European Journal of Immunology

Alternative splice variants of AID are not stoichiometrically present at the protein level in chronic lymphocytic leukemia

Stefan Rebhandl[1], Michael Huemer[1], Nadja Zaborsky[1], Franz Josef Gassner[1], Kemal Catakovic[1], Thomas Klaus Felder[2], Richard Greil[1] and Roland Geisberger[1]

[1] Laboratory for Immunological and Molecular Cancer Research, Department of Internal Medicine III with Hematology, Medical Oncology, Hemostaseology, Infectious Diseases, Rheumatology, Oncologic Center, Paracelsus Medical University, Salzburg, Austria
[2] Department of Laboratory Medicine, Paracelsus Medical University, Salzburg, Austria

Activation-induced deaminase (AID) is a DNA-mutating enzyme that mediates class-switch recombination as well as somatic hypermutation of antibody genes in B cells. Due to off-target activity, AID is implicated in lymphoma development by introducing genome-wide DNA damage and initiating chromosomal translocations such as c-myc/IgH. Several alternative splice transcripts of AID have been reported in activated B cells as well as malignant B cells such as chronic lymphocytic leukemia (CLL). As most commercially available antibodies fail to recognize alternative splice variants, their abundance in vivo, and hence their biological significance, has not been determined. In this study, we assessed the protein levels of AID splice isoforms by introducing an AID splice reporter construct into cell lines and primary CLL cells from patients as well as from WT and TCL1^{tg} C57BL/6 mice (where TCL1 is T-cell leukemia/lymphoma 1). The splice construct is 5′-fused to a GFP-tag, which is preserved in all splice isoforms and allows detection of translated protein. Summarizing, we show a thorough quantification of alternatively spliced AID transcripts and demonstrate that the corresponding protein abundances, especially those of splice variants AID-ivs3 and AID-ΔE4, are not stoichiometrically equivalent. Our data suggest that enhanced proteasomal degradation of low-abundance proteins might be causative for this discrepancy.

Keywords: Activation-induced deaminase (AID) · Alternative splicing · Chronic lymphocytic leukemia (CLL)

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Activation-induced deaminase (AID) mediates class-switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin genes in GC B cells. It does so by deaminating cytosines within genomic immunoglobulin loci, thereby generating uracils. As uracil base pairs with adenine, unrepaired uracils lead to transition mutations from G:C to A:T base pairs. Alternatively, uracil lesions are repaired in an error-prone manner leading to the introduction of mutations at G:C as well as A:T base pairs. GC B cells carrying mutations that result in the generation of higher affine variants of the immunoglobulin are selected

Correspondence: Dr. Roland Geisberger
e-mail: r.geisberger@salk.at

and further differentiate into antibody-producing plasma cells and circulating memory B cells [1]. During CSR, uracils generated by AID within genomic switch regions are removed by the DNA-repair machinery leading to the generation of dsDNA breaks. The linkage of distant switch regions by nonhomologous DNA end joining finally leads to the completion of CSR and to the expression of different immunoglobulin constant regions (IgG, IgE, or IgA isotype) [2]. There is convincing evidence that AID induces a significant amount of genome-wide off-target damage by deaminating genes outside the immunoglobulin loci [3, 4]. In addition, aberrant CSR can result in the translocation of genes into the immunoglobulin locus. C-myc/IgH translocations, for example, are completely dependent on AID activity and recent advances in genome-wide translocation analyses have revealed many AID-dependent translocation hot spots [5–7].

Recent studies have shown that several alternatively spliced AID variants exist, which affect the C-terminal part of the AID protein, while the N-terminal exons 1 and 2 are preserved. Apart from full-length AID (AID-FL; Accession Number NM_020661.2), the splice variants AID-ΔE4 (Accession Number AY536517), AID-ΔE4a (Accession Number AY536516.1), AID-ivs3 (Accession Number AY541058.1), and AID-ΔE3E4 (Accession Number AY534975) were detected (Supporting Information Fig. 1) [8, 9]. Cloning of the splice variants and expression in transfected cell lines revealed that AID-ΔE4, AID-ΔE4a, and AID-ivs3 exhibit altered subcellular localization due to a disturbed C-terminal nuclear export sequence and due to an alpha-helix deletion, respectively [10, 11]. Conflicting data exist regarding the deamination activity of the splice variants. While retroviral assays revealed an increased mutational activity for splice variants AID-ΔE4 and AID-ΔE4a as measured by reversion of an inactive GFP reporter gene, biochemical assays suggested that splice variants are nonfunctional [10–12]. Beyond that, splice variants do not execute CSR [11]. AID splice variants were detected in GC-derived B cells and were also described for chronic lymphocytic leukemia (CLL) B cells [8, 9, 11, 13]. In CLL, AID expression levels correlate with the severity of disease, and hence, AID off-target DNA damage is thought to implicate on CLL progression and pathogenesis [14, 15]. As splice variants have an altered mutational activity, it was suggested that alternative splicing could regulate protein levels of functional AID-FL, or eventually contribute to off-target activity [11]. Also, alternative AID splicing could account for the observed dissociation of CSR and SHM in CLL [16]. However, as alternative splicing alters the amino acid sequence of the resulting peptides, and hence, hampers their recognition by AID-FL-specific antibodies, a thorough analysis of alternatively spliced AID variants on the protein level was so far not carried out.

In this study, we describe the generation of a GFP-tagged AID splice reporter construct (AID-SRC) that allows the detection of all AID splice variants on a protein level, based on an N-terminal GFP-tag that is preserved in all splice variants. We show that the prolymphocytic CLL cell line MEC1 expresses endogenous AID splice variants in a manner comparable to primary CLL cells. We further show that upon stable expression of the GFP-tagged AID-SRC in MEC1 cells, the reporter exhibits the similar splicing pattern as endogenous AID. Despite the presence of alternatively spliced AID transcripts, only AID-FL could be detected on protein level. When inhibiting proteasomal degradation, protein corresponding in size to AID-ivs3 was detected, but less abundant than expected from transcript levels. Our results suggest that alternative splice transcripts of AID are not stoichiometrically equivalent to expressed protein.

Results

Alternative splice transcripts of AID are produced by CLL cells and MEC1 cell line

First, we PCR amplified AID transcripts from primary CLL cells as well as from the prolymphocytic CLL cell line MEC1 [17] and observed several distinct PCR products corresponding in size to previously described splice variants (Fig. 1). Sequencing of PCR products confirmed that MEC1 as well as AID-expressing CLL samples produce the splice variants AID-ΔE4, AID-ΔE4a, and AID-ivs3 aside of AID-FL. However, no band corresponding to AID-ΔE3E4 was detected in our PCR-based assay (Fig. 1). Instead, an additional band at around 800 bp appeared, which did not correspond to any of the splice variants obtained by sequencing (Fig. 1). We next aimed to measure AID protein levels using Western blotting and found that MEC1 cells expressed significant levels of AID protein (Fig. 1). In primary CLL cells, AID protein was only detectable upon stimulation using IL-2, IL-4, and CpG (Fig. 1). However, as the antibody is specific for the C-terminal portion of AID protein and as this part is affected in alternative splice variants AID-ΔE4 and AID-ivs3, we thought that Western blotting likely only revealed the presence of AID-FL and AID-ΔE4a in cell lysates. To test the specificity of the antibody, we cloned cDNAs of the respective AID splice variants in frame with an N-terminal GFP-tag and expressed the respective GFP-AID fusion constructs transiently in HEK293FT cells. Upon immunoblotting of the respective cell lysates, we could show that indeed the AID antibodies failed to detect AID splice variants AID-ivs3 and AID-ΔE4, whereas all splice variants can be detected with an anti-GFP antibody. Furthermore, though the splice variant AID-ΔE4a is hardly discriminable from AID-FL in immunoblotting, AID-ΔE4 and AID-ivs3 are clearly distinguishable from AID-FL based on their different sizes (Supporting Information Fig. 2). Thus, the relative abundance of endogenous alternatively spliced AID variants, notably AID-ivs3 and AID-ΔE4, remained elusive on the protein level.

A GFP-tagged SRC allows detection of AID transcript and protein levels

To enable detection of all AID splice variants on the level of protein, we generated an N-terminally GFP-tagged SRC (AID-SRC, Fig. 2A). Therefore, we cloned the genomic AID locus spanning exons 2–5 in frame with an N-terminal GFP reporter, which is preserved independently of alternative splicing. To confirm

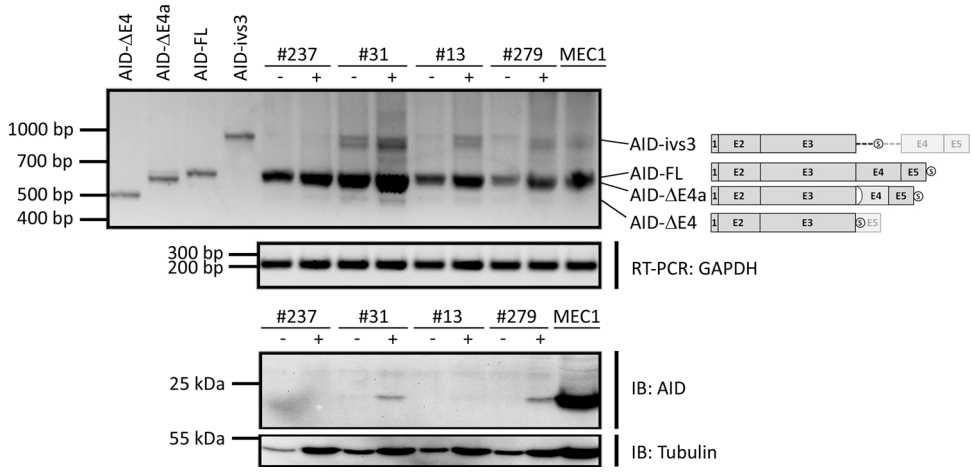

Figure 1. Alternative AID splicing in primary CLL cells and MEC1 cells. RNA and protein were extracted from primary CLL samples that were stimulated with IL-2, IL-4, and CpG (+) or left unstimulated (–) for 6 days. Results of PCR on cDNA using AID-specific and GAPDH-specific primers as loading control are shown (upper panel). Schematic presentations of the alternative splice variants are indicated right to each PCR band. PCR on TOPO-cloned splice variants as templates was performed as size controls and loaded in the first four lanes of the gel. The lower panel shows an immunoblot (IB) on cell lysates using AID- and tubulin-specific antibodies as loading control. Data are representative of two independent experiments.

that AID-SRC-derived primary transcripts are alternatively spliced, we transiently transfected primary CLL cells with AID-SRC and detected AID splice variants using AID-SRC-specific primer pairs. As depicted in Figure 2B, AID-SRC yielded alternative splice transcripts in transfected CLL samples reflected in the appearance of distinct PCR-products corresponding to AID-FL, AID-ΔE4, and AID-ivs3. However, the transgenic AID-SRC could not be detected on protein level, probably due to low transfection efficiency as primary CLL cells are hardly transfectable by conventional transfection methods (data not shown). After confirming correct splicing of AID-SRC in primary CLL cells, we aimed at analyzing protein levels of spliced AID variants by generating MEC1 transfectants. Therefore, we stably transfected MEC1 cells with AID-SRC and analyzed several independent transfectants ($MEC1^{AID\text{-}SRC}$). By using AID-SRC-specific primers, we found that $MEC1^{AID\text{-}SRC}$ showed a similar splicing-pattern for the AID-SRC construct as for the endogenous AID, with a prominent band corresponding to AID-ivs3 being present alongside with a main band for AID-FL (Fig. 2C). Albeit the PCR band corresponding to AID-ΔE4 was less abundant, sequencing of gel-excised PCR products confirmed the presence of splice variants AID-ivs3, AID-ΔE4, and AID-ΔE4a in addition to AID-FL (Sequencing data; Supporting Information Fig. 7). To determine protein levels of AID splice variants, we performed Western blotting on lysates from $MEC1^{AID\text{-}SRC}$ using GFP-specific antibodies. As shown in Figure 2C, only bands corresponding to GFP-tagged AID-FL at around 52 kDa could be detected in 3 of the 12 stable $MEC1^{AID\text{-}SRC}$ transfectants. Notably, five of the eight stable MEC1 transfectants that showed expression of AID-SRC mRNA did not show any AID-SRC protein expression (clones #5, #7, #9, #14, #22; Fig. 2C). While all of these clones were resistant to the selection marker neomycin, only a low percentage of cells showed expression of the GFP-transgene as determined by flow cytometry (0.2–1.7% of cells; Supporting Information Fig. 6) resulting in GFP-AID protein expression below detection limit of immunoblotting in these clones. As $MEC1^{AID\text{-}SRC}$ clone #11 showed the highest percentage of transgene expression (43%; Supporting Information Fig. 6), we used this clone in all further experiments. To increase sensitivity of the immunoblot, we titrated the $MEC1^{AID\text{-}SRC}$ cell lysates and loaded up to 3.5×10^6 cell equivalents onto one slot. As size standards, we loaded lysates from HEK293FT cells transiently transfected with cDNA-GFP fusion constructs encoding the respective splice variants (Fig. 2D). However, in our immunoblot assays, no alternatively spliced AID variants could be detected as translated protein. To assess the sensitivity of our immunoblot setup, we serially diluted lysates from HEK293FT cells transiently transfected with GFP-AID-FL fusion construct, showing that we were able to detect protein of interest down to 1% of an initial amount of protein lysate (Fig. 2E).

The AID-SRC has a preserved alternative splicing pattern

We next tested whether the absence of alternative splice variants on protein level could be due to a low frequency or a low stability of alternatively spliced transcripts, which would both result in a low abundance of steady-state transcript levels. As detection of alternatively spliced transcripts by RT-PCR could possibly lead to a biased amplification and eventually to a misinterpretation of relative abundances, we assessed mRNA levels of AID splice variants by more accurate quantitative methods. Therefore, we first performed RNase protection assays (RPA) using an RNA-probe spanning the exon 4/5 junction including portions of intron 3 and the 3′UTR. This probe was designed to allow distinguishing endogenous as well as transgenic (AID-SRC) AID splice variants, as transcripts descending from AID-SRC lack the

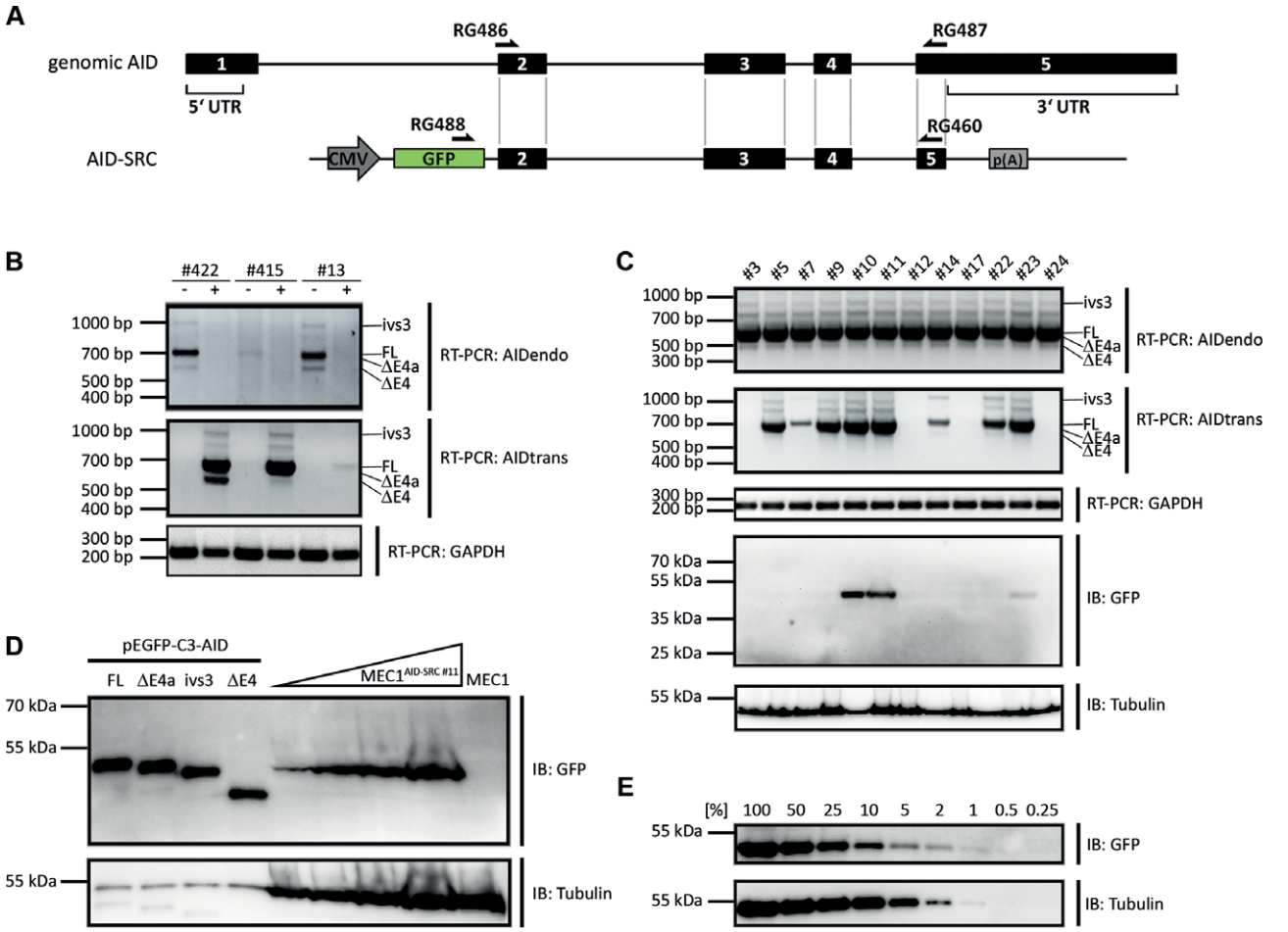

Figure 2. The AID splice reporter construct (AID-SRC) is alternatively spliced in primary CLL cells and MEC1 cells. (A) Schematic representation of the genomic AID locus and AID-SRC. Arrows indicate primer binding sites. (B) Alternatively spliced AID transcripts derived from endogenous AID (AIDendo) or from the transgenic AID-SRC reporter construct (AIDtrans) were RT-PCR amplified from untransfected (–) or AID-SRC transfected (+) primary CLL samples. RT-PCR of GAPDH was performed as control. Data are representative of two independent experiments. (C) MEC1 cells stably expressing AID-SRC were tested for endogenous AID splice variants (AIDendo) and transgenic AID-SRC-derived splice variants (AIDtrans) using PCR. Immunoblot using GFP-specific antibodies (IB) on MEC1 transfectants is shown at the lower panel. Tubulin was used as a loading control. (D) Increasing amounts of MEC1$^{AID\text{-}SRC}$ cell lysates were subjected to SDS-PAGE and immunoblotting as described in (C). As size controls, lysates from HEK293FT cells transiently transfected with constructs encoding the respective splice variant in fusion to GFP were used (lanes 1–4 of the immunoblot). All data in (C–D) are representative for three independent experiments. (E) Serial dilution was performed on whole cell lysates of HEK293FT cells transiently transfected with a GFP-AID-FL cDNA construct, and immunobloting for GFP and tubulin was performed as in (C) and (D). Data are from one experiment.

endogenous 3′UTR and hence lead to the generation of smaller fragments (Fig. 3A). The RPA on RNA from primary CLL samples, MEC1 and MEC1$^{AID\text{-}SRC}$ cells revealed fragments corresponding to the predicted sizes of AID splice variants AID-FL, AID-ivs3, AID-ΔE4, and AID-ΔE4a. Therefore, we could show that alternative splice variants are detectable on RNA level and are thus not a result of PCR artifacts.

In a more quantitative approach, we determined relative splice variant abundances by amplicon next-generation sequencing. We PCR amplified endogenous AID from cDNAs of MEC1 cells, transgenic AID from cDNA of MEC1$^{AID\text{-}SRC}$ cells, and endogenous AID from stimulated CLL #31, using bar-coded primers (listed in Supporting Information Table 2). PCR products were sequenced on a PacBio RS platform and aligned to the reference sequences of the various splice variants. By this method, we obtained a relative quantification of the PCR-amplified splice variants, showing that in all samples analyzed, AID-FL is the predominant splice form followed by AID-ΔE4a, AID-ivs3, and AID-ΔE4 (Fig. 3B).

However, as amplicon sequencing could well result in a biased amplification of the higher abundant templates, we finally set up a splice variant specific quantitative real-time PCR (qRT-PCR) using a set of variant specific reverse primers (Supporting Information Table 2 and Supporting Information Fig. 3), which is certainly the most accurate method to quantify transcript abundances. The accuracy and specificity of the qRT-PCR was evaluated by using cloned cDNA constructs as templates (Supporting Information Fig. 3). By this method, we were able to accurately determine the relative abundances of splice variants and could show that apart from the predominant AID-FL, the variants AID-ivs3 (12.4–27.2% of total AID) and AID-ΔE4a (7.2–16.7% of total AID) account for a

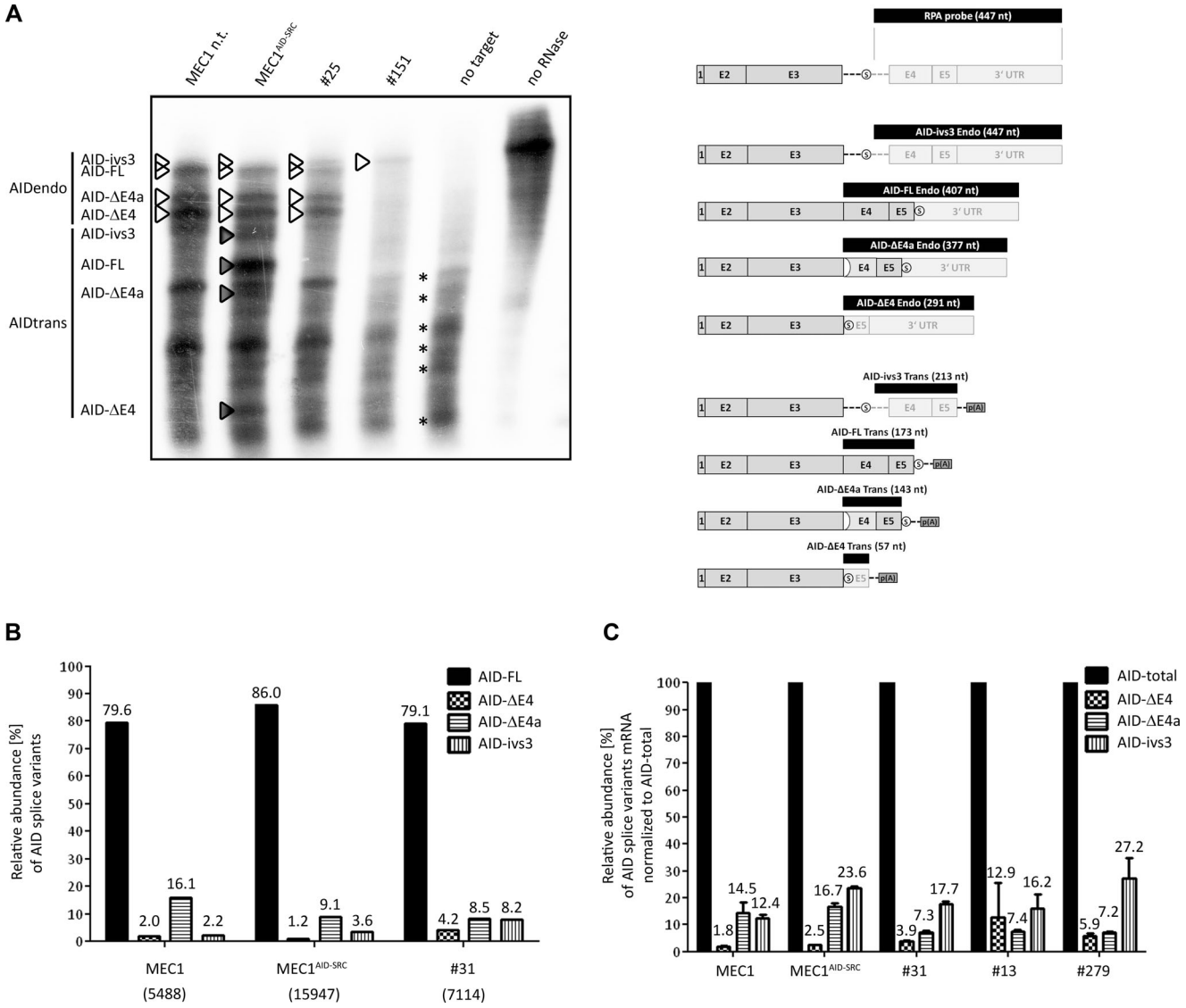

Figure 3. Quantification of AID splice transcripts. (A) RPA was performed on RNA from MEC1 cells, MEC1$^{AID\text{-}SRC}$ cells, and stimulated CLL samples (ID #25 and #151), using an antisense RNA probe spanning intron 3, exon 4/5 junction, and the 3′UTR of endogenous AID. White triangles represent splice variants of endogenous AID (AIDendo), and filled triangles indicate splice variants descending from AID-SRC (AIDtrans). Nonspecific bands that also appear using yeast RNA as control (no target) are indicated with asterisks. Protected fragments are shown to the right of the autoradiograph. Data are representative of two independent experiments. (B) Quantification of PCR-amplified splice variants by amplicon sequencing. AID transcripts were PCR amplified from cDNA of the respective samples (stimulated CLL sample ID #31, MEC1, and MEC1$^{AID\text{-}SRC}$). The PCR products were sequenced and aligned to the reference sequences of splice variants. Based on the relative mapping frequencies, the relative quantities of the PCR-amplified splice variants were calculated. The coverage of uniquely mapped reads (read count) is indicated below each sample. (C) Relative quantification of splice variants was performed using SYBR green qRT-PCR on cDNA from MEC1 cells, MEC1$^{AID\text{-}SRC}$ cells, and stimulated CLL samples (ID #13, #31, and #279). Data show mean + SD of duplicate samples of one experiment.

substantial proportion of total AID in primary CLL as well as MEC1 and MEC1$^{AID\text{-}SRC}$ cells, whereas AID-ΔE4 (1.8–12.9% of total AID) is present at rather moderate levels (Fig. 3C).

Variants AID-ivs3 and AID-ΔE4 are not present as protein at equivalent stoichiometry

To test whether AID transcripts in MEC1$^{AID\text{-}SRC}$ cells are not translated or otherwise immunoblot technique is not capable of detecting low-abundant proteins, we titrated different ratios of lysates of HEK293FT cells transiently transfected with respective GFP-AID cDNA constructs and performed immunoblotting. Aside of AID-FL, also protein for AID-ivs3 and AID-ΔE4 were clearly distinguishable as specific bands down to 2% of total lysate input. Hence, also AID-FL/AID-ivs3/AID-ΔE4 protein ratios corresponding to respective transcript ratios as determined by qRT-PCR on MEC1$^{AID\text{-}SRC}$ cells could be discerned by immunoblotting (Fig. 4A). However, immunoblotting of MEC1$^{AID\text{-}SRC}$ lysates revealed only bands corresponding to AID-FL protein, while bands

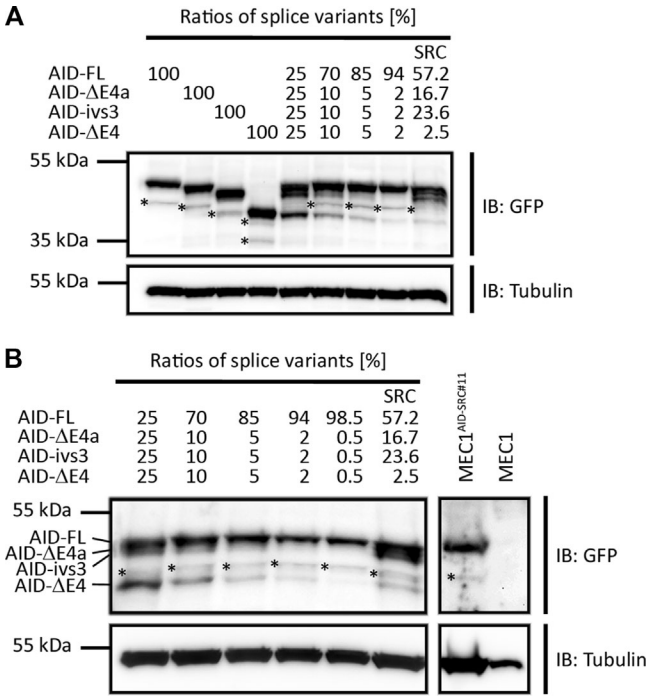

Figure 4. Analysis of AID splice variants by immunoblotting. (A) Whole cell lysates of HEK293FT cells transiently transfected with a GFP-cDNA fusion construct of respective AID variants were subjected to SDS-PAGE and immunoblotting (IB) in indicated ratios with GFP- and tubulin-specific (loading control) antibodies. SRC lane represents percentage composition of MEC1$^{AID\text{-}SRC}$ cells according to qRT-PCR data from Fig. 3C. Lysates of HEK293FT cells transiently transfected with the respective GFP-AID cDNA fusion construct were used as size standards. (B) Immunoblot of MEC1$^{AID\text{-}SRC}$ and MEC1 cell lysates. Western blot sensitivity was titrated by subjecting indicated ratios of transfected HEK293FT lysates (see above) to SDS-PAGE and immunoblotting using indicated antibodies. Asterisks denote nonspecific bands. All data in (A–B) are representative of three independent experiments.

corresponding to AID-ivs3 and AID-ΔE4 were not detectable (Fig. 4B). From these results, we conclude that alternative AID transcripts AID-ivs3 and AID-ΔE4, though present at RNA level, were not detectable as translated protein at equivalent stoichiometry. Splice variant AID-ΔE4a is hardly distinguishable from AID-FL in immunoblotting, and hence, conclusions on its protein level cannot be drawn from our experiments.

To determine whether AID splice variants are not efficiently translated or rapidly degraded, we assessed the effects of proteasome, lysosome, and autophagy inhibition on the steady-state levels of AID proteins in stably transfected MEC1$^{AID\text{-}SRC}$ cells. As splice variants have a disrupted C-terminal nuclear export sequence, they are likely to be trapped in the nucleus. Since nuclear AID was reported to be unstable and rapidly degraded [18], we assumed that alternative splice variants would also be subjected to proteasomal degradation if restricted to the nucleus. Therefore, we treated MEC1$^{AID\text{-}SRC}$ with proteasome inhibitors MG132 prior to analysis by Western blotting. As a control, we generated MEC1 transfectants stably expressing a fusion protein of AID-FL-GFP (MEC1$^{AID\text{-}GFP}$). Again, only bands corresponding to AID-FL-GFP were visible and no other splice variants were detectable despite impaired proteasomal degradation for up to 12 hours, which was verified by accumulation of polyubiquitinated proteins and cleaved poly(ADP-ribose) polymerase 1 in the cell lysates (Fig. 5A). When treating the cells for 24 hours with MG132, a faint band corresponding in size to AID-ivs3 aside of AID-FL was detectable (Fig. 5B).

To further assess the influence of nonproteasomal degradation on the clearance of AID splice variants, we treated MEC1$^{AID\text{-}SRC}$ and MEC1$^{AID\text{-}GFP}$ cells with lysosome and autophagy inhibitors (chloroquine, 3-methyladenine, or ammonium chloride) and determined protein levels by Western blotting. As shown in Figure 5B, treatment with inhibitors, which was verified by accumulation of autophagy light-chain marker LC3B II, did not result in detection of alternatively spliced AID variants.

In addition, we determined the presence of splice variants using fluorescence microscopy. As previous reports suggested that individual cells could express distinct splice variants [11], we tested whether individual MEC1$^{AID\text{-}SRC}$ cells could be discerned, which fail to produce AID-FL but express alternative splice variants that are not recognized by specific antibodies, which are variants AID-ivs3 and AID-ΔE4 (Supporting Information Fig. 2). Such cells could be negative for AID antibody staining but would be positive for GFP. Furthermore, some of the alternatively spliced AID variants exhibit disturbed nuclear export and are thus restricted to the nucleus (AID-ivs3) or show a diffuse subcellular localization (AID-ΔE4) ([10] and Supporting Information Fig. 4). However, MEC1$^{AID\text{-}SRC}$ cells showed a cytoplasmatic localization of the GFP signal that overlapped with the staining pattern observed using AID-specific antibodies (Fig. 5C). Concomitantly, also flow cytometric analysis of MEC1$^{AID\text{-}SRC}$ cells and MEC1$^{AID\text{-}GFP}$ cells as controls did not reveal the presence of GFP^{+}AID^{-} cells beyond background levels. MEC1$^{AID\text{-}SRC}$ cells exhibited the same GFP/AID expression profile as the MEC1$^{AID\text{-}GFP}$ control cells (Supporting Information Fig. 5).

AID is mainly spliced into AID-FL in murine CLL cells and non-B-cell line HEK293FT

To further corroborate our results, we assessed AID splicing in murine B cells and T-cell leukemia/lymphoma 1 (TCL1) mouse model for CLL. Therefore, we transduced primary mouse B cells as well as primary leukemic cells derived from TCL1 mice with an AID-SRC-encoding murine retrovirus vector and determined AID splice variants by performing RT-PCR using PCR primers specific for endogenous murine AID and AID-SRC (Supporting Information Tables 2 and 3), respectively. As shown in Figure 6A, endogenous AID in healthy as well as leukemic murine B cells is mainly spliced into transcripts corresponding to AID-FL. Hence, also transgenic AID-SRC is largely spliced into AID-FL, although we observed a faint band corresponding to AID-ΔE3E4, which did not appear for endogenous AID. Accordingly, aside of unspecific bands, which did not correspond to any splice variant or which also appeared upon loading control lysates, only bands corresponding to AID-FL in fusion to GFP could be detected by Western blotting using

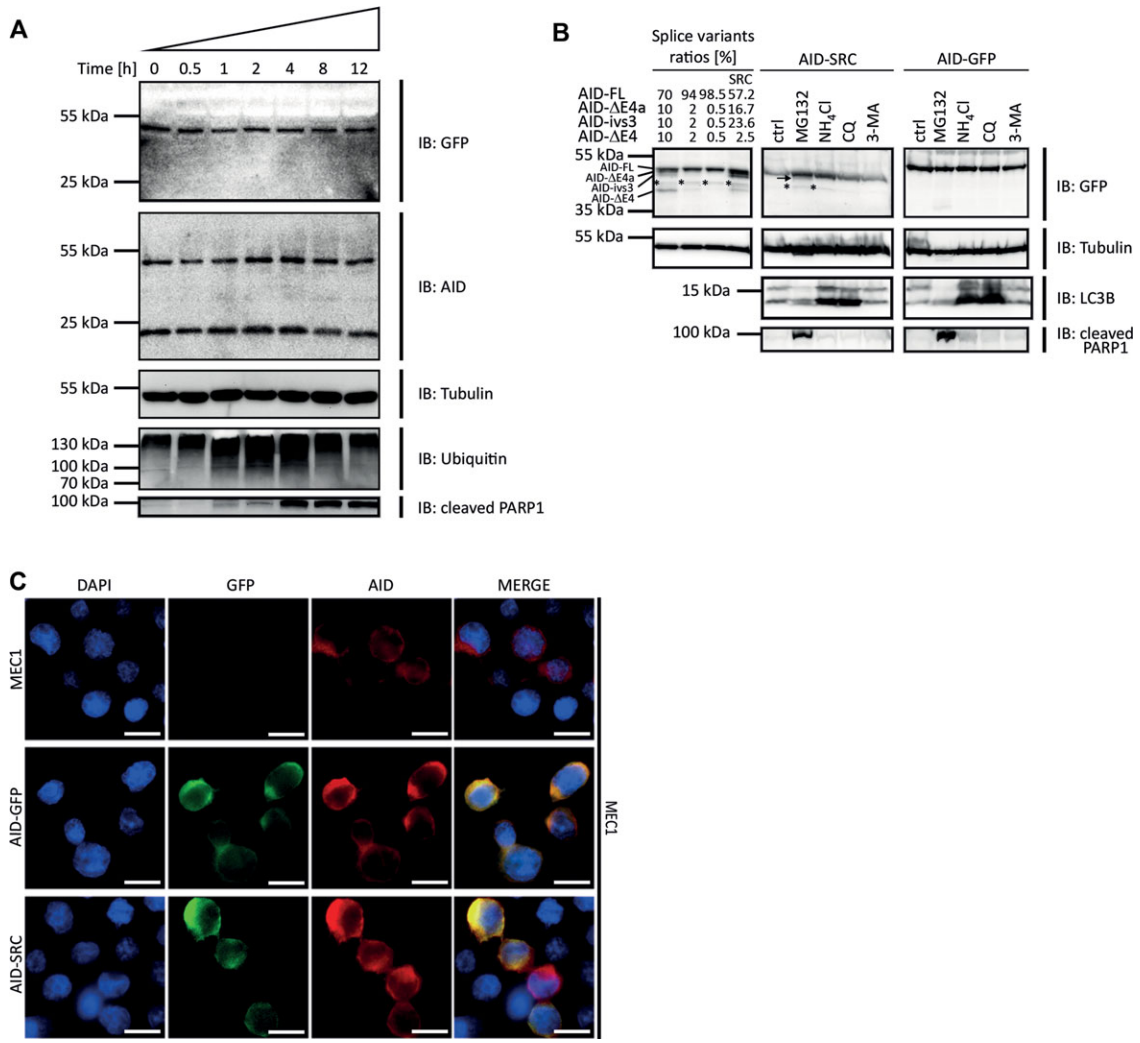

Figure 5. Effect of AID splice variants on protein levels upon inhibition of proteasomal-dependent and -independent degradation. (A) MEC1$^{AID\text{-}SRC}$ cells were treated with proteasome inhibitor MG132 prior to detection of proteins by IB using the indicated antibodies. Tubulin was used as loading control. Data are from one experiment. (B) MEC1$^{AID\text{-}SRC}$ and MEC1$^{AID\text{-}GFP}$ cells were treated with the indicated degradation inhibitors (MG132, ammonium chloride, chloroquin, and 3-methyladenine) or left untreated (ctrl) for 24 hours prior to detection of proteins by IB using the indicated antibodies. Tubulin was used as loading control. Blots were reprobed for LC3B (LC3B I: upper band; LC3B II: lower band) and cleaved poly(ADP-ribose) polymerase 1 as controls for MG132, ammonium chloride, and chloroquin treatment. Arrow marks AID-ivs3 protein. Asterisks indicate nonspecific bands. Data are representative of two independent experiments. (C) MEC1$^{AID\text{-}SRC}$ and MEC1$^{AID\text{-}GFP}$ cells were stained for DAPI and AID using DAPI-containing mounting medium and AID-specific antibodies binding to amino acids 185–198 coded on exon 5 and analyzed by fluorescence microscopy. Transfected MEC1 cells were not stained as controls. Bars: 10 μm (magnification: 60× oil). More than 150 MEC1$^{AID\text{-}SRC}$ cells were analyzed from one experiment.

GFP-specific antibodies on lysates from pMxAID-SRC-transduced B cells (Fig. 6A and B). Notably, transfection efficiency for WT B cells shown in Figure 6A was below the detection limits for GFP-tagged protein. Finally, we assessed alternative AID splicing in non-B cells. To this end, we transiently transfected HEK293FT cells with AID-SRC and assessed the presence of splice variants on RNA and protein level. Our results showed that AID-SRC transcripts are predominantly spliced into AID-FL while PCR products corresponding to AID-ΔE4, AID-ΔE4a, or AID-ivs3 were not detected on the level of RNA (Fig. 7A). Only a faint band corresponding to nonfunctional AID-ΔE3E4 appeared upon high input of cDNA (Fig. 7B). Conclusively, also on protein level, only AID-FL was detectable by Western blotting (Fig. 7A). In addition, fluorescence microscopy on AID-SRC-transfected HEK293FT cells did not reveal the presence of nuclear, and hence, alternatively spliced AID variants and the same staining pattern as for HEK293FT cells transfected with an AID-GFP fusion construct was observed (Fig. 7C).

Discussion

AID is a key enzyme for antibody diversification and due to its mutagenic activity outside immunoglobulin loci it is implicated in lymphomagenesis. Several reports have shown that primary

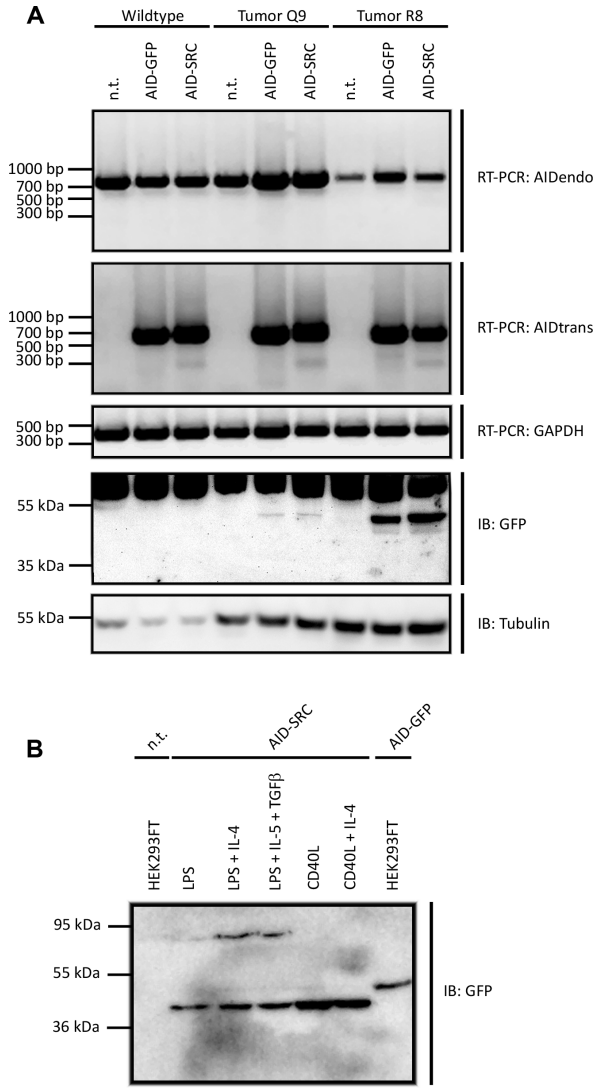

Figure 6. AID splicing in primary mouse B cells and mouse CLL cells. Primary mouse B cells were stimulated with (A) LPS/IL-4 or (B) as indicated and retrovirally transduced with the indicated pMx-based vectors. (A) RT-PCR was performed to amplify endogenous AID (AIDendo) or retrovirally expressed AID-SRC (AIDtrans). As control, RT-PCR of endogenous GAPDH was carried out. In a separate experiment, whole cell lysates of transduced mouse cells were subjected to immunoblotting (IB, lower panel). Transgenic AID was detected using GFP-specific antibodies. Detection of tubulin was used as a loading control. Data are representative of two independent experiments. (B) Immunoblot on lysates from WT mouse B cells were stimulated as indicated and retrovirally transduced with pMxAID-SRC using GFP-specific antibodies. AID-FL bands serve as loading controls. HEK293FT n.t. lane represents negative control to detect nonspecific binding of GFP antibodies. Data are from one experiment.

AID mRNA is alternatively spliced into several variants. Aside of AID-FL, variants with deletions within exon 4 are also produced, yielding variant AID-ΔE4, AID-ΔE4a, AID-ΔE3E4, or AID-ivs3 (Supporting Information Fig. 1) [11]. The physiological relevance of these splice variants has been heavily discussed. Retroviral expression of variants AID-ΔE4 and AID-ΔE4a in cell lines resulted in higher reversion frequencies of a defective GFP reporter, indicating hyperactivity of these splice variants [11]. Conversely, AID-ΔE4 and AID-ΔE4a lacked biochemical activity in vitro when purified from bacterial lysates [10]. It is conceivable that hyperactive variants are implicated in lymphomagenesis by increasing the frequency of off-target DNA damage. Alternatively, splicing into nonfunctional variants could either minimize the abundance of transcripts encoding functional AID-FL or, otherwise, distort the functional AID-FL complex that executes SHM and CSR. In line with that, expression of a catalytically inactive AID in an AID-proficient Burkitt lymphoma cell line resulted in impaired SHM [19]. In CLL, transcripts for AID-FL and its splice variants were present predominantly in samples with unmutated immunoglobulin heavy chains. Thus, it was speculated that presence of nonfunctional AID isoforms in CLL eventually impaired the AID-FL complex, thereby leaving the immunoglobulin genes unmutated [9]. However, although alternatively spliced AID transcripts were found to be present in large abundance in CLL samples and were estimated to comprise up to 25% of total AID transcripts in normal tonsillar B cells [11], their detection as translated proteins was not described, as antibodies used for AID detection recognize the C-terminal part of AID, which is alternated by alternative splicing (Supporting Information Fig. 2). Hence, we constructed an AID splice construct that is N-terminally tagged with GFP, enabling robust detection of all described splice variants. Although our reporter construct is spliced into described variants at significant amounts from 1.8 up to 27.2% of total AID (Fig. 3C) on transcript levels, we could not detect any AID splice variants in freshly prepared cell lysates on the level of protein apart from AID-FL (Figs. 2 and 4), although the sensitivity of our immunoblot assays was sufficient to discern translated splice variants down to 2% of total AID (Fig. 4B and C). As some of the alternative splice variants have a disturbed nuclear export (mainly AID-ivs3 and AID-ΔE4), they are also found in the nucleus [10] (Supporting Information Fig. 4). Since nuclear AID is unstable, rapidly polyubiquitinated and degraded by proteasomal degradation, we assumed that alternative splice variants would accumulate upon inhibition of proteasomes [18, 20–22]. Upon inhibition of proteasomal degradation in MEC1$^{AID\text{-}SRC}$ cells using MG132, splice variant AID-ivs3 aside of AID-FL was detected by Western blotting, although at lower abundance than expected from qRT-PCR data (Fig. 5B). Of note, proteasomal degradation of nuclear AID does also occur in transiently transfected HEK cells [18] and thus, alternative variants expressed in our control lysates prepared from transiently transfected HEK cells underlie the same proteasome-dependent degradative mechanisms as AID variants expressed in the MEC1 cell line. However, it is not inconceivable that proteasomal degradation of nuclear variants is more efficient in MEC1 cells than in HEK cells, which could result in rigorous clearance of nuclear splice variants in MEC1 cells under steady-state conditions.

Apart from proteasomal degradation, it cannot be excluded that also proteasome-independent mechanisms [23] may regulate AID protein levels. However, inhibition of proteasome-independent autophagic and lysosomal proteolysis did not result in detectable AID splice variants in Western blot experiments (Fig. 5B). Finally, we could show that alternative splicing of AID

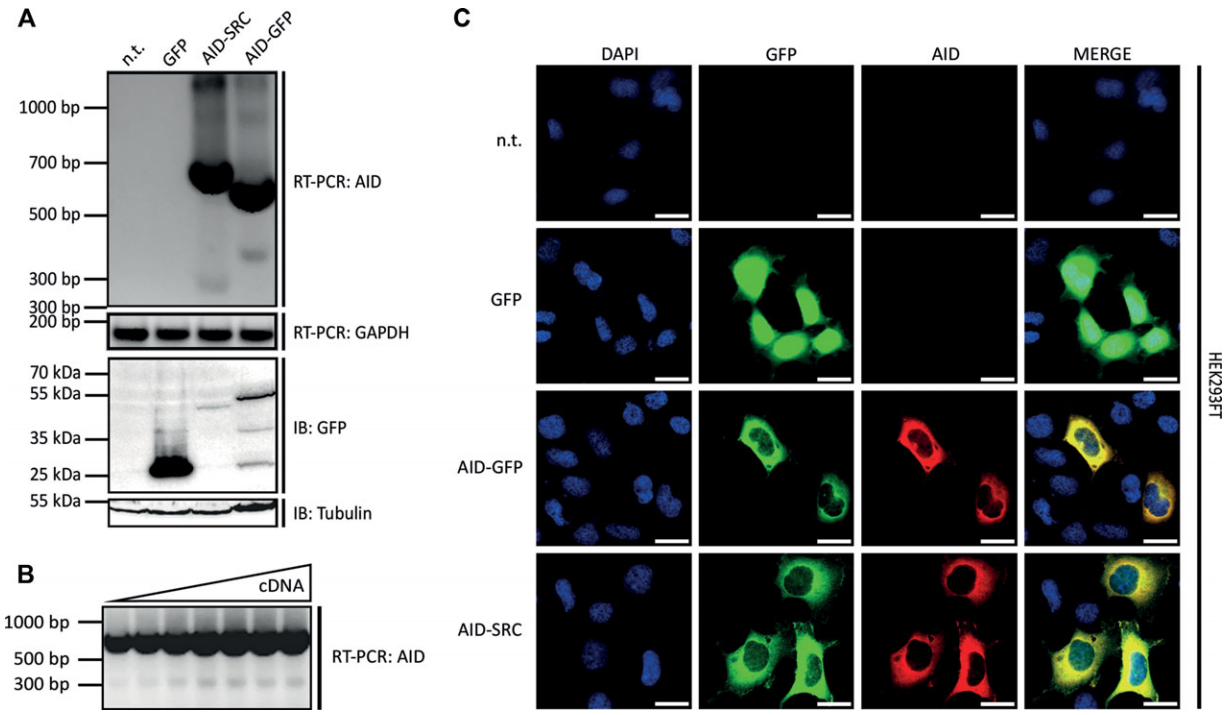

Figure 7. AID splicing in non-B-cell line HEK293FT. (A) The human embryonic kidney cell line HEK293FT was transiently transfected using GFP-, AID-SRC-, and AID-GFP-encoding plasmids. RT-PCR on AID and immunoblots (IB) using GFP- and tubulin-specific (control) antibodies were performed. Data are representative of two independent experiments. (B) RT-PCR was performed on AID (AIDtrans) from different starting amounts of cDNA generated from HEK293FT cells transiently transfected with AID-SRC. Data are from one experiment. (C) Fluorescence microscopy for GFP and AID was performed on HEK293FT cells transiently transfected with GFP-, AID-SRC-, and AID-GFP-encoding plasmids. n.t.: not transfected. Bars: 25 μm (magnification: 20 × oil). More than 120 HEK293FT cells transiently transfected with AID-SRC were analyzed from one experiment.

is only scarcely occurring in mouse B cells (Fig. 6) or in non-B cells (Fig. 7) suggesting that alternative AID splicing is most likely dependent on the presence of cell type specific splicing factors.

Although we cannot exclude the possibility that alternatively spliced AID proteins AID-ivs3 and AID-ΔE4 are below detection limit in MEC1$^{AID\text{-}SRC}$, our data clearly show that these splice variants are not present at protein levels equivalently to transcript abundances as assessed by qRT-PCR. Conversely, cDNA constructs for the respective splice variants yielded abundant protein levels upon transfection into cell lines (Fig. 2D, 4A and B, Supporting Information Fig. 2). It is conceivable that this striking discrepancy might be due to a nonlinear correlation between transcript abundance and translation. Apart from mRNA abundance, translation rates are modulated by many factors such as tRNA and ribosome availabilities, and mRNA levels are largely insufficient to predict protein levels [24, 25]. Thus, it could well be that alternative AID splice variants have to exceed a certain threshold of abundance to be efficiently translated. Alternatively, a selective inhibition of translation could be coupled to alternative AID mRNA splicing, particularly as recent reports showed that splicing factors could modulate translation initiation [26]. Another explanation could be that proteasomal degradation of nuclear AID variants works more efficiently when only low amounts of the respective variants are present, which is the case in primary CLL cells as well as in our MEC1 transfectants. Conversely, HEK cells transiently transfected with cDNA constructs for nuclear variants are overwhelmed with high transgenic protein load whereupon proteasomal degradation might be overstrained and hence less efficient.

Summarizing, we showed a thorough quantification of AID splice variants on transcript and protein levels using a GFP-reporter construct. Our data suggest that AID variants AID-ivs3 and AID-ΔE4 were not present on protein level at the same stoichiometry as on transcript level and that alternative AID splicing is strongly cell type specific. We assume that normal and malignant B cells may primarily exploit alternative splicing of AID mRNA in order to limit the amount of available AID-FL transcripts and hence the abundance of functional AID-FL protein. As AID is a potent genome-wide DNA mutator and implicated in lymphomagenesis, AID-expressing B cells are regulating AID abundance and activity at several layers to allow execution of SHM and CSR while minimizing hazardous off-target activity. These levels comprise transcriptional regulation [27–29], posttranslational modifications [30–36], subcellular localization [37], rapid degradation [18, 20–22], and binding to cofactors (reviewed in [38]). Concomitantly, haploinsufficiency of AID in hemizygote mice suggests that physiological AID levels have to be in a narrow range to ensure antibody production without endangering genome integrity [39]. From our data, we conclude that alternative splicing is a further means to regulate AID activity by regulating the amount of available AID-FL transcripts. Further research will be necessary to elucidate B-cell- or CLL-specific factors that control AID mRNA splicing

and to investigate whether B cells that are disabled in alternative AID splicing are subjected to a higher risk of AID-induced collateral DNA damage.

Materials and methods

Cells and cell lines

Peripheral blood samples from CLL patients from our outpatient care facility at the IIIrd Medical Department were collected upon informed consent in accordance with the Declaration of Helsinki and upon approval by the ethics committee of Salzburg, Austria (Ref. No. 415-E/1287/8–2011). The assessment of immunoglobulin heavy-chain variable region mutational status, CD38 expression, chromosomal aberrations (deletions 11q, 13q, 17p, and trisomy 12), and Rai staging were performed as previously described [40]. Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation using Biocoll (Biochrom AG). For in vitro activation, cells were cultivated in RPMI1640 supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 100 μg/mL streptomycin, 100 U/mL penicillin in the presence of 100 ng/mL recombinant human IL-2 (R&D Systems), 50 ng/mL recombinant human IL-4 (R&D Systems), and 1 μM CpG phosphorothioate oligonucleotide DSP30 [41] (MWG Eurofins) for 6 days. Primary CLL samples (5×10^6 cells) were transiently transfected with 4 μg AID-SRC plasmid using Amaxa nucleofection program U-015 (Lonza). Subsequently after nucleofection, cells were cultivated for 48 hours on a layer of NIH3T3 cells prior to RNA extraction and RT-PCR. The prolymphocytic CLL cell line MEC1 [17] was transfected with 4 μg AID-SRC plasmid using Amaxa nucleofection program U-015 (Lonza) and stable clones were selected on 2 mg/mL neomycin. The human embryonic kidney cell line HEK293FT was transiently transfected in a six-well plate format using genejuice (Novagen) as described by the manufacturer.

For experiments on murine samples, normal B cells and leukemic B cells were isolated from spleens from WT C57BL/6, peritoneal cavity (Q9), and peripheral blood (R8) from TCL1 transgenic mice [42] after suffocation by CO_2 using density centrifugation (LympholyteM, tebu-Bio). To obtain naive resting B cells, samples were further purified by depletion of $CD43^+$ cells (MACS, Miltenyi). For retroviral transfection of mouse samples, WT and TCL1 tumor samples were stimulated using 50 μg/mL LPS (Sigma) and 50 ng/mL recombinant mouse IL-4 (R&D Systems). For retroviral transfection of normal murine B cells, cells were stimulated with either LPS/IL-4 as described above, or with 50 μg/mL LPS, 1 ng/mL TGFβ (R&D Systems), 100 U/mL recombinant mouse IL-5 (R&D Systems), or using a CD40L-expressing fibroblast layer with or without addition of 50 ng/mL IL-4. Retroviral transfections were performed as described previously [18]. Three days after transduction, cells were harvested and subjected to RT-PCR or Western blotting. Mice were maintained at our animal facility according to the guidelines for animal housing and upon approval for animal testing (BMWF 66.012/0009-II/3b/2012)

Construction of reporter constructs

For generation of the AID-SRC, genomic AID (Accession Number NG_011.588.1) spanning exons 2–5 was PCR amplified with a proof reading polymerase (PrimeStar, Takara) using primer RG486 and RG487. The PCR product was gel purified (Qiagen) and cloned into pEGFP-C3 (Clontech) using restriction enzymes HinDIII and BamHI (Fermentas). For generation of cDNA fusion constructs, the coding region of AID-FL was PCR amplified using primers RG210 and RG467 and cloned into pEGFP-N1 using restriction enzymes NheI and BamHI (Fermentas). For cloning into a murine ecotropic retrovirus vector, AID-SRC was PCR amplified using primers RG509 and RG499 and cloned into pMX-Ig [43] using the restriction site Bam HI to obtain pMxAID-SRC. For construction of pMxGFP-AID, a GFP-AID fusion coding region was generated by PCR amplification of GFP from pMx plasmids using primer pairs RG131 and RG133 and of AID using primers RG134 and RG135 followed by a separate PCR using both PCR products as templates and the outer primers RG131 and RG135. The GFP-AID PCR product was cloned into pMx plasmid using BamHI/SalI (primers listed in Supporting Information Table 2)

Cloning of alternative AID splice variants

The alternative AID splice variants were PCR amplified (Phusion Pol, Thermo Scientific) from cDNA of CLL or MEC1 cells using primers RG460 and RG461 followed by TOPO-cloning (Invitrogen). Positive clones were verified by sequencing (MWG Eurofins) and used as templates for size-standard control-PCRs.

For cloning of AID splice variants as C-terminal fusion to GFP, TOPO-cloned splice variants were PCR amplified using primers RG486 and RG487 (Phusion Pol, Thermo Scientific). Gel-purified PCR products (Qiagen) were cloned into the pEGFP-C3 vector using BamHI and HindIII restriction enzymes (Fermentas). Sequences of plasmids were confirmed by sequencing (MWG Eurofins). Primer sequences are listed in the Supporting Information.

RPA

RPA was performed using the Protection RPA III™ Ribonuclease Protection Assay kit (Applied Biosystems/Ambion) according to manufacturer's instructions. DNA template for in vitro transcription of the ^{32}P-labeled antisense RNA probe was generated by nested PCR on pEGFP-C3-AID-SRC plasmid DNA (Phusion Pol, Thermo Scientific) using first-round primers RG655 and RG656. Gel-purified PCR product (Qiagen) was used as template for second-round PCR with primers RG655 and RG654 containing a T7 polymerase promoter sequence overhang at the

5′-end of the reverse primer. ^{32}-P-labeled probes were synthesized using Riboprobe® in vitro Transcription System (Promega) and (α-32P)CTP (111 TBq/mmol; Perkin Elmer). Isotopically labeled probes were gel purified and incorporation of radioactive nucleotides was determined by liquid scintillation counting (Wallac 1450 Microbeta PLU, EG&G Berthold). For RNA hybridization, 20 μg of total RNA was incubated with ~2 × 10^5 cpm ^{32}P-labeled RNA probes at 42°C overnight. Unprotected RNA was digested with 0.5 units of RNase A and 20 units of RNase T1 at 37°C for 30–45 min. RNA hybrids of target RNA and isotopically labeled probe RNA were precipitated and administered to electrophoresis (5% polyacrylamide, 8 M urea), vacuum dried, and exposed to Image Station 2000 Multi-Modal Imaging System (Eastman Kodak Co.) and x-ray films (Kodak). Primer sequences are listed in the Supporting Information (Supporting Information Table 2).

Amplicon sequencing

For amplicon sequencing, AID transcripts were PCR amplified using specific primers containing 5′-sequence tags. PCR was performed on cDNAs from CLL cells (stimulated CLL#31: primers RG657/RG658), MEC1 cells (primers RG659/RG660), and MEC1$^{AID\text{-}SRC}$ cells (primers RG661/RG662; sequences of PCR primers listed in Supporting Information Table 2). PCR products were purified (PCR purification, Qiagen) and a sequencing library was generated from pooled products followed by sequencing using a PacBio RS analyzer (Pacific Biosciences, run mode 1 × 120 min, read length > 3000 bp at GATC Biotech, Germany). Sequences were mapped against reference sequences for splice variants AID-FL, AID-ΔE4, AID-ΔE4a, and AID-ivs3 and uniquely mapping sequences were quantified using CLC Software (Version 6.0.1; performed at GATC Biotech, Germany).

qRT-PCR

For qRT-PCR of AID splice variants, the LightCycler® FastStart DNA Master SYBR Green I kit (Roche) was used. For quantitation of AID variants, primers covering splice variant specific exon–exon or exon–intron junctions were designed (Supporting Information Table 2 and Supporting Information Fig. 3A). The specificity of the PCR was confirmed by using TOPO-cloned splice variants or splice variants cloned into pEGFP-C3 (Clontech) as templates (Supporting Information Fig. 3B). Relative abundances of AID splice variants were calculated by comparing dCt values.

RT-PCR and immunoblot

For RT-PCR, RNA was isolated from cells (High Pure RNA Isolation Kit, Roche) and first strand cDNA was generated (iScrpt, BioRad). For detection of spliced AID-SRC, PCR was performed using a polymerase without 3′-5′ exonuclease activity (HotStar Taq Pol, Qiagen) and AID-SRC-specific primers RG460 and RG488. For detection of endogenous human AID splice variants, the primers RG461 and RG460 or SR43 and SR44 (bind on 5′ and 3′UTR) were used. Endogenous murine AID was identified using primers RG517 and RG522. For PCR-amplification of housekeeping genes, human GAPDH specific primers RG518 and RG519 and mouse GAPDH specific primers RG514 and RG515 were used. For immunoblotting, cells were directly lysed in reducing sample buffer (62.5 mM Tris-HCl (pH 7.5), 10% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.005% bromphenol blue), heat denaturated at 95°C for 5 min, and loaded onto 12% polyacrylamide gels. Proteins were transferred onto Polyvinylidene fluoride PVDF-membranes and detected using specific antibodies for GFP (pAb rabbit anti-GFP HRP #6663, Abcam), AID (mAb EK2 5G9, Cell Signaling), ubiquitin (mAB P4D1, BioLegend), LC3B (pAb #51520, Abcam), poly(ADP-ribose) polymerase 1 (pAb H-250, Santa Cruz), and tubulin (mAb B-5–1–2, Sigma), followed by secondary HRP-conjugated antibodies (pAb anti-rat HRP #7097, Abcam; pAb anti-mouse HRP, DAKO, pAb anti-rabbit HRP #7074, Cell Signaling). To measure proteasomal, lysosomal, and autophagic degradation, cells were incubated for 24 hours or the indicated time at 37°C with proteasome inhibitor MG132 (10 μM, Sigma), lysosome inhibitor ammonium chloride (50 mM, Sigma), lysosome inhibitor chloroquine (100 μM, Sigma), or autophagy inhibitor 3-methyladenine (10 mM, Sigma) as described previously [44]. Cells were then lysed in sample buffer and subjected to immunoblot analyses (primers listed in Supporting Information Table 2).

Sequencing of AID splice variants

PCR products from RT-PCR on AID-SRC and endogenous AID were gel purified (Qiagen) and then TOPO-cloned (Invitrogen). Upon bacterial transformation plasmids containing AID splice variants were isolated (Promega) and then sequenced using primers T3 and T7 (MWG Eurofins).

Flow cytometry and fluorescence microscopy

For flow cytometry, cells were stained intracellularily (BD Cytofix/Cytoperm Kit, BD Bioscience) using anti-AID antibodies specific for exon 5 (mAb EK2 5G9, Cell Signaling) and secondary antibodies (pAb anti-rat AlexaFluor647 #4418, Cell Signaling). For fluorescence microscopy, cells were grown on poly-L-lysine coated slides, fixated using 4% paraformaldehyde, lysed in 0.5% Triton X-100, and blocked using 0.1% Triton X-100 and 1% bovine serum albumin (BSA). Cells were incubated with anti-AID antibodies (mAb EK2 5G9, Cell Signaling) and secondary antibodies (pAb anti-rat AlexaFluor647 #4418, Cell Signaling) and covered in DAPI-containing mounting medium (P-36931, Invitrogen). Cells were analyzed by fluorescence microscopy (60× or 20×, with oil as indicated; Olympus IX81). Three-dimensional

deconvolution was performed using Xcellence (Olympus) software and Near-Neighbor method.

Acknowledgements: This work was supported by grants from the Austrian science fund FWF (P24619 to RGe; SFB021 to RGr; T516-B13 to NZ), the "Klinische Malignom und Zytokinforschung Salzburg-Innsbruck GmbH," and the province of Salzburg.

Conflict of interest: The authors declare no financial and commercial conflict of interest.

References

1 **Di Nola, J. M. and Neuberger, M. S.**, Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* 2007. **76**: 1–22.

2 **Xu, Z. M., Zan, H., Pone, E. J., Mai, T. and Casali, P.**, Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. *Nat. Rev. Immunol.* 2012. **12**: 517–531.

3 **Liu, M., Duke, J. L., Richter, D. J., Vinuesa, C. G., Goodnow, C. C., Kleinstein, S. H. and Schatz, D. G.**, Two levels of protection for the B cell genome during somatic hypermutation. *Nature* 2008. **451**: 841–U11.

4 **Pasqualucci, L., Bhagat, G., Jankovic, M., Compagno, M., Smith, P., Muramatsu, M., Honjo, T. et al.**, AID is required for germinal center-derived lymphomagenesis. *Nat. Genet.* 2008. **40**: 108–112.

5 **Chiarle, R., Zhang, Y., Frock, R. L., Lewis, S. M., Molinie, B., Ho, Y. J., Myers, D. R. et al.**, Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. *Cell* 2011. **147**: 107–119.

6 **Klein, I. A., Resch, W., Jankovic, M., Oliveira, T., Yamane, A., Nakahashi, H., Di Virgilio, M. et al.**, Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. *Cell* 2011. **147**: 95–106.

7 **Robbiani, D. F., Bothmer, A., Callen, E., Reina-San-Martin, B., Dorsett, Y., Difilippantonio, S., Bolland, D. J. et al.**, AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. *Cell* 2008. **135**: 1028–1038.

8 **Albesiano, E., Messmer, B. T., Damle, R. N., Allen, S. L., Rai, K. R. and Chiorazzi, N.**, Activation-induced cytidine deaminase in chronic lymphocytic leukemia B cells: expression as multiple forms in a dynamic, variably sized fraction of the clone. *Blood* 2003. **102**: 3333–3339.

9 **McCarthy, H., Wierda, W. G., Barron, L. L., Cromwell, C. C., Wang, J., Coombes, K. R., Rangel, R. et al.**, High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. *Blood* 2003. **101**: 4903–4908.

10 **van Maldegem, F., Scheeren, F. A., Jibodh, R. A., Bende, R. J., Jacobs, H. and van Noesel, C. J. M.**, AID splice variants lack deaminase activity. *Blood* 2009. **113**: 1862–1864.

11 **Wu, X. S., Darce, J. R., Chang, S. K., Nowakowski, G. S. and Jelinek, D. F.**, Alternative splicing regulates activation-induced cytidine deaminase (AID): implications for suppression of AID mutagenic activity in normal and malignant B cells. *Blood* 2008. **112**: 4675–4682.

12 **van Maldegem, F., Jibodh, R. A., van Dijk, R., Bende, R. J. and van Noesel, C. J. M.**, Activation-induced cytidine deaminase splice variants are defective because of the lack of structural support for the catalytic site. *J. Immunol.* 2010. **184**: 2487–2491.

13 **Marantidou, F., Dagklis, A., Stalika, E., Korkolopoulou, P., Saetta, A., Anagnostopoulos, A., Laoutaris, N. et al.**, Activation-induced cytidine deaminase splicing patterns in chronic lymphocytic leukemia. *Blood Cells Mol. Dis.* 2010. **44**: 262–267.

14 **Patten, P. E. M., Chu, C. C., Albesiano, E., Damle, R. N., Yan, X. J., Kim, D., Zhang, L. et al.**, IGHV-unmutated and IGHV-mutated chronic lymphocytic leukemia cells produce activation-induced deaminase protein with a full range of biologic functions. *Blood* 2012. **120**: 4802–4811.

15 **Palacios, F., Moreno, P., Morande, P., Abreu, C., Correa, A., Porro, V., Landoni, A. I. et al.**, 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* 2010. **115**: 4488–4496.

16 **Oppezzo, P., Vuillier, F., Vasconcelos, Y., Dumas, G., Magnac, C., Payelle-Brogard, B., Pritsch, O. et al.**, Chronic lymphocytic leukemia B cells expressing AID display dissociation between class switch recombination and somatic hypermutation. *Blood* 2003. **101**: 4029–4032.

17 **Stacchini, A., Aragno, M., Vallario, A., Alfarano, A., Circosta, P., Gottardi, D., Faldella, A. et al.**, MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation. *Leuk. Res.* 1999. **23**: 127–136.

18 **Geisberger, R., Rada, C. and Neuberger, M. S.**, 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. USA* 2009. **106**: 6736–6741.

19 **Papavasiliou, F. N. and Schatz, D. G.**, The activation-induced deaminase functions in a postcleavage step of the somatic hypermutation process. *J. Exp. Med.* 2002. **195**: 1193–1198.

20 **Aoufouchi, S., Faili, A., Zober, C., D'Orlando, O., Weller, S., Weill, J. C. and Reynaud, C. A.**, Proteasomal degradation restricts the nuclear lifespan of AID. *J. Exp. Med.* 2008. **205**: 1357–1368.

21 **Uchimura, Y., Barton, L. F., Rada, C. and Neuberger, M. S.**, REG-gamma associates with and modulates the abundance of nuclear activation-induced deaminase. *J. Exp. Med.* 2011. **208**: 2385–2391.

22 **Orthwein, A., Patenaude, A. M., Affar, E., Lamarre, A., Young, J. C. and Di Noia, J. M.**, Regulation of activation-induced deaminase stability and antibody gene diversification by Hsp90. *J. Exp. Med.* 2010. **207**: 2751–2765.

23 **Pyo, J. O., Nah, J. and Jung, Y. K.**, Molecules and their functions in autophagy. *Exp. Mol. Med.* 2012. **44**: 73–80.

24 **Shah, P., Ding, Y., Niemczyk, M., Kudla, G. and Plotkin, J. B.**, Rate-limiting steps in yeast protein translation. *Cell* 2013. **153**: 1589–1601.

25 **Gygi, S. P., Rochon, Y., Franza, B. R. and Aebersold, R.**, Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* 1999. **19**: 1720–1730.

26 **Michlewski, G., Sanford, J. R. and Caceres, J. F.**, The splicing factor SF2/ASF regulates translation initiation by enhancing phosphorylation of 4E-BP1. *Mol. Cell* 2008. **30**: 179–189.

27 **Endo, Y., Marusawa, H., Kinoshita, K., Morisawa, T., Sakurai, T., Okazaki, I. M., Watashi, K. et al.**, Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappa B signaling. *Oncogene* 2007. **26**: 5587–5595.

28 **Crouch, E. E., Li, Z. Y., Takizawa, M., Fichtner-Feigl, S., Gourzi, P., Montano, C., Feigenbaum, L. et al.**, Regulation of AID expression in the immune response. *J. Exp. Med.* 2007. **204**: 1145–1156.

29 **Teng, G., Hakimpour, P., Landgraf, P., Rice, A., Tuschl, T., Casellas, R. and Papavasiliou, F. N.**, MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity* 2008. **28**: 621–629.

30 **Basu, U., Chaudhuri, J., Alpert, C., Dutt, S., Ranganath, S., Li, G., Schrum, J. P. et al.,** The AID antibody diversification enzyme is regulated by protein kinase A phosphorylation. *Nature* 2005. **438**: 508–511.

31 **Cheng, H. L., Vuong, B. Q., Basu, U., Franklin, A., Schwer, B., Astarita, J., Phan, R. T. et al.,** Integrity of the AID serine-38 phosphorylation site is critical for class switch recombination and somatic hypermutation in mice. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 2717–2722.

32 **Gazumyan, A., Timachova, K., Yuen, G., Siden, E., Di Virgilio, M., Woo, E. M., Chait, B. T. et al.,** Amino-terminal phosphorylation of activation-induced cytidine deaminase suppresses c-myc/IgH translocation. *Mol. Cell. Biol.* 2011. **31**: 442–449.

33 **McBride, K. M., Gazumyan, A., Woo, E. M., Schwickert, T. A., Chait, B. T. and Nussenzweig, M. C.,** Regulation of class switch recombination and somatic mutation by AID phosphorylation. *J. Exp. Med.* 2008. **205**: 2585–2594.

34 **McBride, K. M., Gazumyan, A., Woo, E. M., Barreto, V. M., Robbiani, D. F., Chait, B. T. and Nussenzweig, M. C.,** Regulation of hypermutation by activation-induced cytidine dearninase phosphorylation. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 8798–8803.

35 **Delker, R. K., Zhou, Y. J., Strikoudis, A., Stebbins, C. E. and Papavasiliou, F. N.,** Solubility-based genetic screen identifies RING finger protein 126 as an E3 ligase for activation-induced cytidine deaminase. *Proc. Natl. Acad. Sci. USA* 2013. **110**: 1029–1034.

36 **Geisberger, R., Huemer, M., Gassner, F. J., Zaborsky, N., Egle, A. and Greil, R.,** Lysine residue at position 22 of the AID protein regulates its class switch activity. *PLoS One* 2012. **7**: e30667.

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

38 **Besmer, E., Gourzi, P. and Papavasiliou, F. N.,** The regulation of somatic hypermutation. *Curr. Opin. Immunol.* 2004. **16**: 241–245.

39 **Sernandez, I. V., de Yebenes, V. G., Dorsett, Y. and Ramiro, A. R.,** Haploinsufficiency of activation-induced deaminase for antibody diversification and chromosome translocations both in vitro and in vivo. *PLoS One* 2008. **3**: e3927.

40 **Weiss, L., Melchardt, T., Egle, A., Grabmer, C., Greil, R. and Tinhofer, I.,** Regulatory T cells predict the time to initial treatment in early stage chronic lymphocytic leukemia. *Cancer* 2011. **117**: 2163–2169.

41 **Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S. and Lipsky, P. E.,** Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* 1996. **98**: 1119–1129.

42 **Bichi, R., Shinton, S. A., Martin, E. S., Koval, A., Calin, G. A., Cesari, R., Russo, G. et al.,** Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 6955–6960.

43 **Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T. and Kumagai, H.,** Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genornics. *Exp. Hematol.* 2003. **31**: 1007–1014.

44 **Lee, S. M., Olzmann, J. A., Chin, L. S. and Li, L.,** Mutations associated with Charcot-Marie-Tooth disease cause SIMPLE protein mislocalization and degradation by the proteasome and aggresome-autophagy pathways. *J. Cell Sci.* 2011. **124**: 3319–3331.

Abbreviations: **AID**: activation-induced deaminase · **CLL**: chronic lymphocytic leukemia · **CSR**: class-switch recombination · **FL**: full length · **qRT-PCR**: quantitative real-time PCR · **RPA**: RNase protection assay · **SHM**: somatic hypermutation · **SRC**: splice reporter construct · **TCL1**: T-cell leukemia/lymphoma 1

Full correspondence: Dr. Roland Geisberger, Laboratory for Immunological and Molecular Cancer Research, Department of Internal Medicine III with Hematology, Medical Oncology, Hemostaseology, Infectious Diseases, Rheumatology, Oncologic Center, Paracelsus Medical University, Müllner Hauptstrasse 48, Salzburg, Austria
Fax: +43-662-4482-1570
e-mail: r.geisberger@salk.at

Received: 1/7/2013
Revised: 3/2/2014
Accepted: 11/3/2014
Accepted article online: 19/3/2014