DNA transactions, including replication, repair of damaged DNA and recombination (the so-called ‘3Rs’) are crucial processes required for preserving genome integrity and limiting cancer risk. Our previous large-scale studies performed in solid cancers displaying major genetic instabilities, demonstrated that specific misexpression of DNA replication genes could explain cancer-associated genetic changes and predict patients’ outcomes; the overexpression of the unconventional DNA polymerase POLQ being, for instance, associated with poor overall survival in breast and non-small cell lung cancer.

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. Besides chromosome aberrations affecting karyotype integrity, CLL genomes are characterized by elevated frequency of nucleotide point mutations. These different manifestations of genetic instability are not mutually exclusive and may be mechanistically confined to a common pathogenic pathway, which has never been deeply explored.

The clinical course of CLL is highly heterogeneous, ranging from indolent and asymptomatic forms that can remain ‘benign’ for as long as a decade, to more aggressive disease requiring immediate chemotherapeutic treatment. Estimating the course of the disease development at the diagnostic stage has historically relied on clinical staging systems, whereas the mutational status of the immunoglobulin heavy variable gene (IGHV), fluorescence in situ hybridization cytogenetics and presence of common somatic mutations contributed to refining of the prognostication later on. However, the former indicators do not fully explain the heterogeneity of the disease evolution before the therapeutic treatment.

Essential DNA replication processes have, surprisingly, remained an underexplored source of biomarkers and anti-cancer targets for hematological neoplasms, especially CLL, probably because such an explorative approach was not intuitive for this malignancy. Indeed, CLL has been characterized by accumulation of malignant cells resting in quiescent, mostly G0 and early G1 phase of the cell cycle. In contrast to the non-proliferative peripheral blood compartment that is used for diagnostic purposes, a small actively proliferating fraction of CLL cells residing within the lymph nodes contributes to the daily generation of the leukemic clone. However, according to the international ethical guidelines applied, samples originating from the lymph nodes are not always readily available.

Here, we reasoned that the aberrant 3R in CLL and associated occurrence of the ‘replicative stress’ could drive a force of chromosomal instability could represent a major source of genomic variability during the proliferation stages of CLL cells, consequently motoring the heterogeneous evolution of the disease, and could be used as a tool to understand the dynamics of evolution and the clinical outcomes of CLL patients.

CLL patient samples at diagnosis (n = 141) and healthy donor controls (n = 10) were obtained following an informed consent and in accordance with the Declaration of Helsinki. Patients were diagnosed with CLL according to standard clinical and laboratory criteria. Relevant clinical information regarding the cohort is summarized in Table 1. The cohort recapitulates already established correlations between known biological and clinical parameters (P values < 0.05) (Supplementary Figure S1). Purification of B cells from CLL and healthy donors’ samples was performed by an immunomagnetic selection. Purity and viability of B-lymphocyte population was assayed by flow cytometry. RNA extraction and its quality assessment preceded complimentary DNA synthesis.

Gene expression assay was based on a custom selection of 94 probes including, among others, 3 known diagnostic markers, 1 proliferation marker and 82 3R genes (Supplementary Table S1). Detailed sample preparation and gene expression normalization are described in Supplementary Materials and Methods.

Differential gene expression was determined by performing binomial test for CLL vs healthy donor samples and the Mann–Whitney test was used to compare different CLL subgroups (Supplementary Figure S2). Prognostic value of a candidate gene was determined by Kaplan–Meier estimates, whereas marker independence was assessed by the multivariable Cox regression analysis.

We first investigated the expression of 82 genes involved in 3R DNA transactions, that is, initiation of DNA replication (firing and licensing of 50 000 human replication origins), elongation and maintenance of stability of DNA replication forks, signaling and excision/recombination repair of DNA damage, in CLL B lymphocytes compared to healthy B lymphocyte controls. In unsupervised

| Characteristic | Category | n (frequency, %) |
|---------------|----------|-----------------|
| Sex           | Female   | 53/136 (39)     |
|               | Male     | 83/136 (61)     |
|               | Unknown  | 5/141 (4)       |
| Age           | Younger than 65 years or 65 years old | 66/137 (48) |
|               | Older than 65 years | 71/137 (52)    |
|               | Unknown  | 4/141 (3)       |
| Binet stage   | A        | 52/134 (39)     |
|               | B        | 54/134 (40)     |
|               | C        | 28/134 (21)     |
|               | Unknown  | 7/141 (5)       |
| IGHV          | Mutated  | 48/128 (37)     |
|               | Unmutated| 80/128 (63)     |
|               | Unknown  | 13/141 (9)      |
| Cygenetics    | Deletion 13q | 41/113 (36)   |
|               | Unknown  | 28/141 (20)     |
|               | Trisomy 12 | 29/112 (26)    |
|               | Unknown  | 29/141 (21)     |
|               | Deletion 11q | 23/137 (17)   |
|               | Unknown  | 4/141 (3)       |
|               | Deletion 17p | 15/137 (11)   |
|               | Unknown  | 4/141 (3)       |
|               | Deletion 6q | 8/112 (7)      |
|               | Unknown  | 29/141 (20)     |
|               | Complex karyotype (> 3 abnormalities) | 29/112 (26) |
|               | Unknown  | 29/141 (20)     |
| TP53          | Mutated  | 7/98 (7)        |
|               | Unknown  | 43/141 (31)     |
| NOTCH1        | Mutated  | 19/96 (20)      |
|               | Unknown  | 45/141 (32)     |
| SF3B1         | Mutated  | 4/96 (4)        |
|               | Unknown  | 45/141 (32)     |

Abbreviation: IGHV, immunoglobulin heavy variable.
analysis, CLL samples showed a distinct 3R profile and clustered apart from healthy donor CD19+ cells (Figure 1a). Clinical characteristics of CLL clusters are detailed in Supplementary Figure S3. Differential expression data of individual genes deregulated in CLL vs healthy donors are represented in Figure 1b and Supplementary Table S2 (all P values < 0.001). A limited overview of the CLL replisome is illustrated in Figure 1c. Our data showed that genes coding for proteins implicated in the firing of the replication origins, namely CDC7, DBF4B and MCM4, were overexpressed in CLL. Interestingly CDC7 inhibition has been previously proposed as a therapeutic strategy to target CLL. The overexpression of MCM4, on the other hand, could indicate a proliferative potential of neoplastic CLL lymphocytes residing in an early 'in-cycle' G1 state, as it was already shown to be the case with MCM2 expression. Among genes involved in progression and stability of replication forks, PCNA and a component of its alternative loading factor DSSC1, as well as the DNA helicase MCM8, lagging-strand replicative DNA polymerase POLD1 and ASF1A, a component of the replication-dependent chromatin assembly, were upregulated in CLL lymphocytes. Regarding genes implicated in DNA repair processes, HMGA1, known to influence repair of DNA lesions, was downregulated in CLL, whereas XRCC4, functioning in double-strand break repair and the RECQL helicase were overexpressed. Among specialized translesional/repair DNA polymerases, POLH, POLI, POLM and POLN were overexpressed in CLL cells in comparison to healthy donor B lymphocytes. Unlike aforementioned DNA polymerases, POLB was downregulated in CLL, as well as UBE2A gene coding for ubiquitin-conjugating enzyme involved in the post-translational modification of replication enzymes and post-replicative DNA repair synthesis opposite of the DNA damage site.

With the exception of the DNA checkpoint gene, CHEK1, which was downregulated in CLL cells in comparison to healthy donor lymphocytes, most of the DNA damage response genes were overexpressed. These included genes encoding: BRCA1 that functions in replication checkpoint, maintenance of stalled replication forks and homologous recombination repair, single-stranded DNA binding protein RPA1, beta isoform of TP53, as well as TIMELESS and SMARCAL1, both of which are involved in the stabilization of DNA replication forks. The up-regulation of replication origin and DNA damage response genes in CLL may be a general adaptive response to chronic replication stress, activating replication origins to compensate for DNA replication fork stalling and inducing DNA repair to cope with chromosomal breakage.

Next, we investigated whether 3R gene expression signatures correlated to clinical features of CLL patients. Among available clinical parameters of interest (namely, time from diagnosis to initial therapy (TFT) and progression-free survival), we found that...
gene expression level of the replicative histone chaperone ASF1A could define TFT. Indeed, patients with low levels of ASF1A had a shorter treatment-free survival (median TTF was 12 months) than patients with intermediate and high ASF1A levels (median TTF was 30 months) (Figure 1d). Importantly, unlike other existing prognostic markers, ASF1A expression could determine TTF independently of already established clinical parameters (age, Binet staging, IGHV, 17p-, and so on) as revealed by the Cox multivariable analysis (Figure 1e, P < 0.05). Clinical characteristics of ASF1A subgroups of CLL patients are further detailed in Supplementary Figure S4.

Histone chaperone ASF1A interacts with replicative helicase complex MCM2-7, which unwinds the double DNA helix to allow DNA polymerases to replicate single-stranded DNA and coordinates histone supply concomitantly to the course of the replication forks. Through buffering of the histone pool, governing histone post-translational modifications and directing their deposition on the chromatin, particularly in challenging conditions such as in presence of DNA replicative stress, ASF1A has a role in reestablishing the chromatin structure, and thereby, helps maintaining the chromosomal integrity and global genome stability. Strikingly, histone managing driven by ASF1 has recently been implicated in the process of alternative telomere lengthening, which is readily detectable in CLL leukemic cells. Because relative telomere length has been proposed as a marker indicative for TFT, whereas arbitrarily defined telomere dysfunctions can predict CLL progression, whether ASF1A expression can ensure stable telomere length in CLL and reduce the risk of leukemic evolution remains an open question that needs to be further investigated.

3R gene products act as regulators of DNA metabolic processes that are essential for the stable preservation of genetic identity of every cell. CLL is a hematologic disease characterized by genetic instability that can perturb controlled cellular division and motor development of cancer. Here, we investigated 3R profiles of this seemingly indolent disease and revealed strong deregulation of several genes having a role in DNA replication, repair and recombination. Finally, we show that in the 3R genetic background of CLL, ASF1A, which holds principal function in chromatin remodeling during DNA replication, is a novel and independent biomarker determining time to first treatment.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We would like to thank Statitec co. (Labège, France) for assisting in statistical analysis. We also thank La Ligue Nationale contre le Cancer that financially supported JSH’s laboratory (équipe labellisée) and program Le Laboratoire d’Excellence Toulouse-Cancer (TOU-CAN) for funding the project and financing SG’s fellowship.

AUTHOR CONTRIBUTIONS
SG: designed and performed the experiments, analyzed the data and drafted the manuscript. LB: performed part of cell purification and RNA extraction experiments. AQ-M: participated in analyzing the data and editing the manuscript. GL and CR: participated in editing the manuscript. YL: recruited patients, obtained clinical data, participated in editing the manuscript. JSH and CC: conceived the study and participated in drafting the manuscript.

Supplementary Information accompanies this paper on Blood Cancer Journal website (http://www.nature.com/bcj)

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