ARTICLE

SF3B1-mutated chronic lymphocytic leukemia shows evidence of NOTCH1 pathway activation including CD20 downregulation

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by low CD20 expression, in part explained by an epigenetic-driven downregulation triggered by mutations of the *NOTCH1* gene. In the present study, by taking advantage of a wide and well-characterized CLL cohort (n=537), we demonstrate that CD20 expression is downregulated in *SF3B1*-mutated CLL to an extent similar to *NOTCH1*-mutated CLL. In fact, *SF3B1*-mutated CLL cells show common features with *NOTCH1*-mutated CLL cells, including a gene expression profile enriched in *NOTCH1*-related gene sets and elevated expression of the active intracytoplasmic NOTCH1. Activation of the NOTCH1 signaling and downregulation of surface CD20 in *SF3B1*-mutated CLL cells correlate with overexpression of an alternatively spliced form of *DVL2*, a component of the Wnt pathway and negative regulator of the NOTCH1 pathway. These findings were confirmed by separately analyzing the $CD20^{dim}$ and $CD20^{bright}$ cell fractions from *SF3B1*-mutated cases as well as by *DVL2* knockout experiments in CLL-like cell models. Together, the clinical and biological features that characterize *NOTCH1*-mutated CLL may also be recapitulated in *SF3B1*-mutated CLL, contributing to explain the poor prognosis of this CLL subset and providing the rationale for expanding therapies based on novel agents to *SF3B1*-mutated CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by pronounced clinical and biological heterogeneity.[1] Therapy regimens that include monoclonal antibodies against CD20 are widely used, both as single agents and in combination[2,3] although, in specific CLL subsets, the efficacy of anti-CD20 therapy may be reduced by the peculiar dimmer expression of CD20 in CLL[4-6] compared to other lymphoproliferative disorders.[7]

A number of factors have been variously associated with CD20 regulation, e.g., NF-κB signaling, the CXCR4 pathway, B-cell receptor (BCR) signaling, histone deacetylases and activity of multiple transcription factors (such as IRF4, NF-κB, PU.1, OCT1/2);[8] in addition, CD20 expression in CLL can be also affected by mutations of the *NOTCH1* gene whose presence, detected in up to 25% of cases,[9] has been associated with clinical resistance to anti-CD20 immunotherapy both in clinical trials and real-world scenarios.[10-12] We previously demonstrated that the

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reduction of CD20 expression in *NOTCH1*-mutated CLL cells could be due to a *NOTCH1* mutation-driven epigenetic dysregulation involving histone deacetylases.[5,13] Although genetic mutations represent the main contributor to the aberrant activation of the NOTCH1 pathway in CLL,[14] a mutation-independent activation was also reported for a significant fraction of *NOTCH1*-unmutated cases.[15] In that setting, however, nothing is known regarding the mechanism(s) behind the activation of NOTCH1 and the concomitant modulation of CD20 expression.

Another recurrently mutated gene in CLL is the RNA splicing factor 3b subunit 1 (*SF3B1*), found mutated in about 10% CLL cases.[16-19] SF3B1 is a key component of the splicing machinery, responsible of recognizing the branch-point sequences in proximity of the 3′ splice site (acceptor site) allowing intron removal from precursor messenger RNA. Mutations are predicted to alter the protein's tertiary structure, hampering the correct high-affinity recognition of the branch-point sequences and resulting in the selection of alternative 3′ splice sites. This leads to aberrantly spliced transcripts, gain/change/loss-of-function variants, novel stop codons or downregulation of gene expression through nonsense-mediated decay.[20-23]

In CLL, mutations of *SF3B1* have been shown to induce transcriptome-wide alterations of splicing patterns, resulting in an increased frequency of alternative 3′ splice site selection, with functional consequences on several pathways such as DNA damage, telomere maintenance and, possibly, NOTCH1 signaling,[18,19,23,24] although the actual impact of *SF3B1* mutations in the pathobiology of CLL remains to be fully elucidated.

In the present study, by taking advantage of a large cohort of primary CLL cases, we demonstrated that *SF3B1*-mutated CLL have features of NOTCH1 pathway activation and NOTCH1-dependent CD20 downregulation.

Methods

Primary chronic lymphocytic leukemia cells

This study is part of a comprehensive CLL characterization approved by the Internal Review Board of the Centro di Riferimento Oncologico di Aviano (approval n. IRB-05-2010 and IRB-05-2015) upon informed consent in accordance with the Declaration of Helsinki, and included peripheral blood samples from 537 CLL patients (*Online Supplementary Table S1*).[1,25] Primary CLL cells were separated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and either used directly or cryopreserved until use. All studies were performed on highly purified cells (>95% pure). CLL cases were characterized for IGHV mutational status, the main cytogenetic abnormalities, CD49d expression and mutational status of *TP53* as described elsewhere.[26,27] In 382/537 cases, time-to-first-treatment (TTFT) data were available (median TTFT 30 months, 95% confidence interval: 27-34 months).

Next-generation sequencing

Mutational status of *NOTCH1* and *SF3B1* was assessed by next-generation sequencing. An amplicon-based strategy, with at least 2000X coverage, was used for *NOTCH1*, covering the whole of exon 34 and part of the 3′ untranslated region.[9] *SF3B1* mutational status was assessed using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) covering exons 10 to 17. For the purpose of the present study, CLL samples were considered mutated for *NOTCH1* and *SF3B1* if the variant allele frequency (VAF) exceeded 1%[9] or 5%, respectively; in the presence of concurrent mutations, cases were assigned to the SF3B1-mutated category.

Evaluation of CD20 expression

CD20 expression was investigated in the whole cohort by flow cytometry, using an anti-CD20 PE-Cy7 antibody (clone L27, BD Biosciences, Milan, Italy) in the neoplastic $CD19^+/CD5^+$ and in the normal $CD19^+/CD5^-$ compartments. Samples were acquired on a FACSCanto II cell analyzer calibrated with CS&T calibration beads (BD Biosciences) and processed with FACSDiva (BD Biosciences) or FlowJo software (FlowJo LLC, Ashland, OR, USA). A complementary analysis of CD20 distribution was implemented as follows: (i) a linear gate (P1) spanning from the peak value of the histogram and comprising the whole $CD20^{bright}$ population was defined; and (ii) the percentage of the P1-gated population was subtracted from the residual (100-%P1) population.[28]

Gene expression profiling

For gene expression profiling (GEP) experiments, total RNA was labeled, hybridized on oligonucleotide microarray slides (SurePrint G3 Human GE v2 8x60K) and analyzed as previously described.[29] Microarray data are available at the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo/*) under accession number GSE137024.

Statistical analyses

Statistical analyses were performed using Medcalc (Medcalc Software Ltd., Ostend, Belgium) and R (*www.r-project.org*). Data were compared using a two-sided Mann-Whitney rank-test and are presented with Tukey box-and-whisker plots. For correlation analyses, the Spearman rho correlation coefficient was employed. A *P*-value smaller than 0.05 was considered statistically significant and is represented with asterisks: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Further details are provided in the *Online Supplementary Methods*.

Results

SF3B1 and *NOTCH1* mutational status in chronic lymphocytic leukemia

In a cohort of 537 unselected CLL cases, we investigated the mutational status of *SF3B1* and *NOTCH1* by next-generation sequencing. *SF3B1* mutations were present in 48/537 cases (8.9%), with a VAF ranging from 5.0 to 53.0% (mean 32.0%) (*Online Supplementary Table S1*) and all clustered in the previously reported canonical hotspots.[16,19,23] The residues surrounding Lys700 were the most frequently mutated (23/48, 48%), followed by Gly742 (13/48, 27%) (Figure 1A). Multiple mutations were rare, with one case having two concomitant mutations and one case having three of them (cases #3647 and #3301, respectively) (*Online Supplementary Table S1*). *SF3B1* mutations were associated with *TP53* mutations (χ^2 P=0.0313) and with unmutated IGHV status (P=0.0006), but not with specific IGHV alleles. At difference from previous reports,[30,31] *SF3B1* mutations did not show significant associations with classical chromosomal abnormalities (*Online Supplementary Table S2*).

NOTCH1 mutations were present in 89/537 cases (16.6%) and concomitant mutations were more common, with 19 cases (19/89, 21.0%) bearing more than one lesion, increasing the overall mutation frequency to 21.6% (116 mutations in 537 cases). The VAF ranged from 1.6 to

84.0% (mean 33.6%) (*Online Supplementary Table S1*) with 65 canonical c.7541-7542delCT mutations, 35 other truncating mutations (nonsense or frameshift), 12 mutations in the 3′-untranslated region and four missense mutations (Figure 1B). Co-occurrence of *NOTCH1* and *SF3B1* mutations was infrequent, being detected in only six cases (1.1%) (*Online Supplementary Table S1*), suggesting two independent events (χ^2 *P*=0.4268) (*Online Supplementary Table S2*). The remaining 406 cases were unmutated in both genes (wild-type, WT).

TTFT data were available for 382/537 cases: the TTFT intervals for *SF3B1*-mutated and *NOTCH1*-mutated cases

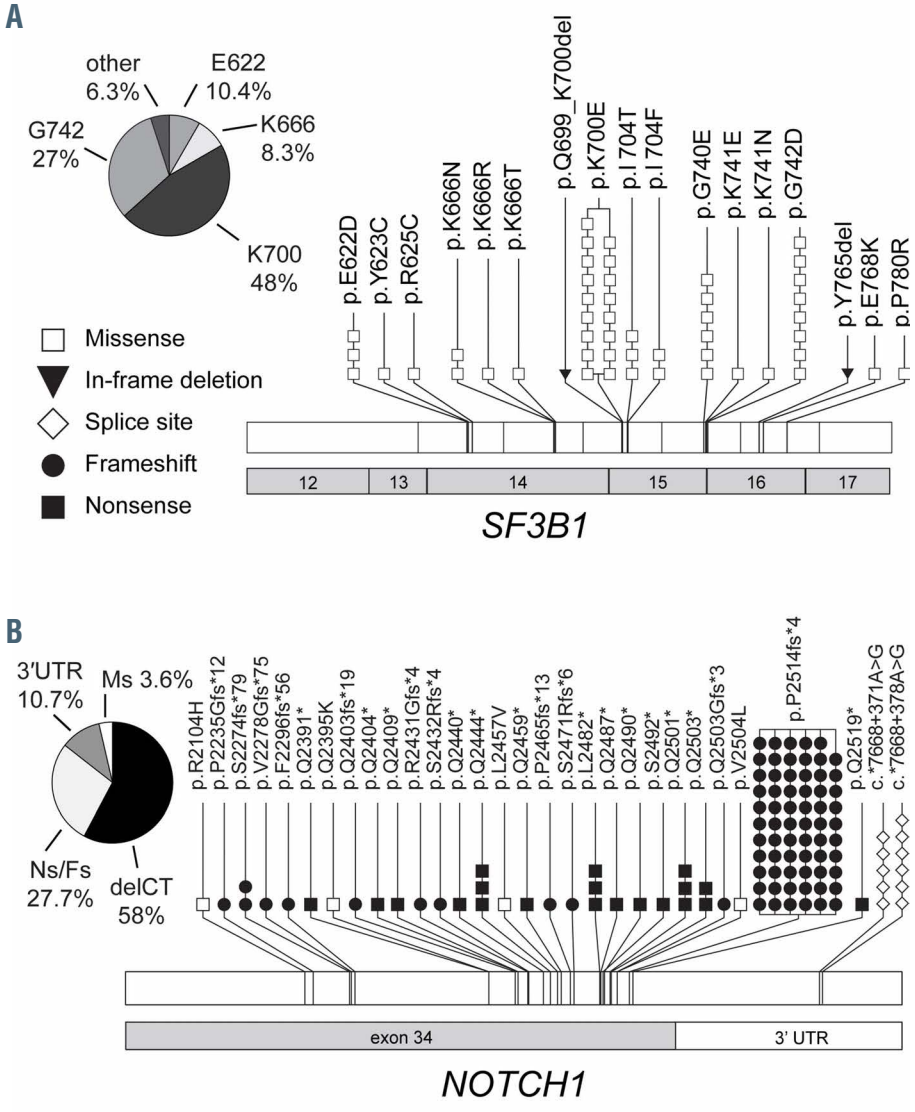

Figure 1. Distributions of mutations in the *SF3B1* and *NOTCH1* genes and impact on time to first treatment. (A) Distribution of mutations of the *SF3B1* gene. The mutated amino acids are indicated and grouped by hotspot. Symbols indicate the different type and number of mutations. The pie chart represents the mutation frequency of each hotspot. (B) Distribution of mutations of the *NOTCH1* gene. The mutated amino acids are indicated. Symbols indicate the different type and number of mutations. The pie chart represents the frequency of each mutation type. delCT: c.7541-7542delCT; Ns/Fs: nonsense or frameshift; 3′UTR: 3′ untranslated region; Ms: missense (C) Kaplan-Meier survival analysis for time to first treatment in 382 cases of chronic lymphocytic leukemia with mutations of *SF3B1* (n=35), *NOTCH1* (n=53) or neither (WT, n=294; *P*<0.0001 for both comparisons, log-rank test).

were similar and significantly shorter than those of cases lacking all these mutations ($P \leq 0.0001$ in all pairwise comparisons with WT) (Figure 1C), in keeping with previously reported studies,[11,16,19,32] irrespective of IGHV status (*Online Supplementary Figure S1A*) or concomitant *SF3B1/NOTCH1* mutations (*Online Supplementary Figure S1B*).

SF3B1-mutated chronic lymphocytic leukemia displays lower expression of CD20 than wild-type cases and similar to *NOTCH1*-mutated cases

Low CD20 expression represents a hallmark of CLL compared to other lymphoproliferative disorders or normal B cells (*Online Supplementary Figure 2A*).[7] We previously showed that a reduction of CD20 could be due to *NOTCH1* mutation-driven epigenetic dysregulation involving histone deacetylases.[5,13] To expand this observation, here we investigated whether low CD20 expression was also associated with *SF3B1* mutations in CLL, possibly via a non-mutational activation of the NOTCH1 pathway.[15] In keeping with previous observations,[5,33] in the whole cohort of 537 cases, CLL bearing trisomy 12 (121/537 cases, 22.5%) had significantly higher expression of CD20 ($P<0.0001$) (*Online Supplementary Figure S2A*) and a strong association with *NOTCH1* mutations (36/121, 29.8%, χ^2 $P<0.0001$); conversely, *SF3B1* mutations were infrequent (6/121, 5.0%, χ^2 $P=0.0815$) (*Online Supplementary Figure S2B, Online Supplementary Table S2*). Therefore, to avoid confounding effects, for the main subsequent analyses we considered only cases without evidence of trisomy 12 (416/537 cases), irrespectively of other concomitant chromosomal abnormalities.

CD20 expression, determined by mean fluorescence intensity, was lower in *SF3B1*-mutated cases than in WT cases (*SF3B1*-mutated, 42 cases, median 7396; WT, 327 cases, median 9538; $P=0.0024$) (Figure 2A), without differences from *NOTCH1*-mutated cases 47 cases, median 8438; $P=0.3748$) (Figure 2A), whose CD20 expression was lower than that of WT cases ($P=0.0248$), as reported previously.[5,13]

CD20 expression is known to be heterogeneous and dispersed in CLL, often spanning a 2- to 3-log range of fluorescence intensity within the $CD19^+/CD5^+$ pathological population.[34,35] We, therefore, implemented a complementary strategy of analysis, evaluating the percentage of the "$CD20^{dim}$ fraction", defined as the differential population fraction between the two sides of the fluorescence distribution with respect to the mode (*Online Supplementary Figure S2C*).[28] As a statistically robust measure of skewness, this method allowed us to discriminate cases with homogeneous or heterogeneous CD20 expression (*Online Supplementary Figure S2D*), with an inverse correlation with mean fluorescence intensity (rho= -0.417, $P<0.001$) (Figure 2B) and was consistent over time in sequential blood samples of treatment-naïve CLL cases (*Online Supplementary Figure S2E*). As shown in Figure 2C, the magnitude of the expansion of the $CD20^{dim}$ fraction was clearly increased in CLL cases with mutations of *SF3B1* (median 15.5) or *NOTCH1* (median 13.4) compared to WT cases (median 8.2; $P<0.001$ for both comparisons) while there were no evident differences between the *SF3B1* mutational hotspots (Figure 2C, right panel). Again, no difference was found between *NOTCH1*-mutated and *SF3B1*-mutated cases ($P=0.6365$). Of note, when analyzed separately, the six cases with concomitant mutations of *SF3B1* and *NOTCH1* showed a trend for a progressive expansion of the $CD20^{dim}$ fraction (median 19.3; $P=0.0466$ *vs.* WT) (*Online Supplementary Figure S2F*). Although excluded upfront from the analyses, the lower expression of CD20 was also confirmed in the trisomy 12 subset in *NOTCH1*-mutated CLL[5,13] and with a trend in the context of the few *SF3B1*-mutated cases (*Online Supplementary Figure S2G*).

SF3B1-mutated cases show evidence of activation of the NOTCH1 pathway

To evaluate whether activation of the NOTCH1 pathway also occurs in primary *SF3B1*-mutated CLL, we performed GEP on 28 cases, 13 WT *versus* nine *SF3B1*-mutated (VAF: range 21-48%, mean 37%) or six *NOTCH1*-mutated (VAF: range 17-51%, mean 30%) cases, all with unmutated IGHV and devoid of trisomy 12.

In the *SF3B1*-mutated category, 585 array probes (502 upregulated and 83 downregulated) corresponding to 443 known genes (402 upregulated and 41 downregulated) were differentially expressed (*Online Supplementary Table S3*) compared to WT samples. On the other hand, when comparing the *NOTCH1*-mutated and the WT categories, we identified 2,097 differentially expressed array probes (1,147 upregulated and 950 downregulated) (*Online Supplementary Table S4*), corresponding to 1,274 known genes (840 upregulated and 434 downregulated). When this *NOTCH1* gene signature was applied to all samples, *SF3B1*-mutated cases clustered with the *NOTCH1*-mutated cases (*Online Supplementary Figure S3A*), suggesting the presence of a common underlying signature. In fact, by merging the 443 *SF3B1*-mutated differentially expressed genes with the 1,274 *NOTCH1*-mutated differentially expressed genes, 419/443 (94.6%) were shared and concordantly regulated in the two signatures (390 upregulated and 29 downregulated) (Figure 3A, B). Consistently, when the *SF3B1* signature was applied to all samples, *NOTCH1*-mutated cases clustered together with *SF3B1*-mutated cases (*Online Supplementary Figure S3B*). GEP data were externally validated by quantitative reverse transcriptase polymerase reaction (RT-qPCR), selecting four genes related to the NOTCH1 pathway (*CD300A, IL1R2, HEY1, HES4*) (*Online Supplementary Figure S3C*). Although in these signatures the differential expression of the single probe dedicated to *MS4A1* (the gene encoding CD20) in our GEP platform (see *Online Supplementary Methods*) was not statistically significant upon correction for the false discovery rate (*Online Supplementary Tables S3* and *S4*), a re-evaluation of *MS4A1* transcript expression by RT-qPCR confirmed its significantly lower levels in *NOTCH1*-mutated *versus* WT cases ($P=0.001$) and in *SF3B1*-mutated *versus* WT cases ($P<0.001$) (*Online Supplementary Figure S3D*).

Gene set enrichment analysis of *NOTCH1*-mutated *versus* WT cases revealed a significant enrichment in 11 out of 17 *NOTCH1*-related datasets (*Online Supplementary Table S5*) linked to NOTCH1 transcriptional activity and overexpression (Figure 3C, *Online Supplementary Figure S4A*). Interestingly, one of the most significantly enriched datasets was a recently published CLL-specific NOTCH1 signature.[15] The gene set enrichment analysis, using the same 17 *NOTCH1*-related datasets, when repeated on *SF3B1*-mutated *versus* WT cases, identified ten datasets as significantly enriched (Figure 3D, *Online Supplementary Figure S4B, Online Supplementary Table S5*), including the

CLL-specific NOTCH1 signature,[15] again suggesting the presence of an active NOTCH1 pathway; it is noteworthy that all ten datasets were included in the 11 datasets enriched in the *NOTCH1*-mutated cases.

Finally, using western blotting, we evaluated the expression of the NOTCH1 intracytoplasmic domain (NICD), the cleaved active form of NOTCH1 in whole cell lysates of 34 CLL cases, of which 13 *SF3B1*-mutated among those with the highest mutational burden and seven *NOTCH1*-mutated cases. We detected a distinct NICD band in all CLL cases with mutations of *NOTCH1*, while the other samples showed a varying degree of NICD staining, in agreement with previous observations of NOTCH1 pathway activation independent of *NOTCH1* mutations.[15] To determine a cutoff for NICD positivity, we defined the threshold as the lowest densitometric intensity among *NOTCH1*-mutated samples (Figure 3E, dashed line). In agreement with GEP data, positive NICD staining could be detected in the majority (8/13) of *SF3B1*-mutated cases but in only 2/15 WT cases (*Online Supplementary Figure S5A*), in keeping with observations of a mutation-independent activation of the NOTCH1 pathway in CLL.[15] The NICD intensity correlated positively with the mutational burden for both *NOTCH1* and *SF3B1* mutations further suggesting, for these events, a modulating role on NOTCH1 signaling (Figure 3E) generally more elevated in NICD-positive samples, as determined by RT-qPCR of the *NOTCH1* target genes *DTX1* and *CD300A* (*Online Supplementary Figure S5B*).

SF3B1 mutations consistently induce alternative splicing of *DVL2*

In CLL, mutations of *SF3B1* have been shown to induce transcriptome-wide splicing alterations in several genes including *DVL2*, a key component of the Wnt pathway, reported to act as a negative regulator of NOTCH1.[36-40]

Using next-generation sequencing in 73 primary CLL cases, we first evaluated *SF3B1*-induced splicing alterations of *DVL2* (Figure 4A) and, as a control, two other highly differentially spliced genes, namely *GCC2* and *MAP3K7* (*Online Supplementary Figure S6A*).[23] For all three genes, the exact reported splicing defects were highly enriched in all SF3B1-mutated cases ($P<0.0001$) (*Online Supplementary Figure S6B*) confirming that these alterations are highly consistent both within and between cohorts.

The expression of alternatively spliced *DVL2* (*altDVL2* hereafter) was then further investigated by RT-qPCR in a wider cohort of 222/537 CLL cases with available RNA,[35] of which with a *SF3B1* mutation, 32 with a *NOTCH1* mutation and 155 WT (Figure 4B). RT-qPCR results were highly concordant with next-generation sequencing data (rho=0.827, $P<0.0001$) (*Online Supplementary Figure S6C*). Expression of altDVL2 was significantly increased in the presence of SF3B1 mutations ($P<0.001$) (Figure 4B) and

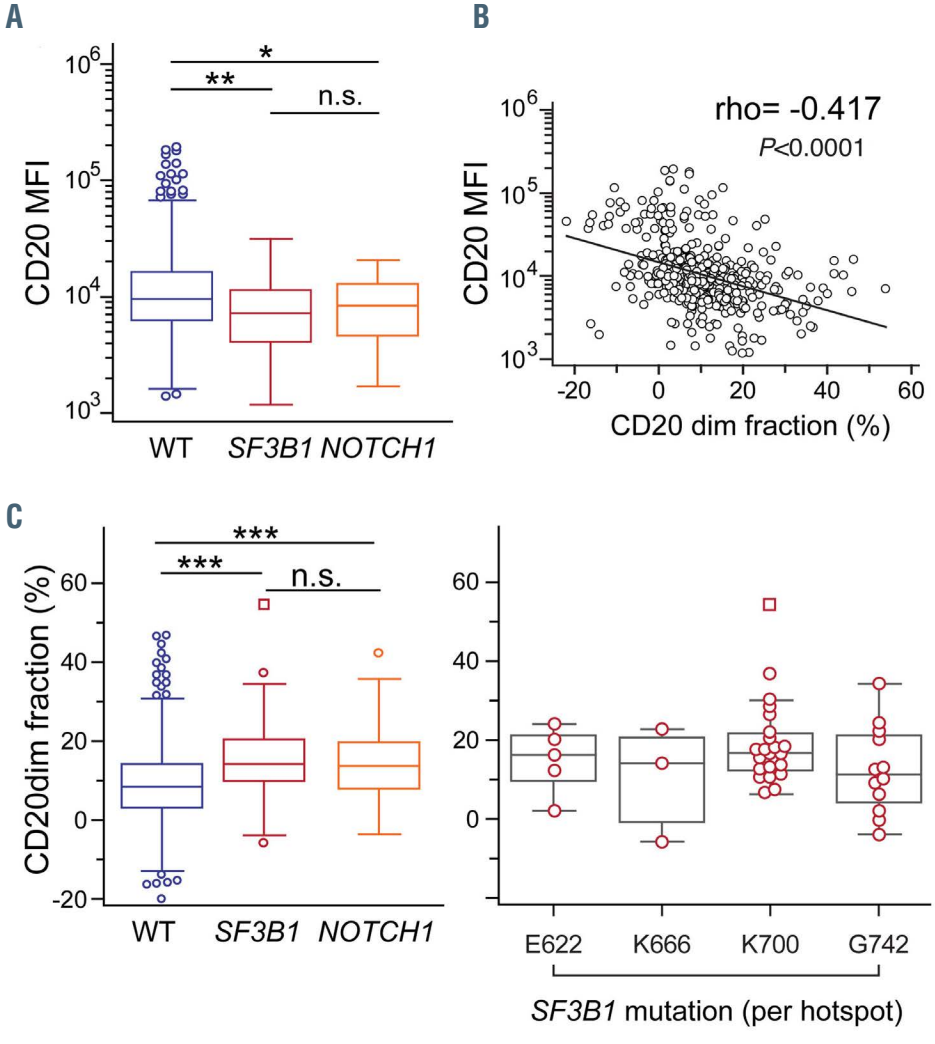

Figure 2. CD20 expression is reduced in chronic lymphocytic leukemia cases with *SF3B1* mutations. (A) Expression of CD20 determined by flow cytometry in *SF3B1*-mutated (*SF3B1*, n=42), *NOTCH1*-mutated (*NOTCH1*, n=47) or unmutated (WT, n=327) cases of chronic lymphocytic leukemia (CLL) without trisomy 12. MFI: mean fluorescence intensity. (B) Correlation between CD20 MFI and the $CD20^{dim}$ fraction in the whole cohort of non-trisomy 12 cases (n=416). The Spearman rank correlation coefficient (rho) and *P*-value are reported. (C) Percentage of the $CD20^{dim}$ fraction in *SF3B1*-mutated (*SF3B1*, n=42), *NOTCH1*-mutated (*NOTCH1*, n=47) or unmutated (WT, n=327) CLL cases. Right panel: percentage of the $CD20^{dim}$ fraction in *SF3B1*-mutated cases with respect to the mutated hotspot. Data are shown by Tukey box and whisker plots. Outliers indicate data outside the 1.5 interquartile range. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$, n.s. not significant, as determined by a two-sided Mann-Whitney test.

highly correlated with the mutational burden (rho=0.694, *P*<0.0001) (Figure 4C). This analysis also evidenced that the increase in *altDVL2* was independent of the type of *SF3B1* mutation, in terms of both mutational hotspot (Figure 4D) or amino-acid residue affected (*Online Supplementary Figure S6D*). Conversely, there was no significant difference between WT cases and cases bearing *SF3B1* or *NOTCH1* mutations in the expression of total *DVL2* (*Online Supplementary Figure S6E*). Of note, we detected a low frequency of alternative splicing for *DVL2* and the other genes also in WT cases (*Online Supplementary Figure S6B*, right inset), suggesting that the *altDVL2* form may be an underrepresented native splicing variation, particularly favored by a splicing machinery containing the mutated form of *SF3B1*.

Alternative splicing of *DVL2* correlates with NOTCH1 signaling and CD20 expression

We then investigated whether *altDVL2* could influence NOTCH1 signaling differently from wild-type *DVL2*. To do so, we overexpressed an exogenous *DVL2*, either wild-type or alternate, into a HEK293 NOTCH1-dependent luciferase reporter cell line; in this context, the transfection of wild-type *DVL2* but not of *altDVL2* was able to

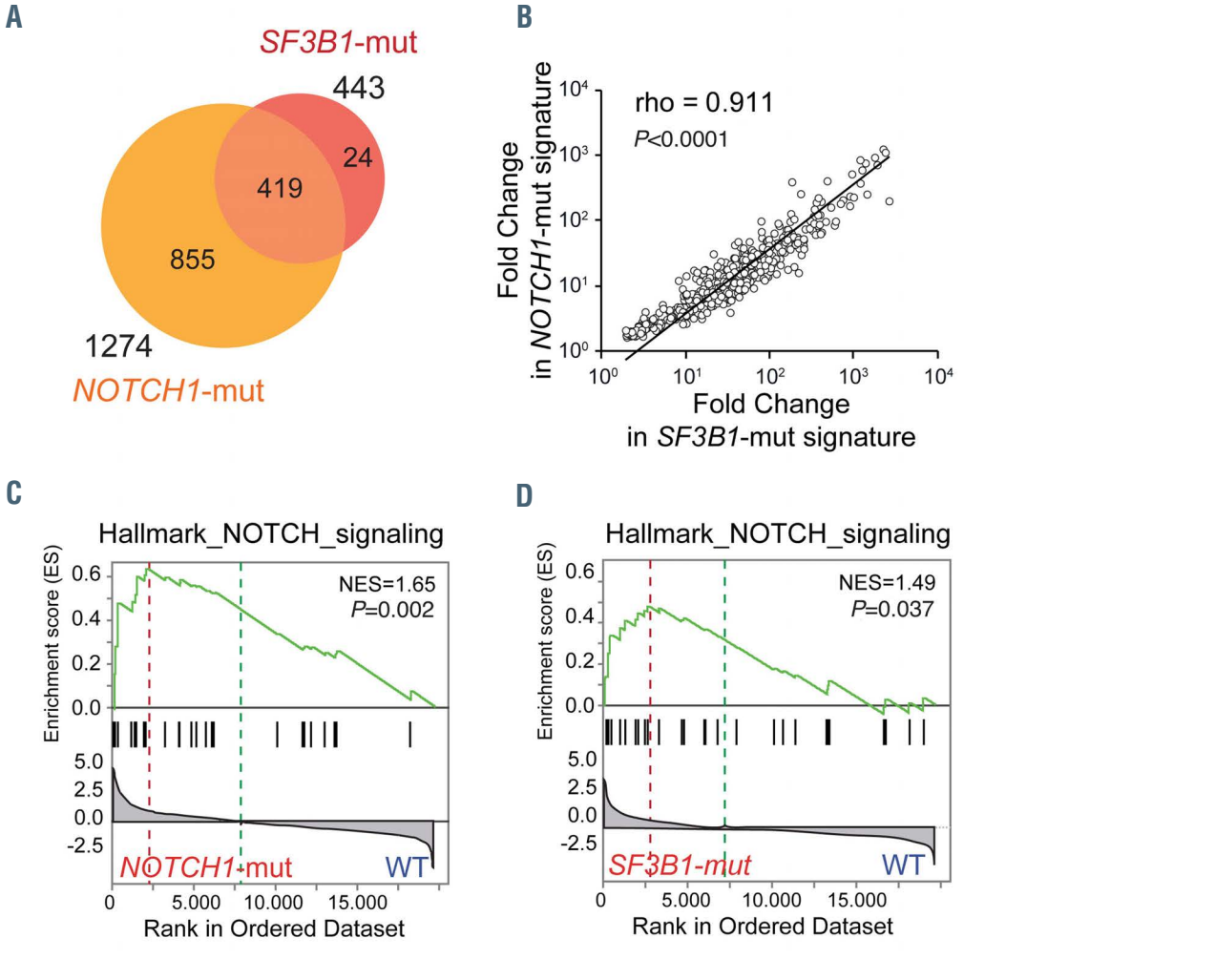

Figure 3. The NOTCH1 pathway is activated in *SF3B1*-mutated cases of chronic lymphocytic leukemia. (A) Venn diagram of the 1,274 differentially expressed known genes in *NOTCH1*-mutated (n=6) *versus* wild-type (WT) (n=13) cases and the 443 differentially expressed known genes in *SF3B1*-mutated (n=9) *versus* WT (n=13) cases. (B) Correlation of the fold change of 419 probes shared between the *NOTCH1*-mutated and the *SF3B1*-mutated signatures. The Spearman rank correlation coefficient (rho) and *P*-value are reported. (C, D) Gene set enrichment analysis enrichment plots tracking the HALLMARK_NOTCH_SIGNALING gene set, significantly enriched in NOTCH1-mutated (C) and *SF3B1*-mutated (D) chronic lymphocytic leukemia. NES: normalized enrichment score. (E) Correlation between NOTCH1 intracytoplasmic domain (NICD) expression, in arbitrary units, and *SF3B1* or *NOTCH1* variant allele frequency; the solid line represents the correlation within the *SF3B1*-mutated cases with the Spearman rank correlation coefficient (rho) and *P*-value reported; the dashed line represents the limit for NICD positivity, defined as the lowest NICD intensity within the *NOTCH1*-mutated cases (see also *Online Supplementary Figure S5A*). a.u.; arbitrary units; VAF: variant allele frequency.

repress the promoter activity (P=0.0244 and P=0.96, respectively) (*Online Supplementary Figure S7A*), in agreement with previous observations[23] and consistent with a defective inhibitory role of *altDVL2* against the NOTCH1-dependent transcriptional machinery. Similarly, after depletion of wild-type *DVL2* by siRNA transfection in the *SF3B1*-wild-type CLL-like MEC1 cell line, NOTCH1 signaling was found to be increased, as demonstrated by *HES1* upregulation (P=0.0176) at 24 h; in addition, CD20 expression was reduced at both protein (P=0.0062) and transcript (P=0.0129) levels (Figure 5A). Taken together, these data corroborate the inhibitory role of DVL2 on the NOTCH1 pathway, counteracted by the presence of its alternate splicing isoform.

In agreement with this observation, *SF3B1*-mutated CLL cases also showed high expression of the NOTCH1 target gene *DTX1* (median *SF3B1*-mutated 0.0029, median WT 0.0013, P=0.0089; rho=0.369, P=0.0348), similar to *NOTCH1*-mutated cases (median *NOTCH1*-mutated 0.0033, median WT 0.0013, P=0.002) (Figure 5B). Notably, in *SF3B1*-mutated cases, the levels of *DTX1* correlated directly with the levels of *altDVL2*, in keeping with the hypothesis of elevated NOTCH1 signaling in the presence of a less functioning alternatively spliced *DVL2* (Figure 5C).

In turn, the expression of *DTX1* correlated with reduced CD20 expression; as summarized in Figure 5D and *Online Supplementary Figure S7B*. *SF3B1*-mutated and *NOTCH1*-mutated CLL cases showed a progressively enriched double-positive $CD20^{dim}$/*DTX1* population compared to WT cases (WT *vs.* *SF3B1*-mutated, P=0.024; WT *vs.* *NOTCH1*-mutated, P<0.001; *SF3B1*-mutated *vs.* *NOTCH1*-mutated, P=0.362; χ^2 test for proportions) (*Online Supplementary Figure S7B*). Accordingly, by performing cell sorting on nine *SF3B1*-mutated CLL cases, all with clonal *SF3B1* mutational burden (VAF 10-50%), we isolated two populations characterized by either high or low CD20 protein and transcript expression. Evaluation of *altDVL2* by next-generation sequencing in the two subfractions revealed an increased frequency of the alterna-

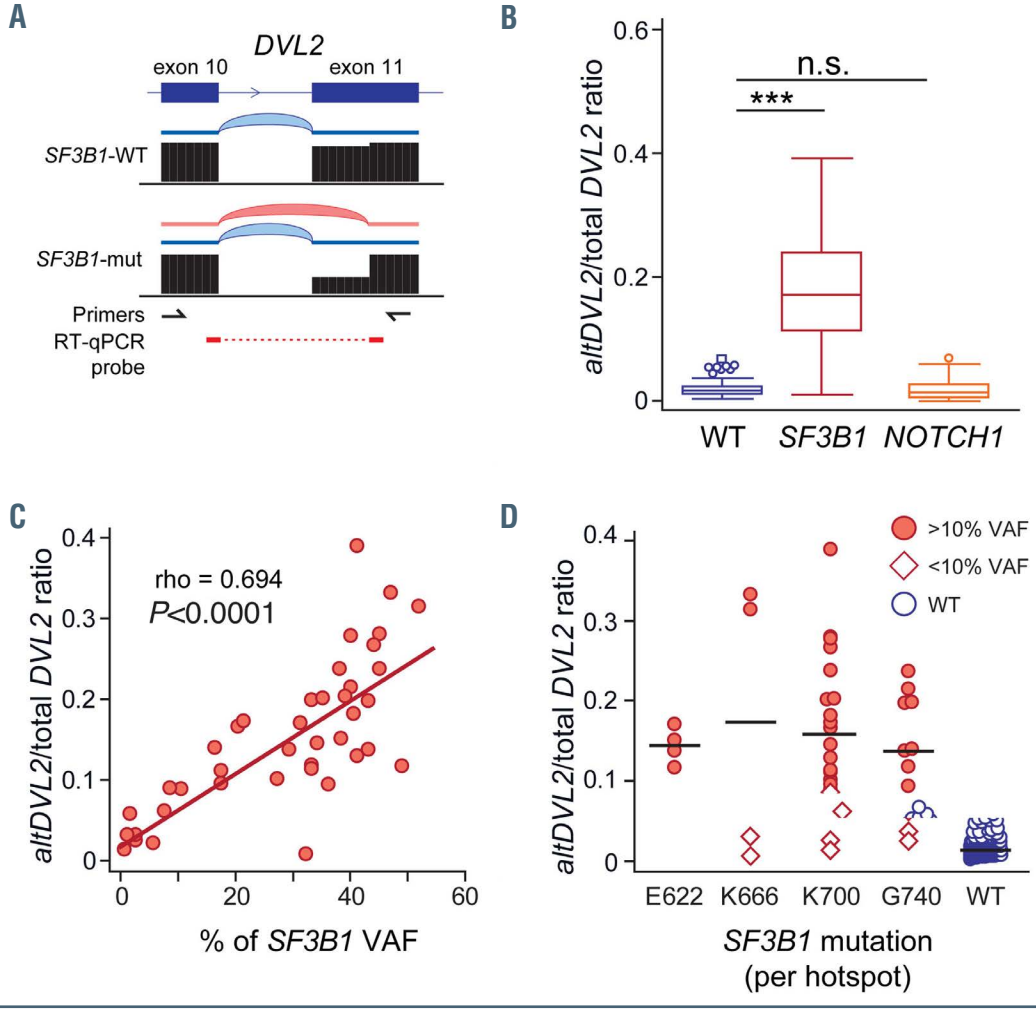

Figure 4. *SF3B1* mutations induce alternative splicing of *DVL2*. (A) Representation of the *DVL2* splicing events determine from next-generation sequencing analysis; arches indicate wild-type (blue) and altered (red) splicing, black bars indicate coverage; the position of primers and the probe for quantitative reverse transcriptase polymerase chain reaction analysis is also reported. (B) Expression of *DVL2* alternative splicing (*altDVL2*) in unmutated (WT, n=155), *SF3B1*-mutated (*SF3B1*, n=35), or *NOTCH1*-mutated (*NOTCH1*, n=32) cases of chronic lymphocytic leukemia. Data are shown by Tukey box and whisker plots. Outliers indicate data outside the 1.5 interquartile range. ***P≤0.001, n.s. not significant, as determined by a two-sided Mann-Whitney rank-test. (C) Correlation of *altDVL2* expression with *SF3B1* mutational burden. VAF: variant allele frequency. The Spearman rank correlation coefficient (rho) and P-value are reported. (D) *altDVL2* expression in relation to *SF3B1* mutations according to the mutational hotspot and burden (n=35). WT cases (n=155) are reported for comparison. Diamonds indicate *SF3B1*-mutated cases with a VAF <10%. Horizontal marks represent the median.

tively spliced isoform in the CD20^{low} fraction compared to the CD20^{high} fraction in most cases (P=0.0078), paralleled by a higher expression of DTX1 (P=0.05).

Finally, when challenged in a rituximab-driven complement-dependent cytotoxicity assay, CLL cells from either *SF3B1*-mutated or *NOTCH1*-mutated samples displayed a lower relative lysis compared to cells from WT samples (*Online Supplementary Figure S7D*), in agreement with the dose-dependent response of the assay from CD20 expression levels (rho=0.745, P=0.0085).

Discussion

The CD20 molecule is one of the preferred therapeutic targets for CLL as testified by the widespread use of humanized anti-CD20 antibodies in different therapeutic regimens,[2,41] although a dim expression of CD20 is among the peculiar features of CLL and part of the so-called Royal Marsden score for the differential diagnosis of CLL from other B-cell lymphoproliferative disorders.[25,42] This notwithstanding, very little is still known about the biological instances that define CD20 expression and determine the stark difference between CLL cells and normal B cells, despite a number of mechanisms having been proposed. Among the many, a few stand out including BCR/CXCR4 signaling,[43] NF-κB signaling and epigenetic/transcription factors such as histone deacetylases.[8,44]

Another of these factors is the presence of mutations of the *NOTCH1* gene,[5] an emerging novel predictive factor for response to anti-CD20 immunotherapy in patients treated with immuno-chemotherapeutic combinations.[11,12] In the present study, by taking advantage of a large cohort of CLL cases, we show that mutations of the *SF3B1* gene represent another factor associated with a reduction of CD20 expression through a mutation-independent activation of the NOTCH1 pathway.

In our cohort, the mutational profile of *SF3B1* was consistent with published data, with every mutation falling inside the canonical hotpots.[16-19] *SF3B1* mutations are consistently associated with an increased rate of aberrant splicing events throughout the transcriptome with varying intensity.[23] By analyzing the relative abundance of different splicing variants, we invariably detected a strong increase of aberrantly spliced transcripts on multiple genes in *SF3B1*-mutated cases, independently of the location of the mutated residue and with a high correlation with the mutational burden. One of these genes was *DVL2*, whose alternatively spliced form, *altDVL2*, consisted in an in-frame loss of 24 amino acids within exon 11. DVL2 is a key mediator of the Wnt pathway and has demonstrated the ability to act as a negative regulator of the NOTCH1 pathway (*Online Supplementary Figure S6A*) by binding the *NOTCH1*-related transcription factor RBPJ.[39] and/or the cleaved active form of NOTCH1 (NICD) itself.[38]

Through co-transfection experiments of exogenous DVL2 in a NOTCH1/RBPJ-dependent luciferase reporter system, we could confirm that the *in vitro* induction of *DVL2* is capable of silencing NOTCH1 signaling, whereas the induction of *altDVL2* did not result in proficient silencing, as previously suggested.[23] Here, we moved further to primary CLL cells and, using GEP, showed that *SF3B1*-mutated cases share a gene signature with *NOTCH1*-mutated cases which drove an unsupervised co-clustering of the two categories, with respect to *NOTCH1/SF3B1*-unmutated cases. In addition, several NOTCH1-specific gene sets were enriched in *SF3B1*-mutated CLL, further suggesting an active commitment of the NOTCH1 transcriptional machinery in this CLL subset; importantly, one of these gene sets was a custom gene set derived from a CLL-specific *NOTCH1* gene signature.[15] Consistently, the NICD was clearly detectable in protein lysates of *SF3B1*-mutated cases, with expression levels often comparable to those observed in *NOTCH1*-mutated cases. The increased NOTCH1 signaling positively correlated with the expression of *altDVL2* and with an elevated CD20^{dim} fraction in *SF3B1*-mutated CLL cases. These data were corroborated by cell sorting experiments of cell fractions with different CD20 expression levels in the context of *SF3B1*-mutated CLL cells, and by functional experiments of *DVL2* knock-out in a *SF3B1*-wild-type CLL cell model.

While the NOTCH1 pathway has for long been recognized as active in CLL,[14] only few mechanisms are known to explain its activation state. *NOTCH1* mutations represent the main but not the sole contributor, as there is evidence of active NOTCH1 signaling occurring in peripheral blood cells of a fraction of *NOTCH1*-unmutated CLL cases,[15] although without a molecular explanation. Our present study may help to sort out at least a fraction of these cases, suggesting that the constitutively activated NOTCH1 pathway can indeed occur in the context of *SF3B1*-mutated CLL, possibly as the result of diminished repression of the dynamic association between NOTCH1 and RBPJ[45] due to higher levels of the inefficient spliced form of DVL2.[15,38,39]

Wang *et al.*[23] speculated that the numerous changes induced by *SF3B1* mutations in the CLL transcriptome may allow neoplastic cells to diversify their evolutionary capacity, most likely through subtle alterations of many gene transcripts rather than through a single fatal lesion. This was hinted by the fact that *SF3B1* mutations, usually a later event in CLL evolution,[46] may not induce *per se* neoplastic transformation in B cells but rather drive a more aggressive and adaptive phenotype. This line of reasoning is in keeping with the hypothesis that the activation of the NOTCH1 pathway in CLL may reflect deregulated expression of a physiological signal, required for B lymphocyte maturation and differentiation.[15,47]

The exploitation of the NOTCH1 pathway by *SF3B1*-mutated CLL cells may well reflect such a strategy by which the proliferative advantages that characterize *NOTCH1*-mutated CLL cells can be incorporated in the complex transcriptomic reshaping occurring in *SF3B1*-mutated CLL, thus contributing to explain the poor prognosis of this CLL subset.[11,16,19,32] Also in keeping with the data presented here, it is likely that the documented activation of NOTCH1 in *SF3B1*-mutated CLL was primarily mediated by the direct involvement of the NICD through the canonical pathway.[45] However, the hypothesis of a NICD-independent activation cannot be excluded firsthand, as Notch-independent activity of the transcription factor RBPJ has been documented.[48]

From a clinical standpoint, the biological similarity of *NOTCH1*- and *SF3B1*-mutated CLL could contribute to explain the poor response of these CLL subsets to chemo-immunotherapy.[49,50] As documented by the CLL8 trial for NOTCH1-mutated cases, the clinical behavior of *SF3B1*-mutated CLL was worse that that of WT CLL,

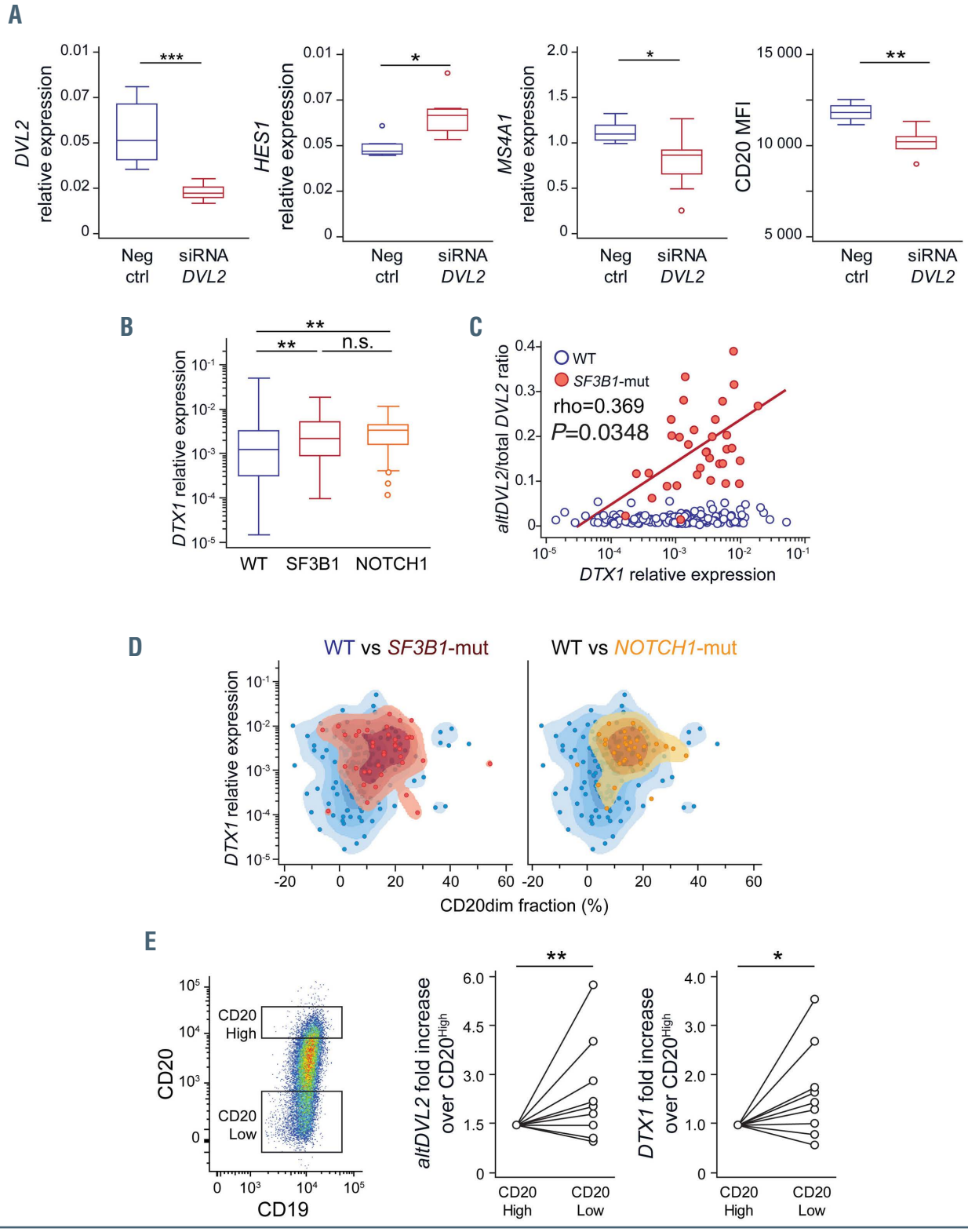

Figure 5. NOTCH1 signaling and *DVL2* silencing correlate with reduced CD20 expression. (A) Expression of *DVL2, HES1, MS4A1* determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) analysis and surface CD20 determined by flow cytometry in MEC1 cells, transfected with negative control (Neg ctrl, n=5) or siRNA for *DVL2* (n=5). Data are shown by Tukey box and whisker plots. MFI: mean fluorescence intensity. (B) *DTX1* expression in wild-type (WT; n=155), *SF3B1*-mutated (n=35) or *NOTCH1*-mutated (n=32) cases of chronic lymphocytic leukemia (CLL). (C) Correlation between *altDVL2* expression and *DTX1* expression in *SF3B1*-mutated (n=35) cases, as determined by RT-qPCR analysis. The Spearman rank correlation coefficient (rho) and *P*-value are reported for *SF3B1*-mutated cases. WT cases (n=115) are reported for comparison. (D) Correlation between *DTX1* expression determined by RT-qPCR and the $CD20^{dim}$ fraction. Density plots (log_{10} density) show clustering of *SF3B1*-mutated and *NOTCH1*-mutated cases as double positive $DTX1^{high}$/$\%CD20^{dim}$ (see also *Online Supplementary Figure S7A*). (E) Left panel: representative dot plot showing the gating strategy for CD20 expression in the $CD19^{+}/CD5^{+}$ CLL population; right panel: evaluation of *altDVL2* by next-generation sequencing or *DTX1* expression by RT-qPCR in the $CD20^{high}$ and $CD20^{low}$ subpopulations of sorted samples (n=9). Data are shown as dot-and-line diagrams of the fold increase over the $CD20^{high}$ fraction. **P*≤0.05, ***P*≤0.01, ****P*≤0.001, n.s. not significant, as determined by a two-sided Mann-Whitney rank-test or paired Wilcoxon signed-rank test.

although observed in the context of both FCR (fludarabine, cyclophosphamide and rituximab) and FC (fludarabine and cyclophosphamide) regimens,[11] in keeping with the presence of additional mechanism(s) of immune-chemo-resistance occurring in *SF3B1*-mutated CLL cells.[23,24]

Collectively, our data may provide the biological basis to promote, for *SF3B1*-mutated CLL patients, the use of novel biological agents, whose activity does not need the addition of anti-CD20 drugs[51,52] or of latest-generation anti-CD20 molecules[53] which operate independently of specific genetic lesions.[54]

Disclosures

No conflicts of interests to disclose.

Contributions

FP contributed to design the study, performed the research, analyzed and interpreted the data and wrote the manuscript; TB performed the research; ET, FV, EV, AZ, RB and FMR contributed to perform the research; LL, GDA, JO, FDR, AC, FZ, GP and GDP provided well characterized biological samples and contributed to writing the manuscript; MDB contributed to design the study; VG designed the study, interpreted the data, wrote and edited the manuscript.

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