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ORIGINAL ARTICLE

Activation-induced deaminase and its splice variants associate with trisomy 12 in chronic lymphocytic leukemia

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Abstract

Activation-induced cytidine deaminase (AID) is a mutator enzyme essential for somatic hypermutation (SHM) and class switch recombination (CSR) during effective adaptive immune responses. Its aberrant expression and activity have been detected in lymphomas, leukemias, and solid tumors. In chronic lymphocytic leukemia (CLL) increased expression of alternatively spliced AID variants has been documented. We used real-time RT-PCR to quantify the expression of AID and its alternatively spliced transcripts (AIDΔE4a, AIDΔE4, AIDivs3, and AIDΔE3E4) in 149 CLL patients and correlated this expression to prognostic markers including recurrent chromosomal aberrations, the presence of complex karyotype, mutation status of the immunoglobulin heavy chain variable gene, and recurrent mutations. We report a previously unappreciated association between higher AID transcript levels and trisomy of chromosome 12. Functional analysis of AID splice variants revealed loss of their activity with respect to SHM, CSR, and induction of double-strand DNA breaks. In silico modeling provided insight into the molecular interactions and structural dynamics of wild-type AID and a shortened AID variant closely resembling AIDΔE4, confirming its loss-of-function phenotype.

Keywords Activation induced deaminase · Chronic lymphocytic leukemia · Complex karyotype · Alternative splicing · Trisomy 12

Abbreviations

AID Splice variants in CLL

Introduction

Chronic lymphocytic leukemia (CLL) is defined as the accumulation of clonal B cells in the peripheral blood (PB), bone marrow (BM), spleen, and lymph nodes (LNs). The disease manifests high clinical and biological heterogeneity and a number of biomarkers have been established to refine patient prognosis: adverse prognostic parameters include the unmutated status of the immunoglobulin heavy chain variable gene (UM-IGHV) [1, 2]; deletions of 17p13 (*TP53*) and 11q22-23 (*ATM*) [3]; mutations in *TP53*, *NOTCH1, BIRC,* and *SF3B1* genes [4]; and the presence of three or more cytogenetic aberrations (i.e., complex karyotype, CK) [5, 6]. CLL with intermediate prognosis often carries trisomy 12 (tri12) while favorable outcome is associated with a normal karyotype or deletion 13q14 present as the sole abnormality [3].

Activation-induced cytidine deaminase (AID) is a DNA mutating enzyme that plays a crucial role in the processes of somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin genes taking place in germinal

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center (GC) B cells during adaptive immune response [7, 8]. SHM enhances antibody affinity towards antigens while CSR diversifies antibody effector functions. AID converts cytosines to uracils in single-stranded DNA exposed during transcription with a preference for RGYW (R = purine, Y = pyrimidine, W = A + T) hotspot motifs [9, 10]. In contrast to the important role AID plays in adaptive immunity, deregulated AID expression and activity is linked to genomic instability, off-target mutations and oncogenic translocations occurring in B cell lymphomas, CLL, Ph-positive acute lymphoblastic leukemia, and chronic myeloid leukemia in a lymphoid blast crisis [11–17]. Aberrant AID expression has also been detected in some solid tumors associated with chronic inflammation, e.g., colorectal cancer, gastric cancer, pancreatic cancer, or hepatocellular carcinoma [18].

In healthy B cells, AID is expressed almost exclusively in germinal centers, and its transcription is tightly regulated [7, 19–23]. In CLL, similar to healthy B cells in germinal centers, the proportion of AID positive cells increases during proliferation [24, 25]. CLL cells enter proliferation centers engaging their CD40 receptor with CD40 ligand presented by Th2 cells and receive other signals which activate AID transcription. Nevertheless, only about 1% of these cells eventually express AID protein [24]. After CLL cells leave the stimulating microenvironment in secondary lymphoid organs and enter peripheral blood circulation, they downregulate AID expression. The highest fraction of PB CLL cells reported to maintain AID transcription was 0.2% [26]; nevertheless, even such a small amount of AID was reported to be associated with adverse survival in CLL patients [24].

The *AICDA* gene itself as well as two genes encoding positive regulators of AID expression, HoxC4 at position 12q13.13 and STAT6 at position 12q13.3, are located on chromosome 12; *AICDA* maps to position 12p13.31, and AID belongs to gene-dosage sensitive proteins [17, 27–29].

Human AID protein is encoded by the *AICDA* gene consisting of five exons, which are translated into a 198 amino acid protein. AID transcripts can undergo alternative splicing giving rise to four variants: AIDΔE4a (deletion of first 10 amino acids from exon 4), AIDΔE4 (deletion of exon 4 leading to a premature stop codon and C-terminal truncation of exons 4 and 5), AIDΔE3E4 (deletion of exons 3 and 4), and AID-ivs3 (retains third intron leading to C-terminal truncation due to a frameshift) (Fig. 1) [26, 30–32]. While GC B cells and memory cells express predominantly the fully functional full-length AID transcript (AID FL), CLL cells express a much higher percentage of alternatively spliced variants [32]. Wu and colleagues reported that AID variants AIDΔE4a and AIDΔE4 had higher mutation activity than standard full-length AID protein [32], which led to speculation that these variants might be implicated in CLL pathogenesis. However, others have shown that AID splice variants lack deaminase activity [31, 33] and their expression at the protein level is too low to be functionally relevant [34]. Our work presented here shows that even though AID splice variants are expressed in CLL cells obtained from peripheral blood, they do not retain function. This result is supported by in silico modeling of an AID variant encompassing amino acids 1–142, which is almost identical to the AID variant ΔE4.

Material and methods

Patients

The cohort included 149 patients with CLL diagnosed at the Department of Internal Medicine—Hematology and Oncology, University Hospital Brno between 1993 and 2014. The study was performed on peripheral blood samples taken with written informed consent provided in accordance

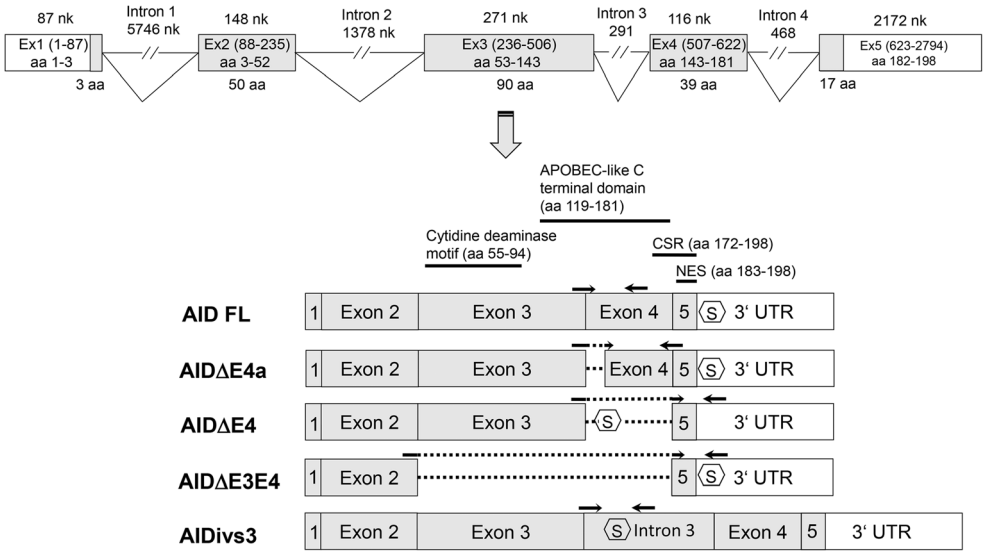

Fig. 1 Scheme of AID variants with positions of RT-PCR primers. Dashed lines (deletions), arrows (primers), gray (open reading frame, ORF), white (untranslated region, UTR), S (stop codon)

with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Tonsillar samples were taken during routine tonsilotomies or tonsillectomies with patients' written informed consent. CLL and control B cells were negatively separated by RosseteSep kits (StemCell). Separation efficiency was verified by flow-cytometry and all tested samples contained ≥ 98% B cells, which corresponded to at least 90% CLL cell purity. Patient samples were selected based on the presence or absence of complex karyotype defined as three or more cytogenetic aberrations. The following molecular characteristics were assessed: the presence of chromosomal aberrations using fluorescent in situ hybridization (FISH) and metaphase cytogenetics, mutational status of IGHV, and mutations in *TP53, SF3B1, NOTCH1,* and *ATM* genes.

Mice

AID knockout mice on a C57BL/6 background were a gift from Michel Nussenzweig (Rockefeller University). AID knockout mice or C57BL/6 controls (WT) between 8 and 12 weeks of age were used for experiments. The mice were bred and maintained under pathogen-free conditions. All animal studies were performed in compliance with the U.S. Department of Health and Human Services guidelines and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Functional analysis of AID splice variants

First, we isolated the individual AID splice variants AIDΔE4a, AIDΔE4, and AIDivs3 from peripheral blood samples of CLL patients and remaining variant AIDΔE3E4 from a tonsil sample, and cloned an open reading frame (ORF) of each variant into a retroviral vector Pmx containing a green fluorescence protein (GFP) marker. We tagged each variant with an N-terminal HA in order to verify protein expression by WB, considering that commercial AID antibodies do not detect all tested splice variants.

Follicular B cells from the spleens of AID knockout mice were purified and stimulated with a combination of lipopolysaccharide (LPS) and interleukin-4 (IL-4) known to induce CSR to IgG1. On the next day, we infected stimulated B cells with retroviruses encoding individual AID splice variants or with an empty vector as a negative control. We cultured the cells in the presence of LPS and IL-4 for an additional 2 to 3 days and then sorted GFP-positive cells. Human MAVER-1 cells were infected with retroviral particles containing individual AID variants. GFP-positive cells were sorted 6 days after infection, cultured for 6 weeks, and then sorted again.

Levels of AID expression were assessed using real-time PCR and western blots. CSR was quantified by flow cytometry, and double-strand breaks (DSBs) were analyzed by ligation-mediated PCR. The number of single nucleotide variants in the Ig Sμ region was assessed by ultra-deep next-generation sequencing (NGS), and the results were analyzed by deep-single nucleotide variant (SNV) algorithm.

Modeling of AID

We created a homology model of human AID using the Modeller 9.11 program [35] and the crystal structure of APOBEC3G (Protein Data Bank code 3IQS) as a template. AMBER package 14 [36] and the force field parm14SB were used for molecular dynamics simulations. We also prepared a shorter model comprising only residues 1–142, which mimics splicing variant AIDΔE4. Control simulations were carried out using a Langevin thermostat. The simulation length was 1 microsecond (μs) (for more details, see Supplementary material).

Statistical analyses

All statistical analyses were performed using R/Bioconductor environment. Two-sided tests were applied and a level of 0.05 was established as statistically significant. The Mann-Whitney *U* test and Kruskal-Wallis ANOVA were performed to clarify the relationships between continuous and categorical variables. Log-rank tests were applied to compare survival rates of different groups of patients, and Kaplan-Meier curves were used for visualizations.

For details on methods, see Supplementary material.

Results

The CLL cohort was selected to contain a comparable number of cases with complex karyotype (CK) (46%) and cases without complex karyotype (non-CK) (54%). Due to this selection, 76% of the samples harbored unmutated IGHV (UM-IGHV). Clinical and biological features of the patients are summarized in Table 1. In line with previous reports [6, 37], the presence of CK was associated with worse overall survival in our cohort ($P = 0.007$) (Suppl. Fig. 1a). Patients with CK had a median overall survival of 39.4 months from the time of sampling versus 62.5 months for the non-CK group. The median overall survival from diagnosis of UM-IGHV patients was 110 months versus 177 months for mutated IGHV (MUT-IGHV) cases ($P = 0.0037$) (Suppl. Fig. 1b).

Alternative AID transcripts were abundantly expressed in our CLL cohort

In order to examine the expression of AID and its splice variants in leukemic peripheral blood B cells from CLL patients, we designed a quantitative real-time RT-PCR assay specific

Table 1 Clinical and biological characteristics of CLL patients

Parameter	Total $n = 149$	(%)
Male vs female	98 vs 51	66 vs 34
Median age at diagnosis (years)	62	
Median age at sampling (years)	67	
Treated vs untreated patients	71 vs 78	48 vs 52
Patients alive 5 years from diagnosis	52	35
Patients alive 10 years from diagnosis	17	11
Complex karyotype yes vs no	69 vs 80	46 vs 54
IGHV unmutated vs mutated vs mixed[a]	113 vs 32 vs 4	76 vs 22 vs 3
Del17p yes vs no[b]	20 vs 129	13 vs 87
Del11q yes vs no[b]	44 vs 100	30 vs 70
Tri12 yes vs no[b]	22 vs 127	15 vs 85
Del13q14 yes vs no[b]	87 vs 62	58 vs 42
Normal karyotype yes vs no	14 vs 135	9 vs 91
Del17p[c]	36	24
Del11q[c]	37	25
Tri12[c]	17	11
Del13q14[c]	37	25
TP53 mutated from total assessed	34/149	23
Notch1 mutated from total assessed	15/99	15
SF3B1 mutated from total assessed	26/127	21
ATM mutated from total assessed	12/38	32

[a] CLL patients with mixed IGHV have both UM-IGHV and MUT-IGHV

[b] Regardless of other aberrations

[c] Hierarchical classification of CAs according to Dohner et al. [3]

for each AID transcript. A schematic layout of AID variants with primer positions is depicted in Fig. 1. There was a continuous distribution of AID expression levels on a log scale spanning a 1000-fold range (Suppl. Fig. 2) pointing not only to different AID transcript levels in the individual cells but likely also to a different proportion of AID-positive cells in each sample. Even though there was no clear cut-off value for AID positivity, we considered samples where 2 out of 3 replicates did not yield a specific PCR product as AID-negative (cycle threshold [Ct] value ≥ 35). cDNA from HEK293T cells not expressing AID was used as a negative control.

We found four out of the five AID variants to be frequently expressed in our cohort (Table 2). The variant missing catalytic exon 3, i.e., AIDΔE3E4, was expressed in the tonsil sample, but only rarely in CLL cells (4% of samples). Therefore, AIDΔE3E4 was not considered in further analyses. Only 13% of the samples did not express any AID transcript. The majority of cases (75%) co-expressed several (two or more) AID variants. Of note, 49% of the samples expressed all four AID transcripts (Table 3). The proportions of AID transcript combinations expressed in CLL samples are listed in Suppl. Table 1. We observed an association between the median expression level of all four AID variants in each sample and a number of different AID splice variants present in that particular sample (Spearman $r = 0.8048$, $P < 0.0001$).

AID expression is associated with complex karyotype, trisomy 12 and UM-IGHV

Next, we analyzed whether AID expression correlates with karyotype complexity and/or the presence of particular chromosomal aberrations (CAs) in CLL cells. We found an association between the presence of CK and expression levels of AID alternative transcripts AIDΔE4a and AIDivs3 and a trend for AID FL and variant AIDΔE4 (Table 4). Concerning links between AID expression and individual chromosomal aberrations (del17p, del11q, tri12, and del13q14), we observed that higher expression levels of all four AID transcripts correlated with the presence of trisomy 12 (Table 4; Fig. 2). This association was confirmed by Kruskal-Wallis ANOVA test (Fig. 3). We also evaluated the previously described association between UM-IGHV and AID expression and confirmed this association for each AID variant tested (Table 4; Fig. 4). Additionally, we detected a higher proportion of UM-IGHV (80% vs 50%; $P = 0.0028$) and CK (50% vs 20%; $P = 0.0149$) in AID-positive samples (Ct < 35) than in AID negative samples. In line with the abovementioned results, especially considering the correlation between AID transcript levels and the presence of CK and IGVH mutation status, we expected that patients with high AID expression would have a more aggressive disease course. However, in this particular CLL cohort, we did not observe any impact of AID expression on patient survival or therapy administration need (data not shown).

AID activity inducing point mutations on a genome-wide scale is well documented in germinal center B cells and in B cell lymphomas and leukemias [12, 24, 38–41]. Therefore, as our next step, we evaluated associations between AID expression and mutations in *TP53*, *NOTCH1, SF3B1,* and *ATM* genes recurrently detected in CLL. We found only two statistically significant correlations: high AIDΔE4a expression associated with wild-type *SF3B1* ($P = 0.0205$) and high AIDivs3 variant level associated with the presence of *NOTCH1* mutations ($P = 0.0487$) (Table 4).

Table 2 Summary of AID expression in the studied CLL cohort

AID variants	AID FL	AIDΔE4a	AIDΔE4	AIDivs3	AIDΔE3E4
Number of cases	109	88	98	117	6
Percent	73	59	66	79	4

Table 3 Summary of AID transcript co-expression in CLL samples

Number of AID transcripts expressed	Cases	Percent
No transcript	20	13
1 transcript	17	11
2 transcripts	14	9
3 transcripts	25	17
4 transcripts	73	49
Total	149	

AID splice variants manifest inactivity in mouse splenic B cells and MAVER-1 cell line

In total, the alternatively spliced AID transcripts were detected in 84% of CLL patients; therefore, we asked whether alternative splicing of AID could affect the function of the resulting AID proteins, potentially making them more mutagenic as reported by Jelinek's group [32]. We designed assays to assess AID function in primary mouse splenic B cells and the human mantle cell lymphoma (MCL) cell line MAVER-1 infected by retroviruses expressing individual AID splice variants.

Protein levels of AID splice variants in the infected murine B cells were variable (Suppl. Fig. 3) possibly in part reflecting the situation in vivo where a final amount of nuclear AID protein is regulated by several posttranslational mechanisms. AID FL was expressed the highest while variants AIDΔE4, AIDΔE4a and AIDivs3 reached only 3%, 21%, and 17% of AID FL expression, respectively.

In concordance with previously published data [31–33, 42–44], we demonstrated that all alternatively spliced AID variants were defective in CSR (Suppl. Fig. 4). It is widely accepted that the AID C terminus (lacking in variants AIDΔE4 and AIDivs3) is necessary for CSR and our results were not surprising in this aspect [32, 44, 45]. An opened question remained whether AID variants are functionally active at least with respect to the induction of point mutations and/or DSBs in AID target sequences. To address this question, we used ultra-deep next-generation sequencing (NGS) to analyze mutability of the mouse immunoglobulin switch region DNA (Ig Sμ), which is known to accumulate high amount of mutations and breaks during B cell cytokine stimulation ex vivo. We infected AID knockout B cells with retroviruses expressing individual AID splice variants. Two sets of biological replicates revealed that only AID FL caused accumulation of point mutations above the background levels (Fig. 5).

To quantify the amount of DSBs in the Sμ region, we performed ligation-mediated PCR (LM-PCR) followed by a Southern blot with Sμ specific probe. As depicted in Fig. 6, only AID knockout B cells infected with AID FL variant manifested increased levels of DSBs. Hence, in our assays, AID splice variants were functionally inactive.

To employ an alternative system, we used the human MCL MAVER-1 cell line, which showed low levels of endogenous AID transcripts compared to other tested B cell lines and no detectable AID protein (Suppl. Fig. 5), while it still expressed the productively rearranged IGH gene providing a bona fide target for AID mutagenesis (Suppl. Table 2). We utilized the ultra-deep NGS approach to assess relative gain in mutation load in IGH V-D-J region between day 0 and 6 weeks after the retroviral transduction of individual AID variants. Both HA-tagged and untagged AID constructs were used; HA-AID proteins were quantified on protein level (Suppl. Fig. 6), and expression of untagged AID variants was measured by conventional PCR (Suppl. Fig. 7). Samples MAVER-5 and MAVER-11 expressed low amounts of AID FL transcripts in addition to the variant containing intron 3 suggesting that this intron has been spliced out in some infected cells.

In line with our results from mouse B cells, none of the alternatively spliced AID variants induced point mutations in the VDJ region of IGH in MAVER-1 cells (Table 5) while both HA-AID FL and untagged AID FL proteins were catalytically active. We conclude that AID splice variants

Table 4 Associations between expression of AID transcripts and biological characteristics of CLL patients

Characteristics of CLL samples	*P* values AID FL	AIDΔE4a	AIDΔE4	AIDivs3
Presence of complex karyotype	0.0745	*0.0184*	0.0992	*0.0382*
Presence of UM-IGHV	*0.0001*	*0.0100*	*0.0001*	*0.0008*
(1) Del17p/TP53 defect	0.7562	0.3313	0.9240	0.9894
(2) Del11q except (1)	0.7878	0.1582	0.8328	0.7480
(3) Trisomy12 except (1, 2)	*0.0021*	*0.0012*	*0.0025*	*0.0024*
(4) Del13q14 (except 1, 2, 3)	0.4814	0.2259	0.2667	0.5274
Presence of TP53 mutation	0.9207	0.6282	0.6283	0.5519
Presence of NOTCH1 mutation	0.1031	0.1485	0.1688	*0.0487*
Absence of SF3B1 mutation	0.8931	*0.0205*	0.6999	0.7133
Presence of ATM mutation	0.7098	0.3623	0.7774	0.4604

CAs are hierarchically ordered [3]

Numbers in italics are statistically significant ($P < 0.05$)

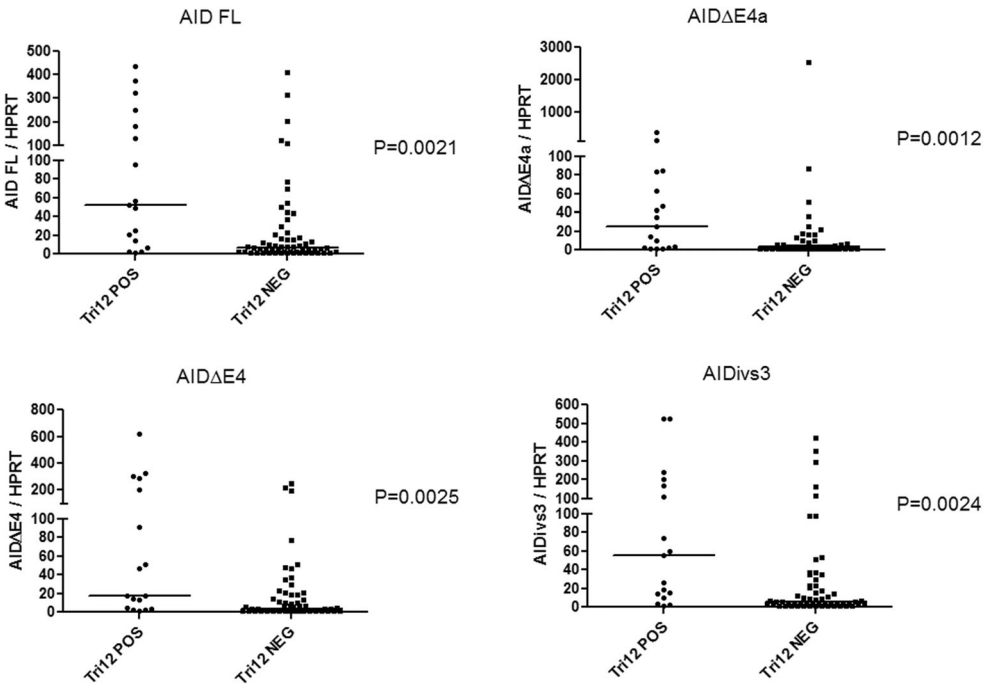

Fig. 2 Association between AID expression and trisomy 12 as determined by Mann-Whitney *U* test. Samples with trisomy 12 were chosen based on the hierarchical model of chromosomal aberrations. Median value of expression for each group is shown

expressed from retroviral vectors in MCL cell line lacked catalytic activity resulting in a complete loss-of-function phenotype.

Modeling of AID structure

To further analyze the loss-of-function phenotype on the molecular level, we modeled AID structure for wild-type protein and AID shortened variant consisting of amino acids 1–142 (AID1–142) closely resembling AID splice variant AIDΔE4. Our in silico modeling relies on atomistic molecular dynamics simulations that provide an insight into molecular interactions and characteristic structural dynamics. Human wild-type AID structure consists of a five-stranded β-sheet flanked by six α-helices (Fig. 7a). The catalytic pocket contains zinc ion coordinated by His56, Cys87, and Cys90 amino acids. During deamination catalysis single stranded DNA is bound to AID so that deoxycytidine (dC) is positioned into the catalytic

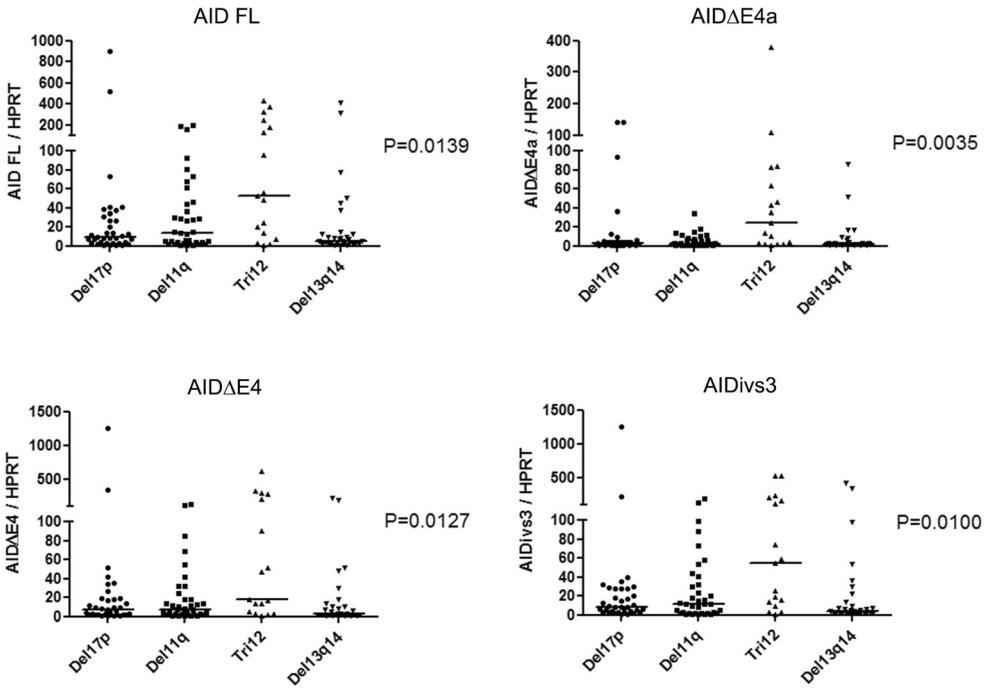

Fig. 3 Kruskal-Wallis ANOVA comparison of AID expression in CLL cells with recurrent chromosomal aberrations. Median values of expression for each AID variant are shown. Hierarchical order of del17p (modified also for the presence of *TP53* mutations), del11q, tri12, and del13q14 aberrations specified in Table 4 was used to assign CLL patients into particular cytogenetic groups

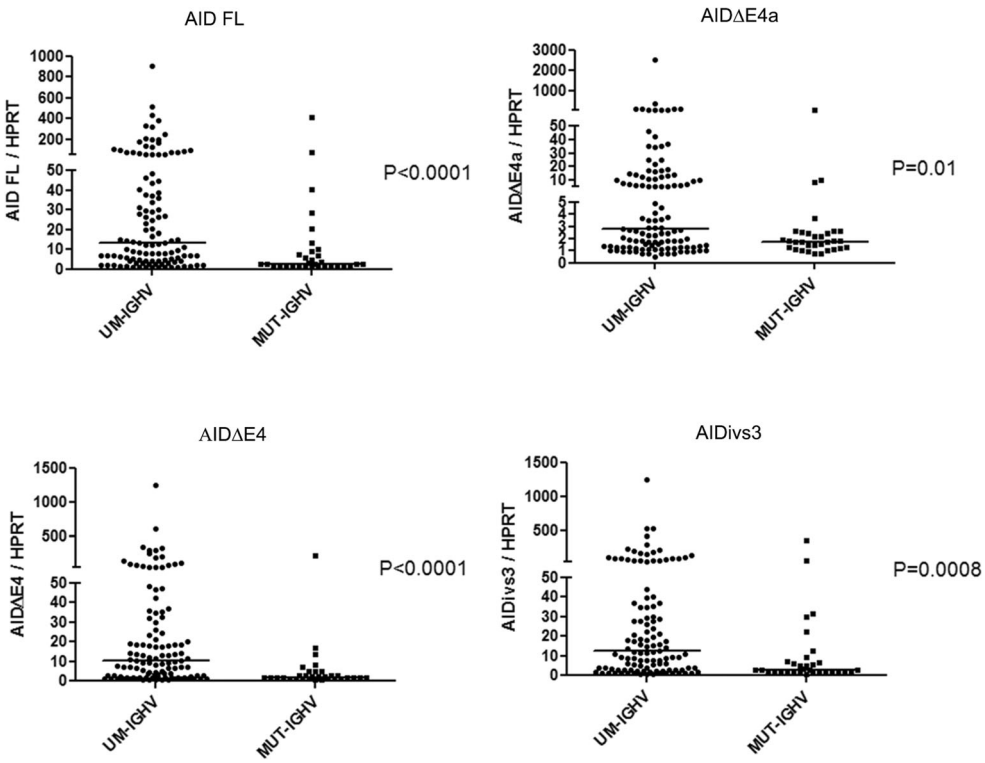

Fig. 4 Expression of FL AID and AID splice variants according to IGHV mutation status. AID relative expression values were evaluated using Mann-Whitney *U* test. Median value of expression for each group is shown

pocket [46]. Molecular dynamics simulations of the wild-type structure were perfectly stable while the shortened AID1–142 variant showed structural rearrangement in the area of the active site and the α1-helix during the first 100 ns (see Fig. 7b–e and Suppl. Fig. 8). Such structural rearrangements might propagate on longer time scales resulting in protein unfolding. We assume that the rearrangement we observed in the simulations most probably affects DNA binding and positioning of dC into the catalytic pocket (for more details, see Supplementary material).

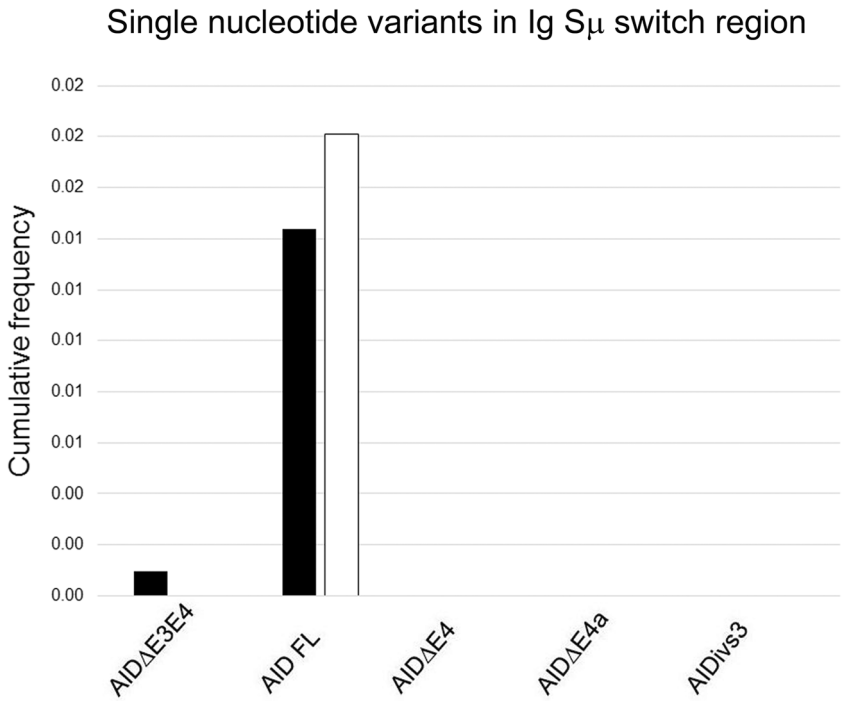

Fig. 5 AID splice variants do not induce point mutations in Ig Sμ switch region. Murine AID knockout B cells were stimulated ex vivo with LPS and IL-4, infected with retroviral AID splice variants, cultured for 2 days, and then sorted for highly positive GFP cells. DNA was subjected to ultra-deep next-generation sequencing to assess the amount of point mutations in each sample. The results were analyzed by deep-SNV algorithm and the cumulative frequency of variant reads was plotted. The experiment was performed in two biological replicates (shown as black and white bars)

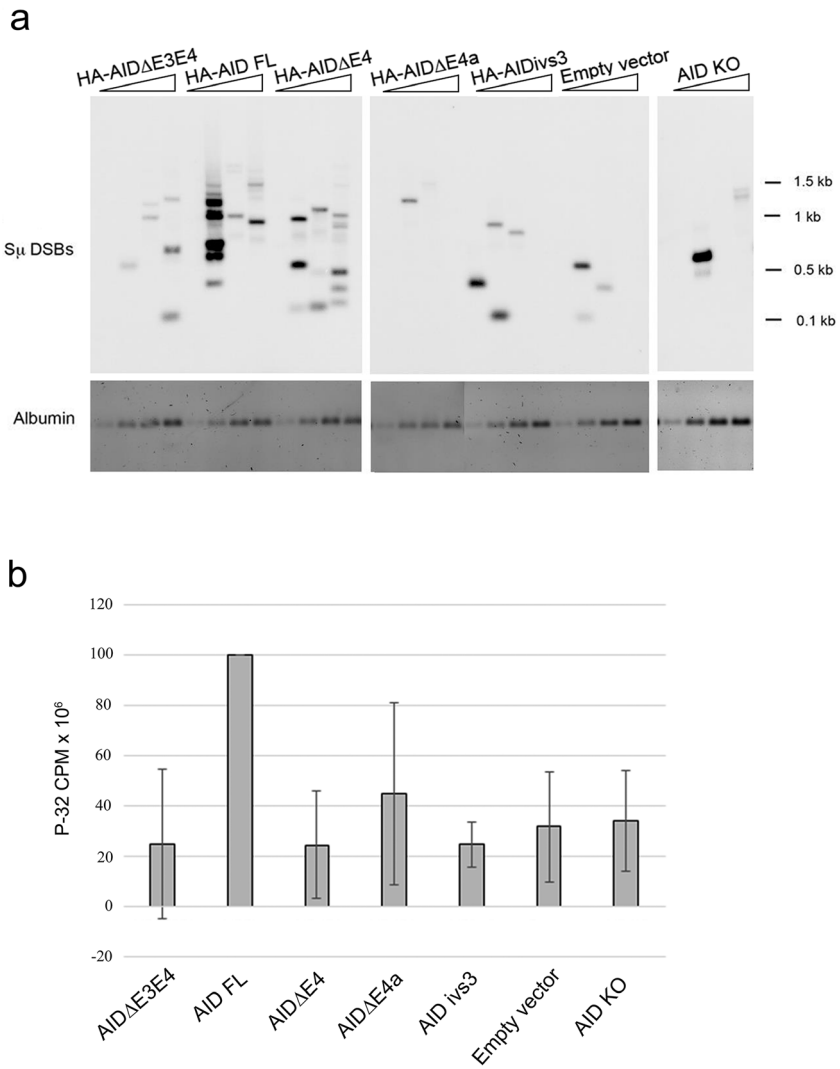

Fig. 6 AID splice variants do not induce DSBs in Ig Sμ switch region. **a** Murine AID knockout B cells were infected with AID splice variants and stimulated with LPS and IL-4. Highly positive GFP cells were sorted the third day of stimulation. DNA samples were normalized to albumin. DSBs were amplified by ligation-mediated PCR followed by hybridization to a radioactively labeled Sμ probe. A representative blot is shown. **b** [32]P signals from 3 different experiments were quantified by Typhoon FLA 9000 laser scanner. Error bars = STDEV

Discussion

Several studies have linked the presence of AID transcripts with more aggressive disease course emphasizing its connection to the activated CLL microenvironment, association with UM-IGHV, chromosomal aberrations, ongoing AID activity in vivo, and increased level of DSBs [24, 38, 47–50]. In addition, Chiorazzi's group demonstrated inferior survival of CLL patients expressing AID mRNA [24].

In our cohort, we found a significant correlation between AID FL and its splice variant expression and the presence of trisomy 12 as well as UM-IGHV and a trend towards

Table 5 AID splice variants did not induce mutations in the variable region of IGH gene rearrangement in MAVER-1 cell line

Sample	Number of variants	Sample	Number of variants
HA-AIDΔE3E4	0	AIDΔE3E4	0
HA-AID FL	2	AID FL	9
HA-AIDΔE4	0	AIDΔE4	0
HA-AIDΔE4a	0	AIDΔE4a	0
HA-AID ivs3	0	AID ivs3	0
HA-Empty vector	0	Empty vector	0

Number of induced single nucleotide variants in the V-D-J region of IGH in the evaluated samples is shown. Control sample DNA was harvested at time 0 before retroviral infection. The evaluated DNA samples were harvested 6 weeks after the retroviral infection from cells sorted for high GFP expression. The counted variants were identified as different from the control sample using customized "tumor/normal" variant calling analysis (specified in Supplementary methods)

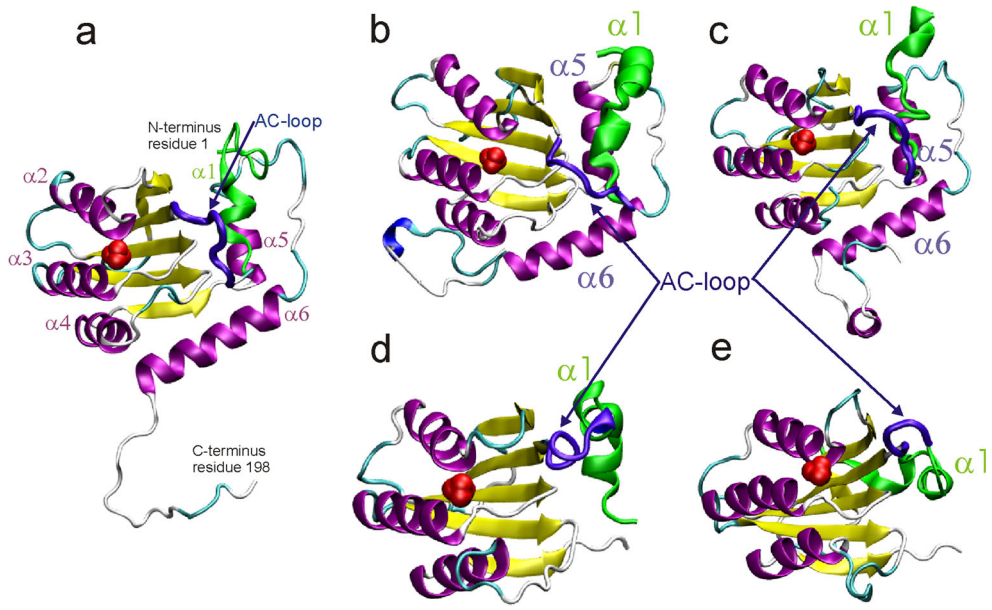

Fig. 7 **a** Homology model of wild-type human AID (residues 1–198). Zinc is represented as tetrahedron-shaped ion in red, α1-helix is green, α-helices 2–6 are marked, and AC-loop (residues 20–26) is blue. In the shorter variant AID1–142, α-helices 5, 6, and the C-terminus are missing. **b**–**e** Wild-type AID and shortened variant AID1–142 after simulating 1 μs, specifically, we visualized structures averaged in the time period 900–1000 ns. **b** Wild-type human AID. **c** Wild-type human AID control. **d** AID1–142 variant. **e** AID1–142 variant control

karyotype complexity. AID transcripts were detected in 87% of patients. This percentage is higher than previously reported in CLL (40% in Albesiano et al., 56% in Heintel et al., and 65% in Marantidou et al.) [26, 30, 47]. Differences in AID expression observed by different groups likely reflect different sensitivities of RT-PCR assays (single-round RT-PCR vs nested RT-PCR vs quantitative real-time RT-PCR performed with TaqMan probes or SYBR Green intercalating dye) as well as distinct CLL cohort compositions. In our study, about half of the cohort was intentionally selected to have complex karyotype which was naturally accompanied by a higher proportion of UM-IGHV (76% of samples) and progressive disease (84% of patients at the time of sampling). Real-time RT-PCR assays showed that the expression of one AID variant was infrequent (11%) while co-expression of all four AID transcripts occurred in almost half of the samples. Only 13% of samples did not express any AID transcript. The AID-negative samples showed a high proportion of MUT-IGHV and a low proportion of CK. On the other hand, AID-positive samples had predominantly UM-IGHV (80% of samples) and an equal proportion of CK.

The positive correlation between the level of expression and the number of expressed AID splice variants that we observed (Spearman $r = 0.8048$, $P < 0.0001$) points to deregulated splicing yielding several AID variants in the same sample rather than to a specific production, processing or selection of a particular AID variant.

The higher AID expression in patients with UM-IGHV compared to those with MUT-IGHV has been repeatedly reported [47, 49–51]. Herein, we also present a significant association between the expression of all four AID variants and UM-IGHV. Although these associations might seem somewhat counterintuitive as AID induces somatic hypermutations in IGHV, a recent proteomic study by Pettitt et al. [52] pointed to a migratory defect of UM-IGHV CLL cells accompanied by their enhanced retention and impaired egress from LNs. Our finding of increased AID transcript levels in CLL cells with UM-IGHV might, therefore, reflect their prolonged exposure to the LN microenvironment where AID expression is induced.

An unexpected result of our study is the strong association between expression of all AID variants and the presence of trisomy 12 (classified according to Dohner's hierarchical model) [3]. CLL patients with trisomy 12 constitute a heterogeneous group with an intermediate prognosis. Trisomy 12 has been associated with mutations in the *NOTCH1* gene [53] as well as with Richter transformation [54], with both events being linked to aggressive disease and inferior clinical outcome. In any case, molecular pathogenesis linked to the presence of trisomy 12 is not well understood. Liso et al. evaluated the proportion of CLL cells with trisomy 12 in three compartments: PB, BM, and LN. Interestingly, LN contained a higher percentage of trisomy 12 positive cells compared to PB or BM [55]. Consequently, it has been demonstrated that CLL cells with trisomy 12 overexpress integrin CD49d, a subunit of the late activation 4 receptor (VLA-4), involved in adhesion to endothelial cells via interaction with VCAM-1, extravasation, and homing of CLL cells to the LNs [56]. In line with these reports, Riches et al. demonstrated that trisomy 12 positive CLL cells have increased expression of β2-integrins involved in trans-endothelial migration of CLL cells from the bloodstream to tissues [57]. Notably, CLL cells infiltrating LNs in Richter syndrome had higher levels of AID as well as *c-MYC, PAX5 and RhoH* mutations in AID hotspot motives comparing to circulating leukemic cells [58, 59]. All the abovementioned facts underscore the LN microenvironment as a key factor in induction of AID expression.

Our functional tests in murine primary B cells and a human MCL cell line did not confirm the hypothesis that AID splice variants might be translated to proteins with increased catalytic activity. We tested SHM, CSR, and DSBs in transduced AID knockout B cells ex vivo because stimulation of murine B cells with LPS and IL-4 turns on processes necessary for AID function (germline transcription and opening of switch regions to AID recruitment). These changes take place rapidly as Ig receptors switched to IgG1 can be detected within 48 h after stimulation. Nevertheless, in vitro stimulated splenic B cells usually die within 5 days of culture which left only a short window for AID induction of SHM and DSBs. Even though we used very sensitive techniques (i.e., ultra-deep next-generation sequencing and Southern blot with a radioactively labeled probe), we did not detect any mutations or DSBs in Ig switch region caused by alternatively spliced AID variants. To confirm this result and leave longer time for AID activity, we infected the human MCL cell line MAVER-1 with HA-tagged or untagged AID variants and harvested DNA after 6 weeks of cell culture. In line with the results from murine B cells, only cells infected with HA-tagged or untagged FL AID retroviruses accumulated mutations in the IGHV-D-J sequence. Interestingly, cells with untagged AID FL gained more IGHV mutations than cells with HA-AID FL, indicating a potential inhibitory effect of the HA tag.

A potential drawback of these functional assays was unequal protein levels of AID variants expressed from a retroviral cassette. We cannot exclude the possibility that each AID variant was translated with different efficiency. In addition, AID proteins are regulated by posttranslational mechanisms which might be responsible for lower levels of AID variants lacking C-terminal domain (AIDΔE4 and AIDivs3). The AID C terminus encodes a conserved nuclear export signal (NES) required for CRM-1 dependent export of AID protein from the nucleus [44, 60, 61]. AID proteins lacking NES stay trapped in the nuclear compartment. Notably, nuclear AID proteins have a much shorter half-life compared to cytoplasmic AID (2.5 h vs. 18 h) as the protein is differentially polyubiquitinated and degraded in these two compartments [42]. Therefore, AIDΔE4 and AIDivs3 lacking NES should be preferentially nuclear as shown in van Maldegem et al. [33] which might reduce their stability. Low protein levels of AID splice variants can also be seen in Rebhandl et al. [34] who expressed AID splice variants from a splice reporter construct and then thoroughly quantified levels of corresponding RNA and translated proteins, showing that AIDΔE4 and AIDivs3 proteins do not stoichiometrically correspond to their RNA counterparts. Our results corroborate these studies suggesting low expression of AID variants at the protein level as one of the potential factors contributing to their loss-of-function phenotype observed in vitro.

To investigate how the lack of exons 4 and 5 affects AID structure, we performed atomistic molecular dynamics simulations and modeled the AID1–142 amino acid protein containing identical sequence as AID variant ΔE4 except for the last three residues (Ala, Pro and Val) at the C-terminus (not present in our model). The comparison of variant AID1–142 with AID FL on a microsecond time scale showed structural rearrangement of the active site area that most probably cannot bind DNA and position dC into the catalytic pocket. Our structural analysis, therefore, supports observations from functional assays, i.e., that the shortened variants highly likely do not exhibit AID deamination activity.

To conclude, we observed an association between higher AID transcript levels and trisomy of chromosome 12 as well as the unmutated IGHV status in CLL patients. However, we have also demonstrated that the alternative AID splice variants do not retain the activity in SHM, CSR, and the induction of double-strand DNA breaks.

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Author contributions KZ designed the research study, performed the experiments, and wrote the paper; KR performed the in silico modeling; VS, JB, KD, TL, MB, KS, AO, and JM performed the experiments; LR, VB, and NT performed the data analysis and biostatistics; MD provided the clinical samples; MLA provided the mice; MLA, MT, JM, and SP designed the research study and wrote the paper.

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Compliance with ethical standards

The study was performed on peripheral blood samples taken with written informed consent provided in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All animal studies were performed in compliance with the U.S. Department of Health and Human Services guidelines and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, Vinciguerra VP, Rai KR, Ferrarini M, Chiorazzi N (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 94(6):1840–1847
2. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK (1999) Unmutated Ig V-H genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 94(6):1848–1854
3. Dohner H, Stilgenbauer S, Benner A et al (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. New Engl J Med 343(26):1910–1916
4. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, Sougnez C, Stewart C, Sivachenko A, Wang L, Wan Y, Zhang W, Shukla SA, Vartanov A, Fernandes SM, Saksena G, Cibulskis K, Tesar B, Gabriel S, Hacohen N, Meyerson M, Lander ES, Neuberg D, Brown JR, Getz G, Wu CJ (2013) Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell 152(4):714–726
5. Baliakas P, Iskas M, Gardiner A, Davis Z, Plevova K, Nguyen-Khac F, Malcikova J, Anagnostopoulos A, Glide S, Mould S, Stepanovska K, Brejcha M, Belessi C, Davi F, Pospisilova S, Athanasiadou A, Stamatopoulos K, Oscier D (2014) Chromosomal translocations and karyotype complexity in chronic lymphocytic leukemia: a systematic reappraisal of classic cytogenetic data. Am J Hematol 89(3):249–255
6. Rigolin GM, del Giudice I, Formigaro L, Saccenti E, Martinelli S, Cavallari M, Lista E, Tammiso E, Volta E, Lupini L, Bassi C, Bardi A, Sofritti O, Daghia G, Cavazzini F, Marinelli M, Tavolaro S, Guarini A, Negrini M, Foà R, Cuneo A (2015) Chromosome aberrations detected by conventional karyotyping using novel mitogens in chronic lymphocytic leukemia: clinical and biologic correlations. Gene Chromosome Canc 54:818–826
7. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102(5):553–563
8. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Lagelouse R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A (2000) Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the hyper-IgM syndrome (HIGM2). Cell 102(5):565–575
9. Bransteitter R, Pham P, Calabrese P, Goodman MF (2004) Biochemical analysis of hypermutational targeting by wild type and mutant activation-induced cytidine deaminase. J Biol Chem 279(49):51612–51621
10. Pham P, Bransteitter R, Petruska J, Goodman MF (2003) Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. Nature 424(6944):103–107
11. Iacobucci I, Lonetti A, Messa F et al (2010) Different isoforms of the B-cell mutator activation-induced cytidine deaminase are aberrantly expressed in BCR-ABL1-positive acute lymphoblastic leukemia patients. Leukemia 24(1):66–73
12. Klemm L, Duy C, Iacobucci I, Kuchen S, von Levetzow G, Feldhahn N, Henke N, Li Z, Hoffmann TK, Kim YM, Hofmann WK, Jumaa H, Groffen J, Heisterkamp N, Martinelli G, Lieber MR, Casellas R, Müschen M (2009) The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. Cancer Cell 16(3):232–245
13. Okazaki IM, Kotani A, Honjo T (2007) Role of AID in tumorigenesis. Adv Immunol 94:245–273
14. Pasqualucci L, Bhagat G, Jankovic M, Compagno M, Smith P, Muramatsu M, Honjo T, Morse HC, Nussenzweig MC, Dalla-Favera R (2008) AID is required for germinal center-derived lymphomagenesis. Nature Genet 40(1):108–112
15. Pasqualucci L, Guglielmino R, Houldsworth J, Mohr J, Aoufouchi S, Polakiewicz R, Chaganti RS, Dalla-Favera R (2004) Expression of the AID protein in normal and neoplastic B cells. Blood 104(10):3318–3325
16. Ramiro AR, Jankovic M, Eisenreich T, Difilippantonio S, Chen-Kiang S, Muramatsu M, Honjo T, Nussenzweig A, Nussenzweig MC (2004) AID is required for c-myc/IgH chromosome translocations in vivo. Cell 118(4):431–438
17. Robbiani DF, Bunting S, Feldhahn N, Bothmer A, Camps J, Deroubaix S, McBride KM, Klein IA, Stone G, Eisenreich TR, Ried T, Nussenzweig A, Nussenzweig MC (2009) AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. Mol Cell 36(4):631–641
18. Kumar R, DiMenna LJ, Chaudhuri J et al (2014) Biological function of activation-induced cytidine deaminase (AID). Biom J 37:269–283
19. Cattoretti G, Buttner M, Shaknovich R et al (2006) Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. Blood 107(10):3967–3975
20. Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, Yokota Y, Shimizu A (2003) The balance between Pax5 and Id2 activities is the key to AID gene expression. J Exp Med 198(9):1427–1437
21. Park SR, Zan H, Pal Z, Zhang J, al-Qahtani A, Pone EJ, Xu Z, Mai T, Casali P (2009) HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. Nat Immunol 10(5):540–550
22. Sayegh CE, Quong MW, Agata Y, Murre C (2003) E-proteins directly regulate expression of activation-induced deaminase in mature B cells. Nat Immunol 4(6):586–593
23. Tran TH, Nakata M, Suzuki K, Begum NA, Shinkura R, Fagarasan S, Honjo T, Nagaoka H (2010) B cell-specific and stimulation-responsive enhancers derepress Aicda by overcoming the effects of silencers. Nat Immunol 11(2):148–U67
24. Patten PEM, Chu CC, Albesiano E, Damle RN, Yan XJ, Kim D, Zhang L, Magli AR, Barrientos J, Kolitz JE, Allen SL, Rai KR, Roa S, Mongini PK, MacCarthy T, Scharff MD, Chiorazzi N (2012) IGHV-unmutated and IGHV-mutated chronic lymphocytic leukemia cells produce activation-induced deaminase protein with a full range of biologic functions. Blood 120(24):4802–4811
25. Rush JS, Liu M, Odegard VH, Unniraman S, Schatz DG (2005) Expression of activation-induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division-linked class switch recombination. Proc Natl Acad Sci U S A 102(37):13242–13247
26. Albesiano E, Messmer BT, Damle RN, Allen SL, Rai KR, Chiorazzi N (2003) Activation-induced cytidine deaminase in chronic lymphocytic leukemia B cells: expression as multiple forms in a dynamic, variably sized fraction of the clone. Blood 102(9):3333–3339
27. de Yebenes VG, Belver L, Pisano DG et al (2008) miR-181b negatively regulates activation-induced cytidine deaminase in B cells. J Exp Med 205(10):2199–2206
28. Sernandez IV, de Yebenes VG, Dorsett Y et al (2008) Haploinsufficiency of activation-induced deaminase for antibody diversification and chromosome translocations both in vitro and in vivo. PLoS One 3(12):e3927

29. Teng G, Hakimpour P, Landgraf P, Rice A, Tuschl T, Casellas R, Papavasiliou FN (2008) MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. Immunity 28(5):621–629
30. Marantidou F, Dagklis A, Stalika E, Korkolopoulou P, Saetta A, Anagnostopoulos A, Laoutaris N, Stamatopoulos K, Belessi C, Scouras Z, Patsouris E (2010) Activation-induced cytidine deaminase splicing patterns in chronic lymphocytic leukemia. Blood Cells Mol Dis 44(4):262–267
31. van Maldegem F, Jibodh RA, van Dijk R, Bende RJ, van Noesel CJM (2010) Activation-induced cytidine deaminase splice variants are defective because of the lack of structural support for the catalytic site. J Immunol 184(5):2487–2491
32. Wu XS, Darce JR, Chang SK, Nowakowski GS, Jelinek DF (2008) Alternative splicing regulates activation-induced cytidine deaminase (AID): implications for suppression of AID mutagenic activity in normal and malignant B cells. Blood 112(12):4675–4682
33. van Maldegem F, Scheeren FA, Aarti Jibodh R, Bende RJ, Jacobs H, van Noesel CJM (2009) AID splice variants lack deaminase activity. Blood 113:1862–1864
34. Rebhandl S, Huemer M, Zaborsky N, Gassner FJ, Catakovic K, Felder TK, Greil R, Geisberger R (2014) Alternative splice variants of AID are not stoichiometrically present at the protein level in chronic lymphocytic leukemia. Eur J Immunol 44:2175–2187
35. Sali A, Blundell TL (1993) Comparative protein modeling by satisfaction of spatial restraints. J Mol Biol 234(3):779–815
36. Case DA, Babin V, Berryman JT et al (2014) AMBER 14. University of California, San Francisco
37. Le Bris Y, Struski S, Guièze R et al (2016) Major prognostic value of complex karyotype in addition to TP53 and IGHV mutational status in first-line chronic lymphocytic leukemia. Hematol Oncol 35:664–670. https://doi.org/10.1002/hon.2349
38. Huemer M, Rebhandl S, Zaborsky N, Gassner FJ, Hainzl S, Weiss L, Hebenstreit D, Greil R, Geisberger R (2014) AID induces intraclonal diversity and genomic damage in CD86(+) chronic lymphocytic leukemia cells. Eur J Immunol 44(12):3747–3757
39. Jiang YW, Soong TD, Wang L et al (2012) Genome-wide detection of genes targeted by non-Ig somatic hypermutation in lymphoma. PLoS One 7(7):e40332
40. Liu M, Duke JL, Richter DJ, Vinuesa CG, Goodnow CC, Kleinstein SH, Schatz DG (2008) Two levels of protection for the B cell genome during somatic hypermutation. Nature 451(7180):841–845
41. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RSK, Küppers R, Dalla-Favera R (2001) Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 412(6844):341–346
42. Aoufouchi S, Faili A, Zober C, D'Orlando O, Weller S, Weill JC, Reynaud CA (2008) Proteasomal degradation restricts the nuclear lifespan of AID. J Exp Med 205(6):1357–1368
43. Geisberger R, Rada C, Neuberger MS (2009) The stability of AID and its function in class-switching are critically sensitive to the identity of its nuclear-export sequence. Proc Natl Acad Sci U S A 106(16):6736–6741
44. McBride KM, Barreto V, Ramiro AR et al (2004) Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. J Exp Med 199(9):1235–1244
45. Zahn A, Eranki AK, Patenaude AM, Methot SP, Fifield H, Cortizas EM, Foster P, Imai K, Durandy A, Larijani M, Verdun RE, di Noia JM (2014) Activation induced deaminase C-terminal domain links DNA breaks to end protection and repair during class switch recombination. Proc Natl Acad Sci U S A 111(11):E988–E997
46. Betts L, Xiang SB, Short SA, Wolfenden R, Carter CW Jr (1994) Cytidine deaminase. The 2.3 A crystal structure of an enzyme: transition-state analog complex. J Mol Biol 235(2):635–656
47. Heintel D, Kroemer E, Kienle D et al (2004) High expression of activation-induced cytidine deaminase (AID) mRNA is associated with unmutated IGVH gene status and unfavourable cytogenetic aberrations in patients with chronic lymphocytic leukaemia. Leukemia 18:756–762
48. Leuenberger M, Frigerio S, Wild PJ, Noetzli F, Korol D, Zimmermann DR, Gengler C, Probst-Hensch NM, Moch H, Tinguely M (2010) AID protein expression in chronic lymphocytic leukemia/small lymphocytic lymphoma is associated with poor prognosis and complex genetic alterations. Mod Pathol 23(2):177–186
49. McCarthy H, Wierda WG, Barron LL, Cromwell CC, Wang J, Coombes KR, Rangel R, Elenitoba-Johnson KS, Keating MJ, Abruzzo LV (2003) High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. Blood 101(12):4903–4908
50. Palacios F, Moreno P, Morande P, Abreu C, Correa A, Porro V, Landoni AI, Gabus R, Giordano M, Dighiero G, Pritsch O, Oppezzo P (2010) High expression of AID and active class switch recombination might account for a more aggressive disease in unmutated CLL patients: link with an activated microenvironment in CLL disease. Blood 115(22):4488–4496
51. Oppezzo P, Vuillier F, Vasconcelos Y, Dumas G, Magnac C, Payelle-Brogard B, Pritsch O, Dighiero G (2003) Chronic lymphocytic leukemia B cells expressing AID display dissociation between class switch recombination and somatic hypermutation. Blood 101(10):4029–4032
52. Eagle GL, Zhuang JG, Jenkins RE, Till KJ, Jithesh PV, Lin K, Johnson GG, Oates M, Park K, Kitteringham NR, Pettitt AR (2015) Total proteome analysis identifies migration defects as a major Pathogenetic factor in immunoglobulin heavy chain variable region (IGHV)-unmutated chronic lymphocytic leukemia. Mol Cell Proteomics 14(4):933–945
53. Balatti V, Bottoni A, Palamarchuk A, Alder H, Rassenti LZ, Kipps TJ, Pekarsky Y, Croce CM (2012) NOTCH1 mutations in CLL associated with trisomy 12. Blood 119(2):329–331
54. Chigrinova E, Rinaldi A, Kwee I, Rossi D, Rancoita PMV, Strefford JC, Oscier D, Stamatopoulos K, Papadaki T, Berger F, Young KH, Murray F, Rosenquist R, Greiner TC, Chan WC, Orlandi EM, Lucioni M, Marasca R, Inghirami G, Ladetto M, Forconi F, Cogliatti S, Votavova H, Swerdlow SH, Stilgenbauer S, Piris MA, Matolcsy A, Spagnolo D, Nikitin E, Zamo A, Gattei V, Bhagat G, Ott G, Zucca E, Gaidano G, Bertoni F (2013) Two main genetic pathways lead to the transformation of chronic lymphocytic leukemia to Richter syndrome. Blood 122(15):2673–2682
55. Liso V, Capalbo S, Lapietra A, Pavone V, Guarini A, Specchia G (1999) Evaluation of trisomy 12 by fluorescence in situ hybridization in peripheral blood, bone marrow and lymph nodes of patients with B-cell chronic lymphocytic leukemia. Haematologica 84(3):212–217
56. Burger JA, Montserrat E (2013) Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. Blood 121(9):1501–1509
57. Riches JC, O'Donovan CJ, Kingdon SJ, McClanahan F, Clear AJ, Neuberg DS, Werner L, Croce CM, Ramsay AG, Rassenti LZ, Kipps TJ, Gribben JG (2014) Trisomy 12 chronic lymphocytic leukemia cells exhibit upregulation of integrin signaling that is modulated by NOTCH1 mutations. Blood 123(26):4101–4110
58. Reiniger L, Bodor C, Bognar A et al (2006) Richter's and prolymphocytic transformation of chronic lymphocytic leukemia are associated with high mRNA expression of activation-induced cytidine deaminase and aberrant somatic hypermutation. Leukemia 20(6):1089–1095
59. Smit LA, van Maldegem F, Langerak AW, van der Schoot C, de Wit MJ, Bea S, Campo E, Bende RJ, van Noesel C (2006) Antigen receptors and somatic hypermutation in B-cell chronic lymphocytic

leukemia with Richter's transformation. Haematologica 91(7):903–911

60. Ito S, Nagaoka H, Shinkura R, Begum N, Muramatsu M, Nakata M, Honjo T (2004) Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. Proc Natl Acad Sci U S A 101(7): 1975–1980

61. Ta VT, Nagaoka H, Catalan N, Durandy A, Fischer A, Imai K, Nonoyama S, Tashiro J, Ikegawa M, Ito S, Kinoshita K, Muramatsu M, Honjo T (2003) AID mutant analyses indicate requirement for class-switch-specific cofactors. Nat Immunol 4(9): 843–848