



OPEN C to U RNA editing of MFN1 is regulated by ADARB1 and associates with favourable prognosis in chronic lymphocytic leukemia

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Cytidine to uridine (C-to-U) as well as adenosine to inosine (A-to-I) RNA editing denotes the posttranscriptional modification of RNA by specialized RNA deaminases. As RNA editing alters the sequence of the RNA, it can affect splicing, stability, miRNA binding and may also lead to recoding of the translated protein. Recently, we analysed recoding A-to-I RNA editing in chronic lymphocytic leukaemia (CLL) and could define prognostically relevant editing patterns. However, disease relevant C-to-U RNA editing in CLL remained unexplored. In this study, we examined C-to-U RNA editing in CLL and discovered a recoding RNA editing site within the *MFN1* gene (hg38; chr3:179,375,230), which has recently been described as RNA editing site in brain samples. We found that *MFN1* editing was not only present in CLL samples but also in naive B cell subsets, primarily occurred at unspliced RNA and correlated with intron retention. We further identified catalytically active ADARB1 as an essential regulator for *MFN1* editing. Finally, *MFN1* editing correlated with prolonged time to treatment and overall survival in CLL patients. Summarizing, we identified a novel ADARB1 function as C to U editing regulator, which regulates *MFN1* splicing and *MFN1* S329L recoding with pathogenic relevance in CLL.

Keywords RNA editing, ADARB1, APOBEC, CLL

Generally, RNA editing comprises posttranscriptional modifications of single bases within RNA molecules. In mRNA, the most prominent RNA editing events are deamination of either adenosine or cytosine, catalyzed by ADARs (adenosine deaminases that act on RNA; ADAR1 and ADAR2) and by AID/APOBEC (activation-induced deaminase/Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family members. While deamination of adenosine yields an inosine (decoded as guanosine), deamination of cytosine leads to uracil, whereupon ADAR dependent RNA editing is referred to as A>I (or A>G) editing and AID/APOBEC dependent editing as C>U editing. While A>I editing is the most common type of RNA editing in metazoan, AID/APOBEC dependent C>U editing is restricted to mammals¹. Recent advances in massive parallel sequencing allowed systematic detection of single base differences from matched DNA/RNA samples, leading to description of an increasing number of individual RNA editing sites in the human genome². Although most of these editing sites are confined to non-coding transcripts and are important to deactivate endogenous retroviral

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elements and transposons, many RNA editing sites were discerned in mRNAs, changing the coding sequence itself or affecting miRNA binding sites or splicing patterns^{1,3}.

In humans, the AID/APOBECs comprise 11 family members, AID, APOBEC1 (A1), A2, A3A, A3B, A3C, A3DE, A3F, A3G, A3H and A4, with all of them (except for A4) showing C-to-U deaminase activity on single-stranded DNA or RNA^{4,5}. So far, RNA editing activity has been reported for A1, A2, A3A and A3G. Originally, A1 was found to edit the RNA of ApoB (apolipoprotein B) in the small intestine, leading to a premature stop codon and expression of a truncated APOB protein, important for lipid metabolism⁶. Since then, A1 was found to have hundreds of targets in RNAs from immune cells and neurons, important for inflammation and brain function^{7,8}. While A2 was originally proposed to have a role in muscle development without evident roles in DNA/RNA editing, A2 transgenic mice showed A2 dependent RNA editing of specific transcripts and contributed to tumorigenesis^{5,9}. Furthermore, A3A was shown to edit hundreds of gene transcripts during macrophage polarization and overexpression of A3A and A3G in cell lines induced C>U editing in thousands of target genes^{10–13}.

It has been noticed that RNA editing is frequently dysregulated in cancer and may critically contribute to cancer pathogenesis or serve as prognostic indicator^{14,15}. Recently, we defined a catalogue of A-to-I editing events in chronic lymphocytic leukemia (CLL), a B cell malignancy of the elderly with high incidence. There, we could characterize CLL-specific RNA and miRNA editing and extracted RNA editing patterns with prognostic impact^{16,17}. In the present study, we analysed C-to-U editing in CLL and discovered a C-to-U editing site within the *MFN1* gene, which leads to S329L recoding of MFN1 (mitofusin-1), a protein critically involved in mitochondria network formation¹⁸. This editing site has been recently detected in brain samples in context of schizophrenia and was shown to affect MFN1 function in mitochondrial fusion¹⁹. Mitochondrial dysfunction has been described in many diseases including cancer²⁰. As mitochondrial networks are implicated in many biological processes including cell death, proliferation, migration and calcium signaling²¹, *MFN1* editing can influence these pathways thereby affecting cellular homeostasis. Here, we show that *MFN1* is highly edited in naïve B cells and in a subset of CLL cell samples. We found that *MFN1* editing is associated with retention of the intron upstream of the editing site and we identified the A>I editing enzyme ADAR1 as a regulator for *MFN1* C-to-U editing. Finally, we found that *MFN1* editing is associated with favourable prognosis in CLL.

Results

C-to-U editing is observed in CLL

We systematically analysed global C-to-U RNA editing in a previously documented cohort of 45 primary chronic lymphocytic leukemia patients (CLL cohort #1, NCBI-SRA accession-number PRJNA540189) by determining single base RNA/DNA differences from matched exome/transcriptome sequencing data^{16,22}. From these 45 samples, we found a total of 5735 non-synonymous C>U differences, which comprised 75 recurrent (present in at least 2 samples), non-synonymous C>U editing events. We then validated these editing sites by screening RNA-seq data from a validation cohort of 98 samples (CLL cohort #2, EGA accession number EGAS00001000374)²³, resulting in 10 remaining validated C>U editing sites, of which 2 showed predicted functional impact (Fig. 1A, Table S1). These 2 C>U sites comprised the genes *MFN1* (hg38; chr3:179,375,230; S329L) and *PLEKHM1* (hg38; chr17: 45,475,685; T113M). We next analysed C>U editing of these 2 sites by Sanger sequencing of genomic DNA and cDNA from CLL samples from our cohort (CLL cohort #1). While *PLEKHM1* editing could not be determined by Sanger sequencing (data not shown), we could clearly verify *MFN1* editing by Sanger sequencing in two samples with high *MFN1* editing from bioinformatic RNA/DNA sequencing data analysis (#219, #224, Fig. 1B). Strikingly, we found that C>U editing of *MFN1* was largely restricted to unspliced *MFN1* transcripts (Fig. 1B). This was consistent with our RNA-seq data from these two patients, in which we observed *MFN1* C>U editing in RNA-seq reads, mostly mapping to unspliced *MFN1* transcripts with retained intron 9 (Fig. 1C and D).

MFN1 editing correlates with intron retention in different cell types

Next, we reanalyzed our RNA-seq data from both CLL cohorts for presence of C>U editing events in reads mapping to spliced or unspliced *MFN1*. Thereby, we detected a clear bias towards editing within the unspliced *MFN1* transcript although editing in spliced *MFN1*, which leads to S329L recoding, was observed in a few cases with IGVH mutated as well as unmutated IGHV (Fig. 2, Table S2). Moreover, we observed significant correlation of *MFN1* C>U editing depth (frequency of edited transcripts) and fraction of unspliced *MFN1* transcripts with intron 9 retention (Fig. 2, Table S2).

We then aimed to analyse *MFN1* editing in healthy tissue. Therefore, we accessed publicly available RNA-seq datasets from stomach, skin, lung, ovary and prostate as well as from B cell subsets (EBV-transformed B cells, naïve, memory and class switched memory B cells) and extracted C>U editing depth at *MFN1* transcripts. Again, we found editing at variable depth in different tissues, with editing frequency correlating with retention of intron 9 (Fig. 3A, Table S3).

Surprisingly, substantial editing in mature spliced *MFN1* transcripts was observed in naïve B cells with S329L recoding frequencies between 17 and 38%, but not in class switched or non-class switched memory B cells (Fig. 3B). This was confirmed by Sanger sequencing of PCR products corresponding to spliced and unspliced *MFN1* amplified from cDNA of purified B cells from healthy donors (Fig. 3C).

ADAR1 supports MFN1 editing

Based on previous reports, we expected expression of the AID/APOBEC member responsible for *MFN1* editing to be associated with editing frequencies¹⁰. Although the *MFN1* editing site is located within a previously defined APOBEC3A consensus motif (5'-UCCG-3') flanked by palindromic sequences¹⁰ (Fig. 4A), *MFN1* editing frequencies in the CLL cohorts and normal B cells did not positively correlate with any APOBEC expression

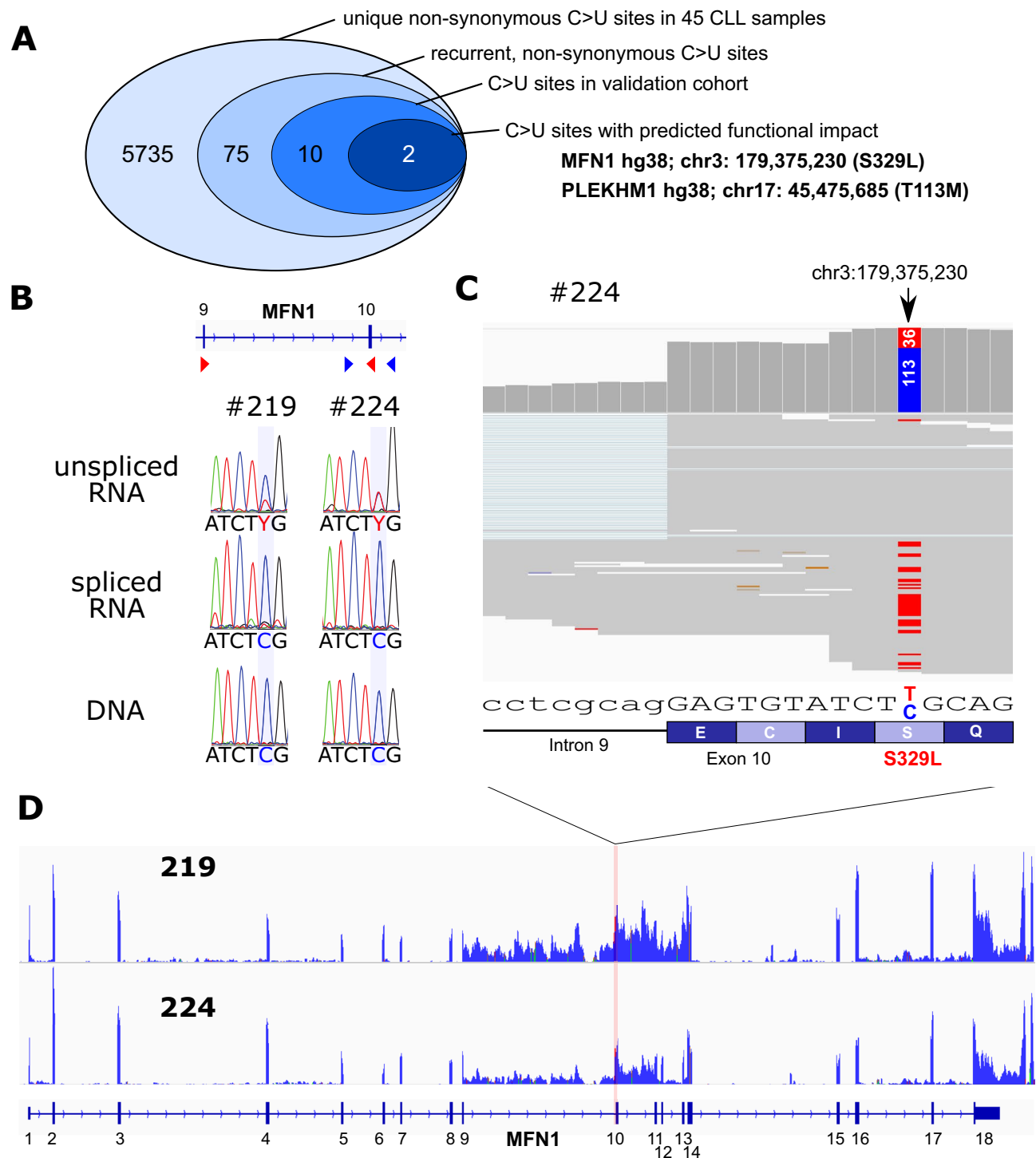


Fig. 1. C>U editing in 2 CLL cohorts. (A) Extraction of recurrent C>U editing sites from 2 independent CLL cohorts. (B) Sanger sequencing confirms C>U editing in *MFN1* transcripts with retained intron 9 (Y denotes presence of a pyrimidine C or T). (C, D) Integrated genome viewer analysis of RNAseq reads mapping to *MFN1* at the C>U editing site (CLL patient #219 and #224).

levels (Fig. 4B). To find candidates for putative positive editing regulators, we performed Pearson correlation analysis using editing frequency and RNA expression from these three cohorts. We screened the top candidates particularly for proteins with known RNA binding activity and found a positive correlation of *MFN1* editing frequency and expression levels of *ADARB1*, an A-to-I RNA editing enzyme (Fig. 4C). To test an influence of *ADARB1* on *MFN1* editing, we cloned Flag-tagged *ADARB1* and a mutant lacking the dsRNA binding motif 1 (Δ DRBM1) together with a catalytically dead mutant *ADARB1*-CD, which has an E396A mutation²⁴ (Fig. 4D). We transfected HEK293 cell lines with the different *ADARB1* constructs and determined editing frequencies of

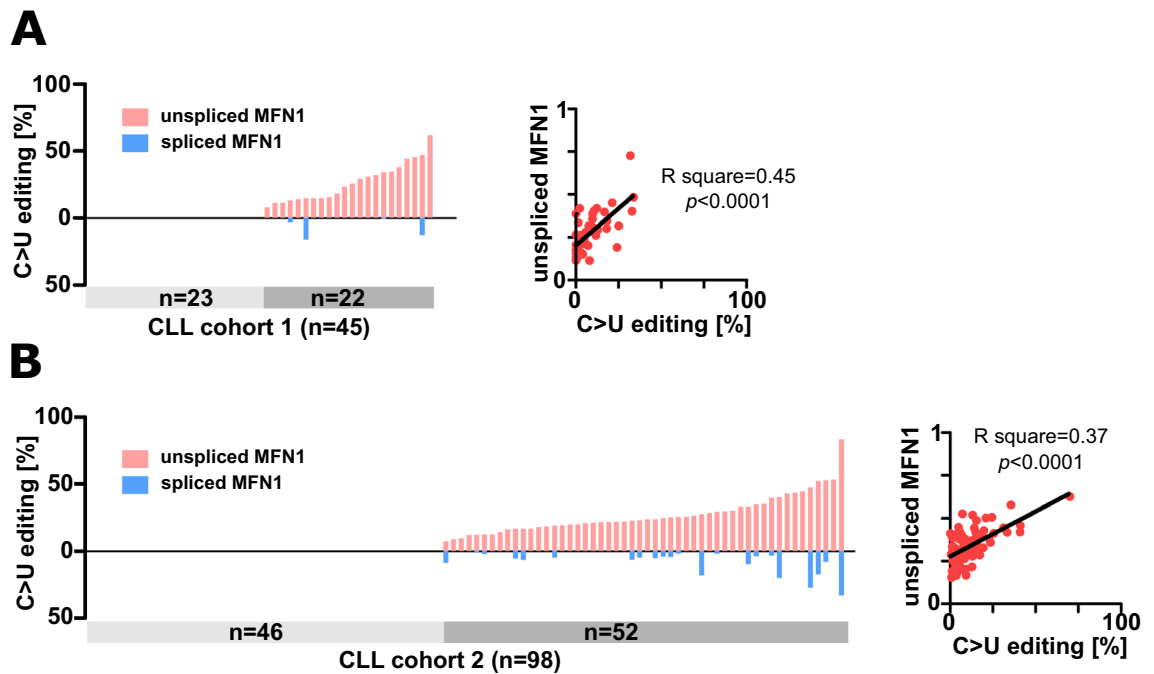


Fig. 2. C>U editing is largely restricted to unspliced *MFN1*. Percentage of C>U editing in reads mapping to spliced or unspliced *MFN1* in CLL cohort #1 (A) and CLL cohort #2 (B). Correlation of C>U editing and *MFN1* splicing is shown on the right.

endogenous *MFN1* transcripts. We found that transient transfection of *ADARB1* was sufficient to induce robust *MFN1* editing in HEK293 cell lines (Fig. 4E, F), showing that *ADARB1* is indeed a factor supporting *MFN1* C-to-U editing. As expected, Δ DRBM1 did not support editing, as this mutant fails to bind RNA. However, surprisingly, also cells transfected with *ADARB1*-CD did not show editing of *MFN1*, indicating that the catalytic domain of *ADARB1* is necessary for *MFN1* C-to-U editing (Fig. 4E, F). To fully clarify, whether also other ADARs can mediate this C-to-U editing event, we also cloned Flag-tagged versions of ADAR1 isoforms p110 and p150 and determined editing frequencies of endogenous *MFN1* transcripts in transfected HEK293 cells. Thereby we could confirm that only *ADARB1* but not other ADAR1 isoforms is competent to induce C-to-U editing in *MFN1* transcripts (Fig. S1).

***MFN1* editing is mediated by APOBEC3 and is coupled to *MFN1* intron retention**

To detect potential transcriptome-wide *ADARB1* dependent C-to-U editing events, we performed RNA-seq from *ADARB1* and control-transfected HEK293 cells and called specific editing events. In line to previous reports, we detected many *ADARB1* induced A-to-I editing events¹ in two independent experiments, whereas C-to-U editing was exclusively restricted to *MFN1*, showing that this is the sole *ADARB1* dependent C-to-U editing site present in RNA from 293HEK cells (RNA editing data from RNAseq and small RNA seq depicted in Table S4, S5). However, RNA-seq analysis revealed many different A-to-I editing sites clustering around the C-to-U site within *MFN1*. Each of the A-to-I editing sites were low frequent and most of them were non-recoding events (Fig S2). Interestingly, four of these A-to-I editing sites, which are closest to the C-to-U editing site were also present in CLL and B cell samples (A-to-I editing sites at position -4, +3, +7 and +10 relative to the C-to-U site) and correlated with C-to-U editing (Fig S2B). Hence, these A-to-I events support the concept of direct binding of *ADARB1* near to the *MFN1* C-to-U editing site. Next, to determine, which cytidine deaminase executes *MFN1* C-to-U editing, we extracted expression levels of AID/APOBEC cytidine/cytosine deaminase family members from the RNA-seq data. From the catalytically active AID/APOBEC members, we did not find transcripts for *AID*, *A1*, *A2* and *A3A* and only very low expression levels for *A3D* and *H*, pointing to *A3B*, *C* or *F* as the likely candidates for catalyzing *MFN1* C-to-U editing (Fig S3, Table S6). To assess whether *MFN1* editing is attributed to C-to-U deamination by *A3B*, *C* or *F*, we performed pooled knockdown experiments of *A3B*, *C* and *F* using small interfering RNAs (siRNAs) in HEK293 cells together with *ADARB1* transfection. Indeed, we observed a decrease in *MFN1* editing frequencies in *ADARB1* transfected cells upon *A3BCF* knockdown compared to control siRNAs, both in spliced as well as unspliced *MFN1* transcripts (Fig. 5A and Fig S4). Notably, also individual knockdowns of *A3B*, *C* or *F* led to decreased C-to-U editing while combined *A3BCF* knockdown had the biggest impact on reduction of editing frequencies (Fig S5). To examine the interrelation of *MFN1* editing and *MFN1* intron retention, we established a taqman based qPCR assay to specifically determine transcript levels of *MFN1* with or without retained intron 9. Although most transcripts remained fully spliced, we found that *ADARB1* expression slightly increased *MFN1* intron retention, which was not observed upon simultaneous knockdown of *A3BCF* (Fig. 5B). Hence, our data promote a model in which *ADARB1* and APOBEC3 cooperate

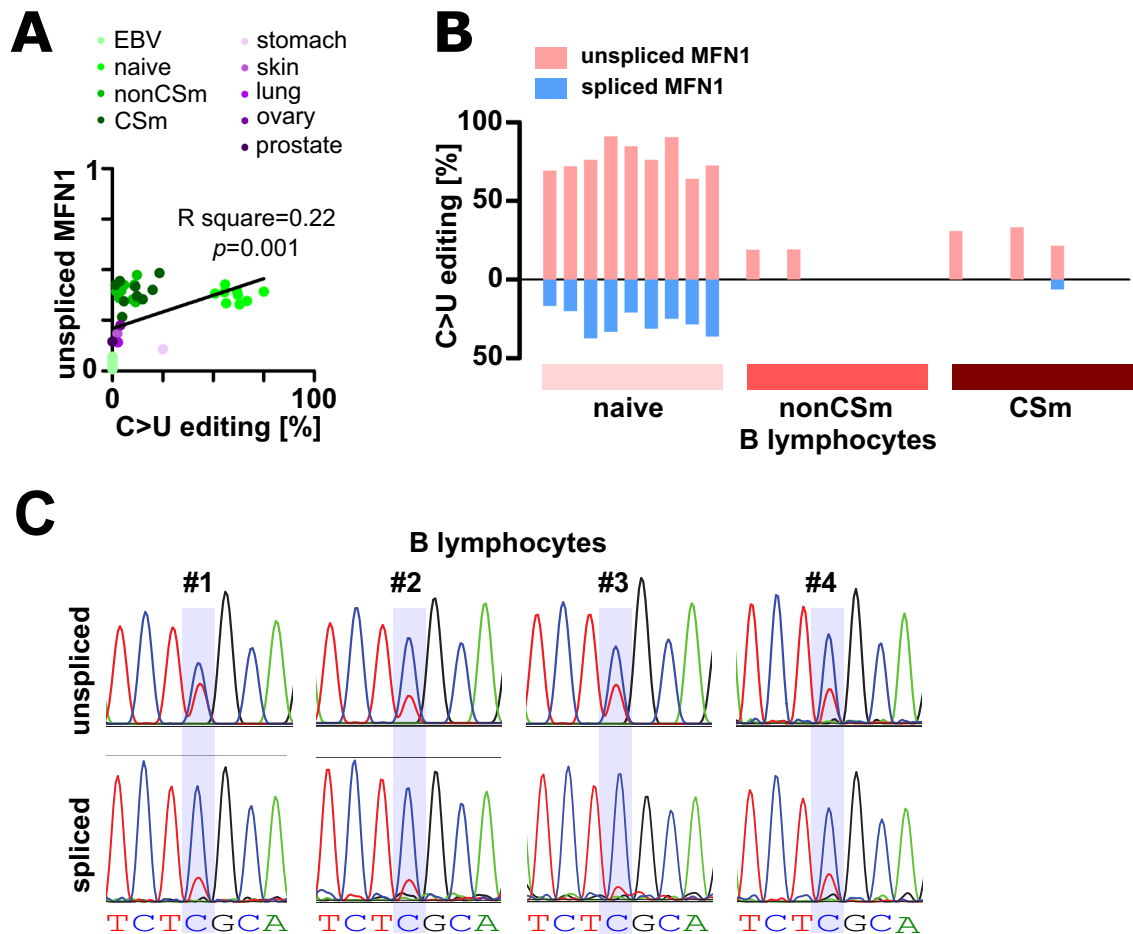
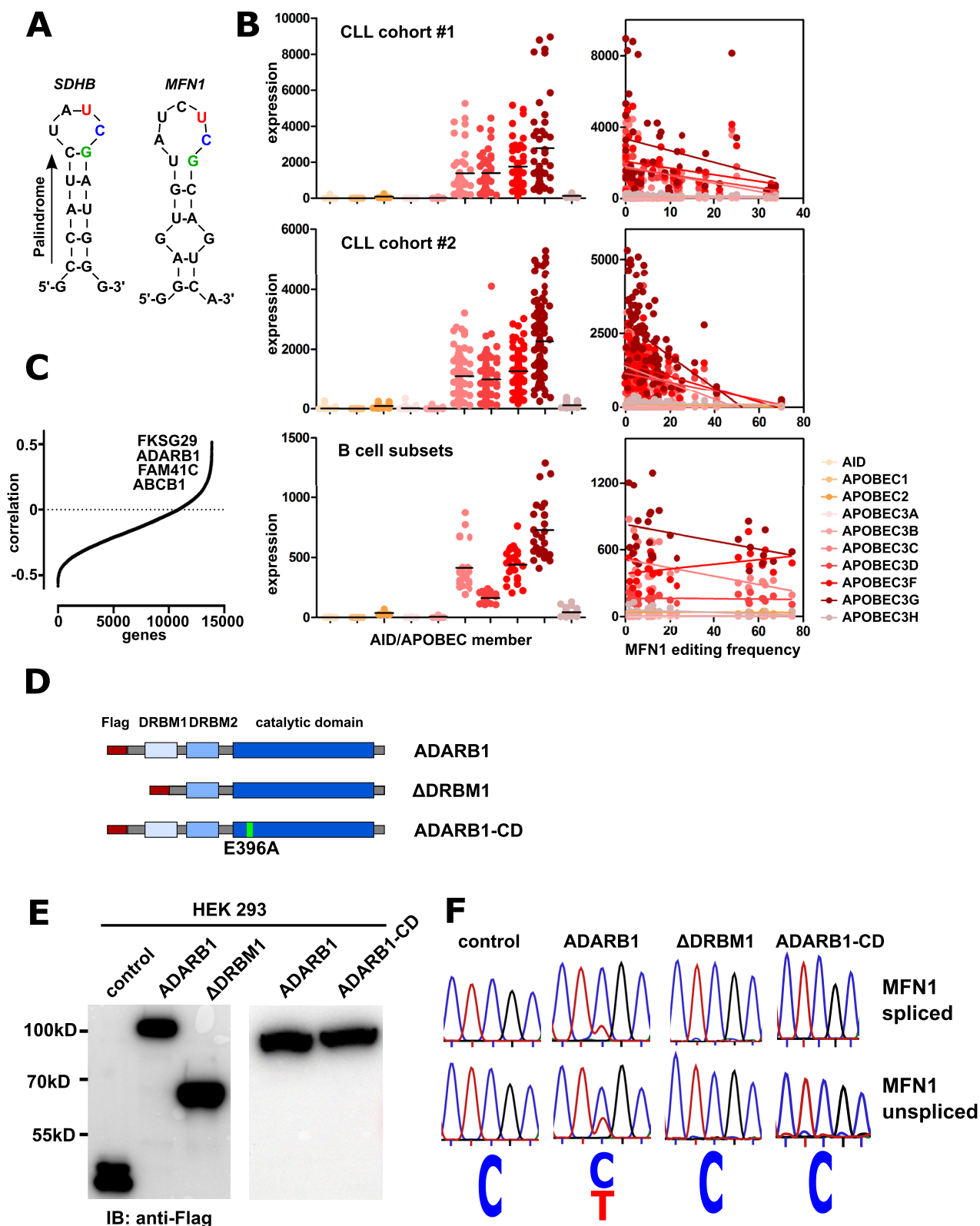


Fig. 3. *MFN1* is robustly edited in B cells. **(A)** Correlation of C>U editing and *MFN1* splicing is shown for different cell types (EBV transformed B cells, naïve B cells, non class switched memory (nonCSm) B cells, class switched memory (CSm) B cells, stomach, skin, lung, ovary, prostate). **(B)** Percentage of C>U editing in reads mapping to spliced or unspliced *MFN1* in B cell subsets. **(C)** Sanger sequencing confirms C>U editing in *MFN1* transcripts in B cells from four different healthy donors.

to highly specifically induce C-to-U editing and intron retention of *MFN1* transcripts to regulate *MFN1* abundance and S329L recoding (Fig. 5C).

MFN1 editing and CLL disease course

MFN1 S329L recoding was previously shown to critically affect mitochondrial fusion and, thus, in turn impacts mitochondria-dependent cellular homeostasis and apoptosis¹⁹. This prompted us to evaluate an association of *MFN1* editing on the course of disease in CLL patients. Therefore, we analysed a pilot cohort of 23 CLL patients, where sampling was done during indolent disease stage (table S7). From this cohort, we performed RNA-seq from CLL samples and determined *MFN1* editing frequencies. Cox-regression analysis and log-rank testing revealed that high frequency of *MFN1* C-to-U editing associated with prolonged treatment-free survival (time to treatment, TTT) from time of diagnosis as well as sampling (Fig. 6A). In addition, overall survival (OS) was significantly prolonged in patients with high frequency *MFN1* editing (Fig. 6A). To corroborate these findings, we analysed *MFN1* editing and course of disease in the validation cohort (CLL cohort #2) of which RNA-seq and clinical data were available²³. In line with findings from the pilot cohort, we observed significantly prolonged TTT as well as OS in patients with high *MFN1* editing frequencies (Fig. 6B). While TTT was not significantly different within the IGHV mutated or unmutated samples, OS remained significantly prolonged in IGHV-unmutated CLL patients with high *MFN1* editing (Fig. 6C). Gene expression analysis from RNAseq data revealed that ADARB1 was highly significantly upregulated in *MFN1*-editing-high samples from the CLL validation cohort. APOBEC3 gene members did not show significant upregulation in *MFN1*-editing-high samples (supplementary Fig S7). Of note, *MFN2* was slightly upregulated in the editing-high group, while *MFN1* levels were similar. However, transcript levels of C-to-U edited *MFN1* were significantly higher in the editing-high group (Fig S7). Finally, gene ontology (GO) term analysis of RNAseq data revealed that many biological pathways were significantly deregulated between editing high and low groups, including those mapping to RNA



metabolisms (splicing, processing). Pathways assigned to cell death or mitochondrial functions were not found significantly deregulated (Table S8).

Discussion

In this study, we show for the first time that the A-to-I editing enzyme ADARB1 plays a novel role in C-to-U RNA editing. Particularly, we found that A3BCF together with ADARB1 cooperate to induce C-to-U editing at *MFN1* transcripts, thereby interfering with *MFN1* splicing and mediating *MFN1* S329L conversion, with robust S329L recoding primarily occurring in naïve B lymphocytes and a subset of primary CLL cells. Although ADARB1

Fig. 4. *MFN1* editing requires ADARB1. (A) *MFN1* editing site is located within a previously defined palindromic consensus motif present in *SDHB* transcripts. (B) Expression levels of AID/APOBEC members and correlation with C>U *MFN1* editing in two CLL cohorts and normal B cell subsets. (C) Mean values from a Pearson correlation from gene expression and *MFN1* editing from two CLL cohorts and B cell samples. The four top candidates positively correlating with editing are indicated in the graph. (D) Schematic representation of Flag-tagged ADARB1, Δ DRMB1 and ADARB1-CD constructs. (E) Western blot analysis of HEK293 cells transfected with the respective Flag-tagged constructs. (F) Representative Sanger sequencing of *MFN1* C-to-U editing sites from spliced and unspliced *MFN1* transcripts in HEK293 cells transfected with the respective Flag-tagged constructs.

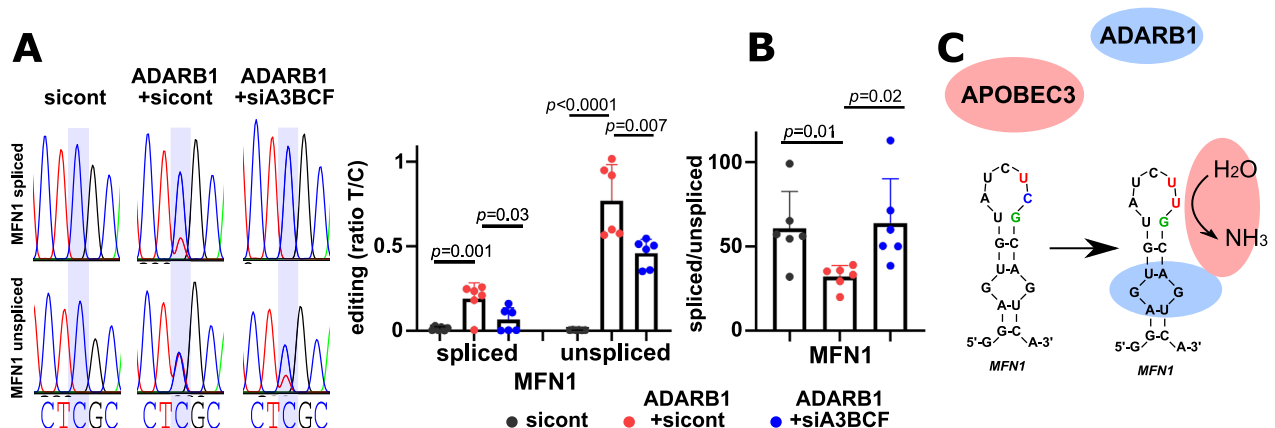


Fig. 5. APOBEC3 proteins and ADARB1 cooperatively induce *MFN1* editing. (A) Representative Sanger sequencing of *MFN1* C-to-U editing sites from spliced and unspliced *MFN1* transcripts in HEK293 cells transfected with control siRNAs (sicont), siRNAs specific for APOBEC3B,C,F (siA3BCF) or Flag-tagged ADARB1 (ADARB1). Graph shows the statistics of editing frequencies from Sanger sequences from 6 independent experiments. (B) Ratio of spliced to unspliced *MFN1* transcripts determined by qPCR from experiments shown in (A). (C) A model illustrating ADARB1 dependent *MFN1* C-to-U editing by APOBEC3 deaminases. In this model, ADARB1 recruits (directly or indirectly) APOBEC3 proteins to *MFN1* pre-mRNAs, thereby enabling C-to-U deamination by APOBEC3 and interfering with splicing. In A and B, mean + SD is shown. Significances calculated using unpaired t-test from 6 independent experiments.

has been previously reported to mediate many A-to-I editing events and to regulate RNA splicing, ADARB1 has so far not been implicated in C-to-U editing^{25,26}. Interestingly, catalytically inactive ADARB1 E396A mutants were not competent for APOBEC3 mediated C-to-U editing of the *MFN1* transcripts. Mechanistically, several models for *MFN1* C-to-U editing can be brought forward: first, the catalytically dead ADARB1 mutant might be compromised in its ability to form a complex with *MFN1*-RNA substrate and the APOBEC3 enzyme. However, we consider this possibility rather unlikely, as the ADARB1 E396A mutant has been shown to retain full RNA binding capacities²⁷. Moreover, a catalytically inactive variant would possibly bind with even higher affinity to RNA targets as A-to-I deamination likely promotes dissociation of ADARB1 from the RNA substrate. Second, ADARB1 might be able to catalyze the C-to-U deamination directly due to a rare and unique conformation of the flipped out cytidine. However, we also consider this possibility rather unlikely, because the C-to-U deamination can be reduced by knockdown of APOBEC3, pointing to APOBEC3 members to catalyze the C-to-U deamination. Third, inactive ADARB1 might lose processivity on RNA targets, which could be necessary for bound APOBEC3 to come in proximity to the C-to-U editing site.

Alternatively, ADARB1 binding to and editing of *MFN1* transcripts could alter the secondary RNA structure, allowing recruitment of A3 members. These possibilities are not mutually exclusive and more research will be necessary to fully delineate the mechanism behind *MFN1*-editing. However, the scattered low-level A-to-I editing events nearby the *MFN1* C-to-U editing site correlate with C-to-U editing, which we interpret as footprint of ADARB1 activity and as evidence that ADARB1 is physically interacting with *MFN1* transcripts in proximity to the C-to-U editing site. Furthermore, our data show that *MFN1* editing is associated with prolonged treatment-free and overall survival in CLL patients. During our study on CLL, another team reported S329L *MFN1* editing in brain samples and hypoeediting of this site in samples from schizophrenia patients¹⁹. There, S329L recoding was shown to affect the function of the *MFN1* encoding protein mitofusin-1. Mitofusin-1 is an important protein mediating mitochondrial fusion and network formation, which alters a diverse set of physiological processes such as mitochondria-dependent cell death, calcium homeostasis, energy metabolism and proliferation^{28–30}. *MFN1* protein, as well as the homolog *MFN2* consists of a GTPase domain, two coiled-coil heptad repeat domains (HR1 and HR2) and a transmembrane domain with the amino and carboxy termini both facing into the cytosol. GTP hydrolysis enables mitochondrial fusion upon interaction of two opposing HR2 domains from homo- or hetero-oligomeric complexes (*MFN1*-*MFN1*, *MFN2*-*MFN2* or *MFN1*-*MFN2*).

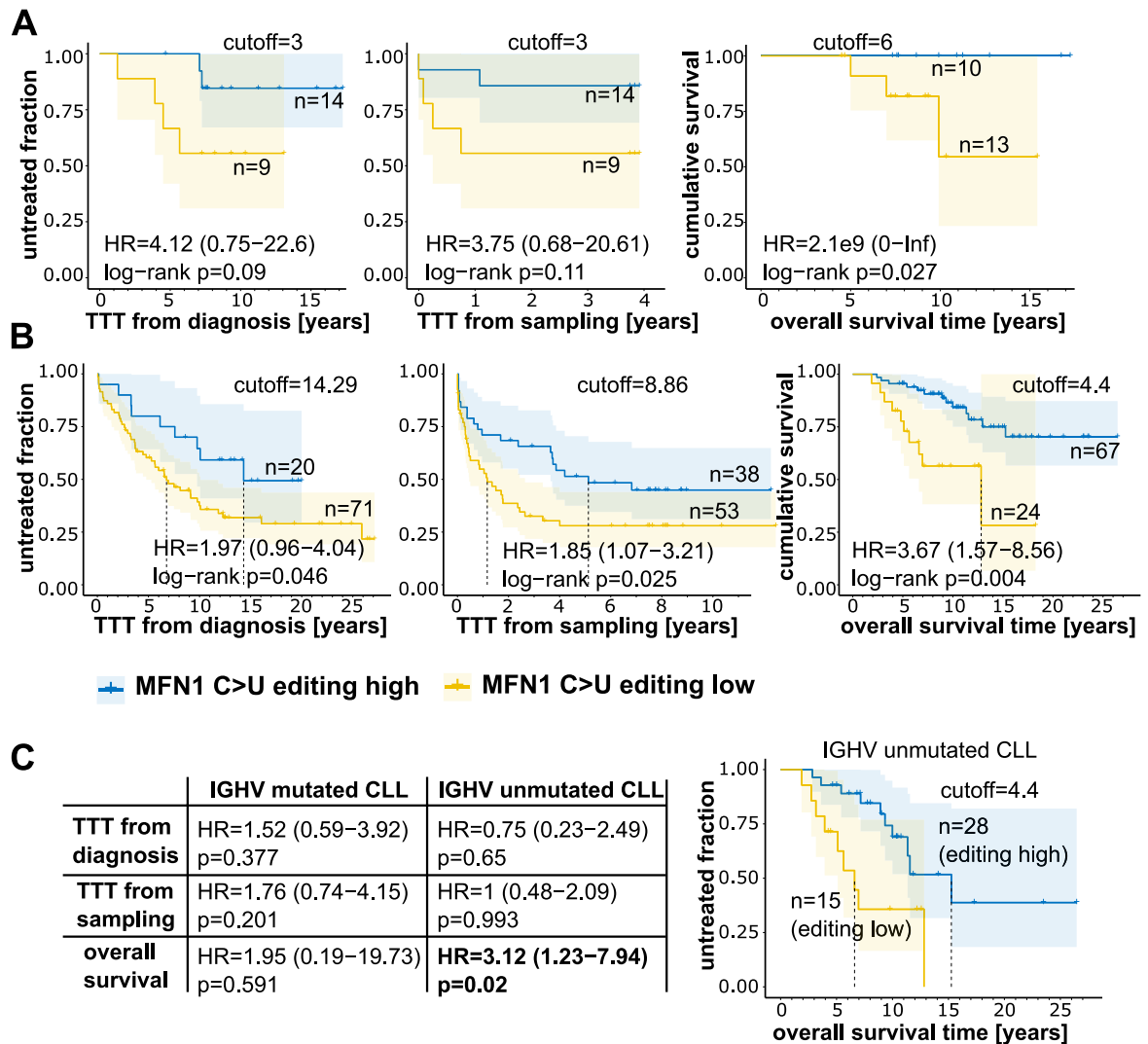


Fig. 6. *MFN1* editing associates with prolonged survival. Kaplan–Meier survival plots show time to treatment (TTT) from diagnosis, from sampling and overall survival in the pilot cohort (A) and the validation cohort (B). Hazard ratios (HR) with confidence intervals (CI), log-rank p-values, cut-offs (%*MFN1* editing) and sample size (n) are indicated within the graphs. (C) TTT and OS in the CLL validation cohort according to IGHV mutation status (cutoffs used as in B).

complexes³¹. Although S329 is not directly located within one of the known *MFN1* domains but between the GTPase and the HR1 domain, S329L conversion was shown to perturb *MFN1* function in mitochondrial fusion and to increase cellular apoptosis¹⁹. Decreased cell fitness upon S329L recoding in leukemic cells would in turn alleviate the disease, which fits to our observed prolonged survival in CLL patients with high *MFN1*-editing frequencies. Nonetheless, a possible benefit of S329L recoding for the respective CLL cells and for naïve B cells remains to be investigated, which likely reflects the complex relationship between mitochondrial morphology and cellular homeostasis in different cell types. Of note, pathway analysis revealed a couple of differentially regulated biological pathways in CLL groups with high versus low *MFN1*-editing, however, we could not find deregulated pathways related to mitochondria dependent functions or cell death regulation.

While it was originally reported that APOBEC2 mediates S329L editing of *MFN1* in brain samples¹⁹, our data suggest that in B cells and CLL cells APOBEC3 family members seem to be responsible for this editing event, as APOBEC2 was not expressed in the cells analysed in our study. This could mean that several APOBEC enzymes can redundantly perform this specific editing, depending on which of them are predominantly present.

Our data further show that S329L recoding is associated with intron retention, which has not been reported so far. Hence, *MFN1* editing and intron retention regulates availability of mature *MFN1* transcripts and in turn *MFN1* protein levels. This is particularly interesting in light of the fact that sole *MFN1* levels critically affect mitochondrial fusion and network formation^{28,32}.

Notably, the extent of intron retention observed in ADARB1 transfected HEK293 cell lines was much less than in ADARB1 positive CLL cells, despite pronounced *MFN1* editing, indicating that additional factors may contribute to intron retention. Our data are especially interesting considering the fact that resistance to current

therapies with BCL2 inhibitors, which promote mitochondria dependent cell death in CLL is also associated with perturbed mitochondria morphologies³³. Hence, a possible role of *MFN1* editing might also be attributed in context of cancer drug resistance, which will be interesting to elucidate in future studies. Since fusion of mitochondria is critical for a diverse set of pathways, *MFN1* editing may be a novel and thus far unexplored mechanism regulating or fine-tuning a diverse set of mitochondria-dependent cellular processes in cancer^{29,31}. In light of the data from our study and the fact that perturbed mitochondrial network dynamics is frequently associated with several disease states such as cancer, diabetes or neurodegeneration^{29,30,34}, aberrant *MFN1* editing might contribute to the pathophysiology of these diseases, warranting a more thorough investigation of *MFN1* editing in future studies.

Methods

Patients

RNA/DNA sequencing data from CLL samples and B cell subsets were downloaded from accession-number SUB5459211 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA540189>)²² and (EGAS00001000374)²³. In addition, RNA sequencing data of normal tissue were downloaded from the European Genome-phenome Archive and the ENCODE database <https://www.encodeproject.org/> (ENCSR000AFG, ENCSR000AFC, ENCSR042GYH, ENCSR495HDM, ENCSR853WOM). For Kaplan–Meier analyses of a pilot CLL cohort, peripheral blood from 23 chemo naïve CLL patients was collected upon informed consent and ethical approval by the Ethics Committee of the Province of Salzburg (415-E/1287/4–2011, 415-E/1287/8–2011, 415-E/1287/20–2018) and was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. CLL cells were obtained by density gradient centrifugation and the B-CLL Cell Isolation kit (Miltenyi Biotec). Cell purity was >90% in all samples. The assessment of prognostic markers was performed routinely at our department as described previously³⁵. B cells from healthy donors were isolated from peripheral blood using magnetic beads (B cell isolation kit, Miltenyi Biotec) (ethics number EP/73/791). RNA was purified using RNeasy Mini kit (Qiagen).

Calling RNA editing events

Matched Exome and Transcriptome bam-files were sorted after alignment to hg38 with bowtie2 and tophat2, respectively, and duplicates were marked by picard-tools (v2.2.2, <https://broadinstitute.github.io/picard/>). The processed bam files were processed using samtools-0.1.19³⁶ mpileup and loci with a mean base quality phred score below 20 or with a coverage of less than 6 reads in either DNA or RNA were omitted. We then used a custom perl script to remove variants that occurred in the first or last base of a read or were strand-biased (present only in either forward or reverse reads). We furthermore omitted variants in deleted or inserted regions and selected only variants uniquely found in RNA by using the somatic variant caller VarScan2 (–min-coverage 8 –min-var-freq 0.05 –strand-filter 1), filtered for high confidence variants (processSomatic, see Koboldt et al³⁷, “Basic Protocol 2”) and subsequently annotated them using ANNOVAR³⁸. A custom perl script and information from hg19.refseq.bed file retrieved from the UCSC database were used to translate variants in genes with reverse strand orientation. Variants in HLA genes were excluded. Functional impact of editing sites was predicted using mutationassessor (mutationassessor.org, accessed February 2019) (Reva et al. 2011).

Calculating MFN1 splicing and editing levels

Downloaded fastq files were aligned to the human hg19 reference genome using the STAR aligner³⁹ (using default options and –outFilterMultimapScoreRange 1). *MFN1* intron/exon intervals (chr3:179,085,824–179,085,891|Exon_9;chr3:179,085,892–179,093,007|Intron_9;chr3:179,093,008–179,093,129|Exon_10;chr3:179,093,130–179,094,829|Intron_10;chr3:179,094,830–179,094,956|Exon_11) were saved in bed format (*MFN1_exons.bed*). GATK DepthOfCoverage (v3.7.0⁴⁰) was used to assess coverage at the indicated introns/exons (–L *MFN1_exons.bed*, –U ALLOW_N_CIGAR_READS).

Samtools (v1.9) view was used to filter reads aligned to the 5' intron/exon border at exon 10 (chr3:179,093,008–179,093,009) and spliced and unspliced reads were separated using “awk ‘(\$6~/N/)’” or “awk ‘(\$6!~/N/)’”, respectively. The resulting files were reconverted to the bam format and variants between spliced and unspliced reads were assessed using samtools mpileup (–r chr3:179,093,018–179,093,018” and varscan mpileup2snp (–min-reads 2 1 –min-var-freq 0.001).

RNA sequencing of transfected HEK293 cells

For RNA sequencing, DNA-free total RNA (300 ng as input) quantified by Qubit 2.0 (Life technologies) and quality checked on TapeStation 4200 (Agilent) was used. The libraries were prepared following NEBNext Poly(A) mRNA Magnetic Isolation Module of Ultra II Directional RNA Library Prep Kit for Illumina (NEB). Libraries were pooled according to the ratio desired read number/number of samples. Denaturation and dilution was conducted following the standard normalization method for NextSeq 500 and NextSeq 550 Sequencing Systems (Illumina), with 1% PhiX library as a sequencing control. The libraries were then sequenced from both ends (paired-end) on Illumina NextSeq500/550 platform with 2 × 150 cycles. Analysis of editing events was performed as described above.

For small RNA sequencing, total RNA was isolated from transfected samples, using Monarch Total RNA Miniprep Kit (NEB) according to the protocol for cultured mammalian cells. The NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) was adapted to convert small RNAs into indexed libraries. The final libraries were diluted to 10 pM according to the recommended standard normalization method for MiSeq System (Illumina). Libraries were sequenced using MiSeq Reagent kit V2 with 50 cycles and 1% PhiX as a sequencing control. Fastq files were trimmed using cutadapt (⁴¹GATCGGAAGAGCACACGTCTGAACTCCAGTCAC, min = 18, max = 300, min_quality = 25) and aligned to hg38 using bowtie1⁴². Samtools³⁶ mpileup (–B

-q 1 -d 100,000) files were generated using the following sample-pairs: siControl-ADA_siControl, siControl-ADA_siBCF2, ADA_siControl-ADA_siBCF2. Edited variants were identified using varscan v2.4.4^{43,44}, somatic -min-var-freq 0.05; processSomatic -min-tumor-freq 0.01 -max-normal-freq 0.05 -p-value 0.05; somaticFilter -min-var-freq 0.01). bam-readcount (<https://github.com/genome/bam-readcount>) was used to calculate read depth in variant sites and varscan ffilter (-dream3-settings 1, -min-var-freq 0.01, -min-var-avgrl 12 min-ref-avgrl 15) was applied to identify high quality variants. Variants with somatic score (SSC) of 30 or higher were considered high quality edited sites. All sequencing data were uploaded to the Sequence Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/sra>) SubmissionID: SUB11167983; BioProject ID: PRJNA814016.

Plasmids and cloning

ADARB1 (encoding 701 amino acids, transcript variant 1, mRNA from RefSeq NM_001112.3) and ΔDRBM1 (lacking amino acid residues 5–196) expressing vectors were generated by PCR amplification of the respective ADARB1 variants from an ADARB1 cDNA-ORF clone (purchased from Sinobiologicals cat# HG18501-UT-1) using primers RG1800/RG1801 or RG1802/RG1801. The backbone of a Flag-tag expression plasmid was PCR-amplified using primers RG1798/RG1799 and fused with inserts using In-Fusion HD cloning (Takara Clontech). ADARB1-CD (catalytically dead variant by mutating E396A) was generated by in vitro mutagenesis using primers RG1841/RG1842. ADAR1 p110 and p150 isoforms were PCR amplified from human cDNA using primers RG2267/RG2268 and RG2269/RG2270 and cloned into the Flag-tag expression plasmid. For control, empty Flag-plasmids (generated by PCR-amplification of Flag-ADARB1 constructs using primers RG1820/RG1821 followed by In-Fusion HD-cloning) or constructs expressing an irrelevant protein (FLAG-RNF126 vector, generated by BamHI/HindIII cloning of PCR-amplified RNF126 from human cDNA using primers RG622/RG623 into a Flag-expression plasmid) were used. The Flag-expression plasmid was a kind gift from Silvestro Conticello⁴⁵. Primers are listed in Table S9.

Cell culture and treatments

HEK293 cells were cultured in RPMI medium (Gibco, ThermoFisher Scientific) supplemented with Fetal Bovine Serum (FBS; 10%), L-glutamine (1%), and Penicillin/Streptomycin (1%). Transfection was carried out using GeneJuice (Merck KGaA, Darmstadt, Germany) according to manufactures instructions. After 48 h, cells were harvested for several experiments.

To knockdown APOBEC family members, siRNAs were purchased from Dharmacon Company using On-TARGETplus technology (individual siRNAs or SMARTpool); a non-targeting siRNA (Table S10) was used as control (siCont). 300,000 cells at 95% confluence were treated with 20 pmol siRNAs using 6ul or 9ul Lipofectamine RNAimax (ThermoFisher Scientific). After 24 h, further transfections with expression plasmids were performed, and 72 h later total RNA was extracted for downstream experiments.

Assessing editing frequency by Sanger sequencing

RNA was extracted from treated cells using High Pure RNA isolation kit (Roche). First strand complementary DNA (cDNA; 500 ng input RNA) was prepared using thermostable Luna Reverse Transcriptase (LunaScript RT SuperMix Kit, NEB) following manufacturer's instructions. Editing sites were PCR-amplified from cDNA using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) using primers RG1464/RG1465 for unspliced (Un), and primers RG1462/RG1475 for spliced (Sp) MFN1. PCR products were gel purified (Macherey-Nagel) and Sanger sequenced (Eurofins, Germany). The Poly Peak Parser Sangerseq R package⁴⁶ was applied to determine the peak amplitude for each base within a basecall window (peakAmpMatrix) from Sanger sequences. T to C ratio was calculated from the ratio of peak amplitude numbers. Primer sequences are listed in Table S8.

Quantitative PCR

Transcript levels of spliced/unspliced MFN1 and APOBECs upon knockdown of APOBECs or overexpression of ADARB1 were assessed by Quantitative PCR (qPCR) using Luna Universal Probe qPCR Master Mix (NEB) on cDNA. Probes for APOBECs and GAPDH were purchased from ThermoFisherScientific. Customized probe (RG1810) and primers for unspliced (RG1464/1465) or spliced (RG1462/1475) MFN1 transcripts were purchased from Eurofins (Ebersberg, Germany) and listed in Table S8. Samples were processed on a ViiA 7 Real-Time PCR system (Applied Biosystems) with GAPDH as internal control, and the comparative C_T method was used for the final evaluation.

Western blot analysis

Cells were harvested in PBS and lysed with RIPA buffer including Protease inhibitor cocktail (Roche) and PMSF for 30 min on ice and vortexing. After centrifugation (10 min, 14000 rpm, at 4 °C), protein concentration was determined applying Pierce BCA Protein assay kit (ThermoFisher Scientific) on Spark multimode reader (Tecan, Trading AG, Switzerland). Protein samples were prepared with 3× Laemmli and RIPA buffer. Total protein (15 µg) of whole-cell extracts were loaded on 10% bis-acrylamide gel by Sodium Dodecyl Sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Expression of FLAG-tagged proteins in HEK293 transfectants was detected by Western blotting using HRP-conjugated anti-FLAG antibody M2 (Sigma-Aldrich Co. LLC), and anti-alpha tubulin antibody (GeneTex) as an internal control. Visualization of protein bands was done on Fusion FX (Vilber) using Amersham™ ECL Prime kit (GE Healthcare). Uncropped full length blots are provided as Fig S4.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism Version 10 (GraphPad Software, Inc.) or R (survminer and survival R packages) using statistical tests indicated in the respective figure legends. Cutt-offs for

Kaplan–Meier graphs were calculated using the maximally selected rank statistics from the ‘maxstat’ R package, providing a value of a cutpoint that corresponds to the most significant relation with outcome. Differential gene expression analysis was performed using the ‘edgeR’ R package⁴⁷. Editing high and low was defined by a cutoff of 14.29% editing frequency. DE genes were ranked by $\log_2FC \times -\log_{10}(p\text{-value})$ and subjected to gene ontology pathway analysis using the R package clusterProfiler⁴⁸. No statistical analyses for sample size estimates were used. No blinding or randomization was used. All available samples from the CLL cohorts were included in the study. Sample sizes, statistical testing, are indicated in each figure legend. All figures were finalized using Inkscape 1.3.2.

Data availability

All sequencing data were uploaded to the Sequence Read Archive (SRA; <https://www.ncbi.nlm.nih.gov/sra>) accession PRJNA814016 and PRJNA1016100.

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Author contributions

AGM conducted main experiments, interpreted data and contributed to figures. FGJ did bioinformatic sequence analyses, interpreted data and contributed to figures. DB, PH, EA, MS, SS and SR performed experiments. FN, EC, RGr, AE and LP provided clinical data. NZ analysed and interpreted data. RGe designed research, interpreted data, contributed to figures and drafted the manuscript. All authors read, revised and approved to the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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