Regulation and Function of the Phosphatase PHLPP2 in Leukemia

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Abstract

PHLPP2, a member of the PHLPP phosphatase family, which targets oncogenic kinases, has been actively investigated as a tumor suppressor in solid tumors. Little was known, however, regarding its regulation and function in hematological malignancies. The first half of this dissertation describes a novel miR-17~92-based mechanism for repression of PHLPP2 protein expression in late differentiation stage acute myeloid leukemia (AML) subtypes. ATRA (all-trans retinoic acid), a drug used for terminally differentiating AML subtypes, was able to induce PHLPP2 protein levels and phosphatase activity significantly by suppressing miR-17-92 expression. The effect of ATRA on miR-17~92 expression was mediated through its target, transcription factor C/EBPβ, which interacts with the intronic promoter of the *miR-17~92* gene cluster to inhibit its transactivation. The second half of this dissertation provides evidence for a novel metabolic function for PHLPP2 and describes the first identification of the energy sensing kinase, AMPK, as a unique PHLPP2 substrate. PHLPP2 could dephosphorylate phospho-AMPK (T^{172}) both intracellularly and *in vitro*. PHLPP2 silencing protected Jurkat T-ALL cells from an apoptotic response to low glucose-induced metabolic stress through activation of AMPK signaling. The pro-survival effect of PHLPP2 knockdown under metabolic stress is likely mediated through AMPK-activated fatty acid oxidation. PHLPP2 regulates AMPK phosphorylation in a variety of tumor types and is the first specific AMPK phosphatase to be identified. These studies on PHLPP2 expression and function expand current knowledge and understanding of the role of PHLPP phosphatases in cancer, and particularly in leukemia. In light of the pivotal role played by AMPK in a number of metabolic diseases, the PHLPP2/AMPK axis is also expected to provide new insights into therapies targeting these diseases.

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Chapter 1

Introduction

Protein phosphorylation: kinases and phosphatases

Protein phosphorylation is a modification of fundamental importance in biological systems and dynamic phosphorylation by kinases and dephosphorylation by phosphatases are major molecular mechanisms for regulating protein function and cell fate in response to extracellular and intracellular stimuli [Hunter, 1995]. In mammals, aberrant activation or inactivation of kinases/phosphatases is often associated with disease occurrence, and particularly with tumorigenesis and tumor progression. A large majority of anti-cancer drugs target these abnormal kinases/phosphatases to inhibit tumor cell proliferation or induce tumor cell death [Zhang et al., 2009; McConnell and Wadzinski, 2009]. A number of new kinases and phosphatases have been discovered in the past two decades; a comprehensive understanding of the expression, regulation and function of these key kinases/phosphatases will provide new insights into signaling pathways within tumor cells and on this basis, lead to novel anti-cancer therapeutics.

PHLPP phosphatase family

The PHLPP (PH domain Leucine rich repeat Protein Phosphatase) serine/threonine phosphatases belong to the metal dependent protein phosphatase (PPM) family, which requires magnesium or manganese for phosphatase activity and is insensitive to phosphatase inhibitor okadaic acid [Warfel and Newton, 2012]. The PHLPP subfamily comprises two members: PHLPP1 and PHLPP2 (PHLPPL), coded by *phlpp1* gene located on chromosome18 and *phlpp2* gene on chromosome16, respectively [Gao et al., 2005; Brognard et al., 2007]. PHLPP1 has two splicing variants: PHLPP1α and PHLPP1β. As shown in Figure 1-1, all three PHLPP proteins share a PH (Pleckstrin Homology) domain, a LRR (Leucine Rich Repeat) domain, a PP2C catalytic phosphatase domain, and a PDZ binding motif at the C terminus. Both PHLPP1β and PHLPP2 have an additional RA (Ras Association) domain. These regulatory domains influence PHLPP phosphatase activity towards their targets [Gao et al., 2005; Brognard and Newton, 2008].

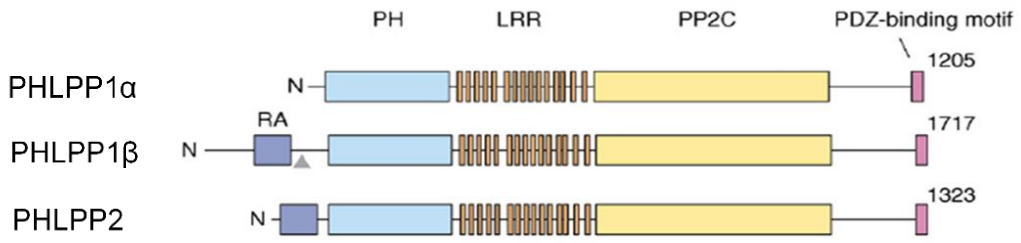

Figure 1-1. The protein structure of PHLPP phosphatase family. PHLPP phosphatase family include three members: PHLPP1α and PHLPP1β two splicing isoforms and PHLPP2. Both PHLPP1β and PHLPP2 have a Ras-Association domain (RA), a Pleckstrin Homology domain (PH), a Leucine Rich Repeats domain (LRR), a PP2C phosphatase domain (PP2C) and PDZ-ligand at the C-terminus. PHLPP1α lacks the RA domain at the N-terminus.

Since the discovery of PHLPP1 as an AKT phosphatase in 2005 and PHLPP2 in 2007 [Gao et al., 2005; Brognard et al., 2007], four direct targets of PHLPP phosphatases - AKT, PKC, P70S6K and MST1- have been identified. All four are key kinases in cell survival or apoptosis pathways [Gao et al., 2005; Gao et al., 2008; Liu et al., 2011; Qiao et al., 2010]. PHLPPs dephosphorylate AKT at Ser^{473}, a critical phosphorylation site, inactivating AKT, which results in attenuation of the amplitude of AKT-mTOR signaling pathway to inhibit cell proliferation [Gao et al., 2005]. Interestingly, PHLPP family members show specificity towards AKT isoforms - PHLPP1 is specific to AKT2 and AKT3, while PHLPP2 is specific to AKT1 and AKT3. This allows PHLPP isoforms to differentially terminate AKT signaling; for example, PHLPP1 regulates AKT2-HDM2 while PHLPP2 controls the activation of the AKT3-p27 pathway [Gao et al., 2005; Brognard et al., 2007]. Unlike PHLPP dephosphorylation of AKT, which regulates its kinase activity, the dephosphorylation of PKC at the hydrophobic motif by PHLPP phosphatases promotes its degradation, which inhibits cell survival [Gao et al., 2008]. P70S6K is also dephosphorylated by PHLPP at a hydrophobic motif (Thr389). Phosphorylated S6K binds to the translation initiation complex and induce translation process, so its dephosphorylation by PHLPP leads to decreased protein translation and reduced cell size [Liu et al., 2011]. When MST1, a pro-apoptotic kinase, is dephosphorylated by PHLPP at Thr387, it becomes activated and promotes apoptosis [Qiao et al., 2010].

Thus, PHLPP phosphatases control the amplitude of several signaling pathways to regulate cell proliferation and apoptosis by regulating the activity and/or stability of their targets. Consistent with its potential tumor suppressor function, PHLPP gene expression level is repressed in various tumors. For example, the expression of PHLPP1 or PHLPP2 is either lost or reduced in 78% and 86% of colon tumor tissues [Gao et al., 2005]. Levels of PHLPP proteins were greatly reduced in human pancreatic ductal adenocarcinoma [Lu et al., 2005]. PHLPP expression was found to be dramatically downregulated in tissue microarrays of breast cancer, pancreas cancer and GI tract tumor tissues [Qiao et al.,

2010]. Moreover overexpression of PHLPP has been shown to inhibit tumorigenesis in several xenograft mouse models [O'Neill et al., 2013].

PHLPP phosphatases have been studied at the molecular and cellular level and in *in vivo* mouse models. However, all of the studies directed at understanding its regulation and expression had been focused on solid tumors, and the understanding of its function been limited to the dephosphorylation of the four known targets and their control of cell proliferation and apoptosis [Warfel and Newton, 2012]. I have focused my dissertation research on the regulation and function of PHLPP2 in hematological malignancies (leukemias) and describe the results of my investigations in the following chapters.

Leukemia

Leukemia is a malignant progressive disease in which the bone marrow and other blood-forming organs produce increased numbers of immature or abnormal leukocytes. These suppress the production of normal blood cells, leading to anemia and other pathological conditions. Leukemia can be of the 'acute' variety where most of the abnormal cells are immature and the disease progresses rapidly, or 'chronic', where the abnormal cells mature partly but not completely [Redaelli et al., 2003]. Disease progression in chronic leukemia can take a long time before they cause problem. Leukemia can also be subdivided to two groups: myeloid leukemia and lymphocytic (or lymphoid) leukemia, based on the lineage of bone marrow cells that are affected. There are totally four major types of leukemia: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

Acute Myeloid Leukemia (AML)

AML is the most severe among the four major types of leukemia, with the lowest 5-year survival rate (26.6 percent in 2016). The two most commonly used classification systems for AML are French-American-British (FAB) system and the World Health Organization (WHO) system. The FAB system divides AML to eight subtypes, M0 to M7, according to the type of cells from which the leukemia develops, their maturity and differentiation levels [Lowenberg et al., 1999]. In general, the more mature or differentiated the AML cell, the later the subtype in the FAB system. The M3 AML subtype is also known as acute promyelocytic leukemia (APL) which typically harbors the t(15:17)(q22:q12) translocation cytogenic signature and is classified as "AML with recurrent genetic abnormalities" in the

WHO classification system. Although treatment of AML is largely limited to traditional chemotherapy and bone marrow transplant, APL patients are treated and often cured with the drug All-Trans-Retinoic-Acid (ATRA). ATRA binds to its nuclear receptor Retinoid Acid Receptor α (RARα) which acts as a transcription factor regulating the expression of the downstream target, such as C/EBPβ, and induces the 'terminal' differentiation of these leukemia cells [Duprez et al., 2003; Huber et al., 2012]. In the APL cells, ATRA also induces the degradation of the oncogenic fusion protein PML/RARα generated from the genetic translocation of t(15:17)(q22:q12), eventually leading cell death [Takitani et al., 2003; Zhang et al., 2000]. The FAB classification system is far from ideal – FAB AML subtypes have varying prognoses and responses to therapy and biomarkers for the different FAB subtypes are still not well categorized. Identifying key regulators of cell differentiation/death will help reveal new biomarkers for the different AML subtypes to facilitate diagnosis and novel anti-cancer therapeutics.

Regulation of PHLPP expression

PHLPP protein expression and activity is suppressed through various mechanisms in cancers. While most studies on PHLPP2 have focused on genetic alterations in solid tumors [O'Neill et al., 2013], PHLPP2 protein expression in small cell lung and colorectal cancers is also restricted through translational suppression [Liu et al., 2011] and post-transcriptional control by microRNAs, such as miR-205 and miR-224 [Cai et al., 2013; Liao et al., 2013]. MicroRNAs (miRNA or miR) are 20~24 nucleotide non-coding RNAs that are derived from hairpin precursors. Mature miRNAs bind to the complementary binding sites in the 3'-Un-Translated Region (3'UTR) of the target mRNAs and suppress gene expression by inhibiting mRNA translation and/or inducing mRNA degradation.

The *PHLPP2* 3'UTR harbors complementary binding sites for a subset of microRNAs belonging to the miRNA-17~92 cluster [Rao et al., 2012]. This cluster comprises six miRNAs on chromosome 13, transcribed as a single polycistronic unit [He et al., 2005; Olive et al., 2013]. Also known as oncomir-1, the cluster enhances cell proliferation or inhibits apoptosis by suppressing targets such as Bim, E2F1 and PTEN and is markedly overexpressed in human cancers [Inomata et al., 2009; Hayashita et al., 2005; Lu et al., 2005; Mogilyansky et al., 2013; Nagel et al., 2009; Tagawa and Seto, 2005; Wang et al., 2013]. Reduced levels of PHLPP2 in chemo-resistant miR-17~92 overexpressing mantle cell lymphomas had suggested that the phosphatase could be a target of oncomir-1 [Rao

et al., 2012]. Known major activators of the miR-17~92 cluster are transcription factor c-MYC and members of the E2F family, while p53 acts as a repressor under hypoxic conditions [O'Donnel et al., 2005; Sylvestre et al., 2007; Woods et al., 2007; Yan et al., 2009].

Since PHLPP2 phosphatases are potential therapeutic targets, it is important to understand mechanisms that regulate PHLPP2 protein expression. As mentioned earlier, most recent investigations of this tumor suppressor family have focused primarily on its regulation in solid tumors and their derivative cell lines, rather than in hematological malignancies. The importance of both PHLPP2 and the miR-17~92 cluster in cell survival/apoptosis signaling prompted our investigation of their coordinate regulation in leukemia. The studies described in Chapters 2 and 3 reveal direct control of PHLPP2 expression through its 3'UTR by the miR-17~92 miRNAs in acute myeloid leukemia (AML) subtypes, and a negative correlation between PHLPP2 protein and miR-17~92 cluster levels in these cancers. These studies also identify a novel pathway for up-regulation of the phosphatase through repression of the oncomir-1 cluster by C/EBPβ, an ATRA-induced transcription factor.

Role for PHLPP2 in metabolism of cancer cells

Previous studies of PHLPP function had centered on its tumor-suppressor role of either inhibiting cell proliferation, or inducing cell apoptosis in solid tumors, via its regulation of four known targets [O'Neill et al., 2013]. There has been as yet no direct association with cancer metabolism as part of PHLPP's tumor suppressor role. The Warburg effect - preferential conversion of glucose to lactate even in the presence of oxygen - also known as aerobic glycolysis, is recognized as one of the hallmarks of cancer [Vander Heiden et al., 2009]. Under these conditions, fuel sources other than glucose are relied upon to satisfy the increased metabolic needs of cancer cells, in terms of energy production, biomass generation and redox homeostasis [Vander Heiden and DeBerardinis, 2017]. Glutamine, acetate, fatty acids, branched chain amino acids are often key supplemental nutrients that allow cancer cells to reprogram their metabolism to survive and proliferate rapidly [DeBerardinis and Chandel, 2016; White 2013]. Purification and mass spectrometric analysis had identified PHLPP2 as a component of a glucose sensitive multi-protein complex in the cytosol of Jurkat T-ALL cells [Lowman et al, 2012 and data

not shown]. This suggested to us that PHLPP2 activity could potentially regulate the response to metabolic stress in the leukemia cells.

AMPK- key energy sensor

Preliminary targeted metabolomics using ^{13}C labelled glucose, carried out to determine whether PHLPP2 affected how the cells utilized glucose (described in Chapter 4), did not reveal much difference in the pathways or glycolytic intermediates between control cells and cells lacking PHLPP2. However, a significant increase in the intracellular AMP/ATP and ADT/ATP ratios in the absence of PHLPP2, suggested it could be involved in the response to metabolic stress in the Jurkat cells. The AMP/ATP ratio is a prime indicator of the energy status and is sensed by AMPK. AMPK (5'-AMP activated protein kinase) is the key energy sensor that regulate cellular energy homeostasis [Carling et al., 2011]. AMPK is a heterotrimer that is composed by three subunits: AMPKα (AMPKα1 and AMPKα2) catalytic subunit and AMPKβ (AMPKβ1, AMPKβ2), AMPKγ (AMPKγ1, AMPKγ2 and AMPK γ3) regulatory subunits [Carling et al., 2012]. When the AMP/ATP or ADP/ATP ratio increases in cells that are under metabolic stress, such as nutrient/ cytokine deprivation or hypoxia, AMP competes with ATP to bind the AMPKγ subunit. The interaction with AMP causes a conformational change in the AMPKγ subunit, which exposes the phosphorylation site at Threonine 172 (Thr^{172}) on the AMPKα subunit (Thr^{183} on AMPKα1 and Thr^{172} on AMPKα2) [Hawley et al, 1996]. Phosphorylation of AMPKα at Thr^{172} activates it to phosphorylate its downstream targets, such as Acetyl-CoA Carboxylase (ACC), ULK1 and TSC2 [Mack et al., 2012; Inoki et al., 2003]. This turns on catabolic pathways such as fatty acid oxidation and autophagy, while switching off anabolic pathways such as fatty acid synthesis and protein synthesis, resulting in an increase in ATP production and a decrease in its consumption, which balances the AMP/ATP ratio and restores energy homeostasis in the cells (see model in Figure 1-2) [Kim and He, 2013].

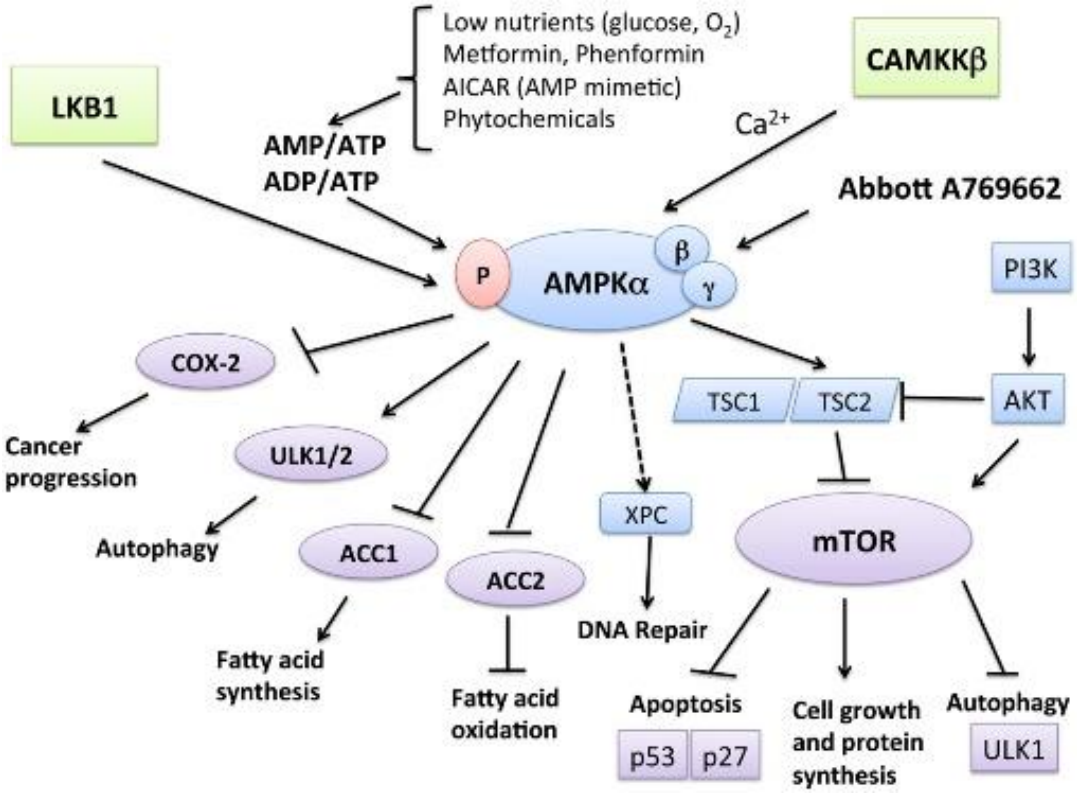

Figure 1-2. Upstream regulation of AMPK and metabolic consequences of AMPK activation.

AMPK is a 'master regulator' of cell metabolism. Its downstream target, ULK1, a key inducer of autophagy, is activated via phosphorylation by AMPK at Ser^{555} [Egan et al., 2011; Kim et al., 2011; Mack et al., 2012]. Acetyl-CoA Carboxylase (ACC), another pivotal target of AMPK and a regulator of fatty acid metabolism, is the enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA [Fullerton et al., 2013]. There are two isoforms of ACC: ACC1 and ACC2, both of which can be phosphorylated, yet inhibited by AMPK. The malonyl-CoA generated by ACC1 in the cytosol is utilized for fatty acid biosynthesis, whereas the malonyl-CoA produced by ACC2 in the outer-membrane of mitochondrial inhibits carnitine palmitoyl transferase 1 (CPT1) and, in turn, fatty acid β-oxidation [Wakil and Abu-Elheiga, 2009]. Thus, by phosphorylating and inhibiting both ACC1 and ACC2, activated AMPK represses fatty acid synthesis while promoting fatty acid oxidation [Fullerton et al., 2013]. AMPK also regulates mTOR-dependent protein synthesis. TSC2, the negative regulator of mTOR activity, is phosphorylated and activated by AMPK [Inoki et al., 2003]. The adapter protein of mTOR1, Raptor, is phosphorylated but inhibited by AMPK [Gwinn et al., 2008]. Besides autophagy,

fatty acid metabolism and protein synthesis, other metabolic pathways such as glycolysis and sterol synthesis are also regulated by AMPK through its downstream targets TXNIP, HMGCR [Wu et al., 2013; Sato et al., 1993].

AMPK activity is regulated by the level of phosphorylation of Thr^{172} on the AMPKα subunit [Willows et al., 2017]. Thus, the AMPK kinase/ AMPK phosphatase axis is important for controlling AMPK activation and function and for terminating AMPK signaling pathways. Upstream kinases of AMPK, the LKB1/STRAD/MO25 complex [Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003] and Ca^{2+}-dependent CaMKKβ kinase, have been well described [Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005]. However, a phosphatase of AMPK has yet to be identified, although previous studies had shown that PP2C family phosphatases could dephosphorylate AMPKα *in vitro* [Stephen et al., 1995; Stein et al., 2000]. We hypothesize that PHLPP2 as a member of the PP2C phosphatase family, functions as an AMPK phosphatase.

The research results described in Chapter 4 and 5 together identify PHLPP2 as a potential regulator of the cellular response to metabolic stress via phosphorylation (and activation) of AMPK. The data also suggest that PHLPP2 regulates fatty acid oxidation and, thus, the utilization of fatty acid to fuel energy production, through an AMPK-ACC route when glucose supply is limited. Thus, our studies not only reveal a new target, they also reveal a novel metabolic function, for the PHLPP2 phosphatase. This would also be the first identification of a specific phosphatase of AMPK. Overall, the research described in these chapters will provide new insights into the regulation of the key energy sensor AMPK, not only in cancer cells, but also in metabolic diseases.

Chapter 2

PHLPP2 is post-transcriptionally regulated by microRNA-17~92 cluster in AML cells

Introduction

The PH-domain Leucine Rich Repeat Protein Phosphatase2, PHLPP2 is a member of the PHLPP family serine-threonine phosphatases that are emerging as central players in cell survival and death regulation [Gao et al., 2005; Warfel and Newton, 2012]. To date four PHLPP substrates, Akt, PKC, Mst1 and S6K1, all kinases involved in cell survival or apoptosis, have been identified [Brognard and Newton, 2008; Brognard et al., 2007; Liu et al., 2011; Qian et al., 2010]. Dephosphorylation inactivates Akt, S6K1 and PKC, and subsequently cell survival signaling, whereas dephosphorylation of Mst1 activates its apoptotic function.

PHLPP protein expression and activity is suppressed through various mechanisms in cancers. While most studies on PHLPP2 have focused on genetic alterations in solid tumors [O'Neill et al., 2013], PHLPP2 protein expression in small cell lung and colorectal cancers is also restricted through translational suppression [Liu et al., 2011] and post-transcriptional control by microRNAs, miR-205 and miR-224 [Cai et al., 2013; Liao et al., 2013]. The *PHLPP2* 3'UTR harbors complementary binding sites for a subset of microRNAs belonging to the miRNA-17~92 cluster [Rao et al., 2012]. This cluster comprises six miRNAs on chromosome 13, transcribed as a single polycistronic unit [He et al., 2005; Olive et al., 2013]. Also known as oncomir-1, the cluster enhances cell proliferation or inhibits apoptosis by suppressing targets such as Bim, E2F1 and PTEN and is markedly overexpressed in human cancers [Inomata et al., 2009; Hayashita et al., 2005; Lu et al., 2005; Mogilyanshky et al., 2013; Nagel et al., 2009; Tagawa et al., 2005; Wang et al., 2013]. Reduced levels of PHLPP2 in chemo-resistant miR-17~92 overexpressing mantle cell lymphomas had suggested that the phosphatase could be a target of oncomir-1 [Rao et al., 2012].

Since PHLPP2 phosphatases are potential therapeutic targets, we were interested in understanding mechanisms that regulate PHLPP2 protein expression. Most recent investigations of this tumor suppressor family have focused primarily on its regulation in solid tumors and their derivative cell lines, rather than in hematological malignancies. The importance of both PHLPP2 and the miR-17~92 cluster in cell survival/apoptosis signaling prompted my investigation of their coordinate regulation in leukemia.

Results

PHLPP2 protein is poorly expressed or absent in specific AML subtypes

We compared PHLPP2 protein levels in a variety of human myeloid and lymphoid leukemia cell lines with those in cell lines derived from solid tumors (Figure 2-1A). Western blots revealed a range of expression, with immortalized B cells (B-NB) showing the highest levels. In three AML cell lines, THP-1, HL-60 and U937, PHLPP2 protein levels were greatly reduced or absent (Figure 2-1A). Under the FAB classification system there are 8 AML subtypes, M0 to M7, classified by myeloid lineage and degree of differentiation of the leukemia cells [Lowenberg et al., 1999]. THP-1 and U-937 are M5 subtypes, while HL-60 is classified as an M3 AML (promyelocytic leukemia) and, more recently, as an M4 AML (myelomonocytic) subtype. Figure 2-1B shows that the PHLPP2 protein was suppressed in the more differentiated (M3/M4 and M5) AML while levels in M0 and M2 lines were comparable to those in K562, a chronic myeloid leukemia (CML) and Jurkat, a T cell acute lymphocytic leukemia (T-ALL). These observations were further supported by PHLPP2 Western blots of patient cells of M0, M1 and M4 AML subtype (Figure 2-1C).

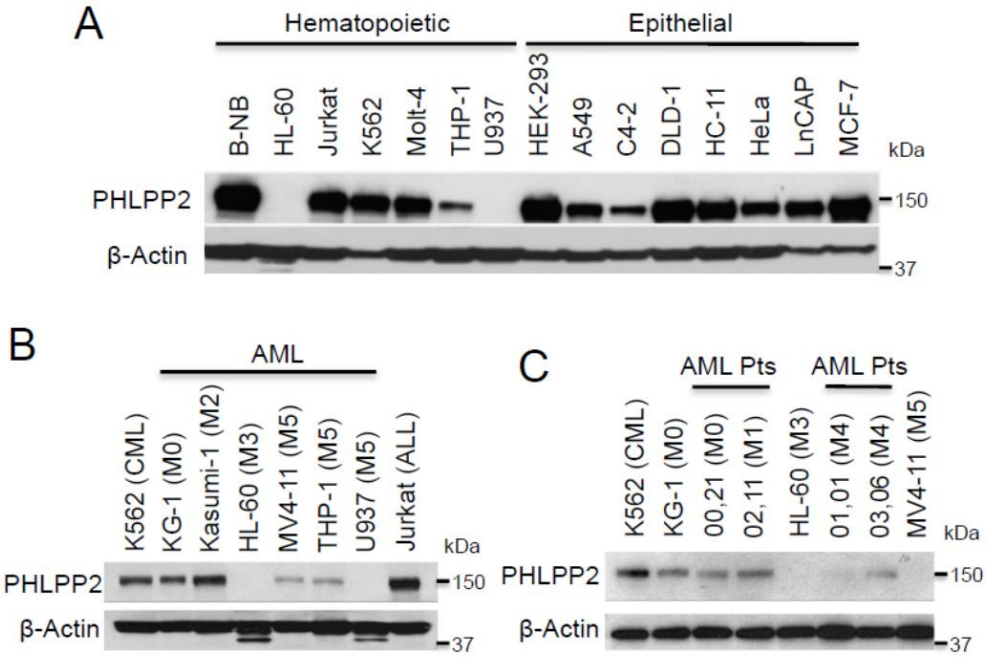

Figure 2-1. PHLPP2 protein is poorly expressed or absent in specific AML subtypes. A. Western Blot (WB) showing PHLPP2 and β-Actin expression in a variety of established

hematopoietic and epithelial cell lines (all human except for HC-11). From left to right: B-NB (Normal B cells), HL-60 (AML), Jurkat (T-ALL), K562 (CML), Molt-4 (T-ALL), THP-1 (AML), U937 (AML); HEK-293 (Human Embryonic Kidney cells), A549 (lung cancer), C4-2 (prostate cancer), DLD-1 (colorectal cancer), HC-11 (mouse mammary epithelial cells), HeLa (cervical cancer), LnCAP (prostate cancer) and MCF-7 (breast cancer). **B.** WB of established AML cell lines (sorted by FAB classification) from L to R: KG-1 (M0), Kasumi-1 (M2), HL-60 (M3), MV4-11 (M5), THP-1 (M5) and U937 (M5). **C.** WB of AML patient samples (sorted by FAB classification) from L to R: "00,21" (M0), "02,11" (M1), "01,01" (M4), "03,06" (M5).

PHLPP2 mRNA is expressed at similar levels in AML

Since *PHLPP2* is deleted or mutated at a high frequency in solid tumors [O'Neill et al., 2013] we checked the TCGA database [Cerami et al., 2012; Gao et al., 2013] to determine whether the poor expression of PHLPP2 in AML was also due to gene mutation or deletion. Our analysis revealed that, compared with breast and prostate tumors, the *PHLPP2* gene was rarely altered in AML, with no mutations and a deletion rate of < 3% (Figure 2-2A). Moreover, there was only one deep deletion and eight instances of upregulated transcripts in a total of 166 AML patient samples in the database (Figure 2-2B), suggesting that gene alteration was unlikely to account for poor expression of PHLPP2 proteins in these cancers.

We checked whether the observed low PHLPP2 protein expression correlated with *PHLPP2* mRNA levels with primer pairs that amplified different regions of the 3.9 kilobase *PHLPP2* transcript (Figure 2-2C). *PHLPP2* mRNA was detected by Q-PCR using either the 5' LRR primer or 3' PDZ-ligand sets (Figure 2-2D) in all the AML lines. mRNA levels in the M5 lines, where PHLPP2 protein levels ranged from low to absent (Figure 2-2B), were comparable to those in high PHLPP2 protein-expressing K562 or Jurkat cells (Figure 2-1B). Notably, *PHLPP2* mRNA levels in three AML patient samples tested, 00,21 (M0 with detectable PHLPP2 protein, Figure 2-1C), and 01,01 or 03,06 (M4 with lower PHLPP2 protein, Figure 2-1C), were also comparable (Figure 2-2E).

The half-life of *PHLPP2* transcripts was similar in both high and low expressing leukemia cell lines (Figure 2-3A), ruling out mRNA degradation as the reason for suppression of PHLPP2 protein in specific AML subtypes. We also treated HL-60 and U937 cells with MG132 and bortezomib (BTZ) to check for increased proteasomal degradation of PHLPP2 protein, as reported previously [Li et al., 2013]. Neither proteasome inhibitor increased PHLPP2 protein expression in the AML cell lines (Figure 2-3B). Thus, these data point to

a significant reduction in PHLPP2 protein levels in specific AML subtypes (M3-M5) via a post-transcriptional mechanism.

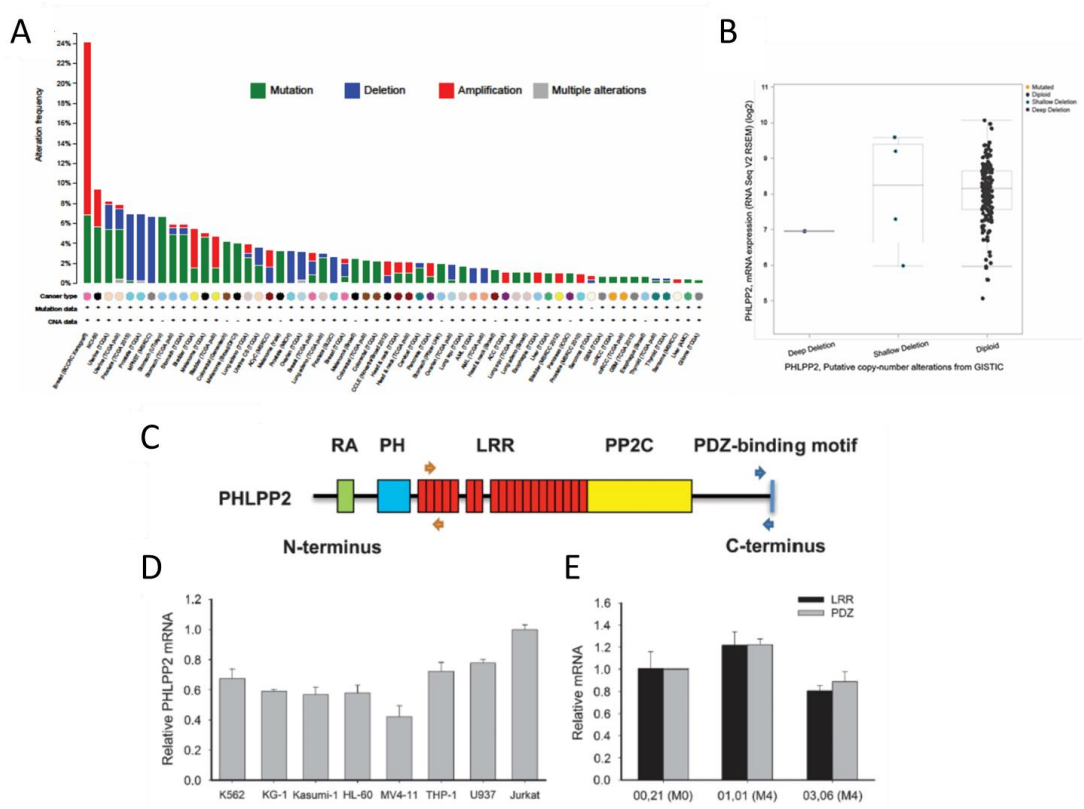

Figure 2-2. PHLPP2 mRNA is expressed at similar levels in AML. A. Schematic of TCGA data (cBioPortal analysis of 106 studies) demonstrating alteration in the PHLPP2 gene across cancers. **B.** TCGA data for PHLPP2 mRNA expression in 166 AML patient samples with 1 deep deletion and 8 instances of mRNA regulations. **C.** The PHLPP2 protein domains (not to scale) and locations of PCR primers. Orange primers amplified a region in the LRR domain; blue primers amplified a region within the PDZ-binding motif at the 3' end. **D.** PHLPP2 mRNA levels were analyzed by real-time PCR (R-PCR) using the PDZ primers as shown in D. PHLPP2 mRNA levels in each of the cell lines shown were normalized to those in Jurkat cells. **E.** PHLPP2 mRNA levels in representative AML patient samples, 00,21 (M0), 01,01 (M4) and 03,06 (M4), were detected by RT-PCR. PHLPP2 mRNA levels in 01,01 and 03,06 were normalized to those in 00,21.

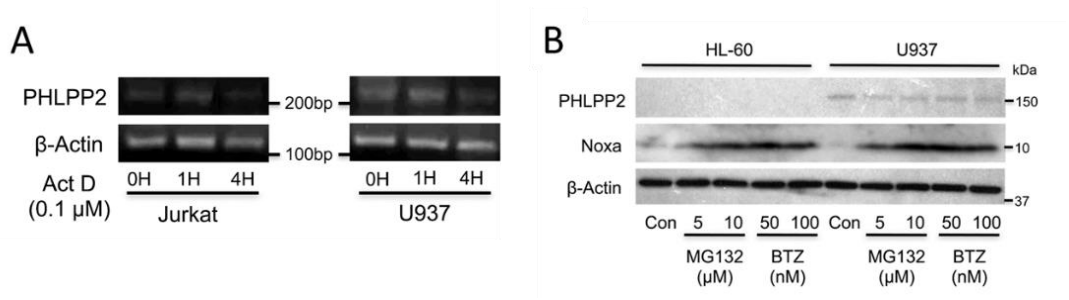

Figure 2-3. the absence of PHLPP2 protein in specific AML subtypes is not due to mRNA degradation or protein degradation. A. PHLPP2 mRNA half-life is comparable in Jurkat and U937 cells. The cells were treated with Actinomycin D (Act D) for 1 hour and 4 hours. At each time point, mRNA were extracted from the cell lysates and LRR primers were used to amplify PHLPP2 mRNA. **B.** The presence of PHLPP2 protein in specific AML subtypes is not due to protein degradation. HL-60 or U937 cells were treated with MG132 (5 or 10 μM) or BTZ (50 or 100 nM) for 6 hours. Then the cells were collected and WB was performed to detect the protein levels of PHLPP2, Noxa and β-Actin.

The low PHLPP2 protein levels are correlated with high miR-17~92 cluster levels in specific AML subtypes

We investigated the possibility that miR-17~92 was regulating translation of the PHLPP2 messenger in AML. The *PHLPP2* 3'UTR harbors complementary binding sites for a subset of microRNAs, miR-17, 19, 18a, 20a and 92a, of the miRNA-17~92 cluster (Figure 2-4).

PHLPP2 was previously shown to be responsive to miR-17~92 miRNAs in transfected HEK 293 cells [Rao et al., 2011]. Our data showed clear negative correlation between PHLPP2 protein levels and miRNA-17~92 levels in AML cell lines and patient samples. Three M5 cell lines with reduced PHLPP2 protein had higher levels of miR-17~92 transcripts than Jurkat cells (Figure 2-5A). Additionally, levels of all individual miR-17~92 miRNAs in M4 patient cells were higher than those in an M0 patient sample (Figure 2-5B).

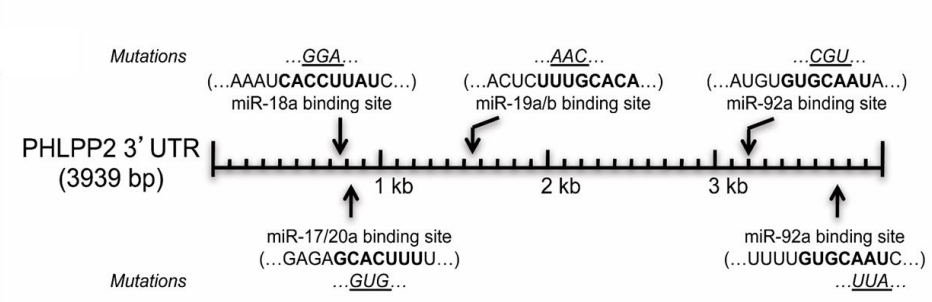

Figure 2-4. PHLPP2 3'UTR (3939 bp) possesses binding sites for miRNAs from the miR-17~92 . Each specific sequence that binds to the miRNA seed sequence within these sites is shown in bold. The mutations for each binding site (three-nucleotide mutation) are shown in underlined italics.

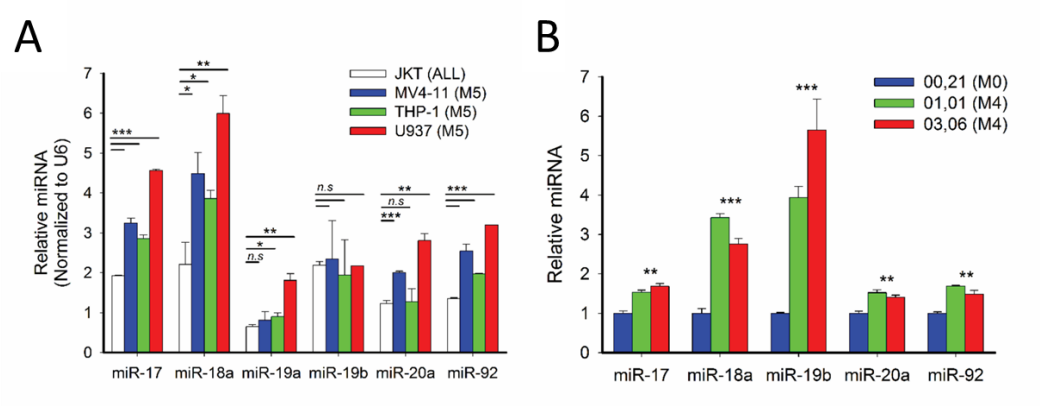

Figure 2-5. the PHLPP2 protein levels are correlated with high miR-17~92 cluster levels in specific AML subtypes. **A.** Expression of miRNAs, miR-17, -18a, -20a, -92, and -19b, from the miR-17~92 cluster was quantified in the indicated cell lines using the miR-17~92 plate assay kit (Signosis). Values were normalized to internal control, U6, in each cell line. *n.s* represents no significant difference; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. **B.** miR-17~92 cluster levels were quantified in the indicated M0 and M4 AML subtype patient samples using the Signosis plate assay.

PHLPP2 protein expression is de-repressed by an shRNA of miR-17~92

To confirm that miRNA-17~92 transcripts were key regulators of *PHLPP2* mRNA in AML, we checked the ability of a tetracycline-inducible construct, expressing three shRNAs against the primary miR-17~92 cluster transcript, to induce expression of PHLPP2 and of known miRNA-17~92 target, Bcl-2 protein Bim [Inomata et al., 2009]. Levels of both proteins were increased in cells transfected with the shRNA (Figure 2-6A), and these were further upregulated by increasing concentrations of doxycycline (Dox) in HEK 293 cells. The inhibitor construct also enhanced Bim and PHLPP2 protein expression in HL-60 and U937 cells (Figure 2-6B). Thus, inhibition of miR-17~92 in AML cells could reverse the suppression of PHLPP2 translation. Attempts to further increase expression with Dox treatment resulted in rapid apoptosis of the AML transfectants (not shown), suggesting these cells were sensitive to increased levels of miR-17~92 targets.

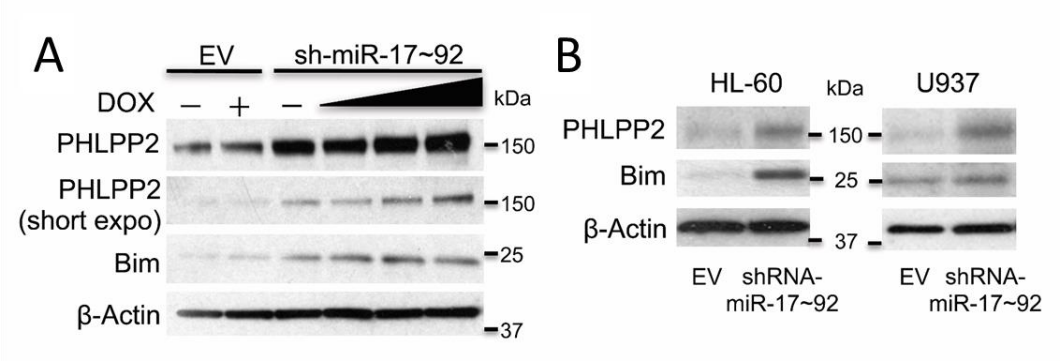

Figure 2-6. PHLPP2 protein expression is de-repressed by an shRNA of miR-17~92 in AML. **A.** WBs of lysates from HEK-293 cells transfected with 2 µg TripZ-shRNA-miRNA-17~92 (sh-miR-17~92) or empty vector (EV), treated with 1 µg/ml Doxycycline (Dox) for EV or 0.3 µg/ml, 1 µg/ml, 3 µg/ml Dox for sh-miR-17~92 24 hours post-transfection and harvested 48 hours later. **B.** WBs of lysates prepared from HL-60 or U937 cells transfected with 3 µg TripZ-empty vector (EV) or TripZ-shRNA-miRNA 17-92 (shRNA). Viable cells were purified by gradient centrifugation within 24 hours and harvested 48 hours later.

Upregulation of PHLPP2 expression by a PHLPP2-3'UTR construct requires intact miR-17~92 binding sites

To assess the ability of the PHLPP2 3'UTR to upregulate PHLPP2 protein by acting as a sponge for miR-17~92 transcripts, we cloned the 3'UTR of PHLPP2 from the genomic DNA of 01,01 (M4) AML patient cells into an expression vector. PHLPP2 protein levels increased in a concentration-dependent manner with increasing amounts of the 3'UTR construct in transiently transfected HEK 293 cells (Figure 2-7A). To check expression of the 3'UTR while differentiating between the endogenous and transfected 3'UTR transcripts, we designed "Internal" and "Overlap" primers (Figure 2-7C). Figure 2-7D shows that stably transfected HL-60 (M3/M4) and U937 (M5) cells expressed higher levels of the internal 3'UTR fragment than control transfectants (CV). Importantly, expression of this exogenous 3'UTR caused upregulation of PHLPP2 proteins, particularly in HL-60 cells (Figure 2-7B) without affecting *PHLPP2* transcript levels in stably transfected cells (Figure 2-7E). Finally, to confirm the specificity of the PHLPP2 3'UTR as a miR-17~92 sponge, we mutated all miR-17~92 miRNA binding sites in the 3'UTR construct (Figure 2-4). The mutated 3'UTR construct no longer induced PHLPP2 expression in either HEK-293 or HL-60 cells (Figure 2-7F). Interestingly, the 3'UTR transfected HL-60 cells with higher PHLPP2 expression showed a slower growth rate than controls (Figure 2-7G). These

results suggest that the miR-17~92 cluster is a major regulator of PHLPP2 protein expression in AML.

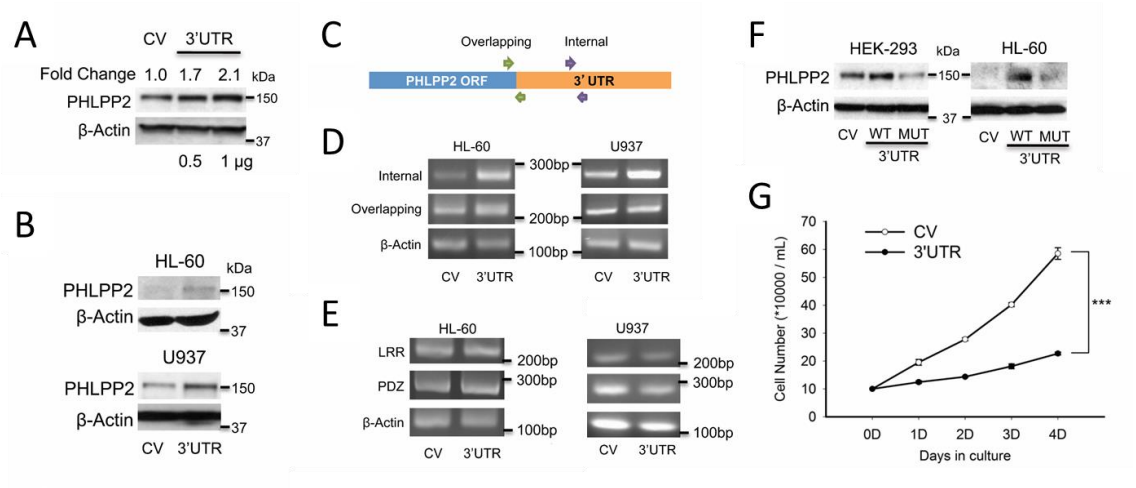

Figure 2-7. Upregulation of PHLPP2 protein expression by a PHLPP2-3'UTR construct requires intact miR-17~92 binding sites. A. Expression of PHLPP2 in HEK-293 cells after transfection with the indicated concentrations of pcDNA3.1 plasmid (CV), or PHLPP2 3'UTR constructs. The PHLPP2 bands were analyzed by densitometry and normalized to the corresponding β-Actin band. **B.** pcDNA3.1 or 3'UTR constructs were transfected into HL-60 and U937 cells. 72 hours after transfection, the cells were cultured in G418-contained medium. The stably-transfected cells were collected to check protein levels. **C.** *phlpp2* gene region and the location of PCR primers. Green primers (Overlapping) amplify a region overlapping the open reading fragment (OFR) and 3'UTR region. Purple primers (Internal) amplify a region internal and exclusive to the 3'UTR. **D.** The mRNA levels of the "Overlapping" and "Internal" fragments were detected. **E.** HL-60 or U937 stably transfected cell were assayed for PHLPP2 mRNA levels. **F.** Expression of PHLPP2 in HEK-293 cells and HL-60 cells after transfection with pcDNA3.1 plasmid (CV), wild type 3'UTR construct (WT) or mutant 3'UTR construct (MUT). **G.** 3'UTR transfected HL-60 cells showed reduced rate of growth. $1x10^5$ cells were put into fresh medium and the cells were counted every 24 hours for 4 days.

Conclusion

As a tumor suppressor PHLPP is deleted in many cancers, while others show reduced mRNA and protein levels. Studies on PHLPP family proteins have focused on solid tumors [O'Neill et al., 2012]. We show, for the first time, that PHLPP2 is suppressed in more differentiated AML subtypes, M3 - M5, identified based on the FAB (French American

British) classification. The differential expression of PHLPP2 in specific AML subtypes suggests it has potential as a biomarker. In both AML cell lines and patient samples, PHLPP2 protein levels are negatively correlated with miR-17~92 cluster levels. In addition, when miR-17~92 cluster is inhibited PHLPP2 expression is de-repressed. In conclusion, this study provides direct physiological evidence that PHLPP2 protein expression is suppressed post-transcriptionally by miR-17~92 cluster in AML.

Chapter 3

MiR-17~92 cluster and PHLPP2 expression are regulated by C/EBPβ during ATRA induced differentiation in AML

Introduction

In the previous chapter, we showed PHLPP2 protein expression was reduced in specific AML subtypes (M3-M5). We also showed that PHLPP2 expression was controlled by miR-17~92 cluster in these AML cells. The cluster, known as oncomir-1, enhances cell proliferation or inhibits apoptosis by suppressing targets such as Bim, E2F1 and PTEN and is markedly overexpressed in human cancers [Mogilyansky and Rigoutsos, 2013]. The important role of miR-17~92 cluster in tumorigenesis prompted us to investigate its regulation in the AML cells. Transcription factor c-MYC and members of the E2F family are the major known activators of the miR-17~92 cluster, while p53 acts as a repressor under hypoxic conditions [O'Donnell et al., 2005; Sylyestre et al., 2007; Woods et al., 2007; Yan et al., 2009]. A TFSEARCH scan of the region 3000 nucleotides upstream of the *miR*-17~92 cluster identified two potential binding sites in the transcription factor C/EBPβ. Notably C/EBPβ is a downstream target of ATRA, a clinical drug used to treat APL (M3 AML subtype) patients. In the studies described below we investigate the regulation of miR-17~92 cluster and subsequent PHLPP2 expression by the transcription factors mentioned above and by the drug ATRA.

Results

Expression of miRNA-17~92 cluster in AML is not controlled by transcription factors c-MYC or E2F

The miR-17~92 cluster was reported to be directly activated by the c-MYC oncogene or E2F transcription factors in a cell type specific manner [Olive et al., 2013; O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007]. We checked whether either of these transcription factors were controlling PHLPP2 expression through miR-17~92 in AML cells. First, silencing c-myc in HL-60 cells (Figure 3-1A) showed no significant effect on the miR-17~92 primary transcript (Figure 3-1B), individual mature miRNAs belonging to miR-17~92 cluster (Figure 3-1C) or on PHLPP2 protein levels (Figure 3-1D). The reduction in Bim protein expression following loss of c-myc, is likely due to the direct regulation of Bim by c-myc rather than miR-17~92 [Campone et al., 2011]. Similarly, c-myc silencing affected neither miR-17~92 transcript nor PHLPP2 protein levels in U937 AML and K562 CML cells (data not shown). We also used siRNA to silence E2F1 and E2F3 expression, individually or together, in HL-60 cells (Figure 3-1E). Again, miR-17~92 primary transcript (Figure 3-

1F) and mature miRNA levels (not shown) were unaffected, indicating that E2F did not control this miRNA cluster in AML. PHLPP2 protein expression also remained unchanged (Figure 3-1E). We conclude that regulation of both the miR-17~92 cluster and subsequent PHLPP2 expression were independent of c-myc and E2F transcriptional factors in AML and, possibly, in other types of myeloid leukemia.

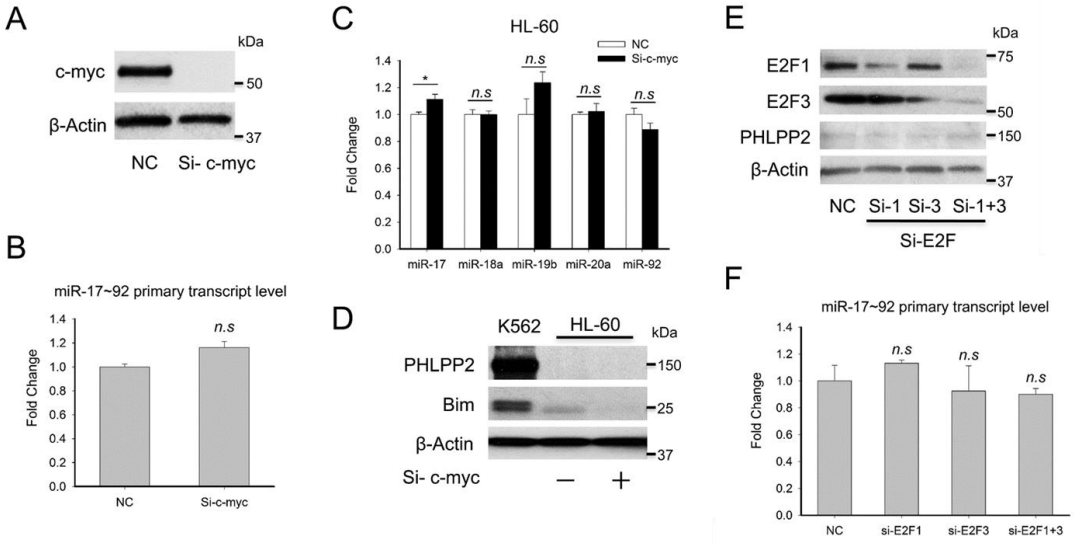

Figure 3-1. Expression of miRNA-17~92 cluster in AML is not controlled by transcription factors c-MYC or E2F. A. WBs showing c-myc levels in HL-60 cells 72 hours after transfection with c-mycsiRNAor control siRNA (NC). **B.** RT-PCR quantification of miRNA-17~92 primary transcript levels in HL-60 cells. **C.** RT-PCR quantification of individual mature miRNAs from the miRNA-17~92 cluster in the transfected HL-60 cells shown in A. miRNA levels were first normalized to the internal control U6 and then individual miRNA levels in si-c-myc cells were further normalized against the negative controls. **D.** PHLPP2 and Bim protein levels in WBs of HL-60 cells 72 hours after transfection with c-myc or control siRNAs. PHLPP2 protein expression in K562 cells was used as a positive control for the PHLPP2 band on the blot. **E.** WBs showing E2F protein levels in HL-60 cells silenced for E2F1 or E2F3 expression, or both, along with negative control, NC. **F.** Fold change in miRNA-17~92 primary transcript levels in HL-60 cells silenced for E2F1 or E2F3 expression individually or together, 72 hours after transfection of the siRNA.

ATRA induces PHLPP2 expression by suppressing the miR-17~92 cluster during granulocytic differentiation

All-trans-retinoic-acid (ATRA) reduces the proliferation rate of HL-60 cells and activates their terminal differentiation into granulocytes [Breitman et al., 1980; Cull et al., 2014;

Degos et al.,1995; Gallagher et al., 1979]. Based on our observation that PHLPP2 3'UTR-transfected HL-60 cells had a reduced growth rate compared to control vector-transfected cells (Figure 2-7G), we asked whether PHLPP2 levels changed during ATRA-induced differentiation and whether this effect was mediated through the miR-17~92 cluster. Figure 3-2A shows that PHLPP2 protein was dramatically induced upon treatment of HL-60 cells with ATRA for 3 to 5 days. Cell surface differentiation marker CD11b correspondingly increased in a concentration- and time-dependent manner (Figure 3-2B). We also detected a reduction in Thr389 phosphorylation of pS6K, a major PHLPP2 substrate, in cells exposed to ATRA for 3 days (Figure 3-2C). However, phosphorylation of Akt Ser 473, a second PHLPP2 target, increased (data not shown). These results were consistent with published studies showing Akt was phosphorylated and activated following ATRA treatment [Billottet et al., 2009] and suggested that pS6K was the primary target of PHLPP2 during ATRA-induced differentiation. Additionally, ATRA had no effect on PHLPP2 mRNA levels (Figure 3-2D) while miR-17~92 cluster family members decreased by almost 50% in HL-60 cells in 3 days (Figure 3-2E). Consistent with the reduction in individual miRNA levels, the primary cluster transcript also decreased with ATRA treatment (data not shown), confirming that ATRA-mediated differentiation inhibits transactivation, rather than processing, of the cluster. Coordinate changes in PHLPP2 and miR-17~92 expression levels during ATRA-induced differentiation offered further evidence of posttranscriptional inhibition of PHLPP2 by the miR-17~92 cluster and supported the hypothesis that this regulation occurred through an ATRA-mediated signaling pathway.

The reduced inhibition of PHLPP2 expression by the miR-17~92 cluster during ATRA treatment, suggests this could be a primary mode of action of the drug. Moreover, the increased rate of differentiation of HL-60 cells stably over-expressing the PHLPP2-3'UTR in response to ATRA (Figure 3-3A and 3-3B), support this possibility. Thus, ATRA exerts its function, at least in part, by reversing the oncomir-1-mediated post-transcriptional inhibition of PHLPP2.

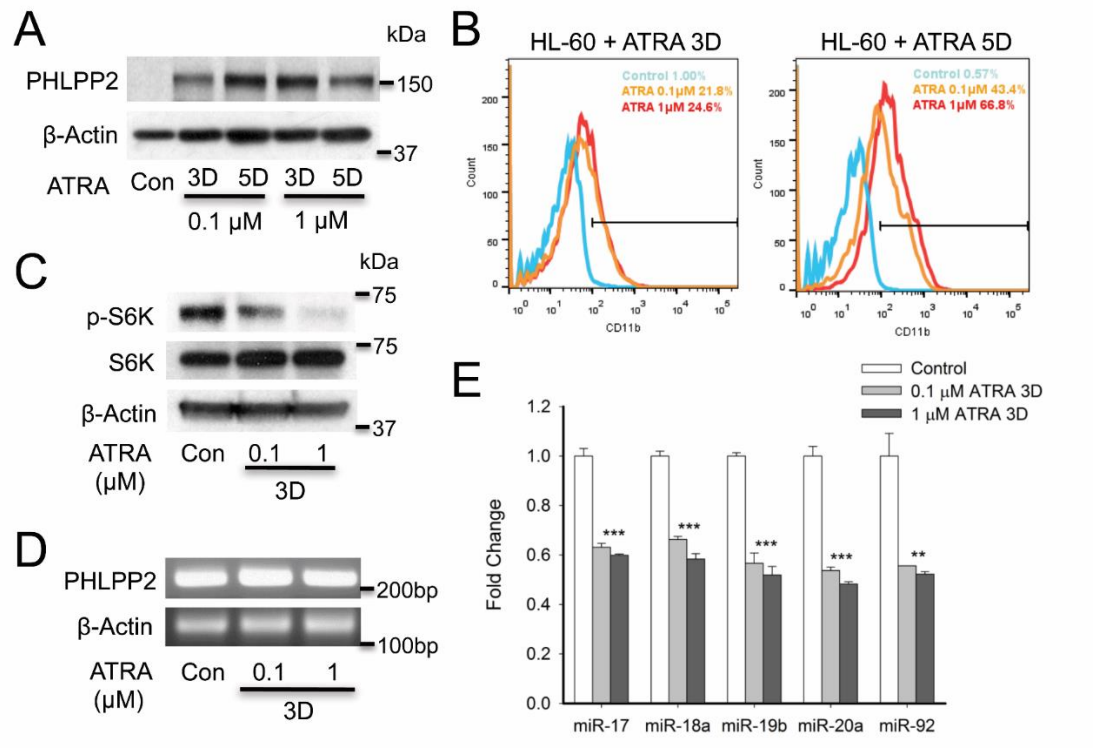

Figure 3-2. ATRA induced granulocytic differentiation in promyelocytic AML is accompanied by PHLPP2 induction and suppression of the miR-17~92 cluster. A. WBs showing PHLPP2 protein levels in HL-60 cells induced to differentiate in the presence of ATRA. **B.** Differentiation of HL-60 cells treated with 0.1μM or 1 μM ATRA for 3 days or 5 days was monitored through flow cytometric detection of CD11b. **C.** Thr^{389}S6K phosphorylation levels were reduced in HL-60 cells treated with 0.1μM or 1μM ATRA for 3 days. **D.** PCR analysis of PHLPP2 mRNA in ATRA-treated HL-60 cells. **E.** miRNA-17~92 cluster levels in response to ATRA treatment. Individual miRNAs from ATRA treated cells and controls were quantified through RT-PCR and normalized to the internal control U6. Shown in the graph is the fold change in individual miRNA levels in ATRA-treated cells compared to untreated controls.

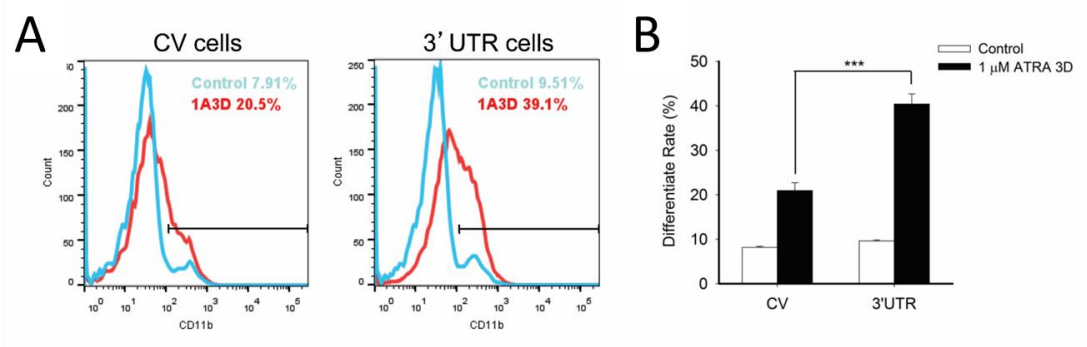

Figure 3-3. ATRA-induced differentiation is promoted when miR-17~92 cluster is inhibited in 3'UTR cells. A. Rate of differentiation of pcDNA3.1 (CV) and 3'UTR transfected HL-60 cells treated with 1 μM ATRA for 3 days was monitored by detection of the surface marker CD11b. Shown are representative data from one of three independent experiments. The results from the three experiments are quantified in **B**.

PHLPP2 protein levels are correlated with miR-17~92 cluster levels in APL patient samples

We also compared PHLPP2 protein expression and miR-17~92 cluster levels in cells from APL (M3) patients with those of a less mature (M0) AML patient sample. Both APL patient samples tested expressed substantially reduced levels of PHLPP2 protein (Figure 3-4A). Furthermore, all four APL samples tested had higher expression of individual miRNAs from the miR-17~92 cluster than M0 AML cells (Figure 3-4B). The consistently negative correlation between PHLPP2 protein levels and miR-17~92 transcripts in APL patient samples underscores the physiological relevance of this regulatory pathway.

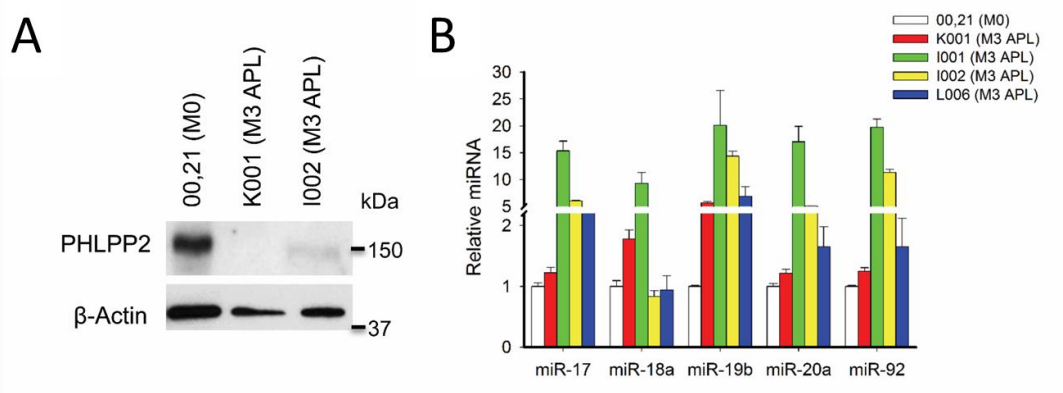

Figure 3-4. PHLPP2 protein levels are correlated with miR-17~92 cluster levels in APL patient samples. A. WBs showing PHLPP2 protein levels in two APL patient samples and an M0 AML patient sample. **B.** Individual miRNA-17~92 cluster transcript levels in four patient samples were quantified using RT-PCR and plotted as a fold change compared with levels in the M0 patient cells.

ATRA induced down-regulation of the miR-17~92 cluster is mediated through transcription factor C/EBPβ

ATRA activates the expression of a number of target genes involved in myeloid cell differentiation through interaction with nuclear receptor and transcription factor RARα [Takitani et al., 2003; Zhang et al., 2000]. A scan of the region upstream of the *miR*-17~92

cluster revealed putative binding sites for CCAATT enhancer binding protein and transcription factor family member, C/EBPβ, a major ATRA target. The activation of C/EBPβ is an early event in the ATRA-induced differentiation response [Duprez et al., 2003; Huber et al., 2012]. We first determined the concentration- and time-dependent induction of C/EBPβ in the HL-60 cell line (Figure 3-5A). Transfection of C/EBPβ siRNA abrogated the drug's ability to upregulate C/EBPβ protein levels (Figure 3-5B) and slowed the rate of ATRA-induced differentiation in the HL-60 cells (Figure 3-5C). Both miR-17~92 primary transcript and mature miRNA levels were reduced in control siRNA expressing HL-60 cells exposed to ATRA, but not in C/EBPβ-silenced cells (Figure 3-5D and 3-5E), suggesting that the ATRA-induced down-regulation of the cluster was dependent on C/EBPβ. The decreased miR-17~92 transcripts in the untreated C/EBPβ knockdown cells remained unresponsive to ATRA and suggest an additional, ATRA-independent mechanism for control of the microRNA cluster by C/EBPβ. PHLPP2 protein was also suppressed in the C/EBPβ-silenced HL-60 cells following ATRA treatment (Figure 3-5F).

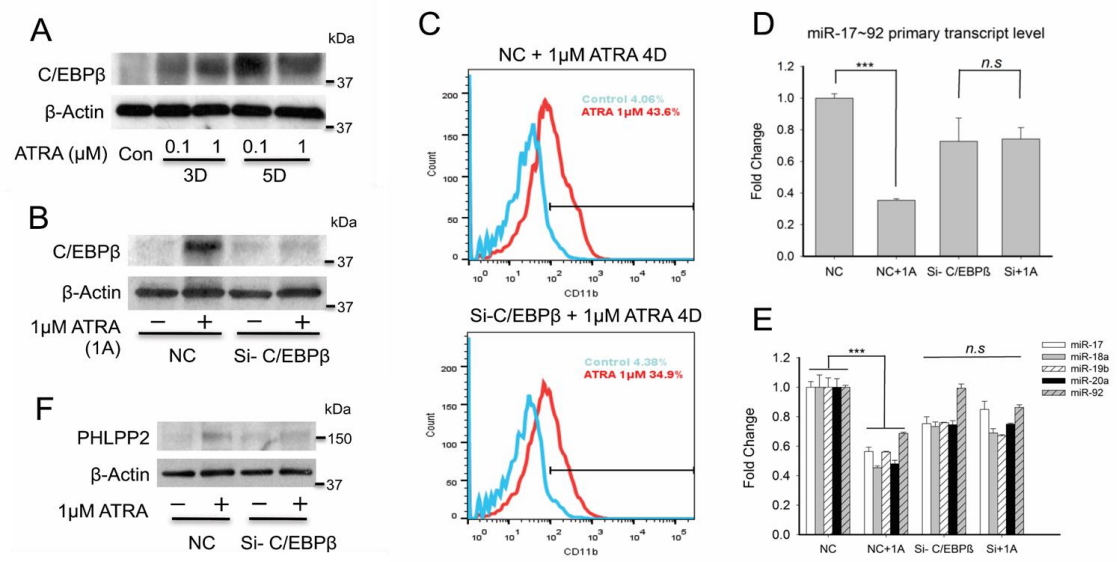

Figure 3-5. ATRA induced down-regulation of the miR-17~92 cluster is mediated through transcription factor C/EBPβ. A. WBs showing C/EBPβ protein levels in HL-60 cells treated with two concentrations of ATRA for 3 and 5 days. **B.** HL-60 cells were transfected with C/EBPβ and control siRNA. 24 hours later cells were treated with 1μM ATRA for 3 days. Induction of C/EBPβ and efficiency of silencing was confirmed by WB. **C.** C/EBPβ silenced cells and controls were treated with 1μM ATRA for 4 days and appearance of the differentiation marker CD11b was detected by flow cytometry. Shown are representative data from three independent experiments.

D. siRNA transfected cells were treated with 1μM ATRA for 3 days and miR-17~92 primary transcript levels were quantified by RT-PCR. **E.** RT-PCR quantification of individual miRNAs in siRNA transfected HL-60 cells with or without ATRA treatment. **F.** WBs showing PHLPP2 levels in control (NC) and C/EBPβ silenced HL-60 cells following exposure to ATRA for 3 days.

C/EBPβ binds to the intronic promoter of the miR-17~92 cluster

The miR-17~92 cluster is an intronic cluster, reported to harbor intronic miRNA promoters [Monteys et al., 2010]. A TFSEARCH scan of the region 3000 nucleotides upstream of the *miR*-17~92 cluster identified two response elements matching the C/EBPβ DNA binding consensus sequence *R*TTGCG*Y*AA*Y* [Osada et al., 1996], roughly 600 bp (-609 to -600) and 1050 bp(-1051 to -1042) upstream of *mir*-17~92, respectively (Figure 3-6A). To determine whether C/EBPβ bound directly to any of these putative response elements, we performed a chromatin immunoprecipitation (ChIP) assay. We designed two PCR amplicons for the putative binding sites and a third for a region without C/EBPβ binding sites. C/EBPβ was enriched at the distal but not at the proximal promoter region, following ATRA treatment (Figure 3-6B). Furthermore, RNA polymerase II was enriched at this region in untreated cells, but not detected following ATRA treatment. The replacement of RNA pol II at this site by ATRA-induced C/EBPβ supports our hypothesis that the transcription factor inhibits *miR*-17~92 transcription. We also detected some, albeit weaker, C/EBPβ interaction with the second predicted (-600bp) element upon ATRA treatment. Although we cannot rule out the presence of C/EBPβ at the proximal promoter we may be detecting distal binding with proximal probes, given the short distance (~450 bp) between the two regions. Regardless, our data strongly support a repressive function for C/EBPβ at the distal promoter. Figure 3-6C shows quantitatively that the interaction between RNA pol II with Amplicon 1 was significantly reduced and interaction of C/EBPβ with this site increased following ATRA treatment. These data demonstrate that C/EBPβ interacts directly with the *mirR*-17~92 intronic promoter and, more importantly, that this interaction represses the transcription of this oncomir. Together with our previous observations (Figure 3-1), the ChIP assay provides strong evidence supporting our hypothesis that transcription factor C/EBPβ, rather than c-MYC or E2F, regulates miR-17~92 cluster expression in AML.

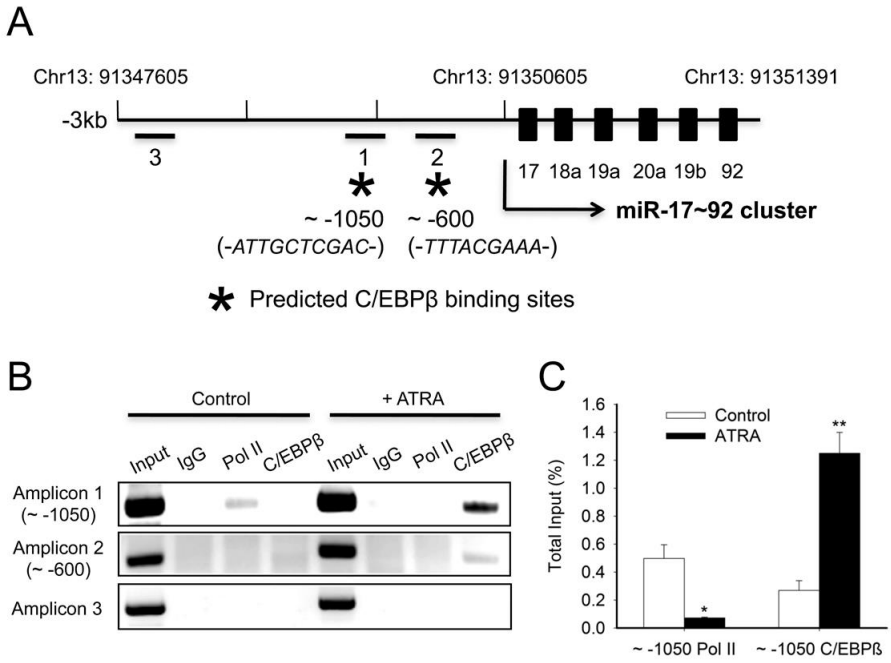

Figure 3-6. C/EBPβ binds to the intronic promoter of the miR-17~92 cluster. A. Schematic representation of the upstream region of *miR*-17~92 cluster (not to scale). Putative C/EBPβ binding sites are shown as asterisks and consensus sequences are italicized in parentheses. PCR amplicons 1, 2, and 3 are shown as numbered lines. **B.** HL-60 cells were treated with 1μM ATRA for 3 days and subjected to a ChIP assay to detect the interaction between RNA polymerase II (Pol II) or C/EBPβ with the amplicons1, 2 and 3 shown in A. **C.** RT-PCR was performed to measure the levels of the binding of Pol II or C/EBPβ with amplicon 1 (containing the 1050 binding site) in HL-60 cells treated with 1μM ATRA for 3 days.

Conclusion

In this chapter, we demonstrate that miR-17~92 cluster levels decrease leading to subsequent PHLPP2 upregulation during ATRA induced granulocytic differentiation in AML cells. Mechanistically, our study showed that the regulation of miR-17~92 and PHLPP2 by ATRA was mediated through a transcription factor C/EBPβ which competed with RNA polymerase II to interact with the intronic promoter of the *mir-17~92* cluster, thus inhibited the transactivation of this microRNA cluster and led to the upregulation of PHLPP2. The model in Figure 3-7 illustrates ATRA mediated regulation of miR-17~92 and PHLPP2 expression in AML.

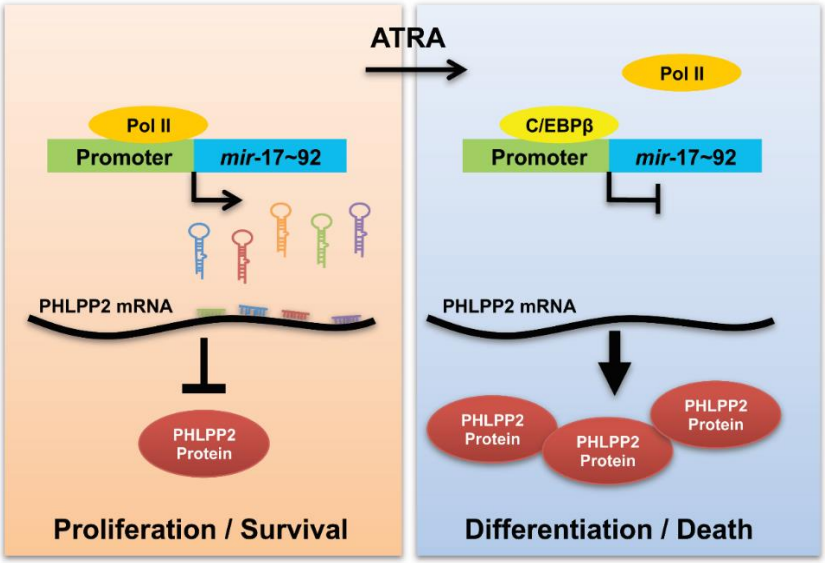

Figure 3-7. A model for ATRA-mediated regulation of tumor suppressor phosphatase, PHLPP2, in AML cells. In unperturbed AML cells RNA Polymerase II binds to and activates the miR-17~92 cluster. These miRNAs (colored hairpins) bind to the 3'UTR of PHLPP2 and inhibit its translation. ATRA treatment leads to the upregulation of C/EBPβ, which binds the *miR-17~92* intronic upstream region distally, preventing RNA Polymerase II from binding and inhibiting transcription of the cluster. This releases the block on PHLPP2 translation allowing the phosphatase to accumulate in the cells and repress growth and proliferation through inactivation of key oncogenic substrates, such as S6K.

Chapter 4

PHLPP2 regulates cellular response to metabolic stress through a novel target - AMPK

Introduction

In this chapter, we continue with our investigation of PHLPP2 regulation in hematological malignancies, such as leukemia. Our previous study focused on its post-transcriptional repression by the miR-17~92 cluster in AML cells, where PHLPP2 protein expression is significantly reduced, and also identified a novel pathway for up-regulation of the phosphatase through repression of the oncomir-1 cluster by C/EBPβ, an ATRA-induced transcription factor. Here we focus on the role and regulation of PHLPP2 in leukemias, such as acute lymphocytic leukemia, which tolerate relatively high levels of the protein. The discovery, through an independent study in the Kelekar lab, that PHLPP2 was a component of a glucose sensitive multi-protein particle in the cytosol of Jurkat T-ALL cells, prompted us to ask whether PHLPP2 played a role in glucose metabolism. Our investigations have led to the identification of the energy sensing kinase AMPK as a new (fifth) target of PHLPP2, and to the discovery of a novel metabolic role for the protein in the cellular response to metabolic stress. We describe these results in the following sections.

Results

Inhibition of PHLPP2 protects against cell death from metabolic stress by activating AMPK

The presence of PHLPP2 in a glucose sensitive multi-protein complex (data not shown) in Jurkat T-ALL cells, suggested to us that PHLPP2 may have a role to play in glucose metabolism. Targeted metabolomic analyses using a [13C]-labeled isotopomer of glucose as a tracer showed no difference in glycolytic intermediates between control cells and PHLPP2 knockdown cells (data not shown). Unexpectedly, however, we observed a dramatic increase in the AMP/ATP ratio and ADP/ATP ratio when PHLPP2 was silenced (Figure 4-1A). We then checked the oxygen consumption rate (OCR), which is associated with ATP production, in Jurkat cells either silenced for PHLPP2 or over-expressing the protein, using a Seahorse Extracellular Flux Analyzer. Basal OCR levels dropped in the absence of PHLPP2 and increased following over-expression of PHLPP2 basal OCR (Figure 4-1B-D), compared to controls, supporting the possibility that PHLPP2 was affecting the AMP/ATP ratio by regulating ATP production.

AMP-activated kinase (AMPK) is the key energy sensor regulating intracellular energy homeostasis [Carling et al., 2011]. Cells undergoing metabolic stress increase their AMP/ATP ratio and activate the AMPK protein though phosphorylation of subunit AMPKα at Thr^{172} [Hawley et al, 1996]. AMPK was previously shown to be de-phosphorylated by PP2C phosphatases, although a specific AMPK phosphatase is yet to be discovered [Stephen et al., 1995; Stein et al., 2000]. Since PHLPP proteins are PP2C-type phosphatases [Warfel and Newton, 2012], we tested the possibility that AMPK was a PHLPP2 substrate.

To expose Jurkat cells to intracellular energy stress, we cultured the cells in medium with 5mM glucose, in addition to the 'complete' 10mM glucose medium. Lower PHLPP2 protein levels allowed the cells to survive better under metabolic stress (Figure 4-2A), but the phosphorylation levels of known downstream targets of PHLPP2, phospho-Akt and phospho-S6K, were unaffected under these conditions (Figure 4-2B). However, as shown in Figure 4-2C, T^{172} phosphorylation of AMPKα significantly increased when PHLPP2 was silenced. The phosphorylation of Acetyl-CoA Carboxylase (ACC) and ULK1, direct downstream targets of AMPK [Mack et al., 2012; Inoki et al., 2003], was also enhanced in the absence of PHLPP2, particularly under low glucose conditions. Thus, the consequence of PHLPP2 silencing in the T-ALL cells was increased phosphorylation, not of known oncogenic kinases Akt and S6K, rather of a novel kinase, AMPK, leading to activation of its downstream signaling pathways.

To determine whether the increased viability of PHLPP2-silenced cells under metabolic stress was due to regulation of AMPK activity and signaling, we repeated the experiments in presence of the AMPK inhibitor, Compound C [Zhou et al., 2001; Handa et al., 2011]. As shown in Figure 4-2D, Compound C abrogated the protective effect of PHLPP2 knockdown in low glucose conditions, suggesting that PHLPP2 was, indeed, regulating cell viability through AMPK. We also exposed Jurkat cells to a small molecule PHLPP2 inhibitor [Sierecki et al., 2010] and, again, detected no change in phospho-AKT levels, but observed, instead, upregulation of phospho-AMPKα and its downstream target, phospho-ACC (Figure 4-2E). Thus, both genetic silencing as well as pharmacologic inhibition of PHLPP2, could increase AMPK phosphorylation.

PHLPP2 is a member of the PHLPP family of phosphatases and PHLPP1 and 2 are known to have common targets [O'Neill et al., 2013]. To check whether PHLPP1 affected viability and AMPK phosphorylation in a manner similar to PHLPP2 we silenced PHLPP1 and PHLPP2, either singly or together, in Jurkat cells (Figure 4-3A). Figure 4-3B shows that only PHLPP2 silencing had the effect of increasing cell viability under metabolic stress. Additionally, PHLPP1 knockdown had no effect on the phosphorylation of AMPKα (Figure 4-3C). We conclude from these data that regulation of AMPKα phosphorylation and cell viability under metabolic stress is unique to PHLPP2.

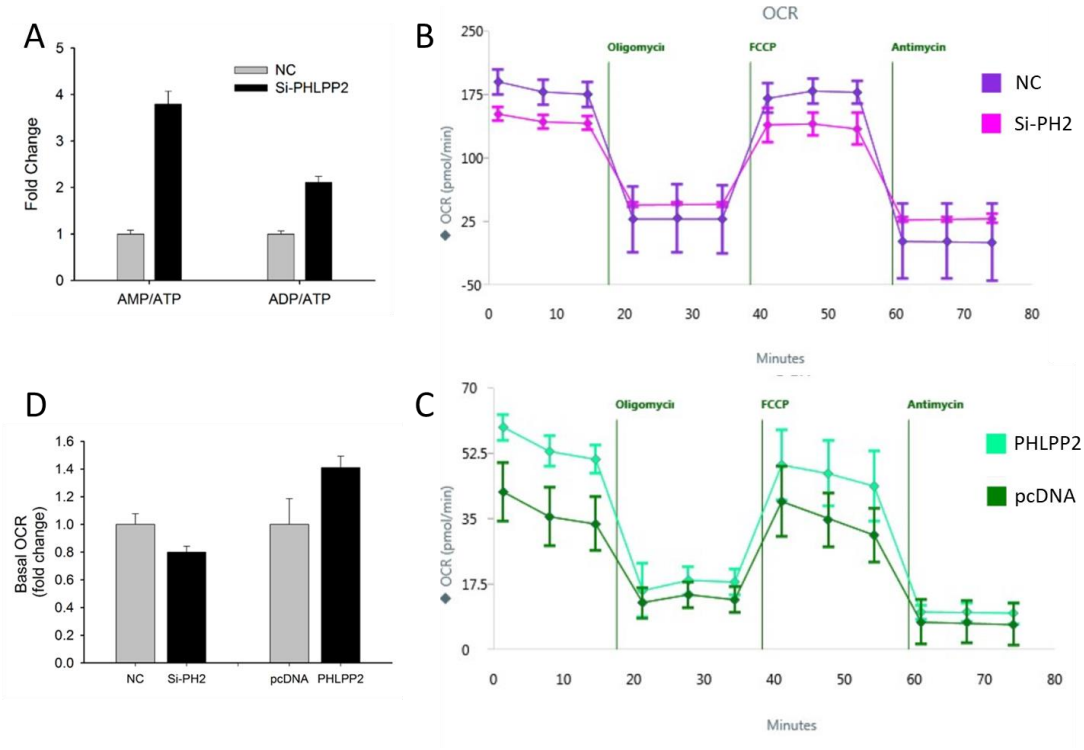

Figure 4-1. PHLPP2 regulates AMP/ATP ratio and oxidative phosphorylation. A. AMP/ATP or ADP/ATP ratios increase in the absence of PHLPP2. The total ATP, ADP and AMP were determined by mass spectrometry in Jurkat T cells transfected with control siRNA or PHLPP2 siRNA. AMP/ATP or ADP/ATP ratios in PHLPP2 knock down cells were calculated and normalized to those in control cells. **B.** Jurkat cells were transfected with control siRNA (NC) or PHLPP2 siRNA and oxygen consumption rate (OCR) was measured 72 hours after transfection using a Seahorse extracellular flux analyzer. **C.** OCR was measured in pcDNA control cells and PHLPP2 over-expressing cells. **D.** Bar graph showing basal OCR from the Seahorse profiles in C and D. The basal OCR in PHLPP2 knock down cells was normalized to that in control siRNA cells (NC) and

the basal OCR in PHLPP2 over-expressing cells was normalized to that in pcDNA transfected control cells.

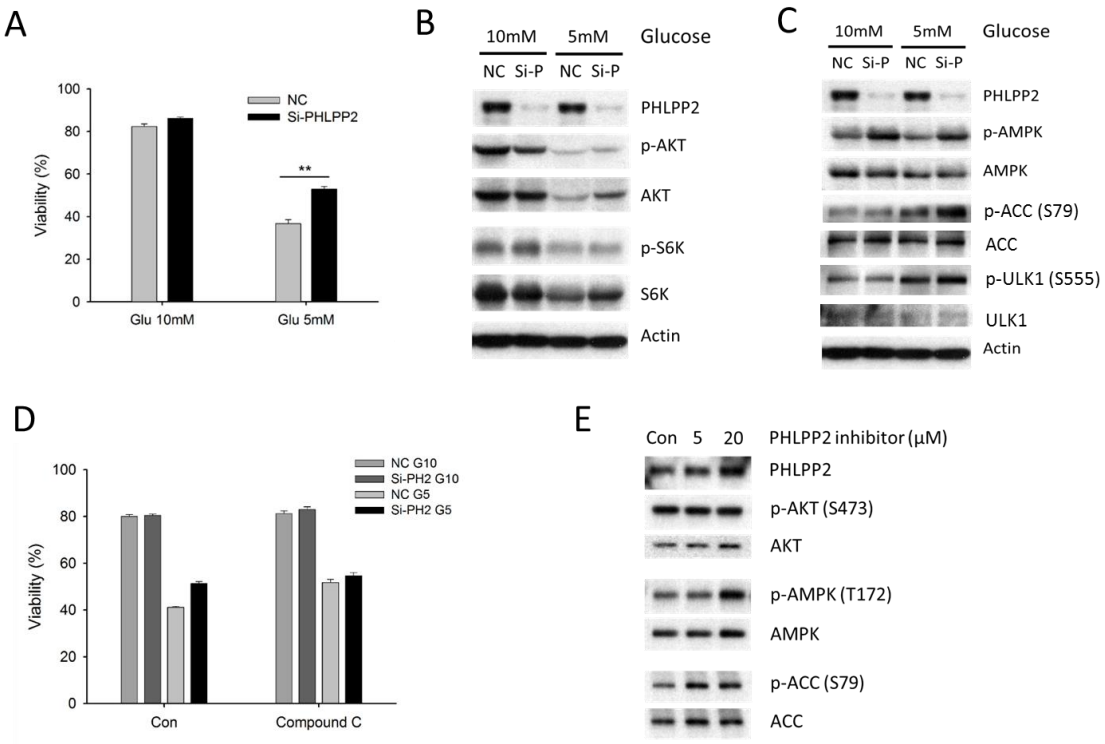

Figure 4-2. PHLPP2 downregulation protects against cell death from metabolic stress by activating AMPK. A. Jurkat cells were transfected with control siRNA (NC) or PHLPP2 siRNA (Si-PHLPP2) and, 24 hours after transfection, cultured in high glucose medium (Glu 10mM) or low glucose medium (Glu 5mM) for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI uptake. **B.** Western blots of phospho-AKT (S473), phospho-S6K (T389) and total AKT and S6K protein levels in control siRNA cells (NC) or PHLPP2 siRNA cells (Si-P) transfected Jurkat cells, 3 days after growth in medium with 10 mM glucose or 5 mM glucose phospho-protein. **C.** Western blots showing phosphorylation of AMPKα (T172), and its downstream targets, ACC and ULK1, in the experiment described in 4-2B above. **D.** The pro-survival effect of si-PHLPP2 under metabolic stress is diminished in the presence of AMPK inhibitor, Compound C. Jurkat cells were cultured for 4 days in 10 mM glucose medium or 5 mM glucose medium with or without 2 μM Compound C 24 hours after transfection with control siRNA (NC) or PHLPP2 siRNA (Si-PH2). Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. **E.** Phosphorylation of AMPKα increases in the presence of a PHLPP2 inhibitor. Jurkat cells were treated with a PHLPP2 inhibitor for 72 hours and phospho-protein and total protein levels were detected by western blot.

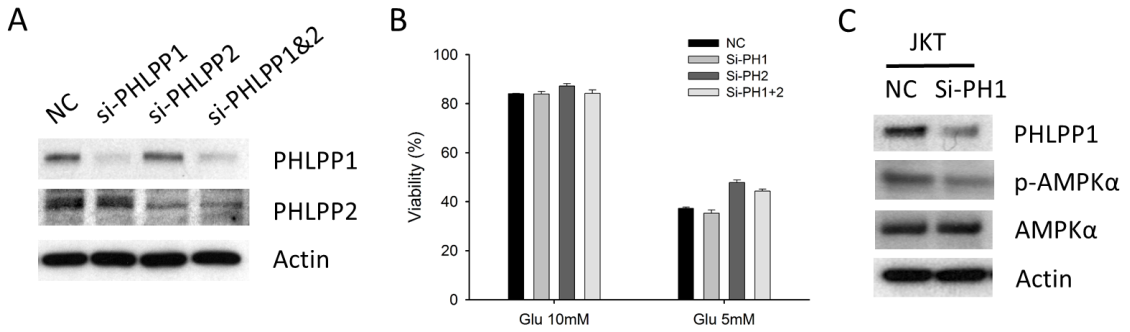

Figure 4-3. PHLPP1 does not affect AMPK-phosphorylation or the cellular response to metabolic stress in Jurkat cells. A. PHLPP1 and PHLPP2 were silenced in Jurkat cells, either singly or together using siRNA. Jurkat cells were transfected with control siRNA (NC), PHLPP1 siRNA, PHLPP2 siRNA or both. 72 hours after transfection. Cells were collected to check the knockdown efficiency. **B.** Cell viability of Jurkat cells under low glucose conditions following PHLPP silencing. Jurkat cells were transfected with control siRNA (NC), PHLPP2 siRNA (Si-PH1), PHLPP2 siRNA (Si-PH2) or both (Si-PH1+2) and 24 hours after transfection the cells were cultured in high glucose medium (Glu 10mM) or low glucose medium (Glu 5mM) for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. **C.** PHLPP1 silencing does not affect AMPKα (T172) phosphorylation level. Western blot shows pAMPKα (T172) levels PHLPP1 protein was silenced.

Absence of PHLPP2 promotes AMPK phosphorylation and prevents the inhibition of AMPK

AMPK, an energy sensing kinase, is phosphorylated and activated under nutrient stress through binding of AMP to the AMPKγ subunit [Hawley et al, 1996]. AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), as an AMP mimetic, also binds to the AMPKγ subunit to induce the phosphorylation of AMPKα and activation of AMPK [Corton et al, 1995]. Both glucose deprivation and AICAR treatment increased phosphorylation of AMPKα (T^{172}) when PHLPP2 was silenced (Figure 4-4A). AICAR also promoted survival of Jurkat cells in low glucose induced stress conditions (Figure 4-4B), indicating that activation of AMPK had a growth promoting effect in Jurkat leukemia cells, consistent with the effects observed with inhibition of PHLPP2 (Figure 4-2A). Next, we looked at the effect of PHLPP2 on AMPK phosphorylation over 24 hours of glucose deprivation. Phosphorylation of AMPK decreased within 2 hours of glucose withdrawal in both control as well as PHLPP2 silenced cells. However, AMPK phosphorylation was restored at an

accelerated pace, within 8 hours, in the absence of PHLPP2, as compared with >12 hours in control cells (Figure 4-4C).

Compound C, as mentioned above, is an AMPK inhibitor that decreases phosphorylation of AMPK [Zhou et al., 2001; Handa et al., 2011]. We showed earlier that, at a concentration of 2μM, Compound C could override the protective effects imparted by loss of PHLPP2 under metabolic stress (Figure 4-2D). We found that lower concentrations (0.5 μM and 1 μM) of Compound C were unable to inhibit AMPK phosphorylation in PHLPP2 silenced cells (Figure 4-4D). At higher concentrations (2μM) Compound C strongly inhibited phosphorylation of AMPK regardless of PHLPP2 protein levels, consistent with the result in Figure 4-2D. However, under low glucose PHLPP2 knockdown was able to protect against the death promoting effects of low concentration of the AMPK inhibitor (Figure 4-2E). Taken together, our data provide evidence that loss of PHLPP2 expression promotes AMPK phosphorylation, prevents AMPK inhibition, and rescues cells from death induced by AMPK inhibition. Thus, the PHLPP2 phosphatase regulates the phosphorylation and activation of AMPK.

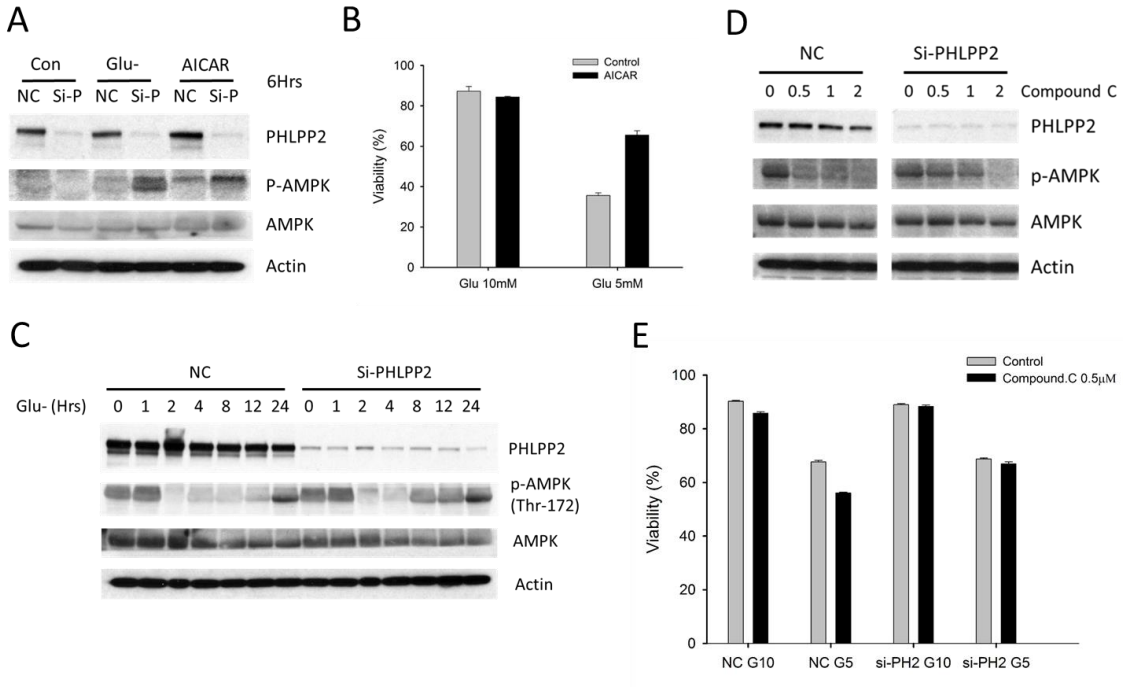

Figure 4-4. Absence of PHLPP2 increases AMPK phosphorylation and prevents the inhibition of AMPK. A. Both glucose deprivation and AICAR treatment promote phosphorylation

of AMPKα in the absence of PHLPP2. 48 hours after transfection with control siRNA (NC) or PHLPP2 siRNA (Si-P), Jurkat cells were cultured in medium without glucose or treated with 0.5 mM AICAR for 6 hours. Protein level changes were analyzed by WB. **B.** AICAR treatment prevents cell death under metabolic stress. Jurkat cells were culture in Glucose 10 mM medium or Glucose 5 mM and at the same time the cells were treated with or without 0.2 mM AICAR for 3 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. **C.** PHLPP2 silencing accelerates AMPKα phosphorylation and activation observed during glucose deprivation. Jurkat cells were transfected with control siRNA (NC) or PHLPP2 siRNA (Si-PHLPP2). Cells were cultured in medium without glucose 48 hours after transfection, and p-AMPKα and total AMPK levels checked at the indicated time points following glucose deprivation. **D.** Absence of PHLPP2 abrogates the inhibitory effect of Compound C on AMPKα. Jurkat cells were transfected with control siRNA (NC) or PHLPP2 siRNA (Si-PHLPP2) and, 24 hours later, were exposed to 0.5, 1, 2 μM of Compound C for a period of 3 days. Protein levels were detected by WB. **E.** The ability of Compound C to promote death under low glucose conditions is abrogated in the absence of PHLPP2. 24 hours after transfection with control siRNA (NC) or PHLPP2 siRNA (Si-PH2) Jurkat cells were cultured for 3 days in 10 mM or 5 mM glucose medium with or without 0.5 μM Compound C. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining.

Over-expression of PHLPP2 promotes de-phosphorylation of AMPKα and cell death under metabolic stress and requires the PH domain for these functions.

The PHLPP2 protein has a Ras-Association domain (RA) at the N terminus, a Pleckstrin Homology domain (PH), a Leucine Rich Repeat domain (LRR), a PP2C phosphatase domain and a PDZ-binding motif at the C terminus (Figure 4-5A) [Gao et al., 2005; Brognard and Newton, 2008]. Previous studies have shown that the ability of PHLPP2 to de-phosphorylate its targets is regulated by the PH domain and PDZ-binding motif [Gao et al., 2005; Gao et al., 2008]. Endogenous phospho-AMPKα T^{172} was significantly reduced in Jurkat cells over-expressing full length PHLPP2, but not in cells expressing ΔPH or ΔPH/LRR deletion mutants (Figure 4-5B). Exogenous full length PHLPP2 induced significant cell death in Jurkat cells under low glucose conditions, and required an intact PH domain for this effect (Figure 4-5C). Since phosphatases are known to interact directly with their targets, we checked whether PHLPP2 could interact with AMPK. As shown in Figure 4-6A, phospho-AMPKα co-immunoprecipitates with endogenous PHLPP2; this binding interaction is impaired in the absence of the PH domain (Figure 4-6B). Thus, these data offer strong support for PHLPP2 as an AMPK phosphatase, and show that the

phosphatase function of PHLPP2, and its ability to promote cell death under metabolic stress and to interact with AMPKα are all dependent on the presence of an intact PH domain.

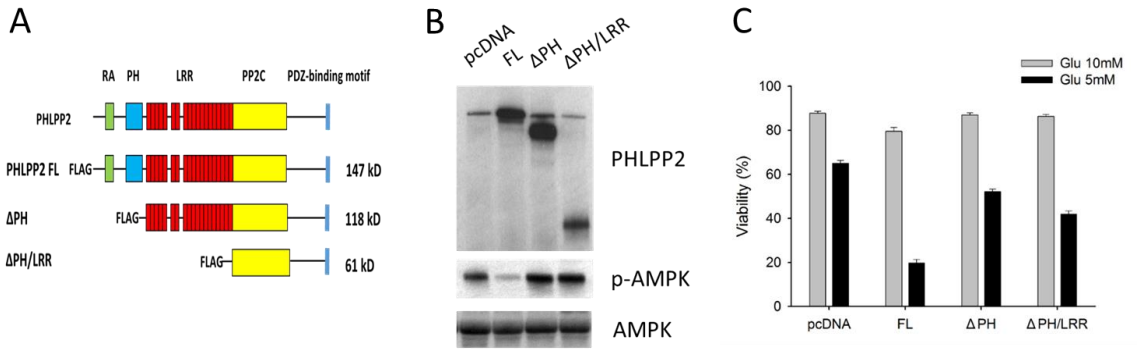

Figure 4-5. Over-expression of PHLPP2 promotes de-phosphorylation of AMPKα and cell death under metabolic stress and requires the PH domain for these functions. A. The protein structure of PHLPP2 and different PHLPP2 inserts in the PHLPP2 constructs. RA: Ras-Associated domain. PH: Pleckstrin Homology domain. LRR: Leucine Rich Repeat domain. PP2C: Protein Phosphatase 2C domain. **B.** Over-expression of full-length PHLPP2, not the PH domain deleted PHLPP2, induces de-phosphorylation of AMPKα in Jurkat cells. The pcDNA empty vector, PHLPP2 full-length (FL), PH domain deleted (ΔPH) and PH/LRR domain deleted (ΔPH/LRR) constructs were transfected into Jurkat cells. 72 hours after transfection, protein levels were detected by WB. **C.** The PH domain is required for the death promoting effect of over-expressed PHLPP2 under metabolic stress. Jurkat cells were transfected with the indicated constructs and 24 hours after transfection the cells were cultured in 10mM or 5mM Glucose medium for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining.

Figure 4-6. PHLPP2 interacts with AMPK and requires the PH domain for the interaction. A. Endogenous PHLPP2 interacted with phospho-AMPKα directly. The interaction between PHLPP2

and p-AMPKα was detected by protein immunoprecipitation. **B.** Full-length PHLPP2, but not the PH domain deleted mutant, interacts with AMPKα. Jurkat cells were transfected with the indicated constructs. 72 hours after transfection, the cells were collected and Flag antibody was used for protein immunoprecipitation with the blot probed with AMPKα and PHLPP2 antibodies.

PHLPP2 dephosphorylates phospho-AMPKα in vitro, and requires the PH domain for this function.

Having shown that PHLPP2 regulates AMPKα phosphorylation intracellularly and can interact with AMPKα protein, we developed an in vitro phosphatase assay to assess the phosphatase activity of PHLPP2 using recombinant active AMPKα as substrate. In this assay, we used either FLAG antibody or PHLPP2 antibody to immuno-precipitate over-expressed PHLPP2 protein from cell lysates and incubated it with a recombinant purified phospho-AMPK protein. After the assay, the phosphorylation level of AMPKα, as an indicator of the phosphatase activity of PHLPP2, was determined by western blot. As shown in Figure 4-7A, phospho-AMPKα T^{172} level was significantly reduced in the presence of full length PHLPP2 protein and this phosphatase activity was dose-dependent (Figure 4-7B). Again, PH domain deleted PHLPP2 was unable to de-phosphorylate p-AMPKα in the in vitro assay (Figure 4-7C); this could be attributed to the impaired interaction between ΔPH PHLPP2 and AMPK shown in Figure 4-6B.

Thus, we offer additional evidence that PHLPP2 is a phosphatase of AMPK in an in vitro assay using a full-length recombinant AMPK protein. Previous studies have used phospho-peptides as substrates to assess phosphatase activity of the PHLPP proteins in vitro [Gao et al., 2005; Brognard et al., 2007]. This would be the first use of a full-length substrate to demonstrate PHLPP2 phosphatase activity.

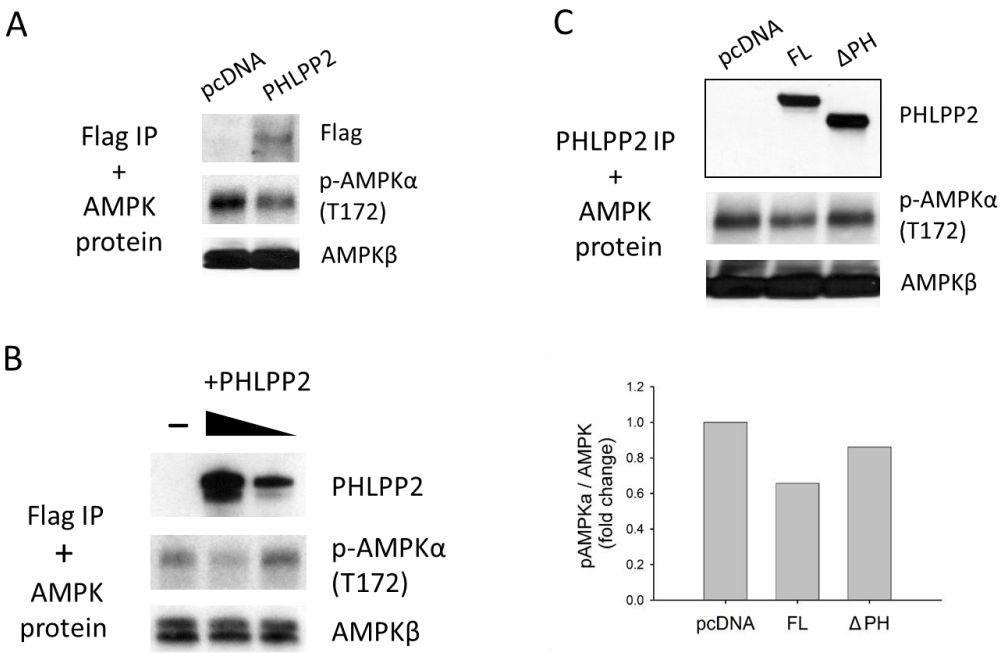

Figure 4-7. PHLPP2 dephosphorylates phospho-AMPKα in vitro, and requires the PH domain for this function. **A.** PHLPP2 de-phosphorylated p-AMPKα in vitro. Jurkat cells were transfected with pcDNA empty vector or PHLPP2 full-length construct. 72 hours after transfection, the cells were collected and over-expressed PHLPP2 proteins were pulled down using FLAG immunoprecipitation and their ability to de-phosphorylate purified recombinant AMPK proteins was tested in an in vitro phosphatase assay. Phosphorylation level of AMPKα and total amount of AMPK proteins (checked by AMPKβ) were measured by WB. **B.** PHLPP2 t de-phosphorylates AMPKα in a dose-dependent manner. Different amounts of FLAG-PHLPP2 proteins were included in the in vitro phosphatase assay. Phosphorylation level of AMPKα and total amount of AMPK proteins (checked by AMPKβ) were measured by WBs. **C.** The ability of PHLPP2 to de-phosphorylate AMPKα was impaired when PH domain was deleted. 72 hours after the Jurkat cells were transfected with the indicated plasmids, PHLPP2 IP was conducted to pull down full-length or PH-deleted PHLPP2 proteins. The ability of these PHLPP2 proteins to de-phosphorylate AMPK was tested through the in vitro phosphatase assay. The graph on the bottom shows the phosphorylation levels of AMPKα normalized to total AMPK level (checked by AMPKβ) after the in vitro assay.

PHLPP2 regulates phospho-AMPK levels in various cancer cell lines.

We have shown that PHLPP2 is an AMPKα phosphatase that can regulate the activation of the AMPK pathway and the cellular response to metabolic stress in Jurkat acute lymphocytic leukemia cells. However, we were unable to detect phosphatase activity on

known targets such as pAKT and pS6K in this leukemia model. We checked whether PHLPP2 could regulate AMPK phosphorylation in other types of tumor cells. As shown in Figure 4-8A, phospho-AMPKα T^{172} levels increased when PHLPP2 was silenced in two leukemia cell lines Molt4 (acute lymphocytic leukemia) and K562 (chronic myeloid leukemia). Importantly, this was also observed in two solid tumor cell lines H522 (non-small cell lung cancer) and DLD1 (colorectal adenocarcinoma), suggesting that PHLPP2 functions as an AMPKα phosphatase in a wide variety of cell types. It must be noted that, while the absence of PHLPP2 protected against cell death induced by metabolic stress in K562 (Figure 4-8C) and H522 cells (Figure 4-8D), it had the opposite effect in Molt 4 (Figure 4-8B) and DLD1 cells (Figure 4-8E). The differential effect of PHLPP2 in regulating cellular response to metabolic stress i.e. whether AMPK has a tumor-suppressive or an oncogenic effect, could depend on cellular context. Moreover, novel, as yet unidentified targets of PHLPP2 could also contribute to the overall effects of this phosphatase in cancer.

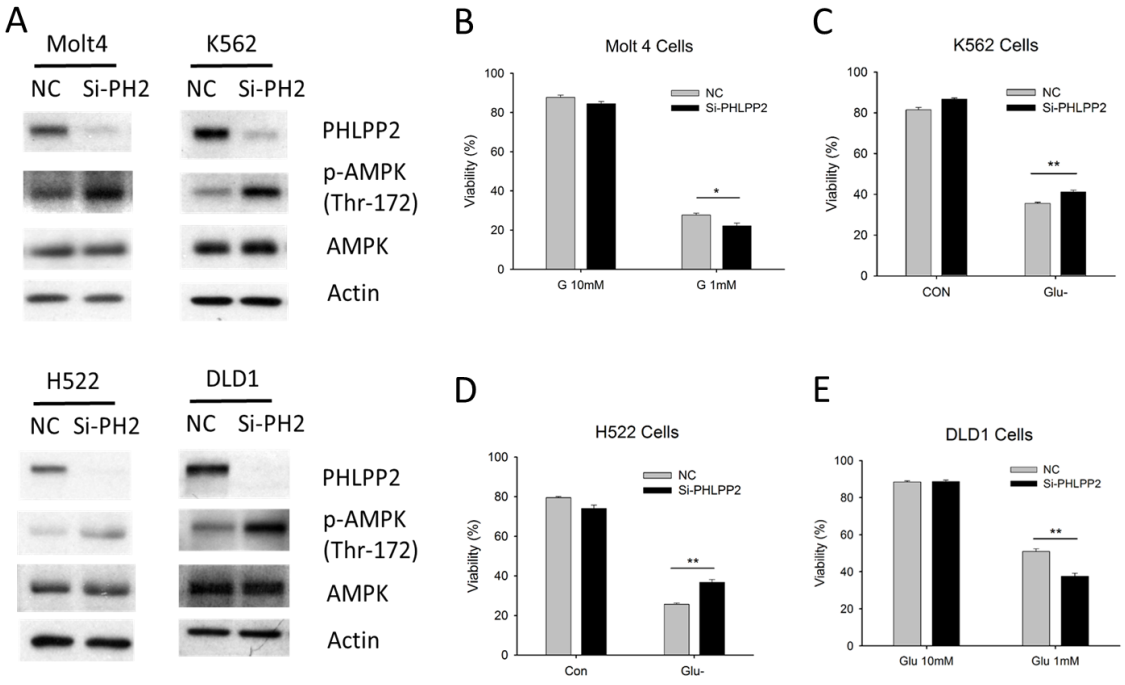

Figure 4-8. PHLPP2 regulates phospho-AMPK levels in various cancer cell lines. A. Phospho-AMPKα levels increased when PHLPP2 was silenced in a variety of cancer cell lines. Molt 4 (acute lymphocytic leukemia), K562 (chronic myeloid leukemia), H522 (non-small cell lung cancer) and DLD1 (colorectal adenocarcinoma) cells were transfected with control siRNA (NC) or PHLPP2

siRNA (Si-PH2). 72 hours after transfection, protein levels were determined by WB. **B.** Cell viability under metabolic stress decreased in the absence of PHLPP2 in Molt 4 cells. siRNA transfected Molt 4 cells were cultured in 10 mM or 1 mM glucose medium for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. **C.** Cell viability under metabolic stress increased in the absence of PHLPP2 in K562 cells. siRNA transfected K562 cells were cultured in 10 mM glucose medium or medium without glucose for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. **D.** Cell viability under metabolic stress increased in the absence of PHLPP2 in H522 cells. siRNA transfected H522 cells were cultured in 10 mM medium or medium without glucose for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining. Glucose. **E.** Cell viability under metabolic stress decreased in the absence of PHLPP2 in DLD1 cells. siRNA transfected DLD1 cells were cultured in 10 mM or 1 mM glucose medium for 4 days. Cell viability was measured by flow cytometric analysis of Annexin V/PI staining.

Conclusion

The studies described here identify a new target and a novel function for the PHLPP2 phosphatase in cancer cell metabolism and energy homeostasis in PHLPP2-expressing hematopoietic cancer cells as well as in cell lines derived from solid tumors. The effects of silencing PHLPP2 on survival of cells undergoing metabolic stress, and on the phosphorylation and activation of the energy sensing kinase AMPK, offer significant insights into a unique role for this phosphatase, that is distinct from PHLPP1, a close family member. Together these studies demonstrate the ability of PHLPP2 to control the cellular response to nutrient stress and to interact with and dephosphorylate AMPK intracellularly and in vitro, and show that the Pleckstrin Homology (PH) domain on the protein is indispensable for all these functions. Thus, AMPK a novel target of PHLPP2 may be the mediator of PHLPP2's role in cancer metabolism. This will also be the first identification of a specific AMPK phosphatase.

Chapter 5

PHLPP2 regulates fatty acid oxidation through AMPK-ACC pathway

Introduction

In the previous chapter, we described studies that led to the discovery of a new target and a new metabolic function for PHLPP2. We showed that PHLPP2 is a phosphatase for AMPK, the key energy sensing kinase, and that it regulates the cell's response to nutrient and energy stress. Under metabolic stress, activated AMPK phosphorylates its downstream targets to switch on catabolic pathways and switch off anabolic metabolism and restore energy homeostasis [Garcia and Shaw, 2017]. We found that under low glucose conditions phosphorylation of AMPK targets, ULK1, and particularly ACC1/2, increased significantly with PHLPP2 inhibition or loss (Figure 4-2C and 4-2E). Phosphorylation of ACC by activated AMPK inhibits its activity which leads to increased fatty acid oxidation and decreased fatty acid synthesis [Fullerton et al., 2013]. The increase in phospho-ACC levels in response to PHLPP2 protein loss suggested to us that the effect of PHLPP2 on cell survival under metabolic stress might be mediated via its regulation of fatty acid metabolism through an AMPK-ACC pathway. Studies described in this chapter investigate this possibility.

Results

PHLPP2 silencing does not affect fatty acid synthesis but increases endogenous fatty acid oxidation under nutrient stress.

To determine whether PHLPP2 influenced fatty acid synthesis we measured the incorporation of [14C]-acetate into triacylglycerols (TAG) and phospholipids (PL). Knocking down PHLPP2 expression in Jurkat cells did not significantly affect the rate of *de novo* lipogenesis as shown in Figure 5-1A. To check fatty acid oxidation (FAO) rate we determined the sensitivity of the oxygen consumption rate (OCR) to FAO inhibition. Long chain fatty acids are transported to the mitochondrial matrix through carnitine palmitoyl transferase (CPT) system [Wakil and Abu-Elheiga, 2009]. In the mitochondria, fatty acid is oxidized to generate acetyl-CoA which enters the TCA cycle to be further oxidized. Electrons derived from these oxidation steps are passed to O2 in the mitochondrial respiratory chain, providing the energy for ATP synthesis. For the FAO assay using Seahorse, cells are treated with Etomoxir (ETO), a CPT1 inhibitor, which blocks long chain fatty acids from entering the mitochondria. Thus, a decrease in OCR with ETO treatment is a reliable indicator of intracellular fatty acid oxidation. Carrying out the assay with cells

cultured in nutrient-limited medium offers a clue to how the cells utilize fatty acid under energy stress. As shown in Figure 5-1B, the OCR in PHLPP2 silenced Jurkat cells was significantly more sensitive to ETO (or FAO inhibition) compared to the control group. These results suggest that PHLPP2 knockdown promotes fatty acid oxidation in cells that are under metabolic stress.

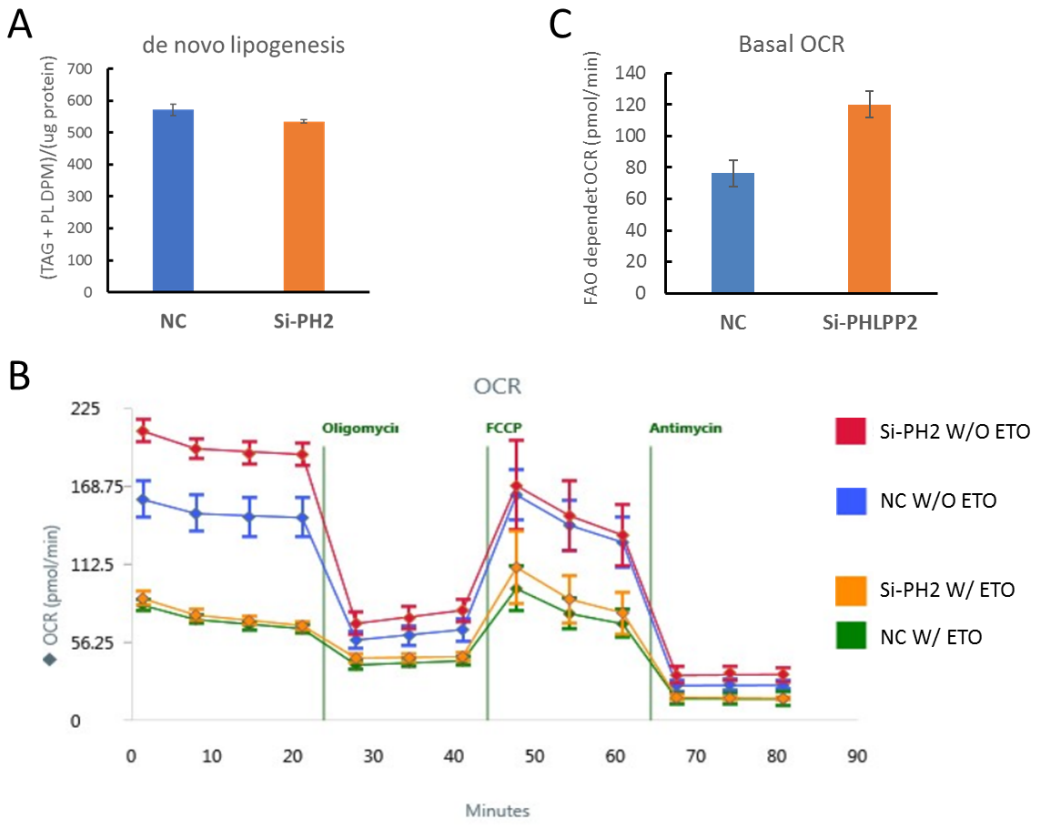

Figure 5-1. PHLPP2 silencing does not affect fatty acid synthesis but increases endogenous fatty acid oxidation under nutrient stress. A. The incorporation of [14C]-labelled acetate into triacylglycerols and phospholipids was measured to check de novo lipogenesis. **B.** To evaluate fatty acid oxidation using the Seahorse Analyzer, cells (NC or si-PHLPP2 cells) were cultured in nutrient-limited medium the previous day. The assay was carried out in the presence or absence of Etomoxir to measure the FAO-dependent oxygen consumption rate (OCR). **C.** Bar graph of FAO dependent OCR values (basal OCR W/O ETO minus basal OCR W/ ETO) in NC cells or Si-PHLPP2 cells.

PHLPP2 regulates fatty acid oxidation through AMPK

To determine whether PHLPP2's effect on fatty acid oxidation was mediated through the AMPK pathway, we inhibited AMPK with Compound C. As shown in Figure 5-2A, 5-2C, without Compound C treatment the FAO dependent OCR increased significantly in PHLPP2 silenced cells. In the presence of Compound C, however, PHLPP2 knockdown could no longer promote fatty acid oxidation (Figure 5-2B, 5-2C), strongly supporting a role for PHLPP2 in regulating fatty acid oxidation through an AMPK-ACC route in metabolically stressed cells.

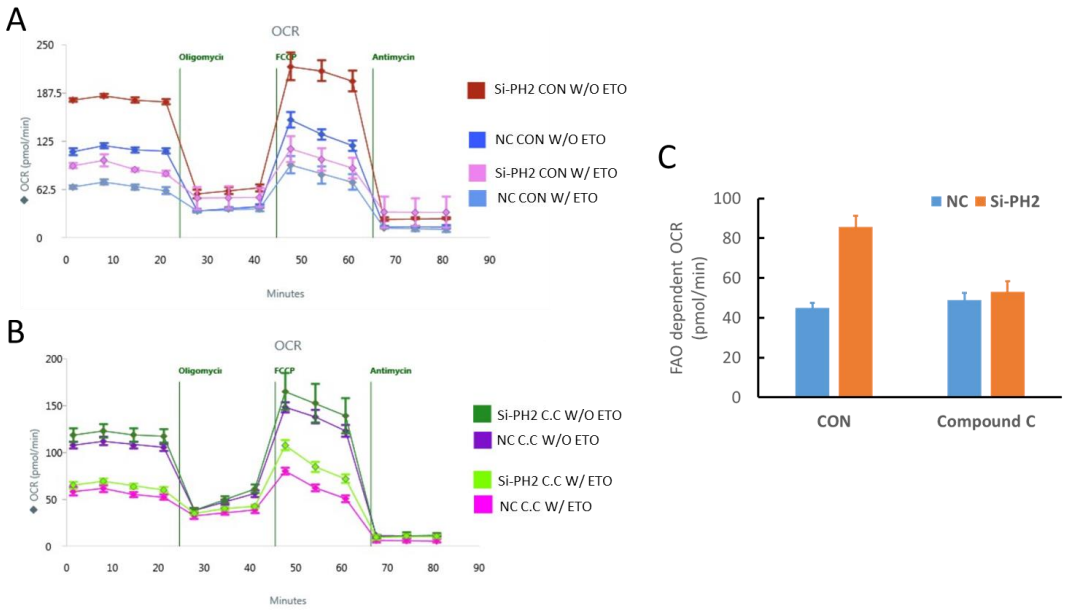

Figure 5-2. PHLPP2 regulates fatty acid oxidation through AMPK. A. Fatty acid oxidation was measured using a Seahorse FAO assay. The contribution of FAO to the OCR level is determined by the reduction in OCR following Etomoxir treatment. **B**. The increased FAO observed in the absence of PHLPP2 was reversed by AMPK inhibitor Compound C. Jurkat cells was transfected with control siRNA (NC) or PHLPP2 siRNA (si-PH2) and 24 hours after transfection, treated with Compound C. 24 hours later, the cells were cultured in nutrient-limited medium for one day and subjected to a Seahorse assay to measure FAO dependent OCR. **C**. Bar graphs of FAO dependent OCR values (basal OCR W/O ETO minus basal OCR W/ETO) in NC cells or Si-PHLPP cells without or with Compound C from the experiment profiled in A and B.

PHLPP2 knockdown promotes cell survival under metabolic stress by increasing fatty acid oxidation

In the previous chapter, we showed that the absence of PHLPP2 promoted cell survival under energy stress by inducing the phosphorylation and activation of AMPK. Here we asked whether the increased fatty acid oxidation was contributing to the pro-survival effect of PHLPP2 knockdown. We found that the cell survival effect of PHLPP2 knockdown under low glucose conditions was dampened in the presence of the fatty acid oxidation inhibitor, ETO (Figure 5-3A), which suggested that the effect of PHLPP2 on the cellular response to metabolic stress was mediated, at least in part, through its effect on fatty acid oxidation.

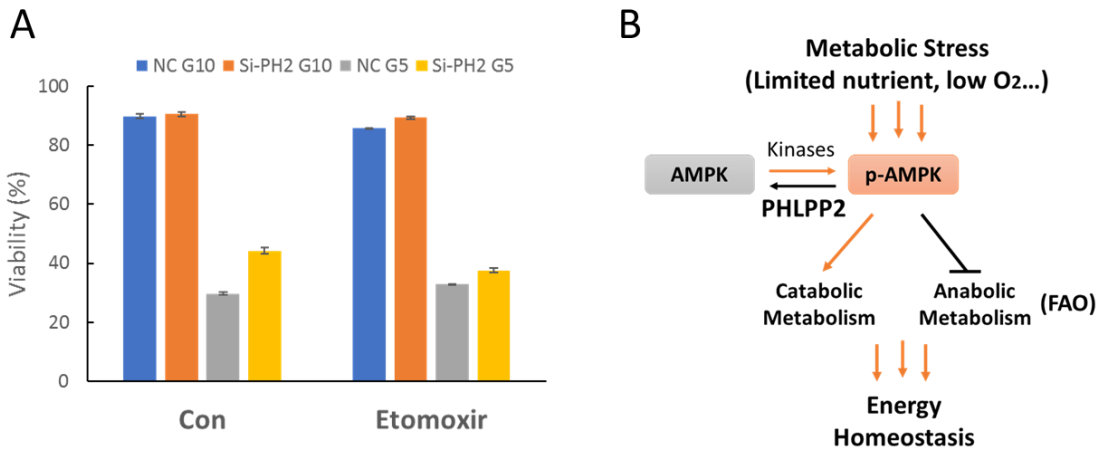

Figure 5-3. PHLPP2 knockdown promotes cell survival under metabolic stress by increasing fatty acid oxidation. A. The pro-survival effect of PHLPP2 knockdown was reversed by FAO inhibitor Etomoxir. Jurkat cells were transfected with control siRNA (NC) or PHLPP2 siRNA (Si-PH2) and, 24 hours post-transfection, cultured for days in medium containing either 10mM or 5mM glucose, in the presence of absence (Con) of Etomoxir, before analysis of cell viability was analyzed by flow cytometry. **B**. simplified model depicting our current understanding of the role of PHLPP2 in modulating energy homeostasis under metabolic stress, through regulation of AMPK and fatty acid oxidation (FAO).

Conclusion

The experiments described in this chapter provide the first evidence of a role for PHLPP2 in regulating fatty acid oxidation (FAO) under conditions of nutrient limitation, and demonstrate that this role is mediated through AMPK signaling. When glucose supplies are low, Jurkat cells can use FAO to support ATP production, and for survival, only in the absence of PHLPP2, suggesting that PHLPP2 promotes cell death under nutrient stress

at least in part by preventing the switch to FAO. Additional pathways that are regulated by AMPK may also contribute to the effect of PHLPP2 on the cellular response to energy stress and will be investigated. The model in Figure 5-3B illustrates our current understanding of how PHLPP2 modulates energy homeostasis under metabolic stress though regulation of AMPK and fatty acid metabolism.

Chapter 6

Discussion

Phosphatases are central players in signaling pathways that antagonize both cell survival and the cancer phenotype and the PHLPP family of serine threonine phosphatases, in particular, with oncogenic kinases as targets [Brognard and Newton, 2008], are emerging as important tumor suppressors [O'Neill et al., 2013]. The focus of this dissertation is the phosphatase, PHLPP2, in myeloid and lymphoid leukemia cells. This study makes major contributions to our understanding of the regulation, expression and function of PHLPP2, identifying a novel regulatory mechanism, a prominent energy sensing kinase as a new substrate, and a novel metabolic function for the phosphatase.

Regulation of PHLPP2 in AML

Studies on PHLPP family proteins have focused on solid tumors, such as prostate, colorectal and breast cancers, as well as melanomas and non-small cell lung cancers [O'Neill et al., 2013]. As a tumor suppressor PHLPP is deleted in many cancers, while others show reduced mRNA and protein levels. Here we demonstrate for the first time, that PHLPP2 is suppressed in the more differentiated AML subtypes, M3, M4 and M5, by miRNAs belonging to the miR-17~92 cluster, oncomir-1. The FAB system [Lowenberg et al., 1999] sorts AML on the basis of morphology, cell type and degree of differentiation in contrast to the broader WHO classification, which takes into account factors that impact prognosis. The differential expression of PHLPP2 in specific AML subtypes underscores its potential as a biomarker and a therapeutic target. Differential expression of the miR-17~92 cluster across FAB subtypes also suggests developmental regulation. We show that the phosphatase is significantly upregulated in AML cells induced towards granulocytic differentiation by ATRA, a drug used for treating AML, through repression of the miR-17~92 cluster. Finally, we demonstrate a novel role for transcription factor C/EBPβ, a mediator of ATRA–induced differentiation, in direct repression of the miR-17~92 cluster leading to upregulation and activation of PHLPP2. These findings are summarized in the model depicted in Figure 3-7.

The miR-17~92 cluster is frequently amplified in several lymphomas and in lung cancer [Hayashita et al., 2005; Ota et al., 2004], and aberrant overexpression of the miR-17~92 polycistron is detected in many cancers [Garofalo et al., 2011]. Retinoblastoma-like 2 protein (p130, Rbl2), Cdk inhibitor p21CIP1, transforming growth factor β1 (TGFβ1), pro-apoptotic Bcl-2 protein Bim, the tumor suppressor PTEN and transcription factor E2F1, are some of the cluster's targets [Olive et al., 2013]. A previous study on drug resistant

mantle cell lymphomas with an amplified oncomir-1 cluster had suggested that the chemo-resistance was mediated largely through PHLPP2 suppression, although this was not directly tested in the mantle cell lymphoma model [Rao et al., 2012]. Our studies offer the first direct evidence that the miR-17~92 cluster controls PHLPP2 protein levels through the 3'UTR of its mRNA.

The regulated expression of miR-17~92 across AML subtypes, suggests a developmental role for these miRNAs. This cluster has already been implicated in the survival signal that promotes progression from the pro-B- to pre-B-cell stage during normal B cell development, in part through its suppression of Bim protein expression [Ventura et al., 2008]. Downregulation of the miR-17~92 cluster is essential for induction of normal myeloid differentiation orchestrated by myeloid transcription factor PU.1 [Alemdehy et al., 2012]. Thus, upregulation of the cluster could lead to a differentiation block in specific AML subtypes. Our studies suggest that increased expression of the miR-17~92 cluster is not controlled by c-MYC or E2F1/3, transcription factors known to upregulate the cluster in solid tumors and in some B lymphomas [O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007], although loss of a repressor in the PU.1 signaling pathway is being not ruled out. We also show that ATRA-induced activation of differentiation in HL-60 cells causes significant suppression of the miR-17~92 cluster (Figure 3-2B and 3-2E). Suppression and subsequent de-repression of miR-17~92 targets, including PHLPP2 (Figure 3-2A), are likely to be critical for mediating ATRA's differentiating effect. The increased sensitivity of PHLPP2 3'UTR "sponge"-transfected cells to ATRA (Figure 3-3A and 3-3B) support this possibility.

We show that ATRA target, C/EBPβ, mediates the repression of the oncomir-1 cluster in HL-60 cells by interacting directly with a binding site in the intronic promoter of the *miR-17~92* gene (Figure 3-6). Previous studies showed that transcription factors c-MYC and E2F bind to the promoter region of the host gene, MIR17HG [O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007]. In AML cells, neither c-MYC nor E2F silencing affected miR-17~92 expression, but C/EBPβ binding to the intronic promoter could repress the cluster, suggesting that transcriptional regulation of the miR-17~92 cluster in AML is independent of its host gene. C/EBPβ is a member of the C/EBP family of basic region/leucine zipper transcription factors, important for differentiation of liver, adipose tissue and hematopoietic cells [Duprez et al., 2003]. The C/EBPβ promoter contains

binding sites for several transcription factors including retinoic acid receptor a (RARa). C/EBPβ can promote either activation or repression of its target genes in a cell- and tissue-specific manner [Duprez et al., 2003; Huber et al., 2012]. It is one of the major proteins induced in response to ATRA, and is essential for granulocytic differentiation. We show that the ATRA-induced reduction in miR-17~92 levels requires the presence of C/EBPβ (Figure 3-5). The initial drop in miR-17~92 levels in untreated HL-60 cells expressing C/EBPβ siRNA compared to controls suggests that the transcription factor may function as an activator of miR-17-92 at low concentrations. However, untreated levels were unaffected by the addition of ATRA (Figure 3-5D). C/EBPβ has been attributed a tumor-promoting role in breast and prostate cancer [Bundy et al., 2003; Kim et al., 2008; Zahnow et al., 1997] and shown to be a negative regulator of miR-145, a tumor suppressor miRNA often downregulated in breast cancer [Sachdeva et al., 2012].

Interestingly, C/EBPβ that is upregulated in ATRA-treated cells can also bind and activate the promoter for master hematopoietic transcription factor PU.1 [Mueller et al., 2006]. Suppression of PU.1 is critical to the maturation block at the promyelocytic stage in APL, and restoration of its expression by ATRA was suggested to be a major mechanism leading to granulocytic differentiation. The significant reduction in miR-17~92 levels and in the primary transcript in ATRA treated HL-60 cells (Figure 3-2E and 3-5D) supports the possibility that C/EBPβ suppresses miR-17~92 via two pathways – indirectly through induction of PU.1 expression, where it functions as an activator, and more efficiently, through direct interaction with the miR-17~92 promoter, as we have demonstrated, where it functions as a repressor. Overall, these studies establish the significance of PHLPP2 regulation during AML development and terminal differentiation and provide a foundation for future studies aimed at therapeutic targeting of the phosphatase, either directly or via C/EBPβ and/or miR-17~92.

Despite growing interest in their potential therapeutic value as tumor suppressors, investigations of PHLPP phosphatases have focused largely on solid tumors. We were interested in identifying mechanisms regulating the widely different expression patterns of PHLPP2 in leukemia. We believe post-transcriptional inhibition by the oncomir -1 cluster, described in chapters 2 and 3, is just one of multiple cell type-specific, regulatory mechanisms in leukemia. The second half of my dissertation focuses on a different leukemic cell type in which the PHLPP2 protein is constitutively expressed, likely to be

regulated via post-translational modifications, and responsive to alterations in the metabolic state of the cell.

PHLPP2 plays a novel metabolic role as a phosphatase for AMPK

In identifying an important energy sensing kinase, AMPK, as the newest target of PHLPP2, our studies also reveal a novel metabolic role for this phosphatase in regulating the cellular response to energy stress through AMPK signaling. We showed that PHLPP2 could dephosphorylate AMPKα (T^{172}) both in cells and in vitro, and phosphorylation and activation of intracellular AMPK protein increased with loss in PHLPP protein levels. PHLPP2 loss promoted survival of glucose deprived Jurkat T-ALL cells, suggesting a role for the protein in metabolic reprogramming. Most importantly, PHLPP2 regulation of AMPK was observed not only in leukemia cells but also in solid tumor-derived cancer lines. Our findings from these studies are summarized in a model in Figure 5-3B.

We provide evidence that AMPK, the new target of PHLPP2, mediates its novel metabolic function. When cells are under energy stress and low in ATP, they increase their AMP/ATP ratio, causing AMPK to be phosphorylated at T^{172} by upstream kinases [Carling et al., 2011]. AMP competes with ATP to bind AMPKγ subunit, causing a conformational change and exposing the T^{172} on AMPKα to phosphorylation by kinases [Hawley et al, 1996]. The interaction between AMP and AMPK protein also renders the protein a better substrate for kinases, but less than ideal for phosphatases [Gowans et al., 2013]. PHLPP2 regulates the intracellular AMP/ATP ratio through its effect on oxidative phosphorylation and ATP production (Figure 4-1). Our studies showed PHLPP2 regulates phosphorylation and activation of AMPK by dephosphorylating T^{172} on the alpha subunit, while PHLPP2 silencing promotes the phosphorylation of AMPK by upstream kinases. Thus, as an AMPK phosphatase, PHLPP2 may act as master regulator of AMPK phosphorylation and activation through modulation of the intracellular AMP/ATP ratio.

PHLPP phosphatases inhibit cancer cell proliferation by dephosphorylating Akt, PKC and S6k, and induce cell apoptosis by activating Mst1 [O'Neill et al., 2013]. Here, using a Jurkat T leukemia cell model, we show that the *loss* of PHLPP2 promotes cell survival under metabolic stress, by *increasing* AMPK phosphorylation/activation. AMPK activator AICAR, had a similar effect under these conditions while AMPK inhibitor Compound C promoted death (Figure 4-4B, 4-4E). The above results point to an oncogenic role for AMPK in Jurkat

cells. One study [Jeon et al., 2012] has suggested that AMPK promoted lung tumor cell survival during energy stress, supporting our conclusions. Another showed AMPK protecting leukemia-initiating cells from metabolic stress in AML [Saito et al., 2015]. Yet another, however, showed that AMPKα is a negative regulator of the Warburg effect, and that its loss accelerates Myc-driven lymphomagenesis [Faubert et al., 2013]. The opposing roles for AMPK in cancer may depend on several factors, including cell type, context and the AMPK isoform that is expressed. Emerging evidence suggests that AMPKα2 acts as a tumor suppressor, but AMPKα1, the most commonly expressed isoform, is an oncoprotein [Hardie, 2017]. However, this needs to be further investigated with respect to PHLPP2 regulation of AMPK in Jurkat cells.

AMPK exerts its oncogenic or tumor suppressive function by regulating glucose metabolism, redox homeostasis and fatty acid synthesis/oxidation [Garcia et al., 2017]. In Jurkat cells, the increased phospho-AMPKα in PHLPP2 silenced cells significantly increased phosphorylation of Acetyl-CoA Carboxylase (ACC) (Figure 4-2C). Previous studies have shown that phosphorylation of ACC1 by AMPK inhibited its activity and led to a decrease in fatty acid synthesis, and phosphorylation of ACC2 by AMPK led to increased β-oxidation [Fullerton et al., 2013]. The studies described in Chapter 5 point to a role for PHLPP2 in regulating fatty acid β-oxidation, rather than fatty acid synthesis, in Jurkat cells. Under low glucose availability, cells were able to reprogram their metabolism to use more fatty acid for ATP production and for survival, only if PHLPP2 was silenced. However, the data not rule out the possibility that other AMPK associated pathways could also be mediating the metabolic function of PHLPP2.

In conclusion, the research described in this dissertation makes significant inroads into our understanding of the phosphatase PHLPP2, not only in leukemia, but in solid tumors as well. These studies provide evidence for a novel miRNA based regulatory mechanism for PHLPP2 expression in AML, identify a key energy sensing kinase as a substrate for PHLPP2 phosphatase activity and reveal a new role for the PHLPP2 in regulating cell survival and death during metabolic stress.

Chapter 7

Materials & Methods

Reagents, Chemicals and Antibodies

All-trans retinoic acid (ATRA) was purchased from Enzo Life Sciences. MG-132 was purchased from Calbiochem. BTZ was from LC Laboratories and G418 (Geneticin) was purchased from Gibco. The antibody directed against PHLPP1 and PHLPP2 (PHLPPL) was purchased from Bethyl Laboratories. Compound C was purchased from Sigma and AICAR was purchased from Cell Signaling Technology. Antibodies for Bim, c-myc, E2F1, P70S6K, phospho-P70S6K (Thr389), Akt, phospho-Akt (Ser473), ACC, phospho-ACC (Ser79), AMPKβ were from Cell Signaling Technology. The antibody for E2F3 was purchased from Sigma-Aldrich, Antibodies for C/EBPβ, Noxa, AMPKα, phospho-AMPKα (Thr172), and β-Actin were from Santa Cruz Biotechnology. The flow cytometry antibody for CD11b was purchased from Biolegend. The antibodies for ULK1 and phospho-ULK1 (Ser555) were a kind gift from Do-Hyung Kim lab.

Cell lines, patient cells, cell culture and cell differentiation

HL-60, U937, THP-1, Jurkat, Molt4, K562, H522 and DLD1 cells were grown in RPMI-1640, supplemented with 10% FBS, 2 mM L-glutamine and non-essential amino acids. Kasumi-1 cells were grown in RPMI-1640 supplemented with 20% FBS and 2 mM L-glutamine. MV4-11 cells were grown in Iscove's MDM (Sigma-Aldrich) supplemented with 10% FBS and 2 mM L-glutamine. KG-1 cells were grown in IMDM supplemented with 20% FBS and 2 mM L-glutamine. 293 cells were grown in DMEM with 10% FBS and 2 mM L-glutamine. All cells were supplemented with 100-units/mL penicillin and 100 ug/mL streptomycin and maintained in a humidified atmosphere of 5% CO2 at 37°C.AML patient samples were obtained from the Leukemia MDS Tissue Bank of the University of Minnesota. The frozen patient samples, highly enriched of leukemic cells, were thawed and used for Western blot and microRNA quantification.

For cell differentiation studies ATRA-treated HL-60 cells were collected and stained with CD11b antibody in FACS buffer (2% FBS and 0.1% sodium azide in PBS). Cells were washed twice in FACS buffer and fluorescence was detected using the BD LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software.

mRNA Isolation and RT-PCR

mRNA was extracted from $1x10^6$ - $5x10^6$ suspension leukemia cells with TRIZOL® reagents (Invitrogen) and cDNA was synthesized from 500 ng - 800 ng mRNA template

using the DyNAamo cDNA Synthesis Kit (Thermo Scientific), according to manufacturers' protocols. The cDNA was amplified through PCR primers (see Supplemental Information for primers). The PCR products were run on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

microRNA Quantification

Total miRNAs were extracted from cells using mirVana™ miRNA isolation kit (Life Technologies). cDNA was synthesized by reverse transcription using the miScript II RT kit (QIAGEN). Finally, miScript SYBR® Green PCR kit (QIAGEN) was used to quantify the levels of individual miRNA in miR-17~92 cluster. The miRNA levels were normalized to an U6 internal control. Except for the miR-17~92 cluster measured in leukemia cell lines for Figure 2B, which was performed with the MiR-17~92 cluster plate assay kit (Signosis Inc.), all measurements of miR-17~92 cluster levels were performed using the miScript® miRNA PCR assay (QIAGEN).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation was performed using the Millipore ChIP Assay Kit according to the manufacturer's protocol. Briefly, 37% formaldehyde-cross-linked HL-60 cells were collected, lysed, sonicated to approximately 500bp segments and subjected to immunoprecipitation with RNA polymerase II, C/EBPβ antibodies or IgG control antibody (Santa Cruz). Immunocomplexes were collected on protein A/G agarose beads blocked with salmon sperm DNA and eluted. Cross-links were reversed by incubating at 65°C overnight. PCR was performed to amplify different portions of the miR-17~92 promoter (primer sequences are in Supplemental Information). PCR products were run on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. For quantification of fold enrichment in Pol II or C/EBPβ binding, 5ng of total chromatin was used as control and quantitative real time PCR was performed using SYBR Green.

Western Blotting

For Western blotting, cells were lysed in RIPA buffer (50 mMTris-HCl (pH 7.5), 150 mMNaCl, 0.5% v/v sodium deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated with specific antibodies and chemiluminescent reactions were carried out using the ECL Plus kit

(Amersham). Blots were stripped for reuse by washing for 30 min in TBS-T buffer (pH 2.5–3.0).

Protein Immunoprecipitation

For immunoprecipitation, cells were lysed in Buffer A (100 mM HEPES; 0.15 mM MgCl2; 100 mM KCl; 0.5% IGEPAL) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem). Cell lysates were incubated with 1-5µg/ml of antibody at 4°C overnight and complexes were captured with protein G Agarose beads (Invitrogen). For the immunoprecipitation of the Flag-tagged protein, anti-Flag M2 affinity gel (Sigma) were added to the cell lysates directly and incubated at 4°C for 2 hours. Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated with specific antibodies and chemiluminescent reactions were carried out using the ECL Plus kit (Amersham).

In-vitro Phosphatase Assay

PHLPP2 protein was immunoprecipitated from the cell lysates according to the protein immunoprecipitation method. 2x phosphatase buffer (0.1M Tris-HCL PH7.4; 2mM DTT; 10mM MnCl2), 25ng AMPKα1β1γ2 recombinant protein (ThermoFisher) were added to the immunoprecipitates. The in-vitro phosphatase assay was conducted at 30°C for 15min and 2 x Sample buffer (without 2-Mercaptoethanol) was added to stop the reaction. The levels of phospho-AMPK α1 and total AMPK protein in the phosphatase reaction products were analyzed through Western Blotting.

Plasmid Constructs

The PHLPP2 3’ UTR region was amplified using high fidelity Taq polymerase from genomic DNA of AML patient leukemia cells using DNeasy® Blood & Tissue Kit (QIAGEN). The primers used were: forward 5’GAGCTCGGATCCGAAACAGCACCAGGACAGC3’; reverse 5’GAGCTCGCGGCCGCGTTTGTTCTCACTTATCTTC3’. PCR products were cloned into the control vector (CV) pcDNA3.1(+) using BamH1/ Not1. DNA sequencing of the 3’UTR was carried out by Sanger sequencing and no mutations were found. Briefly, the miR-17~92 shRNA plasmid was generated by inserting three individual shRNAs into the TripZ vector. The sequences of the shRNAs are:

shRNA1: AATAAGATGTAACTCATACAATGTGCTTGTATGAGTTACATCTTATTAA;

shRNA2: TAAATGAGAACTAATTTTGGATGTGCTTCAAAATTAGTTCTCATTTATT;

shRNA3: TTATACTGCCAAATCTGACACTGTGCTTGTCAGATTTGGCAGTATAAAT.

Cell Transfection

Transfections of PHLPP2 3'UTR constructs into U937 or HL-60 cells, and transfection of PHLPP2 full length construct or truncated constructs into Jurkat cells were performed using a BioRad Electroporator as described previously (51) or through the Neon™ Transfection System (Invitrogen) using the manufacturers protocol. FuGENE® Transfection Reagent (Roche) was used for HEK293 cell transfections according to the manufacturer's protocol. Negative control siRNA, C/EBPβ siRNAs, PHLPP1 siRNA, PHLPP2 siRNA (GE Dharmacon) and siRNA for c-myc, E2F1, E2F3, (Santa Cruz Biotechnology) were introduced into HL-60, U937 or Jurkat cells using the Neon System.

Cell Death Assay

For cell viability studies, cells were collected under indicated conditions, washed once with cold PBS and stained with Annexin V-FITC and Propidium iodide (PI). The flow cytometry data were analyzed using FlowJo software (Tree Star, Inc).

Seahorse Extracellular Flux Assay

Seahorse base medium (Agilent, Santa Clara, CA USA) was supplemented with 10mM glucose, 1mM sodium pyruvate and 4mM L-glutamine and adjusted to pH 7.4 with 2Mm NaOH. Cells were plated at 200,000 cells per well and adhered to the Seahorse 96 well culture plate using CellTak (Corning, Oneonta, NY USA). Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse XFe96 (Agilent, Santa Clara, CA USA).

Fatty Acid Oxidation Assay

The oxidation of fatty acids was measured by a modified method with the XF Cell Mito Stress Test (Agilent). 24 hours prior to the Seahorse assay, replace growth medium with substrate-limited medium (0.5 mM glucose, 1 mM GlutaMAX, 0.5 mM carnitine and 1% FBS). 45 min prior to the assay, wash the cells once with FAO assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 2 mM MgSO4, 1.2 mM NaH2PO4. 2.5 mM glucose, 0.5 mM carnitine and 5 mM HEPES, PH 7.4). Cells were plated at 200,000 cells per well and adhered to the Seahorse 96 well culture plate using CellTak (Corning, Oneonta, NY

USA) and incubated in a non-CO2 incubator for 30-45 min at 37°C. Load the assay cartridge with XF Cell Mito Stress Test compounds (final concentration: 2.5 µg/ml oligomycin, 2 µM FCCP, 2 µM rotenone/4 µM antimycin A). 15 min prior to starting the assay, add Eto (final concentration: 40 µM) or vehicle to each well. And incubate for 15 min at 37 °C in a non-CO2 incubator. Insert the XF Cell Culture Microplate into the XF96 Analyzer and run the XF Cell Mito Stress Test to measure oxygen consumption rate (OCR). The OCR level that was decreased due to the Eto treatment would indicate the level of fatty acid oxidation.

Metabolic labeling experiments in cell culture

Live, healthy cells were recovered by Ficoll density gradient centrifugation within 24 hours prior to experiment. At 10E6 cells per sample were pelleted and washed in glucose/glutamine free medium. For glucose labeling experiments, cells were resuspended at 1E6 cells/ml in complete glucose free medium supplemented with 10% dialyzed FBS, NEAA, and 4mM L-glutamine for 1 hour. After starvation, cells were supplemented with 10mM [1,2-^{13}C] labeled glucose for 24 hours. Cells were then pelleted and washed once with cold PBS. Pellets were then resuspended in 100-200µl -20°C methanol, snap frozen and stored at -80C. Liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) were used for identification and quantification of labeled metabolites of all samples. [1,2-^{13}C] glucose was purchased from Cambridge Isotopes (Tewksbury, MA USA).

RT-PCR Primer Sequences

Chromatin Immunoprecipitation Primers

DNA was amplified with the following primer sets:

Amplicon 1(~ -1050 binding site): forward 5'GCTGCTGATCATAATCAAGTAT3',

Reverse 5' AGAATACACTTACCCATTCCA3';

Amplicon 2 (~ -600 binding site): forward 5'TGGAATGGGTAAGTGTATTCT3',

Reverse 5' TTATCAGTCAATTGTAGCAGAAC3';

Amplicon 3 (none binding site): forward 5'CTTGTCCGTATTTACGTTGA3',

Reverse 5' CAGACTCACCAGGAGGAGTA3'.

Statistics

All averages are presented as mean ±SE. Student's two-tailed test were used for all analyses: *P < 0.05; **P < 0.01; ***P < 0.001.

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