVA or unpaired t-test. Asterisks indicate significant difference from PIP2 to other conditions. ***, $p < 0.0001$ (G) The ACLY full length protein binds to PIP_2 in Src protein tyrosine kinase-dependent and PI 3-kinase-dependent manner. HEK293T cells were transfected with full length ACLY-HA alone or in co-transfection of active SRC kinase. After 36 h of transfection cells were subjected to DMSO or Dasatinib (2 uM) or BKM120 (2 uM) for 15 h and lysed in cell lysis buffer and followed the PIP_2 immunopreciptation and western blotting. The phospho-ACLY bound to PIP_2 were quantified using IMAGE software to analyze densitometry values for quantitation using PRISM Graphpad statistical analysis tool. Asterisks indicate significant difference from DMSO to Dasatinib (Src inhibitor) or BKM120 (PI3K inhibitor). **, $p < 0.001$.

experiment (Figure 2F) indicated that PI(4,5)P_2 selectively bound to the CoA-binding domain (peptide-2), but not to the ATP-binding domain (peptide-1) of ACLY. The differences detected by this binding assay between PI(4,5)P_2 and seven other control phospholipids were highly statistically significant. The ACLY peptide binding results on the Cova PIP Specificity Plates were consistent with our data obtained with five other assays: 1. the trifunctional PIP_2/PIP_3 derivatives binding assay in living cancer cells (Figure 1 A,B), 2. protein co-immunoprecipitation by Western blotting (Figure 1C), 3. protein co-localization by immunofluorescence (Figure 1D), 4. membrane lipid strips binding assay (Figure 2A-C), 5. the phospho-ACLY binding to PIP_2 in transfected cells (Figure 2G). Taken together, the mechanistically distinct experimental approaches and multiple data indicate consistently that ACLY directly binds to PIP_2 and PIP_3 and the specific association with PIP_2 is mediated through the ACLY CoA-binding domain (Figure 2E,F).

2.3. ACLY is phosphorylated on tyrosine residues by Lyn in AML

We observed that ACLY-mediated production of Acetyl-CoA is sensitive to Lyn tyrosine kinase inhibitor in AML (Figure 4 A-C). To determine whether Lyn plays a role in ACLY activation, we transfected kidney embryonic HEK293T cells either with HA-tagged ACLY alone or with HA-tagged ACLY and Src. Figure 3A shows that we could specifically precipitate 120-kDa strongly tyrosine phosphorylated ACLY protein with HA-conjugated agarose and that this phosphorylation only took place in cells co-transfected with Src. This observation was confirmed by *in vitro* tyrosine kinase assay on purified ACLY protein and Lyn immunoprecipitates from HL-60 AML cells. In the presence of active pY396-Lyn the ACLY was tyrosine phosphorylated and this process was sensitive to Lyn tyrosine kinase inhibitor (Figure 3B) (Methods). These findings show that SFK-dependent pathway, Lyn in AML cells, induces the ACLY activity in protein tyrosine kinase-dependent manner.



Lyn kinase directly phosphorylates ACLY on the tyrosine residues

Annotated Sequence	Modifications	XCorr score
EGD**Y**VLFHHEGGVDVGDVDAK	Y131	1.56
VDATAD**Y**ICK	Y227	1.65
EAYPEEA**Y**IADLDAK	Y252	2.02
RGGPN**Y**QEGLR	Y384	3.19
LYRPGSVA**Y**VSR	Y659	3.51
TTDGV**Y**EGVAIGGDR	Y682	1.77

SRC kinase directly phosphorylates ACLY on the tyrosine residues

Annotated Sequence	Modifications	XCorr score
VDATAD**Y**ICK	Y227	1.46
EAYPEEA**Y**IADLDAK	Y252	1.46
TTDGV**Y**EGVAIGGDR	Y682	2.06
QHFPATPLLD**Y**ALEVEK	Y1006	1.29

Figure 3. Lyn directly interacts and phosphorylates the tyrosine residues of ACLY (A) Src family kinase phosphorylate ACLY on the tyrosine residues. ACLY-HA and ACLY-HA + SRC transfected human HEK293T cells were lysed, precipitated with HA or IgG control antibody and blotted for p-ACLY (pan Tyrosine Y100), p-SRC (Y416) and HA. Cells transfected with Src showed remarkable induction of ACLY tyrosine phosphorylation and phosphorylated SRC (Y416) was present in ACLY-HA precipitates. The input lysate was also analyzed. Results are representative of two independent experiments (Methods) (B) LYN directly phosphorylates ACLY on the tyrosine residue. *In vitro* tyrosine kinase assay on ACLY (left panel): recombinant HA-tagged ACLY (non-phosphorylated form) was purified as described in the method section and was incubated with immunoprecipitated Lyn (from total lysates of HL-60 AML cells treated with DMSO or 500 nM Bafetinib for 16h) in an *in vitro* kinase assay buffer and subsequently blotted with the indicated antibodies. The ACLY protein is phosphorylated on tyrosine residue only in the presence of active LYN (pY396) and this tyrosine phosphorylation of ACLY is prevented by the Lyn kinase inhibitor, Bafetinib. As indicated in the right panel, ACLY is present in Lyn immunoprecipitates in HL-60 AML cells (5% input) (C-D) Phosphoproteomics analysis of ACLY in vitro phosphorylated samples. Active recombinant Lyn or Src kinase directly phosphorylated purified His-tagged ACLY at tyrosine residues in an in vitro kinase assay (Methods). Phosphorylated Lyn (Y396) and Src (Y416) and also ACLY were detected by pan phospho-Tyrosine antibody (pY100) (C). *In vitro* tyrosine phosphorylated ACLY samples were resolved on 10% Novex gels and stained with colloidal blue (D, left panel). The bands were excised and samples were evaluated by phosphoproteomics analysis. Phosphoproteomics Analysis (Methods) resulted in identification of novel Lyn kinase or Src kinase mediated tyrosine phosphorylation sites of ACLY. Sites on ACLY which are common in both Lyn and Src are highlighted in red (D, right panel). See also Figure 6A and Table S3. The full, non-adjusted western blot images are in Figure S7.

2.4. Identification of the tyrosine residues of ACLY that are phosphorylated by Lyn and/or Src

Next, we examined whether any of the tyrosine residues of ACLY could be directly phosphorylated by Src family kinases Lyn or Src. We performed in vitro tyrosine kinase assays on bacterially expressed and purified recombinant full length ACLY protein in the presence of active recombinant Lyn or Src and determined that active recombinant Lyn or Src directly phosphorylated purified ACLY at tyrosine residues (Figure 3C). The phosphoproteomics analysis of ACLY *in vitro* phosphorylated samples indicated that Lyn and Src directly phosphorylated ACLY on six and four tyrosine residues, respectively (Figure 3D). The three ACLY tyrosine residues, Y682, Y252, Y227, were common for Lyn and Src and were located in the catalytic domain, the citrate-binding domain and the ATP-binding domain, respectively (Figure 3D right panel, Figure 6A, Table S3).

2.5. ACLY enzyme activity and Acetyl-CoA production are inhibited by PI3K and LYN inhibitors in AML cells

To determine whether PI3K and Lyn activity could affect ACLY-mediated synthesis of Acetyl-CoA in AML, we treated HL-60 cells for 16 h with the specific Lyn inhibitor (Bafetinib) or two structurally and mechanistically distinct inhibitors of PI3K (LY294002 or BKM120), and then we measured ACLY enzyme activity and acetyl-CoA levels (Methods). As shown in Figure 4B, each of the three inhibitors significantly prevented the synthesis of Acetyl-CoA in AML cells. The corresponding control experiments indicated statistically significant inhibition of ACLY enzyme activity in these HL-60 cell lysates (Figure 4A). Coupled with the fact that PIP_2 and PIP_3 are directly associated with Lyn-phosphorylated ACLY (Figures 1, 2, and 3) and ACLY is a major enzyme for Acetyl-CoA synthesis (Cai et al., 2011; Zaidi et al., 2012; Sivanand et al., 2017) these findings strongly indicate that over-activated PI3K and Lyn in leukemia cells (Ptasznik et al., 2002, 2004; Dos Santos

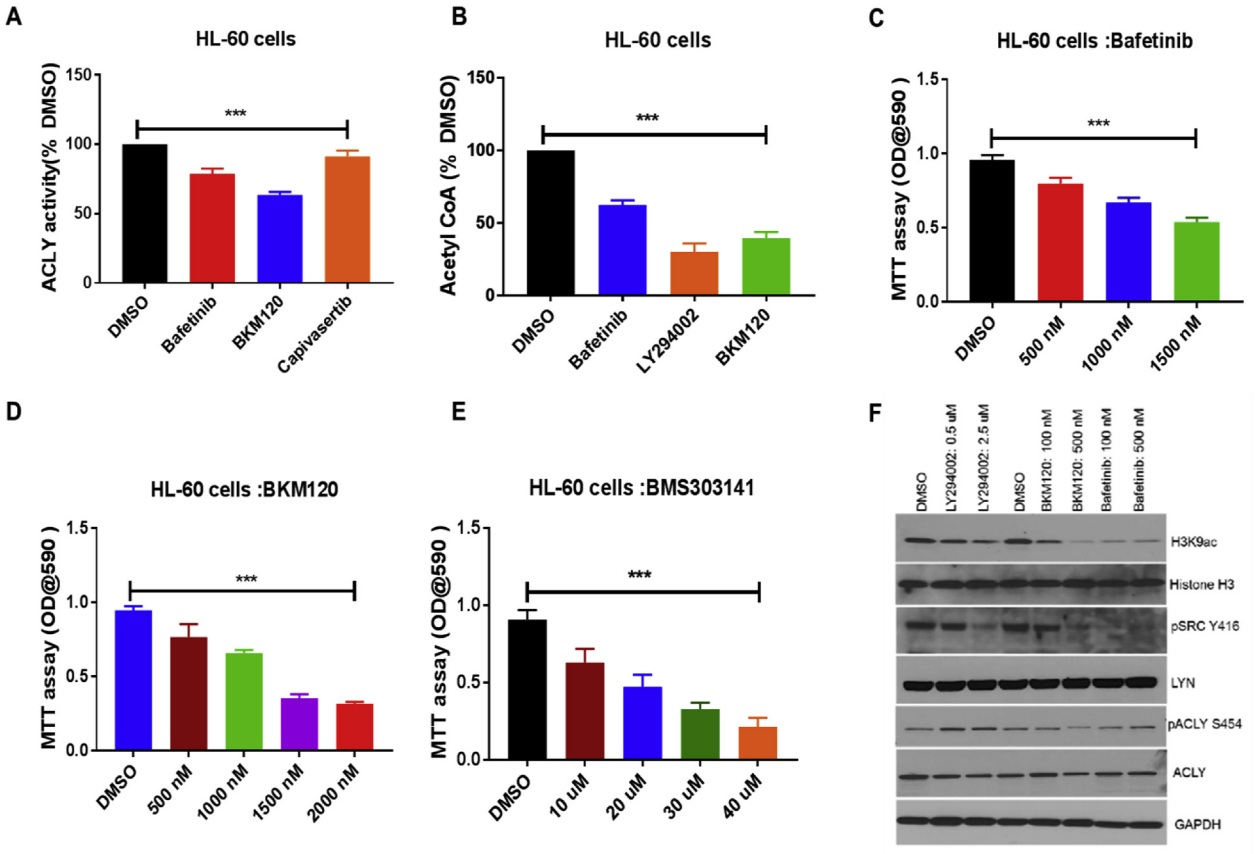


Figure 4. PI3K and Lyn inhibitors suppress the ACLY enzyme activity, synthesis of Acetyl-CoA and Acetyl-CoA-dependent downstream activities (histone acetylation, cell growth) in AML cells (A-B) Effect of PI3K and Lyn inhibitors on the ACLY enzyme activity and synthesis of Acetyl-CoA (A) The ACLY enzyme activity assay on HL-60 cells treated with DMSO, LYN kinase inhibitor, Bafetinib (1.0 uM) or PI3Kinase inhibitor BKM120 (2.0 uM) or AKT inhibitor, Capivasertib (5 uM) for 16 h and lysates. Error bars, S.E.; n = 3, one-way ANOVA analysis. Asterisks indicate significant difference from DMSO/Capivasetib to Bafetinib/BKM120. ***, $p < 0.0001$ (B) HL-60 cells were treated with Lyn inhibitor (Bafetinib) or PI3K inhibitors (LY204002, BKM120) or vehicle for 16 h for Acetyl-CoA measurement (Methods). Control values were the means of 3 DMSO control samples. Student's unpaired t-test was used to compare the DMSO control vs inhibitor treatment group. Data are shown as mean ± SEM; ***$p < 0.0001$, n = 3 (C-E) Effect of Lyn, PI3K and ACLY (BMS303141) inhibitors on AML cell growth. HL-60 cells were treated with various concentrations of the inhibitors or vehicle (0.1% DMSO) in the presence of 10% FBS in RPMI media for 72 h for MTT assay (Methods). Error bars, S.E.; n = 3, one-way ANOVA analysis. Asterisks indicate significant difference from DMSO to Bafetinib or BKM120 or BMS303141. ***, $p < 0.0001$ (F) Effect of Lyn and PI3K inhibitors on Histone H3 acetylation. HL-60 cells were treated with the Lyn inhibitor or PI3K inhibitors or vehicle in the presence of 10% FBS in RPMI for 16 h and then were blotted for H3K9ac, p-SRC Y416 (p-LYN Y396) and p-ACLY S454. Densitometric analysis showed that Histone H3 acetylation was effectively blocked by the treatment of cells with Lyn inhibitor Bafetinib (90%) and PI3K inhibitors, LY294004 (60%) or BKM120 (97%). The treatment with inhibitors did not suppress serine-threonine phosphorylation of ACLY (p-ACLY S454), which is an AKT-mediated event. The pan-PI3K inhibitors at higher concentrations (2.5 uM LY294002 or 500 nM BKM120) also partially suppressed the Lyn activity (~40–50%), as expected, since Lyn is coupled to PI3K in HL-60 cells (Ptasznik et al., 2002) and Src family kinases can be phosphorylated by PI3K (Ptasznik et al., 2002; Watson et al., 2016) (Methods). Notably, figures A and F indicate that ACLY is stimulated by Lyn and PI3K in AKT-independent manner in HL-60 AML cells (see also in Discussion). The full, non-adjusted western blot images are in Figure S7.

et al., 2008; Goncalves et al., 2018) stimulate the ACLY-mediated Acetyl-CoA production.

2.6. Growth of AML cells is strongly suppressed by Lyn, PI3K and ACLY inhibition

ACLY/Acetyl-CoA provides pro-growth and pro-survival signals to the cells, by providing acetyl groups that are required for histone acetylation at growth genes and fatty acids in phospholipid synthesis (Wellen et al., 2009; Cai et al., 2011; Zaidi et al., 2012; Migita et al., 2014). In the present study, we confirmed that the ACLY inhibitor BMS303141 inhibited within 72h growth of HL-60 AML cells with an IC50 of ~10-20uM (Figure 4E). This was lower than the effective doses reported in literature for ACLY-associated growth inhibition in other cells (Solomon et al., 2017). The similar pattern of growth inhibition within 72h was observed with the Lyn inhibitor and PI3K inhibitor (Figure 4 C,D). Thus, prolonged inhibition of Lyn, PI3K and ACLY can profoundly suppress AML cell growth. These results show that Lyn/PI3K and

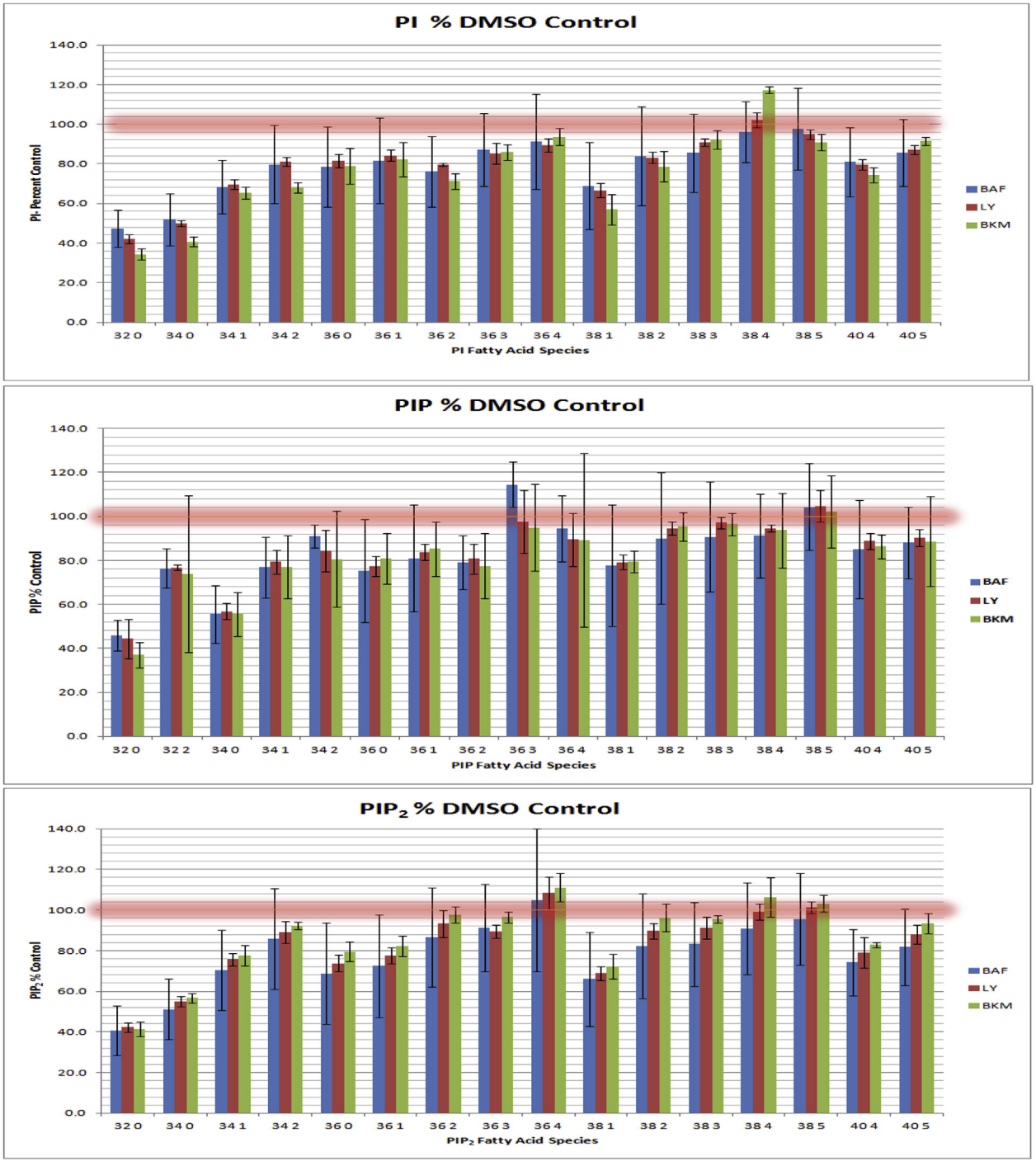


Figure 5. Effect of Lyn and PI3K inhibitors on Fatty Acid composition of PI, PIP, and PIP_2. HL-60 cells were treated with Lyn inhibitor (Bafetinib, BAF) or PI3K inhibitors (BKM120, BKM or LY294002, LY) or vehicle in the presence of 10% FBS in RPMI for 16 h for lipidomic analysis (Methods). The treatment with the inhibitors resulted in an overall decrease in levels of total PI/PIP/PIP2 (as compared to DMSO control – 100%) and the species of PIs with shorter fatty acid chains (32:0, 34:0) were most affected by the inhibitors, in a manner consistent with ACLY inhibition (see also explanation in Results). Control values were the means of 3 DMSO control samples against which values from individual treated samples were calculated. Data are means +/- SD, n = 3 (see additional results and statistics in Table S2).

ACLY/Acetyl-CoA provides pro-proliferation and pro-survival signals in AML cells.

2.7. H3K9 acetylation is prevented by PI3K and LYN inhibitors in AML cells

ACLY/Acetyl-CoA is required for histone acetylation by providing acetyl groups and initiates cell growth by promoting acetylation of histones specifically at growth genes (Wellen et al., 2009; Cai et al., 2011; Wan et al., 2017). The active oncogenic N-RAS and other oncogenes, that are expressed in our patient-derived primary AML cells and HL-60 AML cell line (SupplementalInformation), can increase H3K9ac (Wan et al., 2017; Yi et al., 2018). Acetylation of H3K9 is particularly important, since it is present almost exclusively at growth genes and is highly correlated with active promoters of oncogenes (Wan et al., 2017; Yi et al., 2018). Since we observed that the PI3K and Lyn inhibitors prevented ACLY-mediated production of Acetyl-CoA (Figure 4), we examined whether these inhibitors could also suppress acetylation of H3K9 in AML cells. Indeed, Figure 4F shows that both Lyn tyrosine kinase and PI3K inhibitors almost totally blocked H3K9 acetylation in AML cells. These data (together with data in Figure 4 A-E) indicate that over-activated PI3K and Lyn in leukemia cells increase histone acetylation and gene activation through stimulating the synthesis of Acetyl-CoA.

2.8. Phosphoinositide fatty acid composition is altered by PI3K and Lyn inhibitors in AML cells in a manner consistent with ACLY inhibition

The production of fatty acids/phospholipids requires ACLY/Acetyl-CoA (Zaidi et al., 2012). Since we found that ACLY enzyme activity and production of Acetyl-CoA were blocked by PI3K and Lyn inhibitors (Figure 4 A,B), and PIP_2/PIP_3/Lyn were directly associated with ACLY (Figures 1, 2, and 3), we used mass spectrometric analysis to examine whether these inhibitors affected the fatty acid moieties of phosphoinositides in HL60 AML cells. Inhibitors suppressed PI, PIP and PIP_2 formation, especially saturated and monounsaturated species with shorter fatty acid chains (Figure 5, Supplementary Table 2). Specifically, 32:0, 34:0 and 36:0 PI, PIP and PIP_2 decreased most dramatically, according to the following order (32:0 > 34:0 > 36:0; PI > PIP > PIP_2) (Figure 5, Table S2). This differential inhibition is consistent with ACLY/Acetyl-CoA inhibition since ACLY activity generates shorter chain

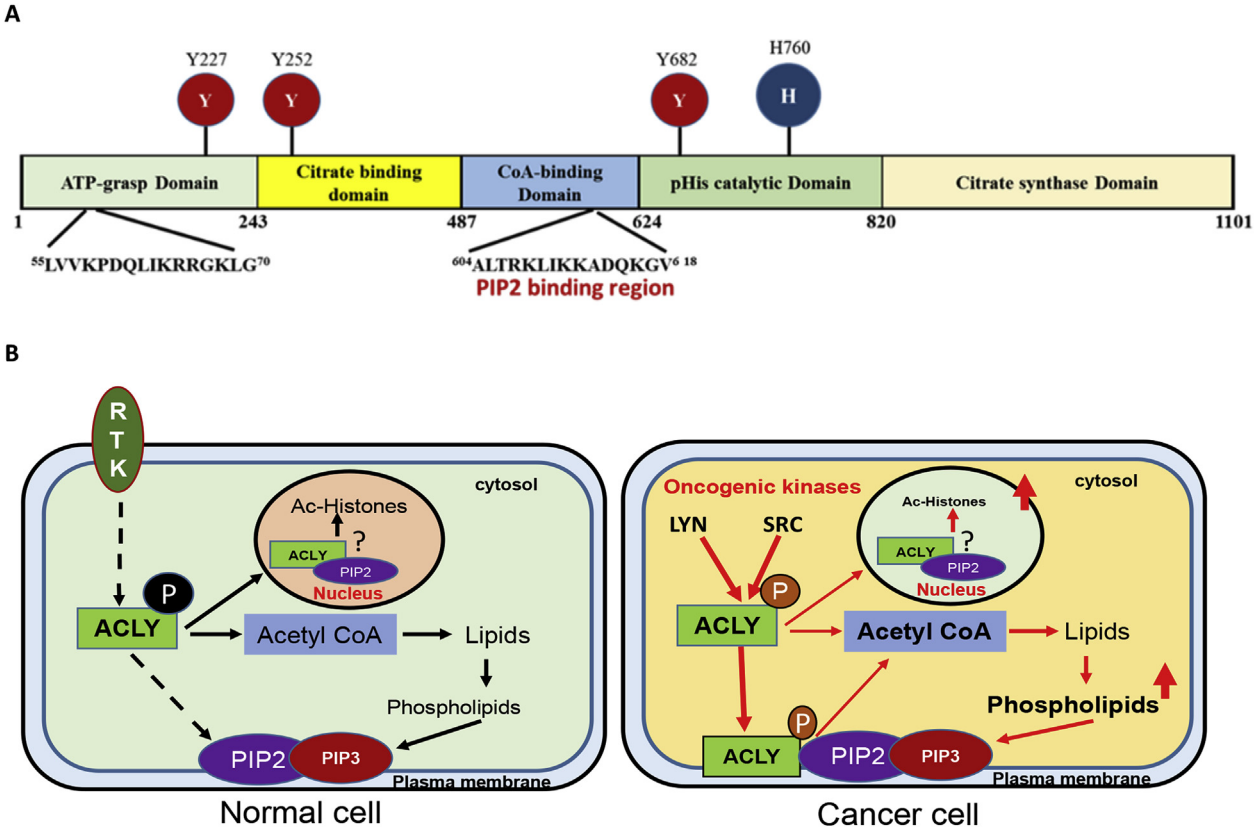


Figure 6. (A) Schematic presentation of the ACLY PIP_2 binding region and the novel Lyn/Src-dependent tyrosine phosphorylation sites. The three tyrosine phosphorylation sites identified in our study and common in Lyn/Src kinase mediated ACLY phosphorylation are shown, including Y682 (catalytic domain), Y252 (citrate-binding domain) and Y227 (ATP-binding domain). The ACLY peptides which were used in our experiments are shown (peptide-1 in the ATP-binding domain sequence and peptide-2 in the CoA-binding domain sequence). The PIP_2 binding motif on ACLY, which was detected using the ACLY peptide-2, is shown. See also Table S3 for the detailed information about the phosphorylation and binding sites (B) Proposed model for interaction between oncogenic signal transduction pathways and Acetyl-CoA metabolic pathway in transformed cells. PI3K and Src family kinases-mediated pathways are the most frequently activated signaling pathways in cancer. As indicated on the right, we propose that Lyn/Src oncogenic kinase-mediated tyrosine phosphorylation of ACLY (Figure 3) induces its interaction with phospholipids where ACLY directly binds to $PI(4,5)P_2$ and other phospholipids in cancer cells (Figure 1 and 2). These interactions lead to increased ACLY-dependent Acetyl-CoA synthesis (Figure 4), which may in turn lead to the increase of phospholipid synthesis (including PIP_2) (Figure 5, Table S2) and protein acetylation in cancer cells (Figure 4F). The basis for a persistent interaction of PIP_2/PIP_3 with ACLY remains to be defined, but it could result from Lyn/Src-mediated phosphorylation of ACLY (Figure 3) and/or increased Lyn/PI3K-mediated PIP_2 synthesis (Figure 5, Table S2) and/or direct oncogene-mediated alteration of PIP_2 (Cao et al., 2019; Choi et al., 2019) in the cell membrane and/or the nuclear compartment of cancer cells. Thus, we propose a Src family tyrosine kinase and PI3K-dependent mechanism whereby oncoproteins hijack a major, Acetyl-CoA-mediated, metabolic pathway fueling synthesis of phospholipids and growth of cancer cells. This paradigm may provide further insight into the striking ability of PI3K and Src kinases to transform various cell types (red color on the right stands for the oncogenic constitutive activation pathways in cancer cells; RTKs on the left in normal cells: Receptor Tyrosine Kinases, the dashed line represents a regulatory temporary stimulation via cytokine/RTK and other receptors in normal cells).

fatty acids first which are the precursors for longer chain fatty acids (Migita et al., 2014). Thus, the inhibition remodeled the overall phosphoinositide fatty acid profile and reduced total levels of phosphoinositides. Both mechanistically distinct inhibitors of PI3K and the Lyn inhibitor dramatically reduced PI/PIP/PIP_2 synthesis in leukemia cells (Table S2). These findings indicate that over-activated PI3K and Lyn in leukemia cells (Ptasznik et al., 2002, 2004; Dos Santos et al., 2008; Goncalves et al., 2018) augment phosphoinositide synthesis (including PIP_2) through activation of ACLY/Acetyl-CoA.

3. Discussion

We demonstrate here that ACLY, a key enzyme generating Acetyl-CoA, directly interacts with crucial signaling molecules, PIP_2/PIP_3 and Lyn, in cancer cells. We show that the direct binding of Lyn to ACLY and the tyrosine phosphorylation and enzymatic activity of ACLY, are mediated by Lyn tyrosine kinase activity (Figures 3A, B, C, 4 and 6). The direct binding of PIP_2/PIP_3 to ACLY and activation of ACLY are mediated by both Lyn and PI3K activity (Figures 2G, 4, 6). We identify the PIP_2-binding region in the key CoA-binding domain of ACLY and the Lyn/Src-regulated tyrosine residues also in the critical domains of ACLY (the catalytic domain, citrate-binding domain and ATP-binding domain) (Figure 6A). These data indicate that over-activated Lyn/Src and PI3K-PIP_2/PIP_3 in cancer cells can directly bind to and modify the ACLY enzyme activity and Acetyl-CoA synthesis (Figure 6B).

Based upon the responses described herein to the PI3K and Lyn inhibitors, and several Lyn/PIs/ACLY binding experiments, we postulate that Lyn directly activates ACLY through a tyrosine kinase phosphorylation-mediated mechanism. Further, we propose that ACLY is activated in parallel by Lyn and PI3K-PIP_2/PIP_3, both contributing to ACLY activation. This scenario is consistent with the mutational pattern of AML cells showing multiple pathogenic mutations in PI3K pathway-activating proteins, including oncogenic N-RAS, which is frequently found in AML cases (Supplemental Information, Table S1; Figure S1). Also Lyn is highly expressed and constitutively phosphorylated in most, if not all, AML cases, as opposed to normal hematopoietic progenitors (Dos Santos et al., 2008).

Our results also suggest that AKT is bypassed by Lyn and PIP_2/PIP_3 in AML cells. First, Lyn and PI3K inhibitors reduce the ACLY enzyme activity, but the AKT inhibitor Capivasertib does not reduce significantly ACLY activity in HL-60 AML cells (Figure 4A). Also, PI3K and Lyn inhibitors do not reduce AKT-dependent serine-threonine phosphorylation of ACLY at S454 in HL-60 cells, a recognized site of AKT-mediated phosphorylation (Figure 4F) (Sivanand et al., 2017). Second, AKT is not a hit target of trifunctional derivatives of PIP_2 and PIP_3 in AML patient-derived cells (Figure 1 A,B, Figure S2 and data not shown). Third, PIP_2 and PIP_3 directly bind to ACLY, as we demonstrate by several different approaches, both in living cell and *in vitro* cell-free systems (Figure 1A-D and 2). Notably, it is also known that a specific and strong inhibition of Lyn expression in AML cell lines and multiple AML patient samples using siRNA does not affect AKT phosphorylation (Dos Santos et al., 2008). Together, this demonstrates that AKT and PI3K/Lyn can operate independently of each other, as previously reported in other malignancies (Mahajan and Mahajan, 2012). These findings indicate that PIP_2/PIP_3 and Lyn couple directly to ACLY inducing its activity through a novel tyrosine kinase-dependent mechanism in AML cells and thus *independent of AKT,* which is a serine/threonine-specific kinase. We conclude that, in addition to well documented AKT-mediated pathways for ACLY activation (Sivanand et al., 2017), the additional tyrosine kinase-mediated pathway exists, as we demonstrate in this paper in NRAS-mutant positive AML primary samples and HL-60 AML cell line (Table S1; Figure S1).

In conclusion, our results demonstrate a pathway which is based on the SFKs/PI3K-mediated regulation of functions underlying cancer growth through modulation of ACLY/Acetyl-CoA-dependent activities such as fatty acid/phospholipid synthesis and histone acetylation. This signaling paradigm (Figure 6) provides a tyrosine kinase dependent mechanism coupling oncogenic signal transduction to alterations in key metabolic pathways in cancer.

Declarations

Author contribution statement

J. Basappa and A. Ptasznik: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Citir: Conceived and designed the experiments; Performed the experiments.

Q. Zhang, H. Wang, X. Liu, O. Melnikov and H. Yahya: Performed the experiments.

F. Stein: Analyzed and interpreted the data.

R. Muller and C. Schultz: Contributed reagents, materials, analysis tools or data.

A. Traynor-Kaplan: Performed the experiments; Analyzed and interpreted the data.

M. Wasik: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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