DNA polymerase ν gene expression influences fludarabine resistance in chronic lymphocytic leukemia independently of p53 status

Srdana Grgurevic,[1]* Patricia Montilla-Perez,[1] Alice Bradbury,[2] Julia Gilhodes,[3] Sophie Queille,[1] Sandrine Pelofy,[4] Aurélien Bancaud,[4] Thomas Filleron,[3] Loïc Ysebaert,[5] Christian Récher,[5] Guy Laurent,[5] Jean-Jacques Fournié,[1] Christophe Cazaux,[1,†] Anne Quillet-Mary[1] and Jean-Sébastien Hoffmann[1]*

[1]CRCT, Université de Toulouse, Inserm, CNRS, UPS, France; Equipe Labellisée Ligue Contre le Cancer, Laboratoire d'Excellence Toulouse Cancer, France; [2]University of Newcastle, UK; [3]Clinical Trials Office - Biostatistics Unit, Institute Claudius Regaud, Institute Universitaire du Cancer Toulouse-Oncopole (IUCT-O), Toulouse, France; [4]LAAS, Toulouse, France and [5]Department of Hematology, Institut Universitaire du Cancer Toulouse-Oncopole, Toulouse, France

*SG and J-SH contributed equally to this work

† In memoriam

ABSTRACT

Alteration in the DNA replication, repair or recombination processes is a highly relevant mechanism of genomic instability. Despite genomic aberrations manifested in hematologic malignancies, such a defect as a source of biomarkers has been underexplored. Here, we investigated the prognostic value of expression of 82 genes involved in DNA replication-repair-recombination in a series of 99 patients with chronic lymphocytic leukemia without detectable 17p deletion or *TP53* mutation. We found that expression of the *POLN* gene, encoding the specialized DNA polymerase ν (Pol ν) correlates with time to relapse after first-line therapy with fludarabine. Moreover, we found that *POLN* was the only gene up-regulated in primary patients' lymphocytes when exposed *in vitro* to proliferative and pro-survival stimuli. By using two cell lines that were sequentially established from the same patient during the course of the disease and Pol ν knockout mouse embryonic fibroblasts, we reveal that high relative *POLN* expression is important for DNA synthesis and cell survival upon fludarabine treatment. These findings suggest that Pol ν could influence therapeutic resistance in chronic lymphocytic leukemia. (Patients' samples were obtained from the CLL 2007 FMP clinical trial registered at: *clinicaltrials.gov identifer: 00564512*).

Introduction

Chronic lymphocytic leukemia (CLL) is the most common hematologic malignancy in the Western hemisphere.[1] With a median age at diagnosis of 70 years, CLL is considered to be a disease of the elderly. The malignancy is characterized by the accumulation of mature B lymphocytes that carry B-cell markers, such as CD23, CD19 and CD20, along with a T-cell marker, CD5. Leukemic cells have a defective apoptosis pathway disabled by, among others, overexpression of Bcl-2 protein.[2] However, a prolonged life-span of leukemic B cells is not the only cause of disease progression. Several studies have shown that proliferation also plays an important role in the development and clinical course of CLL.[3] CLL cells proliferate in so-called proliferation centers (pseudofollicles) of the lymph nodes where they are provided anti-apoptotic and proliferative stimuli from other accessory cells, such as CD40 ligand from helper T lymphocytes or interleukin-2.[4] In contrast to the lymph node compartment, the majority of leukemic lymphocytes in the peripheral blood are arrested in the quiescent G0 or G1 phase of the cell cycle.[5]

CLL is a highly heterogeneous disease in terms of its clinical course and outcome.

Many patients diagnosed with CLL survive for several years without needing any treatment while others develop an aggressive form of the disease and require immediate therapeutic intervention. Apart from Binet and Rai staging, clinicians take into consideration several other biological characteristics, such as mutational status of the immunoglobulin heavy chain variable gene segment (*IGHV*) and genomic aberrations. Approximately 4 out of 5 CLL patients harbor at least one chromosomal aberration in their leukemic clone and these, likewise, have a clear clinical impact on patients' survival.[6] Recent studies have also revealed other genomic defects in CLL, including mutations in genes involved in RNA processing, such as *SF3B1*[7] and, in general, in B-cell development,[8] and mutations in non-coding regions that potentially affect the clinico-biological course of CLL.[9] Nevertheless, few of these markers are used in routine clinical practice and even fewer of them have been able to demonstrate any predictive value in terms of response to chemotherapy, such as 17p-deletion[6] and mutational status of the *TP53* gene.[10]

Genomic instability is a prominent source of genetic diversity within tumors, generating a different cell population that can be subject to selection in a given microenvironmental or therapeutic context. The past decade has seen a dramatic advance in our understanding of how genomic integrity is related to cancer and has revealed that replication stress in the S phase of the cell cycle is an important feature,[11,12] placing studies on replication stress at the forefront of cancer research.[13] The genomic instability in CLL may indicate the presence of underlying replication stress. In addition, the backbone of the standard, first-line treatment for CLL patients is fludarabine,[14] which leads to excessive replication stress by inhibiting ribonucleotide reductase, an enzyme involved in the production of precursors essential for DNA synthesis. In contrast to solid cancer, DNA replication defects as a source of markers in hematologic malignancies have been poorly

explored. It was only recently that we revealed the importance of the DNA replication checkpoint *CHEK1* as a predictor of survival in acute myeloid leukemia and resistance to therapy with cytarabine.[15]

Here, we hypothesized that mis-expression of DNA replication-repair-recombination (3R) genes could occur during CLL leukemogenesis and contribute to the evolution of the pathology. We reasoned that a modified DNA replication program might, likewise, have an impact on the response to fludarabine which targets several steps in chromosome duplication. We also speculated that specific 3R gene expression signatures would be relevant in the context of relapse, representing potential predictors of the patients' outcome. Our data show that, among more than 80 3R genes analyzed in a series of primary samples from 99 patients with CLL prior to treatment, only expression of the *POLN* gene encoding the specialized A-family DNA polymerase nu (Pol ν), with yet unknown function, can forecast the time to relapse after first-line therapy. We also demonstrate that relatively high *POLN* expression protects CLL cells against replicative stress caused by fludarabine by allowing efficient DNA replication in the presence of an unbalanced nucleotide pool.

Methods

Patients with chronic lymphocytic leukemia

RNA from patients' samples were obtained from the CLL 2007 FMP clinical trial (*clinicaltrials.gov identifier: 00564512*).[16] In accordance with the Declaration of Helsinki, informed consent to participation in the trial was obtained from each patient. Patients recruited for the trial were medically fit, previously untreated, diagnosed with Binet stage B or C CLL, under 65 years of age and without detected 17p deletion or *TP53* mutation. Patients were randomized into two treatment arms: fludarabine-cyclophosphamide-campath (FCCam) and fludarabine-cyclophosphamide-rituximab (FCR). The primary outcome, namely, progression-free survival at 36 months, was the same for both treatment arms (*P*=0.21). Relevant data regarding the cohort of patients are summarized in Table 1.

Cell lines and culture
MEF cells and culture

Primary mouse embryonic fibroblasts (MEF) were derived from e13.5 embryos with genotypes *Poln*^{+/+}, *Poln*^{+/-} and *Poln*^{-/-} (C57BL/6J strain). Exon 2 containing the initiation codon was deleted in the knockout allele. Primary MEF were cultured in medium containing a high concentration of glucose, glutamax-Dulbecco modified Eagle medium (Invitrogen), 15% Hyclone fetal bovine serum (Thermoscientific), non-essential amino acids, sodium pyruvate, modified Eagle medium vitamin solution, and penicillin/streptomycin (Invitrogen) and was maintained in air-tight containers filled with a gaseous mixture of 93% N_2, 5% CO_2 and 2% O_2 (Praxair) at 37°C. Immortalized MEF were cultured in medium containing a high concentration of glucose, glutamax-DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologics) and penicillin/streptomycin, and maintained in a humidified incubator in 5% CO_2 at 37°C.

Cell culture

MEC-1 and MEC-2 cell lines (DSMZ, Braunschweig, Germany) were cultured in Iscove modified Dulbecco medium with glutamax supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at a density of $5x10^5$ and $3x10^5$ cells/mL for

Table 1. Clinical characteristics of the chronic lymphocytic leukemia patients.

Characteristic	Category	N. (frequency, %)
Sex		
Female		30/99 (30)
Male		69/99 (70)
Age		
≤ or 57 years old		51/99 (52)
> 57 years		48/99 (48)
Binet stage		
B		78/99 (79)
C		21/99 (21)
IGHV		
Mutated		43/99 (43)
Unmutated		56/99 (57)
Cytogenetics		
Deletion 13q	Detected	52/99 (53)
	Not present	42/99 (42)
	Unknown	5/99 (5)
Deletion 11q	Detected	21/99 (21)
	Not present	78/99 (79)
	Unknown	-
Trisomy 12	Detected	13/99 (14)
	Not present	80/99 (80)
	Unknown	6/99 (6)

MEC-1 and MEC-2, respectively. MEF cell lines were grown in Dulbecco modified Eagle medium with glutamax supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at a density of 1.5×10^5 cells/10 cm dish. Primary peripheral blood mononuclear cells from CLL patients were cultured in Roswell Park Memorial Institute medium supplemented with 1% glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin at a density of 1×10^7 cells/mL allowing long-term viability.

DNA synthesis

DNA synthesis was monitored by a Click-iT EdU imaging assay (Invitrogen, Saint-Aubin, France). 5-ethynyl-2'-deoxyuridine (EdU) was added at the concentration of 25 μM for 30 minutes (min) as indicated. During DNA staining, cells were incubated with propidium iodide at a concentration of 50 μg/mL, 0.1% RNase A and 0.1% Triton X-100 in a phosphate-buffered saline solution for 10 min away from light.

DNA combing in nanochannels

Cells were incorporated into agarose plugs (200,000 cells/plug) and each experiment was carried out with half of an agarose plug deposited in a 1.5 mL tube filled with 800 μL of 0.5X TBE buffer supplemented with 2% poly-vinylpyrrolidone (40 kDa, below the overlapping concentration of 7%) and 5% dithiothreitol. Agarose was melted by heating the tube at 70°C for 15 min. The temperature was set at 42°C, and 2 μL of β-agarase (New England Biolabs) were added for overnight agarose digestion. Finally, DNA was stained with 2.5 μM fluorophore SYTOX-orange (Molecular Probes). Nanofluidic chips were fabricated using a two-step photolithography process to generate the array of nanochannels by projection lithography (ECI 1.2 μm photoresist, Stepper Canon 3000i4); these were then transferred into silicon by Reactive Ion Etching over a depth of 250 nm. Conventional photolithography was then performed to etch lateral microchannels of 20 μm in width and 7 μm in depth. For optical mapping, we used hydrodynamics to force the uptake of genomic DNA in nanochannels. Imaging was performed with a wide-field inverted Zeiss microscope equipped with a 40X lens (NA=1.4). The light source was a LED engine (Lumencor) with 542/33 nm emission with the filter sets Cy3-4040C (Semrock) for SYTOX-orange visualization. Images were collected with an Andor Zyla camera operating with a 2x2 binning (pixel size = 325 nm). The velocity of molecules was set at 200 ± 20 μm/s using a pressure source operating at 80-100 mbar. All images presented in the manuscript were filtered using the FFT bandpass filter implemented in ImageJ using minimum and maximum cut-offs of 3 and 40 pixels, then subtracting the background.

Statistical analysis

The patients' clinical data are summarized by frequency and percentage for categorical variables and by median and range for continuous variables. Time to progression was defined as the period from the first-line therapy to progression or last follow up (censored data) and estimated by the Kaplan-Meier method with 95% confidence intervals. The minimum *P*-value approach was used to dichotomize *POLN* expression,[17] which selects the threshold that best discriminates patients' outcomes. Selected values of the prognostic factor are examined as candidates for the threshold, after eliminating the top and bottom 10% of the extreme values. The value that best separates patients' outcomes according to a minimum *P*-value obtained by the log-rank test is chosen. The *P*-value is adjusted using the Altman correction to account for the problem of multiple testing. Stability of the threshold was assessed using bootstrap internal validation. Backward selection was performed with a Cox proportional hazards model to identify clinical factors

associated with time to progression. Multivariate analysis was also performed using a Cox proportional hazards model to study the influence of *POLN* on time to progression after adjusting for the clinical factors previously identified. Two-sided *P*-values of less than 0.05 were considered statistically significant. All statistical analyses were performed using STATA 12.0 software. Viability data obtained by the MTS assay were analyzed in GraphPad Prism using an ANOVA multiple test. The length of replicated domains assessed in the DNA combing in nanochannels experiments was analyzed in GraphPad Prism using the Mann-Whitney rank-sum *t*-test. FACS data were analyzed in GraphPad Prism using a paired or unpaired Student *t*-test depending on the experimental conditions.

Results

POLN determines time to progression in chronic lymphocytic leukemia

In order to investigate the implication of 3R genes in the clinical course of CLL, we first performed a large, high-throughput quantitative reverse transcriptase polymerase chain reaction-based gene expression analysis on 99 primary samples obtained from treatment-naïve patients included in the CLL 2007 FMP clinical trial (see above). The patient's baseline characteristics are summarized in Table 1. Unsupervised clustering on 3R gene expression values confirmed, as previously published,[18] clear underlying differences between samples from healthy donors and CLL lymphocytes (Figure 1A) and showed a significantly higher expression in CLL of *POLN*, the gene encoding for the specialized DNA polymerase ν (Figure 1B), as compared to most of the 3R genes, including other specialized DNA polymerases or genes involved in double-stranded break repair (*Online Supplementary Figure S1*). Next, we performed a cross-analysis on the gene expression information obtained and the patients' clinical data. The results of this analysis reveal that among all 3R genes, *POLN* was the only one linked to the therapeutic outcome of CLL. In univariate analysis, a high level of *POLN* expression was associated with a shorter time to relapse after first-line therapy, i.e. time to progression (threshold=11.9×10^{-2}, stability=47.42%; *P*=0.0009, adjusted *P*=0.0227), as shown in (Figure 1C). Interestingly, we found that *POLN* expression maintained a significant correlation with time to progression in multivariate analysis after adjustments using previously identified prognostic factors (Table 2). The adjusted Hazard Ratio (HR) was 4.14 with a 95% Confidence Interval of 1.60-10.72 and a *P*-value of 0.003, defining *POLN* expression as the strongest prognostic marker of time to progression after fludarabine-based treatment, independently of Binet stage or *IGHV* mutational status.

Proliferating chronic lymphocytic leukemia lymphocytes over-express *POLN*

Leukemic lymphocytes circulating in the CLL patients' peripheral blood are arrested in the G0 and G1 phases of the cell cycle.[5] To proliferate, CLL lymphocytes need to receive proliferative and pro-survival stimuli released by the accessory cells residing in the lymph node pseudofollicules.[4] In order to investigate CLL replication parameters, we mimicked the proliferative lymph node microenvironment by stimulating primary CLL lymphocytes with interleukin-2 and DSP30. After obtaining proliferating leukemic cells, as confirmed by the CFSE dilution assay

(Figure 2A and *Online Supplementary Figure S2*), we analyzed the full 3R gene expression profile of non-cycling and cycling purified CLL lymphocytes. Our data revealed that actively dividing primary CLL cells modified expression of only 11 out of a total of 82 3R genes (Figure 2B). Ten of these 11 3R genes were down-regulated, including genes involved in DNA damage response, such as *FANCD2, PRKDC, SIN3B, TIMELESS* and *LIG3*, and several genes implicated in global DNA replication, e.g. *SHPRH, CDC25B, CHTF18, POLE* and *MCM9*. In contrast, the genes encoding factors of replication origin licensing and firing, such as *CDC6, CDT1,* and *GINS4,* remained stable (Figure 2B). Interestingly, *POLN* was the only 3R gene found to be up-regulated in proliferating CLL cells. This finding led us to postulate that *POLN* might contribute, from the origin, to disease evolution, namely, in the proliferating leukemic lymphocyte.

POLN expression increases during disease progression according to a cellular model

MEC-1 and MEC-2 are two CLL cell lines that were established from the same CLL patient sequentially during the course of the disease.[19] More precisely, the MEC-1 cell line was established during an early stage of the disease, while the MEC-2 cell line was established later when the patient developed a more aggressive form of the disease. Based on the clinical characteristics of the patient at the time of sampling, as well as the cellular immunophenotype, it is considered that these two cell lines reflect the clinical progression of CLL.[20] In order to employ this cellular model in our further investigations, we first decided to analyze the 3R gene expression profiles of the two cell lines. This analysis revealed that the two cell models share a high level of similarity in their 3R gene profiles as they differ in the expression levels of only 6 3R genes (Figure 3).
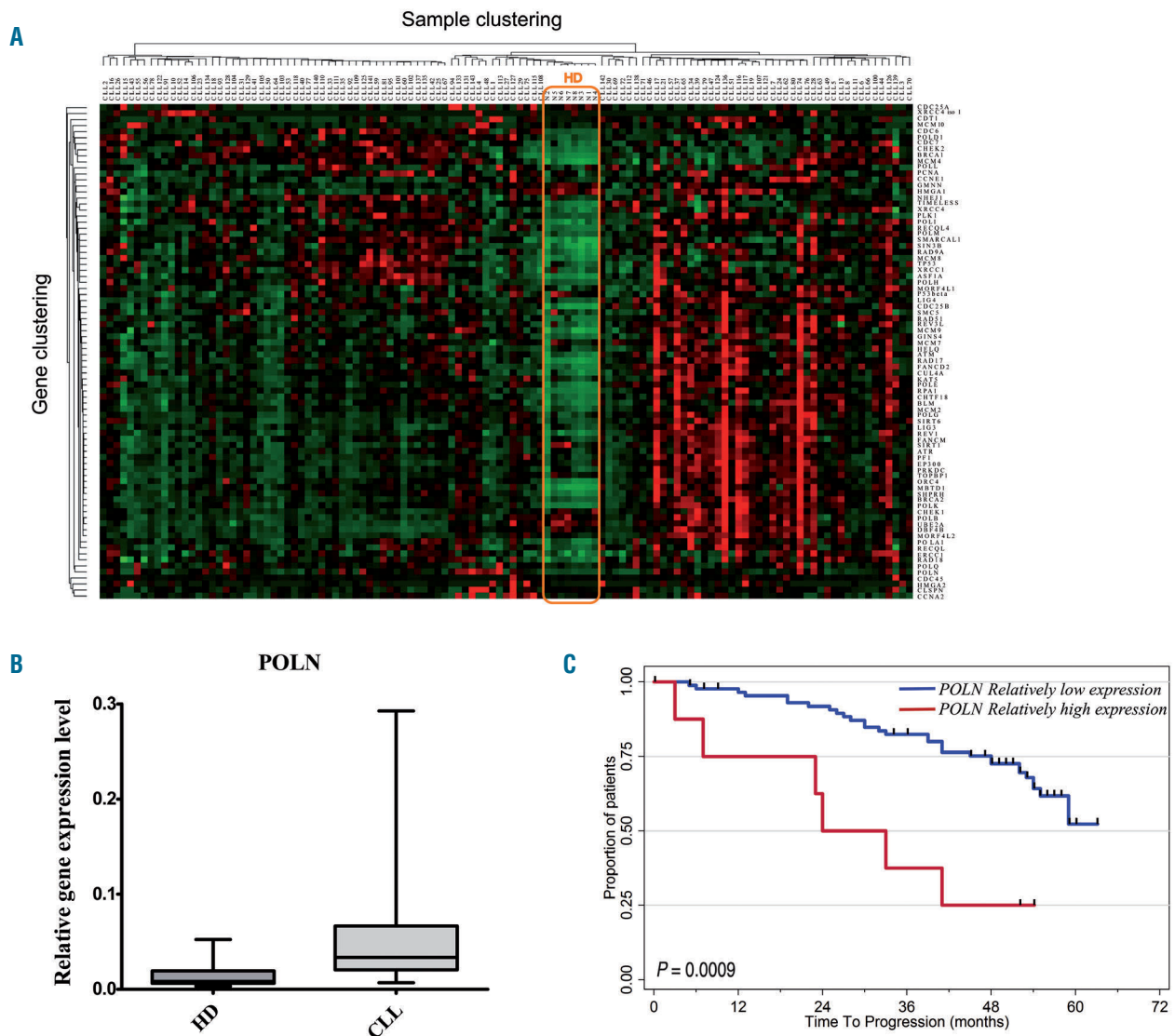

Figure 1. *POLN* gene expression as an independent prognostic marker of time to progression in chronic lymphocytic leukemia (CLL). (A) Unsupervised clustering of CLL and healthy donor (HD) samples based on 3R gene expression data. (B) *POLN* gene expression in CLL primary samples in comparison to HD samples. (C) Kaplan-Meier graphical estimates of time to progression (TTP) according to *POLN* expression level. The cut-off between low and high expression is 11.9×10^{-2}. The expression number ranges in under-expressed and over-expressed groups are 0.008-0.118 and 0.119-0.272, respectively.

These include the β isoform of the tumor suppressor *TP53* β and the *LIG4* gene coding for a protein required in double-stranded break repair through the non-homologous end joining pathway, which were up-regulated by the MEC-2 subclone, along with *MCM10* and *GINS4*, coding for factors implicated in initiation of replication and *POLH*, a translesion synthesis DNA polymerase gene, which were slightly down-regulated (less than 2-fold) in the MEC-2 subclone. Strikingly, with more than a 5-fold higher expression level in the aggressive MEC-2 subclone, *POLN* was the gene we found to be most deregulated between the two CLL cell lines (Figure 3). These results suggest that *POLN* could be an advantageous trait for the CLL clone and could be selected during the evolution of the disease.

Relatively higher *POLN* expression contributes to fludarabine chemoresistance

We next evaluated the sensitivity of the MEC-1 and MEC-2 cell lines in the presence of fludarabine. Cells were treated with different doses of the nucleoside analog for 24 h and cellular viability was measured at the end of the treatment period. MTS data analysis showed that the MEC-2 cell line was more chemoresistant than the MEC-1 cell line, as indicated by higher cellular viability and fludarabine EC_{50} values (239.2±2.16 μM for MEC-1 and 433.3±2.38 μM for MEC-2) (Figure 4A).

In order to gain deeper insight into the molecular mechanism by which MEC-2 cells maintained higher survival than MEC-1 in the presence of fludarabine, we next evaluated DNA replication efficiency in these two cell lines upon 2 hours (h) of treatment with fludarabine at the doses of 180 μM and 600 μM. The nucleoside analog EdU was added at the end of the treatment to visualize DNA synthesis and cells were stained with propidium iodide. When normalized to untreated conditions, flow cytometry cell cycle analysis showed a higher percentage of EdU-positive cells among the chemoresistant MEC-2 cells (Figure 4B and *Online Supplementary Figures S3 and S4*), suggesting that

Table 2. Multivariate analysis of time to progression by Cox proportional hazard models.

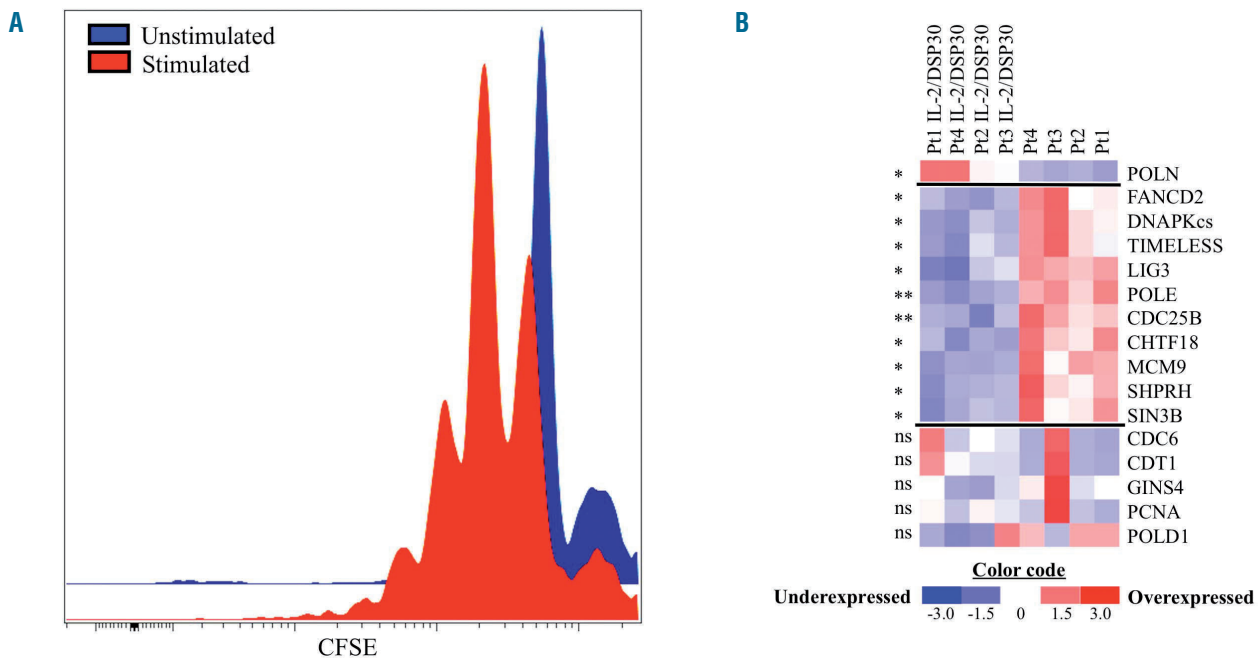|	Parameter	Number	Hazard Ratio (HR)	95% HR Confidence limits	P
POLN	High expression	90	4.14	1.60-10.72	0.003
	Low expression	9	1 (base)		
IGHV status	Unmutated	56	2.04	1.02-4.12	0.045
	Mutated	43	1 (base)		
Gender	Male	69	2.11	0.92-4.83	0.078
	Female	30	1 (base)		
BINET stage	C	21	1.61	0.76-3.42	0.214
	B	78	1 (base)		

Figure 2. Proliferation and 3R gene expression profiles of primary sample from patients with chronic lymphocytic leukemia (CLL). Peripheral mononuclear cells from CLL patients were cultured *in vitro* in the absence or presence of proliferation stimuli (interleukin-2/DSP30) for six days. (A) CFSE-labeled unstimulated/non-proliferating and stimulated/proliferating primary CLL lymphocytes. (B) Unsupervised clustering according to 3R gene expression in non-proliferating and proliferating purified CLL cells. Out of 82 3R genes, only one gene was up-regulated upon proliferation (*POLN*), 10 genes were down-regulated and the other 71 genes remained stably expressed.

MEC-2 cells were able to sustain DNA synthesis more efficiently than the chemosensitive MEC-1 cells in the presence of the drug.

To test this hypothesis in more detail, we evaluated DNA replication parameters in both cell lines upon fludarabine treatment by using an innovative genome-wide DNA combing nanofluidics-technology developed recently in our laboratory.[21] By applying differential pressure between the entry and exit microchannels, individual DNA molecules are directed into and stretched on their way through the nanochannels. The strategy allows us to monitor the length of the *in cellulo*-replicated domains at the level of individual replicating DNA molecules. The results of this analysis showed that treating chemosensitive MEC-1 cells with fludarabine reduced the length of the replicated domains. In contrast, the size of the replicated domains in MEC-2 cells was not decreased upon fludarabine treatment in comparison to the untreated condition. Track length data (scatter and bar plots), representative photographs of tracks and their quantified histograms are presented in Figure 4C.

As we found that *POLN* expression is greater in the MEC-2 cell line than in the MEC-1 cell line, and since we observed relatively higher *POLN* expression to be a marker of negative patients' outcome after fludarabine-based first-line therapy in our CLL cohort, we hypothesized that relatively higher *POLN* expression might contribute to enhanced DNA replication ability and cell survival upon fludarabine treatment. To test this, we used independent MEF expressing normal (MEF$^{+/+}$) and relatively low (MEF$^{+/-}$) levels of *POLN*, as well as knockout *POLN* MEF (MEF$^{-/-}$) (*Online Supplementary Figure S5A*). These cell lines were treated with fludarabine for 4 h, then exposed to EdU for 30 min and stained with propidium iodide. DNA synthesis efficiency was then assessed by flow cytometry. Analysis of cell cycle and quantification of the proportion of EdU-positive cells confirmed that POLN-proficient cells (MEF$^{+/+}$) could indeed sustain DNA synthesis more efficiently than MEF$^{+/-}$ or MEF$^{-/-}$ cells in the presence of fludarabine (Figure 5A and *Online Supplementary Figures S4 and S5B*). We did not observe any significant differences in fludarabine-mediated DNA replication inhibition between the MEF$^{-/-}$ and MEF$^{+/-}$ cell lines (*Online Supplementary Figure S5B*). This might be due to the poor expression of *POLN* in mouse fibroblasts, and consequently a non-significant differential expression between heterozygous and knock-out cells (*Online Supplementary Figure S5A*). Collectively, these results suggest that relatively high expression of *POLN* could contribute to higher replication efficiency and sustained cell survival upon fludarabine treatment.

POLN-mediated resistance to fludarabine is related to dNTP pool starvation

In order to investigate whether *POLN* could play a role in limiting replication stress specifically upon decrease of dNTP, we used the ribonucleotide-reductase inhibitor hydroxyurea, which mimics the effect of fludarabine on the dNTP pool. MEF$^{+/+}$ and MEF$^{+/-}$ cells were treated with different concentrations of hydroxyurea for 4 h and pulsed with EdU for 30 min at the end of the treatment. DNA synthesis profiles were analyzed by flow cytometry, schematically represented in *Online Supplementary Figure S7*. We observed that the *POLN*-expressing MEF$^{+/+}$ cells sustained DNA synthesis upon hydroxyurea treatment more efficiently than their *POLN*-deficient MEF$^{+/-}$ counterparts (Figure 5B and *Online Supplementary Figures S8 and S9*), implying that Pol ν

could limit replication stress caused by fludarabine in the condition of dNTP starvation.

In order to confirm that fludarabine chemoresistance driven by *POLN* was specifically related to the condition of a reduced dNTP pool, we performed a rescue experiment by supplementing MEF cells with deoxynucleosides in the presence of fludarabine. MEF$^{+/+}$ and MEF$^{+/-}$ cells were treated with a lower concentration of fludarabine (30 µM) for a longer period (8 h) in order to allow rescue of DNA synthesis with deoxynucleosides, which were added during the last 4 h of treatment. Finally, cells were pulsed with EdU for the last 30 min of the incubation period. Flow cytometry analysis showed no significant impact on the DNA synthesis rate in either cell line when the deoxynucleosides were added in the absence of fludarabine. As expected, in the absence of supplemental deoxynucleosides, inhibition of DNA synthesis by fludarabine was more pronounced in the MEF$^{+/-}$ cell line than in the MEF$^{+/+}$ cell line (Figure 5C and *Online Supplementary Figures S10 and S11*). Importantly, we found that addition of deoxynucleosides could rescue DNA synthesis in the *POLN*$^{+/-}$ cell line abolishing the difference between two MEF cell lines (Figure 5C and *Online Supplementary Figures S10 and S11*). This result confirms that *POLN* reduces the impact of dNTP starvation on DNA synthesis. Collectively, these data suggest that a relatively high level of *POLN* might contribute to cell survival and provide advantages, not only upon endogenous replicative stress related to a pathology-associated dNTP pool imbalance, but also when CLL cells are treated with therapeutic agents that further affect dNTP metabolism, such as fludarabine.

Discussion

In our study, we report that peripheral blood leukemic lymphocytes from CLL patients express *POLN* at a relatively high level and that the relative expression of *POLN* is a
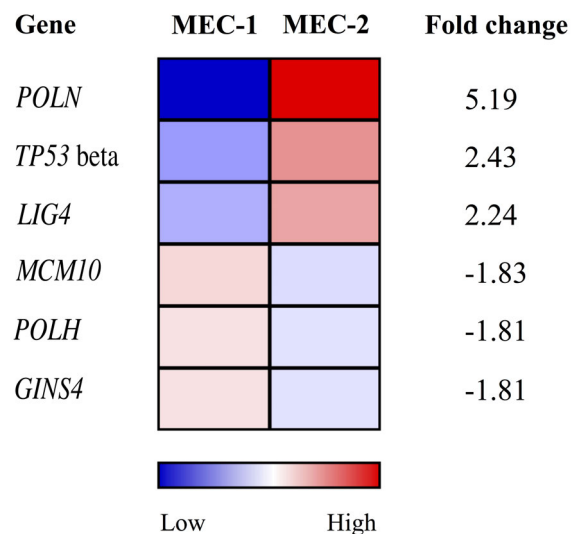
Gene	MEC-1	MEC-2	Fold change
POLN			5.19
TP53 beta			2.43
LIG4			2.24
MCM10			-1.83
POLH			-1.81
GINS4			-1.81

Low   High

Figure 3. 3R gene expression profile change during clinical progression of chronic lymphocytic leukemia (CLL) based on a CLL cellular model. MEC-1 and MEC-2 cell lines were established sequentially from leukemic lymphocytes obtained from the same CLL patient. MEC-1 was isolated at the beginning of the disease while MEC-2 was isolated at the onset of active disease and represents a more aggressive CLL subclone. Expression fold change for each 3R gene was calculated using the comparative Ct method.
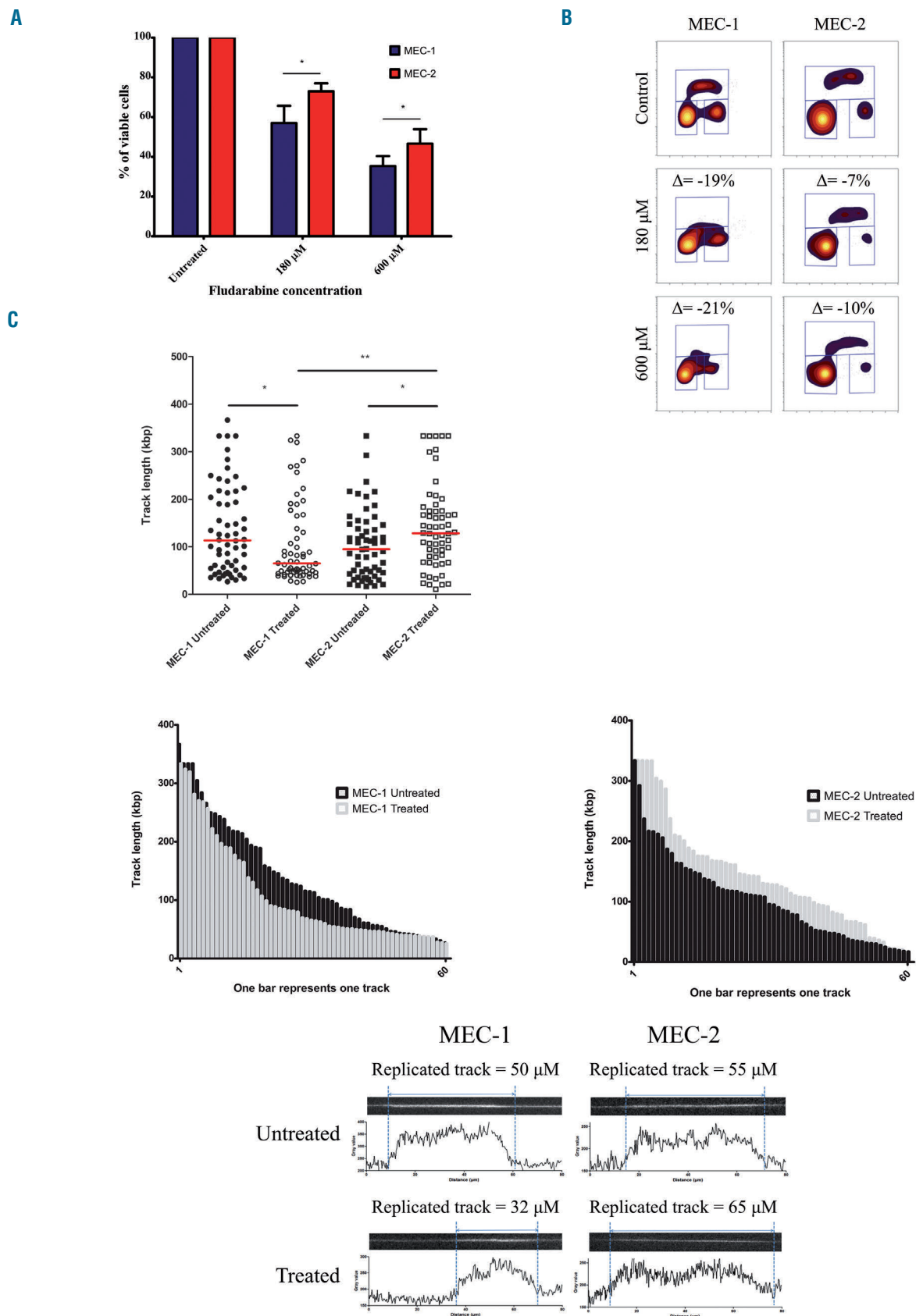
Figure 4. *POLN and fludarabine chemoresistance in vitro.* (A) MTS viability assay of MEC-1 and MEC-2 cell lines in the absence or presence of fludarabine for a treatment period of 24 hours (h). The percentage of viable cells is expressed as the mean ± Standard Deviation of three independent experiments. The fludarabine EC_{50} value for the MEC-1 cell line was 239 μM and that for the MEC-2 cell line was 433 μM. (B) Flow cytometry cell cycle analysis of MEC-1 and MEC-2 cell lines, control and treated with fludarabine for 2 h. Delta (Δ) is calculated as the difference of percent of cells present in the EdU+ gate by control condition (representative of three experiments). (C) DNA combing in nanochannels. The length of replicated domains is expressed in kilobase pairs (kbp). The number of tracks measured in all conditions was 60. The concentration of fludarabine used was 600 μM; the duration of treatment was 4 h. Medians in the dot plot are indicated by red lines.

marker determining CLL patients' outcome and fludarabine chemoresistance. By stimulating *in vitro* proliferation of primary CLL samples, we also revealed that *POLN* is the only up-regulated 3R gene among 82 genes analyzed, suggesting that higher expression of *POLN* could be important for CLL proliferation and progression, independently of external treatment. In an independent approach, using two cell lines established sequentially from the same CLL patient which reflected clinical progression of the disease, we found that the most striking difference between the two cell lines was, again, the relatively enhanced expression of *POLN* in the more aggressive CLL clone. Cell survival and DNA synthesis experiments with CLL cell lines and murine homozygous, heterozygous and knockout MEF for *POLN* demonstrated that relatively high expression of *POLN* conferred higher viability following nucleotide pool starvation by fludarabine, the backbone of therapeutic treatment in CLL. The link between fludarabine resistance and expression of *POLN* was further confirmed in the high *POLN*-expressing CLL cells by showing that 30% *POLN* deple-

tion by a small interfering RNA in these cells resulted in a mild but significant decrease in fludarabine sensitivity (*data not shown*). Collectively, these results suggest a possible role of *POLN* in the natural course of CLL and in cell survival upon external stress affecting the dNTP pool balance. This led us to reason that these two mechanisms might be mutually associated as endogenous replicative stress frequently results in dNTP pool starvation when replication origins are over-used. Upon nucleotide starvation by fludarabine, a high *POLN* level allowed globally higher DNA replication efficiency and, conversely, *POLN* depletion led to increased perturbation of replication dynamics. Importantly, rescue experiments by supplementing *POLN*-depleted cells with deoxyribonucleosides in the presence of fludarabine showed a complete restoration of the rate of DNA synthesis.

POLN encodes a low fidelity A-family Pol ν, whose cellular role still remains elusive although some repair activity has been proposed.[22,23] Taking into consideration the high sequence homology between Pol ν and the polymerase
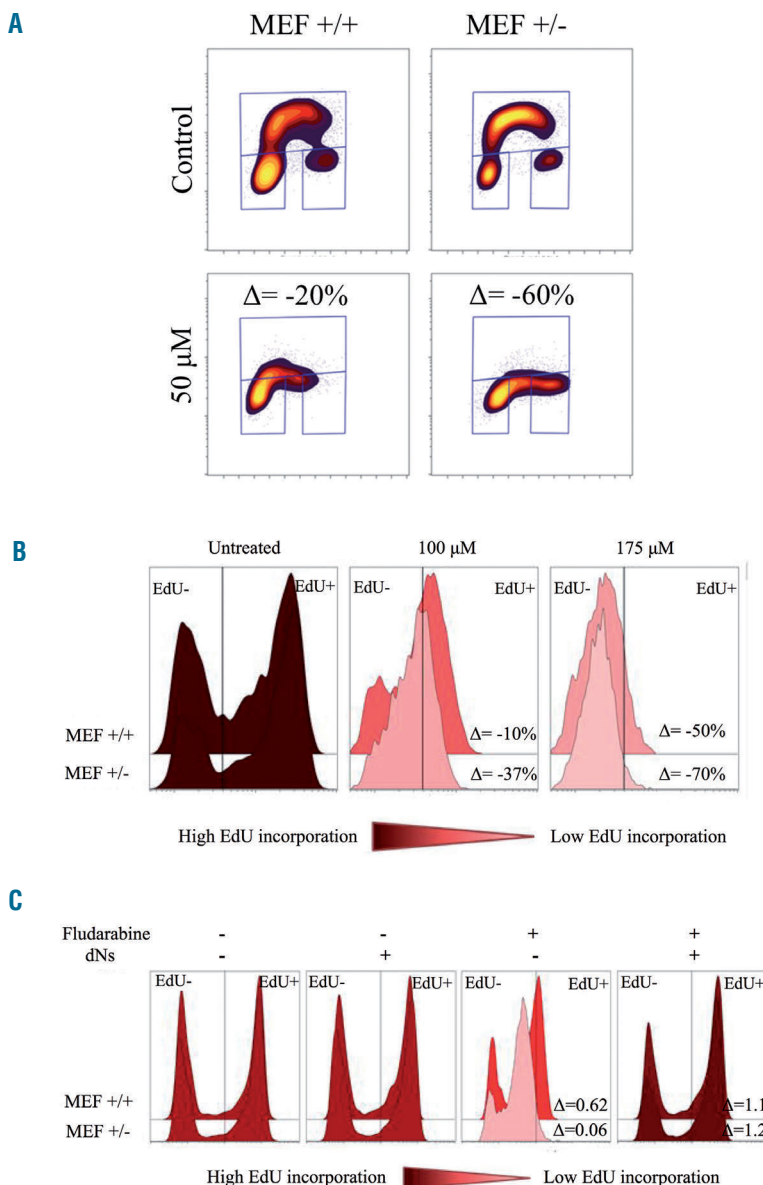

Figure 5. *POLN* **and DNA synthesis in the presence of replicative stress due to a limited dNTP pool.** (A) Flow cytometry cell cycle analysis of MEF *POLN*^{+/+} and MEF POLN^{+/-} cell lines in the control condition or treated with fludarabine for 4 hours (h). Delta (Δ) is calculated as the difference of percent of cells present in the EdU[+] gate by control condition (representative of 3 experiments). (B) Flow cytometry DNA synthesis analysis of MEF POLN^{+/+} and MEF POLN^{+/-} cell lines, control and treated with hydroxyurea for 4 h. Delta (Δ) is calculated as difference of percent of cells present in the EdU[+] gate by control condition, (representative of 3 experiments). (C) Flow cytometry DNA synthesis analysis of MEF POLN^{+/+} and MEF POLN^{+/-} cell lines, control and treated with fludarabine for 8 h at 30 μM concentration and supplemented or not with deoxynucleosides (dNs). Delta (Δ) is calculated as a fold change of EdU incorporation by control condition (representative of 3 experiments).

domain Pol θ, whose function in DNA double-stranded break repair has been more thoroughly established (reviewed by Wood and Doubliè[24]), one could suppose that Pol ν might play partially similar roles as its paralog. Enhanced repair of double-stranded breaks by an excess of Pol ν in CLL might contribute to maintaining cell survival in managing double-stranded breaks occurring at stalled replication forks upon endogenous replication stress and fludarabine treatment.

We recently reported that, besides its documented microhomology-mediated end joining repair activity, Pol θ is capable of regulating the activity of replication origins by interacting with replication origin licensing factors and regulating the timing of replication initiation.[25] Whether Pol ν could also regulate origin activity will be another issue to explore in the future since it may also give an alternative mechanistic basis for adaptive response to replication stress.

During disease pathogenesis, CLL leukemic cells succumb to an underlying level of replication stress. This fact is evidenced by the presence of markers of genomic instability, e.g. recurrent chromosomal abnormalities and common somatic mutations. We could hypothesize that, for the CLL cell entering the cell cycle and starting division inside the lymph node pseudofollicule, enhanced expression of *POLN* could be an adaptive mechanism to limit replication stress caused by a suddenly elevated requirement for dNTP. Our study suggests that the MEC-2 cell line, which shows characteristics of a CLL subclone that has recently exited the lymph node, expresses a higher level of *POLN*, allowing leukemic cells to surpass replication stress imposed by treatment with fludarabine. In this context, we could hypothesize that enhanced expression of *POLN* could be a characteristic acquired in the lymph node as an adaptive mechanism to endogenous replication stress and an advantageous trait once the leukemic cell has experienced treatment with fludarabine.

In conclusion, the A-family DNA polymerases, whose enhanced expression is observed frequently in solid cancers and, as we reveal here for the first time, in hematologic neoplasia, could be considered as a response to replication stress, contributing to both cancer progression and therapeutic resistance, which makes these enzymes attractive targets for future anti-cancer therapies.

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