of the disease. Manageable toxicities and observed activity of elacytarabine CIV in the dose range 875–2000 mg/m^2 per day may allow more flexible administration and dosing than for ara-C.

AUC values for both substances and the increased initial half-life of ara-C after elacytarabine administration (2.0 h versus 0.1–0.2 h),[12] indicate that treatment with elacytarabine provides plasma levels of both elacytarabine and ara-C in the concentration range associated with cytotoxicity that is, concentrations of ara-C that are effective in patients with AML.[13] This enables leukemia cells to maintain a high level of intracellular ara-C and consequently a high level of the active metabolite, ara-CTP. These findings are supported by *in vitro* data in tumor cells after treatment with elacytarabine.[8] Elacytarabine administered at the recommended dose and schedule may provide a better exposure of leukemic cells to ara-CTP than that provided by ara-C. The acceptable safety profile and clinical anti-AML activity documented on this study support further evaluation of elacytarabine, particularly in patients with hENT1 deficiency.[14] Fatty acid esterification of nucleoside analogs is a promising approach to circumvent clinically important mechanisms of resistance.

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Mechanisms and consequences of the loss of PHLPP1 phosphatase in chronic lymphocytic leukemia (CLL)

The PHLPP (pleckstrin homology domain and leucine-rich repeat protein phosphatase) family of novel Ser/Thr phosphatases serve as important regulators of cell survival and apoptosis.[1,2] These proposed tumor-suppressors lead to inactivation of Akt, degradation of PKC and negatively regulate ERK1/2 activation.[2–4] Furthermore, reductions in PHLPP1 and/or PHLPP2 expression have been detected in several cancers and linked to cancer progression.[4–7]

Chronic lymphocytic leukemia (CLL) results in the accumulation of monoclonal B cells owing to enhanced responsiveness to survival cues and apoptotic resistance *in vivo*. Activation of survival pathways such as Akt and ERK1/2 by factors produced in the microenvironment, including the chemokine CXCL12, protects CLL cells from apoptosis.[8,9] Consequently, alterations in the expression or function of proteins regulating these survival pathways, including the PHLPP phosphatases, can influence how CLL cells respond to growth/survival cues and contribute to CLL pathogenesis.

Recently, a reduction in PHLPP1 protein levels in CLL cells was reported.[10] Similarly, we observed a loss of PHLPP1 expression in 39 out of 43 CLL samples (loss in >90% of patients), although it was detected in all B cells from healthy donors (*n* = 6) and was highly expressed in the Ramos Burkitt's lymphoma line. However, PHLPP2 and PTEN expression were detected in all CLL cells probed (representative blot Figure 1a).

To elucidate potential consequences of the loss of PHLPP1, CLL cells were adenovirally transduced to express PHLPP1 or GFP control, stimulated with CXCL12 and probed for phosphorylation and activation of Akt (S473) and ERK1/2. PHLPP2 expression was not altered upon PHLPP1 adenoviral expression (data not shown) and CLL cells transduced with PHLPP1 expressed comparable levels of PHLPP1 mRNA relative to healthy B cells (1.05 ± 0.48). Strikingly, forced expression of PHLPP1 in CLL cells dramatically reduced the CXCL12-induced phosphorylation of Akt and ERK1/2 without affecting total levels of Akt or ERK (representative blot from four independent CLL samples Figure 1b, Supplementary
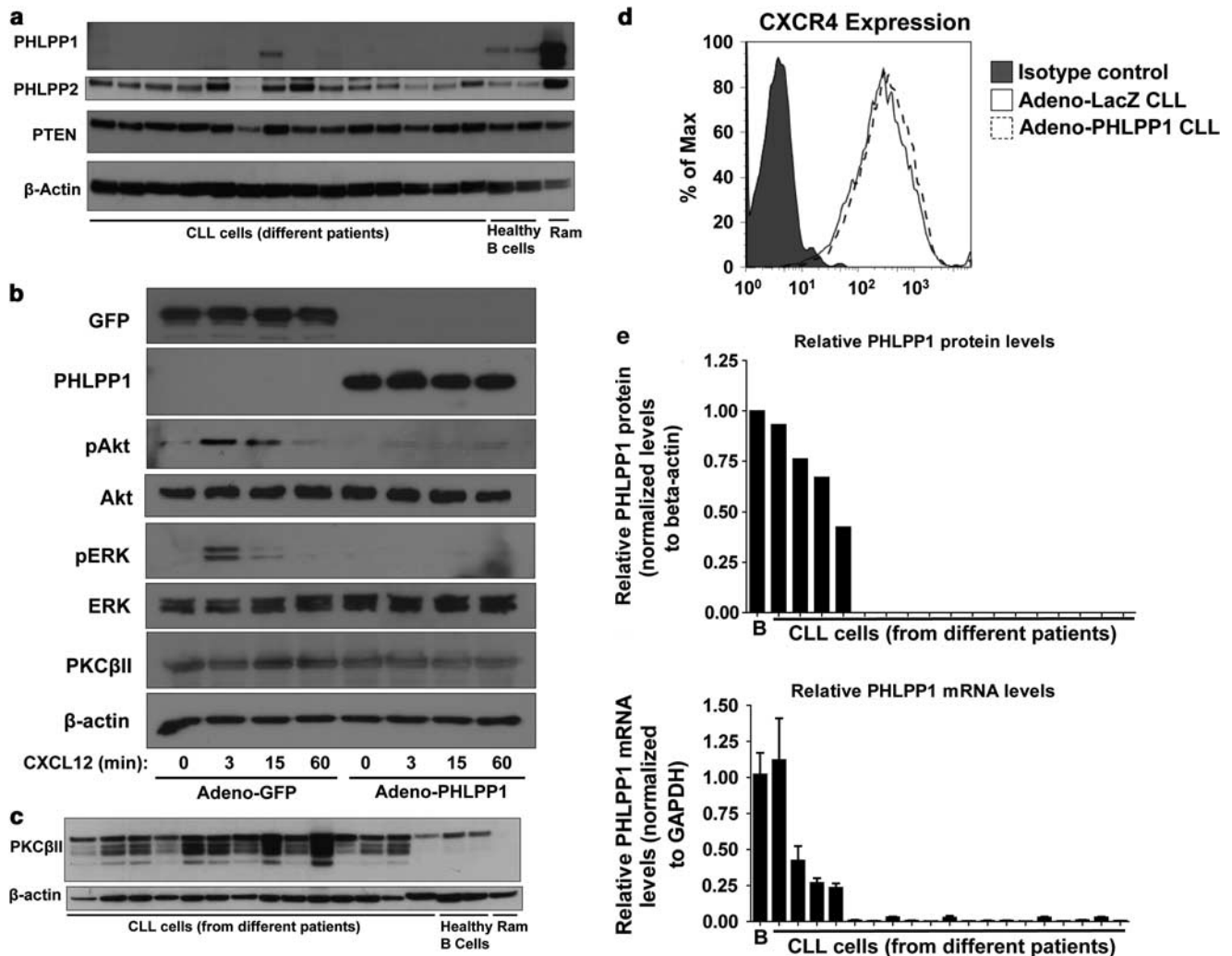
Figure 1. PHLPP1 expression is undetectable in over 90% of primary CLL cells and its enforced expression reduces CXCL12-induced Akt and ERK1/2 activation. (**a**) Representative western blot profiling PHLPP1, PHLPP2 and PTEN expression in primary CLL cells ($n = 14$) compared with B cells from healthy donors ($n = 2$) and a Ramos B cell line (Ram). β-actin served as a loading control. (**b**) Western blot comparing phospho-Akt (S473), phospho-ERK1/2 and PKC-βII levels in a representative ($n = 4$) CLL patient's cells adenovirally transduced with PHLPP1 or GFP control and unstimulated or stimulated with 30 nM CXCL12 for 3, 15 or 60 min. β-actin served as a loading control and GFP and PHLPP1 panels demonstrate successful adenoviral transduction. (**c**) Representative western blot profiling PKCβII levels in CLL cells ($n = 14$), healthy B cells ($n = 2$) and Ramos B cells. (**d**) CLL cells were infected for 72 h with an adenovirus encoding PHLPP1 or LacZ as a control. The cells were then stained for surface expression of CXCR4 (Adeno-PHLPP1-dotted line, Adeno-LacZ-solid line) or an IgG isotype control (solid gray fill) and analyzed by flow cytometry. (**e**) The top panel shows PHLPP1 protein expression quantified by densitometry from western blots of healthy B cells (B, $n = 6$), cells from the four CLL patients expressing endogenous PHLPP1, followed by a panel of other CLL patients' cells ($n = 15$). The corresponding mRNA expression levels from these cells determined by quantitative RT-PCR are shown in the bottom panel. Data presented is an average of three independent experiments performed in triplicate and normalized to GAPDH, and the values indicated are relative to an average of normal B cells (mean ± s.d.).

Figure 2). Although PHLPP1 regulates PKC stability,[1] PKCβII levels were maintained comparable to controls (Figure 1b). However, as PKC has a very stable half life,[11] we also compared endogenous levels of PKCβII and observed a stronger signal and banding pattern of PKCβII expression in CLL cells compared with normal B cells, corresponding with the loss of PHLPP1 (Figure 1c). Furthermore, based on flow cytometry and quantitative PCR analyses, we determined that the effects of forced PHLPP1 expression on Akt and ERK1/2 activation in CLL cells were not related to alterations in CXCR4 levels (receptor for CXCL12) (Figure 1d and data not shown).

To assess whether the loss of PHLPP1 protein was because of a decrease in transcript levels or reduced protein stability, we performed quantitative reverse-transcription PCR (RT-PCR) on RNA isolated from purified CLL cells and B cells from healthy donors. In healthy B cells and cells from the four CLL patients

in which PHLPP1 protein levels were detectable (top panel Figure 1e), significant levels of mRNA were observed (bottom panel Figure 1e). However, CLL cells with undetectable PHLPP1 protein had significantly reduced or undetectable PHLPP1 mRNA levels. A similar correlation between PHLPP1 protein and mRNA expression levels were observed in various CLL cell lines as well (Supplementary Figure 1). These results suggest that the mechanism for PHLPP1 reduction could be due to genetic mutation(s), epigenetic modification (for example, DNA methylation) or alterations in mRNA stability rather than decreased protein stability; this is consistent with findings from Suljagic et al.[10] in which calpain and proteasomal inhibitors had no effect on PHLPP1 protein accumulation in CLL cells.

To address genetic mutation as the source of decreased PHLPP1 levels in CLL, we sequenced the 17 exons that

comprise the *PHLPP1* gene. Twenty-three PCR reactions covering 14 693 bp of the *PHLPP1* genomic sequence including the longer PHLPP1β splice variant were carried out on 15 CLL samples, 3 healthy B-cell controls and Ramos cells. Subsequent DNA sequencing analysis uncovered several alterations in the exonic sequence, all of which had been previously characterized as single-nucleotide polymorphisms (according to the NCBI database), indicating that these changes were present in normal B cells and that there were no somatic mutations in *PHLPP1* in the CLL samples (Supplementary Table 1). As we were able to amplify each exon, it is unlikely that bi-allelic loss of the *PHLPP1* gene was responsible for the absence of PHLPP1 mRNA and protein in the CLL cells.

We next examined whether methylation of the *PHLPP1* promoter or gene could account for the reduced transcript levels since hypermethylation of tumor suppressor genes, resulting in loss of expression, is frequently observed in cancers.[12,13] Bisulfite treatment of DNA from purified CLL cells ($n = 13$), healthy B cells ($n = 3$) and B-cell lines was performed (Figure 2a, see Supplementary Information for detailed methods and Supplementary Figure 3). This analysis revealed that the majority of methylation was detectable in the last ~800 bp of exon 1,

although little was observed in the promoter region, consistent with observations from Dr Christiane Knobbe and Dr Guido Reifenberger on *PHLPP1* methylation analysis of glioblastoma samples (personal communication). These results indicate the end of exon 1 is potentially important for regulation of *PHLPP1* gene expression.

We focused on a portion of the highly methylated region represented by the last 340 bp of PHLPP1 exon 1 to quantitatively compare the degree of CpG methylation between CLL cells and normal B cells (highlighted in Figure 2b). We found that the CLL cells with no/reduced PHLPP1 expression also had a significantly higher degree of CpG methylation and a more extensive distribution of methylated CpG sites compared with healthy B cells and CLL cells-expressing PHLPP1 (Figures 2c and d). Additionally, Ramos cells and the WaC3CD5+ CLL line (endogenously high PHLPP1) had no/very low levels of methylation, whereas the EHEB CLL line (reduced PHLPP1) exhibited a higher degree of *PHLPP1* methylation (although still not as extensive as that observed in the primary CLL cells) (Figure 2c). In summary, there is a strong correlation between the loss of PHLPP1 protein expression and a high degree of *PHLPP1* methylation at the end of exon 1.
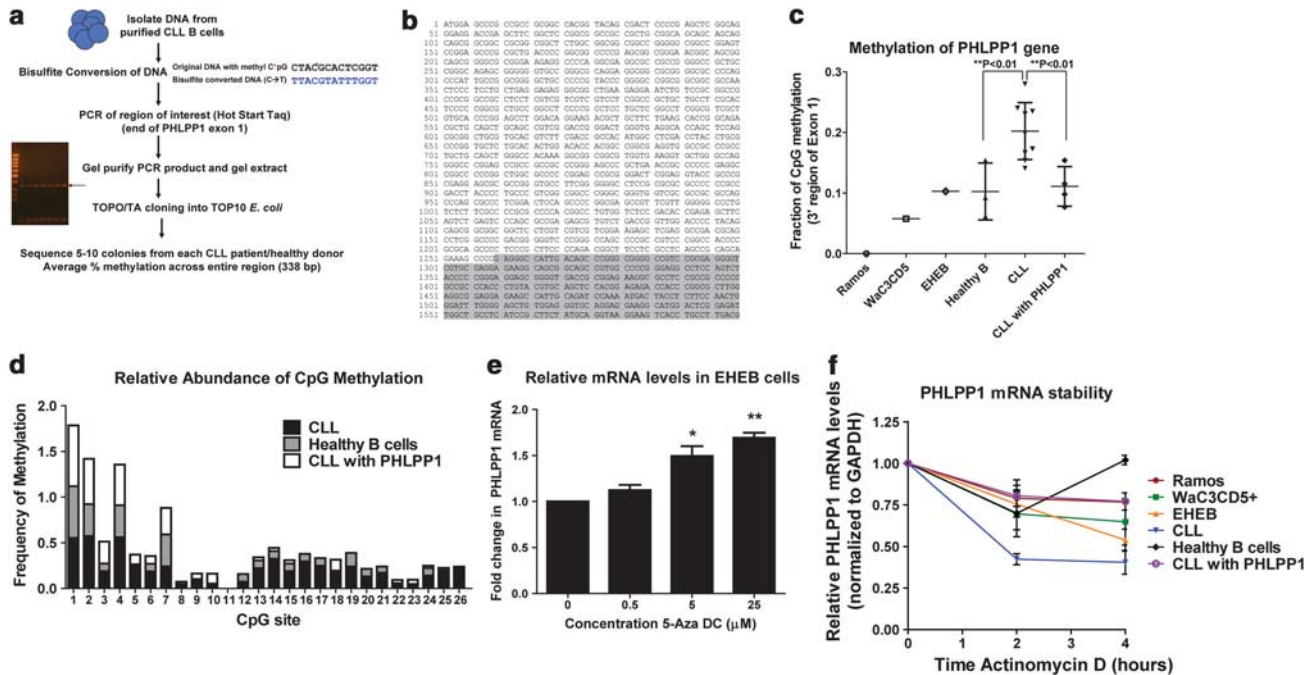
Figure 2. CLL cells lacking PHLPP have a higher degree of DNA methylation and decreased transcript stability compared with healthy B cells. (**a**) Flow diagram of the experimental procedure for methylation analysis of the *PHLPP1* gene: DNA from purified CLL B cells was bisulfite converted and PCR amplified over the region of interest (end of exon 1), PCR products were gel purified, cloned into a TOPO/TA vector and 5–10 colonies from each sample were sequenced and the fraction of CpG methylation was quantified. (**b**) Region of *PHLPP1* gene analyzed for quantitative methylation comparison. Highlighted in gray is the region of the *PHLPP1* gene (non-bisulfite converted) near the end of exon 1 that exhibited significant levels of CpG methylation and was probed for quantification. (**c**) The average fraction of CpG sites methylated across the entire region of interest highlighted in (**b**) (quantified from 5 to 10 colonies corresponding to each sample) is shown for Ramos cells, WaC3CD5+ cells, EHEB cells, healthy B cells ($n = 3$), CLL cells-expressing PHLPP1 ($n = 4$) and a set of CLL cells with low/no PHLPP1 expression ($n = 9$). Each dot represents a separate patient/donor and the bar indicates the average ± s.d. A student's *t*-test was performed to determine statistical significance. (**d**) Relative abundance of methylation at individual CpG sites within the region of interest off *PHLPP1* that was probed. Fraction of cells exhibiting methylation at each CpG site in the region highlighted in (**b**) is compared between CLL cells (black), healthy B cells (gray) and PHLPP1-expressing CLL cells (white). The data represent an average (of the different patients/donors) of the frequency at which methylation is observed at the individual CpG sites. (**e**) EHEB cells were treated with varying concentrations of 5-Aza DC from 0.5 to 25 μM or dimethylsulphoxide control for 72 h and levels of PHLPP1 mRNA were compared by quantitative RT-PCR. Values indicate an average (from four independent experiments performed in triplicate) fold change in PHLPP1 mRNA levels after normalization to GAPDH relative to dimethylsulphoxide control treatment. Error represents the s.d. of the mean: *$P < 0.05$ and **$P < 0.01$. (**f**) Levels of PHLPP1 mRNA relative to GAPDH mRNA at 0, 2 and 4 h following 5 μg/ml Actinomycin D treatment were compared between Ramos (red), WaC3CD5+ (green), EHEB (orange), B cells from healthy donors (black), CLL cells expressing endogenous PHLPP1 (purple) and CLL cells expressing low, but detectable, PHLPP1 mRNA (average of four independent CLL patients, blue). Data represent the mean and s.d. of normalized PHLPP1 mRNA levels from two to three independent experiments performed in triplicate as assessed by quantitative RT-PCR.

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To more directly assess the effects of DNA methylation on protein expression, the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza DC), is generally employed. However, 5-aza DC only works on dividing cells and primary CLL cells are non-dividing. Although CLL cell lines offer an alternative, the PHLPP1 levels are higher and methylation lower in these lines compared with primary CLL. Despite these caveats, we examined the effects of 5-aza DC on PHLPP1 mRNA levels in the EHEB cells. As shown in Figure 2e, a small dose-dependent increase in PHLPP1 mRNA expression was observed in the EHEB cells (1.72 fold increase following a 25-μM treatment with 5-aza DC for 72 h); no significant increases in PHLPP2 mRNA levels were observed (Supplementary Figure 4), suggesting that the methylation inhibitor may modulate the expression of PHLPP1, but not PHLPP2.

As an alternative to 5-aza DC, valproate (VPA) has been shown to indirectly induce active replication-independent DNA demethylation.[14] Therefore, we tested varying concentrations of VPA from 0 to 20 mM for 72 h on CLL cells from two different patients to determine whether demethylation would result in an increase in the PHLPP1 mRNA expression. Although the dimethylsulphoxide control treated CLL cells maintained ~90% viability over this 72 h time course based on trypan blue staining, the VPA-treated cells were <10% viable and so the effects could not reliably be assessed (Supplementary Figure 5). Nevertheless, the dramatic reduction in cell viability upon VPA treatment suggests that histone acetylation and DNA methylation may regulate growth and survival promoting genes in CLL cells. Overall, our results suggest that methylation is a plausible mechanism that may contribute to the loss of PHLPP1 protein expression in CLL cells.

Lastly, as differences in mRNA stability could also account for the decrease in PHLPP1 transcript in CLL cells, we assessed the decay of PHLPP1 mRNA relative to GAPDH mRNA following inhibition of transcription by Actinomycin D. CLL cells expressing low, but detectable PHLPP1 mRNA, exhibited a rapid reduction in PHLPP1 mRNA levels within 2 h following Actinomycin D treatment. In contrast, B cells from healthy donors, CLL cells-expressing PHLPP1 and the cell lines exhibited a much slower decay profile (Figure 2f, differences were statistically significant, $P < 0.01$). Furthermore, upon direct comparison of PHLPP1 and PHLPP2 mRNA stability in the CLL cells, there appeared to be a more rapid degradation of PHLPP1 transcript compared with PHLPP2, consistent with the loss of PHLPP1, but not PHLPP2, in these cells (Supplementary Figure 6). These data suggest that a decrease in PHLPP1 mRNA stability may also contribute to the lower levels of PHLPP1 transcription and protein expression observed in CLL.

In summary, in agreement with Suljagic et al.,[10] we observed the loss of protein expression of the tumor suppressor PHLPP1 in CLL cells and also demonstrated a corresponding loss/decrease in PHLPP1 mRNA. Our results suggest a novel methylation-mediated mechanism for repression of PHLPP1 expression and a decrease in PHLPP1 transcript stability in CLL cells, allowing for increased responsiveness of CLL cells to CXCL12 and potentially other signals from the microenvironment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MO designed and performed experiments and wrote the manuscript. MN and VMN designed and performed the DNA sequencing experiments. JFF, TJK, DM and TMH contributed reagents, ideas/experimental design and reviewed the manuscript.

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