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Bidirectional linkage between the B-cell

receptor and NOTCH1 in chronic lymphocytic leukemia and in Richter's syndrome: therapeutic implications

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Abstract

NOTCH1 mutations in chronic lymphocytic leukemia (CLL) lead to

accumulation of NOTCH1 intracellular domain (NICD) and prolong signaling. These mutations associate with a more aggressive disease compared ~~with~~to wild-type (WT) CLL. In this work we demonstrate a bidirectional functional relationship between NOTCH1 and the B cell receptor (BCR) pathways. By using highly homogeneous cohorts of primary CLL cells, activation of NOTCH1 is shown to increase expression of surface IgM, as well as *LYN*, *BTK,* and *BLNK*, ultimately enhancing BCR signaling responses, including global mRNA translation. Upon BCR cross -linking, NOTCH1 itself is actively translated and increased on cell surface. Furthermore, BCR ligation induces calcium mobilization that can facilitate ligand-independent NOTCH1 activation. These data suggest that the two pathways are functionally linked, providing a rationale for dual inhibition strategies. Consistently, addition of the γ-secretase inhibitor DAPT to ibrutinib significantly potentiates its effects, both ~~in vitro~~*in vitro* and in a short-term patient-derived xenograft model. While this observation may find limited applications in the CLL field, it is more relevant for Richter's Syndrome (RS) management, where very few successful therapeutic options exist. Treatment of RS-patient-derived xenografts (RS-PDX) with the combination of ibrutinib and DAPT decreases disease burden and increases overall survival.

AQ1

AQ2

Introduction

NOTCH1 is a surface protein and ligand-activated transcription regulator that controls gene expression directly, by recruiting a transcription complex, and indirectly, by affecting a complex nuclear balance that involves both transcription activators (MAML and p300) and inhibitors (HDAC1/2 and SPEN) [1]. *NOTCH1* represents the most commonly mutated gene in patients with chronic lymphocytic leukemia (CLL) [2]. Prevalence is ~10% at diagnosis, increasing in high-risk patients and affecting one-third of CLLs transforming to a diffuse large B-cell lymphoma (Richter's syndrome, RS) [3, 4]. Two main mutation hot spots were identified: a two-nucleotide deletion in exon 34, which leads to the generation of a premature STOP codon and a splice variant in the 3′ UTR. Both mutations generate a truncated protein lacking the PEST domain that regulates NOTCH1 ubiquitination and degradation [5, 6]. The absence of the PEST domain prolongs the half-life of

the active NOTCH1-intracellular domain (NICD) resulting in a quantitative modulation of the transcriptional program under the direct and indirect control of NOTCH1. Our previous data highlighted proliferation and chemotaxis as the two most affected cellular processes. The presence of a longer-lasting mutated NICD results in a decreased availability of RBPJ, an NICD binding protein, for the HDACs, which in turn complex with DNA methyl transferases. The subtle modulation in gene expression can significantly affect cellular responses to chemokines, and particularly to CCL19, increasing homing of CLL cells to lymphoid organs, where the proliferative core of the disease is hosted [7, 8, 9]. In the tumor-friendly environment of the proliferation center, CLL cells receive signals from the B cell receptor (BCR), which controls genetic programs resulting in altered balance between proliferation and death [10], metabolic adaptation [11], and chemokine secretion [12]. Importantly, one of the mechanisms of action of ibrutinib, a Btk inhibitor yielding significant clinical responses in patients, is to cause release of leukemic cells from peripheral lymphoid organs into the blood, where they are less protected and likely more prone to apoptosis [13, 14, 15]. For these reasons, the question at the base of this paper is whether there is a functional cooperation between NOTCH1 and the BCR in CLL patients and whether it may be therapeutically relevant to target both pathways.

AQ3

Methods

CLL cohort

Peripheral blood samples from CLL patients were obtained after informed consent, in accordance with Institutional Guidelines and the Declaration of Helsinki. We examined a cohort of 21 *NOTCH1*-mutated (M) and 23 *NOTCH1*-WT samples. All samples carried unmutated immunoglobulin heavy-chain variable region (*IGHV*-UM) genes [16, 17] and had a normal FISH profile or a 13q14 deletion as the sole abnormality. A cut-off of ≥30% variant allele frequency (VAF) was applied to restrict analyses to clonal *NOTCH1*-M samples (Table 1). Analyses were performed either immediately after thawing cells (+1-h recovery) or after 16-h recovery from anergy resulting from prior antigen stimulation ~~in vivo~~ *in vivo* [18]. This second time point was the best window to discriminate between *NOTCH1*-M- and -WT-dependent effects [7]. In fact, while NOTCH1 activation in the absence of stimuli is rapidly lost in WT samples it still persists in -M cells.

Table 1

CLL samples cohort

#	SAMPLE ID	~~NOTCH1~~*NOTCH1* c.7541_7542delCT (VAF)	IGHV	% homology	FISH	$CD5^+/CD19^+$
1	ID1213	>30% if possible, please align to the center all the percentages referring to NOTCH1 (VAF)	UM	100	del11; del13	87.70
2	ID425	>30%	UM	100	del13	95.4
3	ID283	>30%	UM	100	del13	98.1
4	ID982	>30%	UM	100	del13	89.2
5	ID233	>30%	UM	98.3	del13	88.5
6	PMN246	>30%	UM	99.60	del13	97.6
7	ID561	>30%	UM	100	del13	87.1
8	PMN336	>30%	UM	100	Normal	93.5
9	PMN209	>30%	UM	100	del13	94
10	GML064	>30%	UM	100	del13	94
11	ID959	>30%	UM	100	Normal	86.6
12	ID114	>30%	UM	100	Normal	93.8
13	ID1318	>30%	UM	100	Normal	93.30
14	PMN150	>30%	UM	99	Normal	88.5
15	GML120	>30%	UM	98.8	Normal	93.6
16	PMN236	>30%	UM	100	Normal	95.40
17	TRN245	>30%	UM	100	Normal	97.90
18	PMN335	>30%	UM	100	Normal	86.60
19	TRN257	>30%	UM	100	Normal	85.5
20	GML068	>30%	UM	100	del13	88.2

Clinical and molecular features of the samples included in this study. Asterisks mark c

VAF variant allele frequency, *UM* unmutated, *y* years, *UnTX* untreated Please add the f
TTFT time to first treatment
OS overall survival

#	SAMPLE ID	~~NOTCH1~~*NOTCH1* c.7541_7542delCT (VAF)	IGHV	% homology	FISH	$CD5^+/CD19^+$
21	PMN200	>30%	UM	100	Normal	90
22	ID758	<1%	UM	100	del13; del11	96.9
23	PMN116	<1%	UM	100	del13	96.4
24	ID148	<1%	UM	100	del13	93.5
25	ID1153	<1%	UM	100	del13	87.5
26	ID1173	<1%	UM	100	del13	96.1
27	ID1178	<1%	UM	98.9	del13	97.2
28	ID262	<1%	UM	99.2	del13	97
29	ID780	<1%	UM	100	del13	98
30	ID288	<1%	UM	100	del13	90.5
31	PMN239	<1%	UM	98.5	del13	97.6
32	PMN132	<1%	UM	98.2	del13	98.4
33	PMN306	<1%	UM	100	del13	98.6
34	PMN315	<1%	UM	100	Normal	91.6
35	PMN106	<1%	UM	100	del13; del11	85
36	ID763	<1%	UM	99.7	Normal	97.3
37	ID1195	<1%	UM	100	Normal	96.6
38	ID1368	<1%	UM	100	Normal	94.50
39	TRN228	<1%	UM	100	Normal	98.6
40	NPL274	<1%	UM	100	del13	98.5
41	GML125	<1%	UM	100	del13	94
42	PMN217	<1%	UM	100	del13	97
43	GML077	<1%	UM	100	Normal	98
44	PMN337	<1%	UM	100	del13	89

Clinical and molecular features of the samples included in this study. Asterisks mark

VAF variant allele frequency, *UM* unmutated, *y* years, *UnTX* untreated Please add the f
TTFT time to first treatment
OS overall survival

AQ4

AQ5

AQ6

Reagents and antibodies

A complete list of reagents and antibodies is provided in Supplementary Information (SI).

Intracellular Ca^{2+} measurement

Ca^{2+} fluxes were measured using the Fluo-3 AM calcium indicator (Thermofisher, Monza, Italy), as described [17]. Samples were acquired using a FACSCantoII (BD Biosciences, Milan, Italy) and analyzed with FlowJo v9.7.6 (FlowJo, Ashland, OR). Percent Ca^{2+} mobilization was calculated as [peak (all events)—mean Y (unstimulated cells)/%$CD19^+$ cells] × 100, where %$CD19^+$ cells was the percentage of $CD19^+$ cells in the live lymphocyte gate of the test sample.

Phosflow assay

Phosphorylation of downstream players in BCR signaling cascade was evaluated using a Phosflow assay [19]. CLL cells (10^6) were stimulated with α-IgM [5 µg/ml, $F(ab)_2$, 10 min]. Fixed and permeabilized cells were stained with antibodies listed in SI. Where indicated, cells were pretreated with ibrutinib and/or DAPT.

AQ7

Flow cytometric assessment of global mRNA translation

Global mRNA translation was evaluated using the Click-iT™ Plus OPP Alexa-Fluor-647 Protein Synthesis Assay Kit (Invitrogen, Thermofisher) [20]. Full details in SI.

Polysome fractionation

Ribosomes were separated into polysomal and subpolysomal fractions using a sucrose density gradient centrifugation [21]. Full details in SI.

~~In vivo~~*In vivo* experiments

Primary CLL cells and RS cells were intravenously (i.v.) injected in NOD/SCID/γ-$chain^{-/-}$ (NSG) immunocompromised female mice. All the ~~in~~

~~vivo~~*in vivo* experiments were approved by the Italian Ministry of Health (Authorization n.12/2016-PR). Full details in SI.

Supplemental methods

Methods fully described in the SI include: EDTA stimulation, RNA extraction and qRT-PCR, western blot, immunohistochemistry, apoptosis assay, ~~in vivo~~*in vivo* experiments.

Statistics and drug combination analyses

Statistical analyses were performed with GraphPad v6 (GraphPad Software Inc, La Jolla, CA, USA). Data are expressed as fold change over unstimulated or untreated cells (FC/NT) to highlight modulation induced by α-IgM and/or treatments. In the figures, data are presented as mean ± SD. The Mann–Whitney or Wilcoxon matched-pairs signed rank test was used to determine statistical significance. In the case of grouped analyses, the two-way ANOVA test was preferred. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Analysis of drug combination effects was performed by calculating a Combination Index (CI) based on the Bliss Independence model, which assumes that the examined drugs act independently on different sites of actions, but each contributes to a common result [22]. CI was calculated as:

$$\mathrm{CI} = \frac{E_\mathrm{A} + E_\mathrm{B} - E_\mathrm{A}E_\mathrm{B}}{E_\mathrm{AB}}$$

Where E_A, E_B and E_AB indicate the effects of either drug used as single agents or drug combination effects, respectively. A CI value <1 indicates synergism, >1 indicates antagonism, while values around 1 indicate an additive effect.

Results

Activation of NOTCH1 signaling in *NOTCH1*-M samples induces BCR and downstream interactors

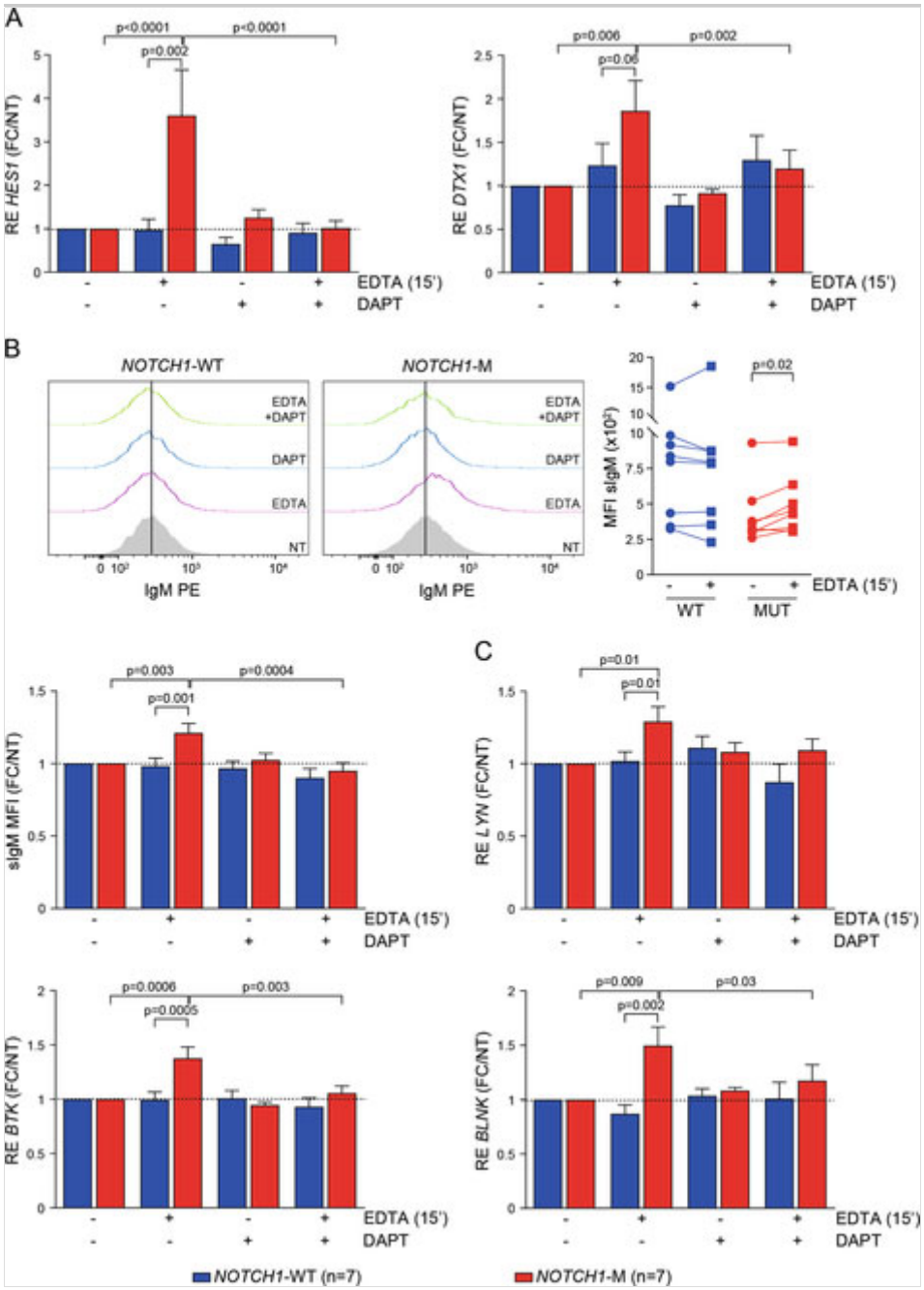
We first tested whether activation of NOTCH1 pathway impacted on the expression of BCR signaling members. To do this, we artificially induced NOTCH1 cleavage on primary CLL cells using EDTA that releases surface NOTCH receptors in a ligand-independent way [23]. We found that short EDTA treatment (15 min) was sufficient to trigger NOTCH1 activation in -M but not in -WT samples, as indicated by mRNA levels of *HES1* and *DTX1*

(Fig. 1a). Under these conditions, we observed significantly increased surface IgM (sIgM) levels in *NOTCH1*-M samples compared with the counterpart (Fig. 1b). No differences in the background staining, i.e., isotype controls (I.C), were observed after EDTA treatment (not shown). Furthermore, NOTCH1 activation led to increased transcription of *LYN*, *BTK* and *BLNK* (Fig. 1c), consistent with recent observations highlighting a NOTCH1-regulated BCR gene signature in CLL [24]. Accordingly, pretreatment with the γ-secretase inhibitor (DAPT), which prevents NICD cleavage, completely abrogated these effects (Fig. 1a–c). When maintaining EDTA treatment for 6 h, we obtained appreciable levels of NOTCH1 activation also in -WT CLL samples, leveling differences with -M samples (Supplementary Fig. 1a). Prolonged EDTA stimulation increased sIgM levels, as well as *LYN*, *BTK,* and *BLNK* expression, independently of *NOTCH1* mutational status, recapitulating the effects that were already visible in -M samples in more stringent experimental conditions (Supplementary Fig. 1b, c). The inference of these results is twofold: on one side, they indicate that NOTCH1 might directly modulate BCR signaling by regulating the expression of multiple elements in its signaling cascade; on the other side, they suggest the existence of a threshold of activation required to observe NOTCH1-mediated effects and that weaker or transient stimuli are sufficient to trigger signaling in -M samples, but not in -WT, likely due to NICD accumulation as a consequence of the mutation [7].

Fig. 1

Activation of NOTCH1 signaling increases expression of the BCR and related genes. **a** Short-term EDTA treatment (15 min) of primary CLL samples increases expression of *HES1* and *DTX1*, selected as representative NOTCH1 target genes, in *NOTCH1*-M samples, but not in *NOTCH1*-WT. Analyses were performed by treating cells with 5 mM EDTA for 15 min and waiting 2 h after EDTA wash out. Treatment with the γ- secretase inhibitor DAPT completely abolishes *HES1* and *DTX1* induction. A total of 7 cases from each category were analyzed. **b** Representative flow cytometry profiles of sIgM expression following the indicated treatments. A representative case in the *NOTCH1*-WT and -M categories is shown. The plot on the right shows cumulative results obtained from the eight patients analyzed. The histogram below flow cytometry profiles shows the selective increase in sIgM expression in *NOTCH1*-M vs -WT cases, following treatment with EDTA, expressed as fold change over unstimulated cells (FC/NT). Where indicated, cells were pretreated with DAPT, which was maintained during EDTA incubation and in the following 6 h and which completely prevented sIgM levels increase. sIgM levels were analyzed 6

h after EDTA wash out. **c** RT-PCR data showing expression levels of genes belonging to the BCR pathway, including *LYN*, *BTK*, and *BLNK*. Increased expression is only observed in ~~NOTCH1~~*NOTCH1*-M cases and is completely prevented by DAPT administration. Seven different primary samples for each category were analyzed. Analyses were performed by treating cells with 5 mM EDTA for 15 min and waiting 2 h after EDTA wash out. RE: relative expression



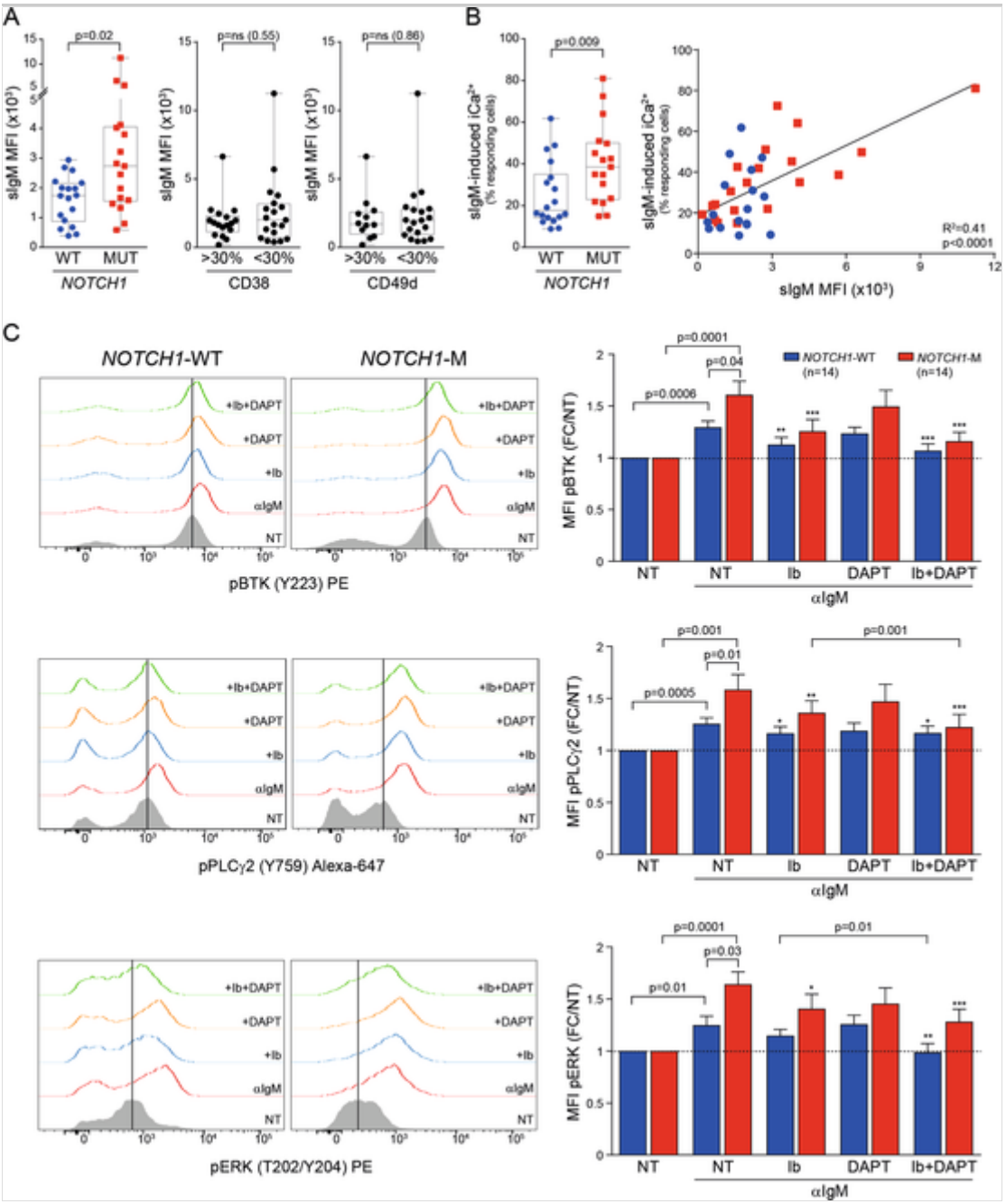
NOTCH1-M samples show increased sIgM levels and signaling capacity

We then compared baseline expression and signaling through sIgM in our cohort of *IGHV*-UM selected CLL samples. Flow cytometry analysis,

performed shortly after thawing to recapitulate ~~in vivo~~ *in vivo* conditions, indicated that sIgM levels were variable across samples in both subsets. Nevertheless, *NOTCH1*-M cells showed significantly higher sIgM compared ~~with~~to the WT counterpart (Fig. 2a, left panel). Importantly, stratification of samples according to surface levels of CD38 and CD49d (Fig. 2a, middle and right panels), to ZAP70 expression or Rai classification of disease stage (Supplementary Fig. 2), failed to reveal significant associations.

Fig. 2

NOTCH1-M cases show constitutively higher levels of sIgM and are more responsive to IgM cross--linking. **a** sIgM levels determined by flow cytometry immediately after thawing in a cohort of 35 CLL patients, 17 of which harboring *NOTCH1*-M. Results indicate that the only significant association is between *NOTCH1* mutational status and sIgM levels, while CD38 and CD49d surface expression are not significantly associated. **b** Left panel. *NOTCH1*-M primary cells mobilize significantly more Ca^{2+} ions in response to BCR ligation signaling compared with the counterpart. Right panel. Regression line showing a direct relationship between sIgM levels and intracellular Ca^{2+} fluxes in response to BCR cross--linking. Each dot identifies a different primary case. *NOTCH1*-M cases are in red. **c** BCR cross--linking in *NOTCH1*-M cases results in higher signaling performances compared with *NOTCH1*-WT cases, as determined by Phosphflow assay. Specific readouts include pBTK (top), pPLCγ2 (middle) and pERK1/2 (bottom) evaluated 16 h after thawing to allow complete recovery from ~~in vivo~~*in vivo* anergy. Representative profiles for *NOTCH1*-M and -WT cases are reported on the left, while histogram bars show cumulative data. The effects of ibrutinib (Ib), DAPT and their combination is shown. Red bars identify *NOTCH1*-M samples. Star marks above each bar indicate significance referred to corresponding (paired) α-IgM NT condition



Consistently, *NOTCH1*-M CLLs were also significantly more responsive to sIgM engagement than -WT cells, as witnessed by intracellular calcium release in response to α-IgM triggering of BCR after 1 h (Supplementary Fig. 2B) and 16 h (Fig. 2b, left panel), and by the robust correlation between sIgM levels and calcium mobilization in our cohort (Fig. 2b right panel). We next compared critical responses occurring downstream to BCR signaling activation in the *NOTCH1*-WT and -M sample subsets. BCR engagement triggers a phosphorylation cascade of mediators ultimately leading to the activation of MAPK and NFκB signaling [25, 26]. Upon short soluble α-IgM stimulation (10 min), *NOTCH1*-M samples showed significantly increased phosphorylation of BTK, PLCγ2, and ERK compared ~~with~~to -WT, as

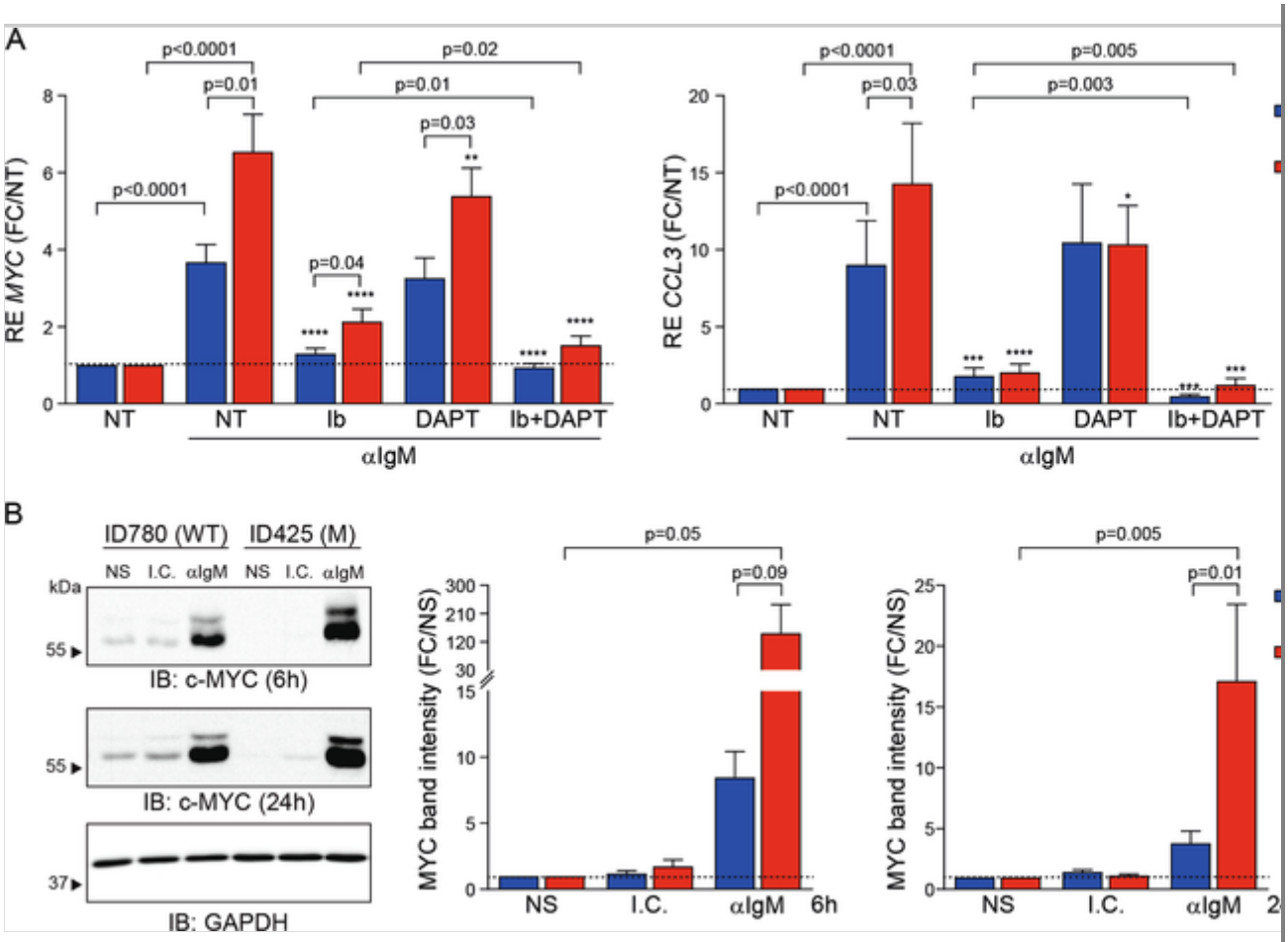
evaluated by a Phosflow assay. Pretreatment with ibrutinib efficiently prevented the phosphorylation cascade in both subsets, whereas blocking NOTCH1 activation with DAPT had no measurable effects, indicating that NOTCH1 itself does not directly regulate early events downstream to BCR ligation (Fig. 2c).

NOTCH1-M samples show enhanced induction of BCR dependent targets

Genetic reprogramming was evaluated after 24 h stimulation with plate-bound α-IgM, to induce persistent activation of BCR signaling [16]. mRNA levels of *MYC* and *CCL3* were analyzed as representative BCR target genes [27, 28, 29], and were robustly increased upon α-IgM stimulation in both *NOTCH1*-WT and -M samples (Fig. 3a). However, both genes were significantly more induced in the *NOTCH1*-M CLL subset. Interestingly, BCR stimulation in the presence of DAPT significantly restricted *MYC* and *CCL3* induction acting selectively in -M samples and reducing, at least in part, the difference between the two subsets. Furthermore, when used in combination, DAPT enhanced the effects of ibrutinib in both *NOTCH1*-WT and -M samples (Fig. 3a), indicating that the two pathways cooperate independently of the mutation and that sustained NOTCH1 activation, as in the case of PEST domain mutations, may confer further advantage to CLL cells.

Fig. 3

NOTCH1-M cases show higher levels of ~~MYC~~*MYC* and ~~CCL3~~*CCL3* induction compared with ~~NOTCH1~~*NOTCH1*-WT in response to IgM cross~~-~~-linking. **a** Histograms show *MYC* (left) or *CCL3* (right) RNA levels in *NOTCH1*-M compared with *NOTCH1*-WT cases. Data are represented as fold change over the untreated condition (FC/NT). Ibrutinib (Ib), DAPT or their combination were added to the cultures where indicated. Star marks above each bar indicate significance referred to corresponding (paired) α-IgM NT condition. ~~(~~RE: relative expression.~~)~~ ~~(~~**b**~~,~~ (left panel) Western blot showing c-MYC expression levels before and after IgM cross~~-~~-linking in representative *NOTCH1*-M or -WT cases. Histogram plots reporting intensity of the c-MYC bands in 5 *NOTCH1*-M or -WT cases at 6-h (middle panel) and at 24-h stimulations (right panel). Band intensities were measured using ImageJ and are shown as fold change over the band referring to unstimulated cells (FC/NS). Red bars identify *NOTCH1*-M samples



In line with RT-PCR data, MYC was strongly induced at the protein level upon BCR ligation and significantly more in the *NOTCH1*-M subset (Fig. 3b). MYC induction was already evident after 6 h stimulation, indicating that it is an early event in BCR signaling.

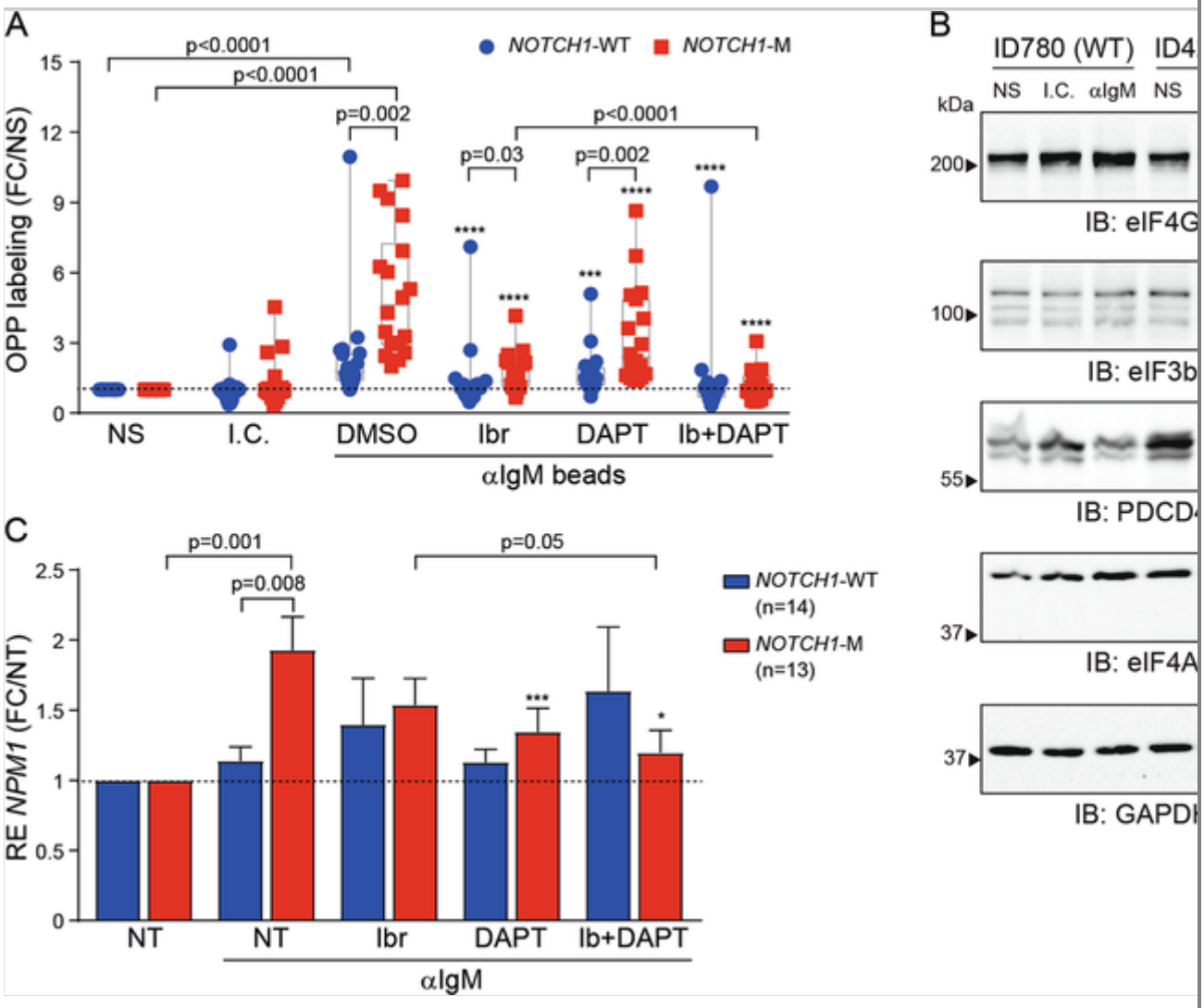
NOTCH1-M CLLs exhibit increased global mRNA translation upon sIgM engagement

BCR signaling is considered the driving force for CLL cell proliferation and survival and operates through the modulation of protein translation [20], which represents the cell core-power. We compared global mRNA translation in *NOTCH1*-WT and -M samples after 24 h BCR engagement with bead-bound α-IgM, to induce sustained signaling by preventing receptor internalization, similarly to plate-bound α-IgM. Compared ~~with~~to WT samples, *NOTCH1*-M CLLs showed increased BCR-induced global mRNA translation (Fig. 4a). Ibrutinib alone did not completely shut down translation in the -M subset, whereas it was sufficient to fully inhibit it in -WT cells. Interestingly, DAPT also significantly inhibited global mRNA translation in response to BCR in both subsets, suggesting a major contribution of NOTCH1 pathway. In *NOTCH1*-M samples, addition of DAPT potentiated the effects of

ibrutinib alone and decreased mRNA translation levels to those of unstimulated cells, abrogating the difference with -WT cells (Fig. 4a).

Fig. 4

NOTCH1-M samples show significantly higher levels of global mRNA translation compared with the *NOTCH1*-WT counterparts. **a** *O*-propargyl-puromycin (OPP) labeling test show increased global mRNA translation in *NOTCH1*-M (red) compared with *NOTCH1*-WT (blue) primary samples. Cells were treated with plate-bound α-IgM for 24 h, with the indicated drugs added at the beginning of the culture. **b** Expression levels of the elongation factors (eIF4G, eIF3b, PDCD4, and eIF4A) in *NOTCH1*-M or -WT representative samples. **c** mRNA expression levels of *NPM1* in *NOTCH1*-M vs *NOTCH1*-WT samples treated as indicated. Star marks above each bar indicate significance referred to corresponding (paired) α-IgM NT condition. RE: relative expression, Ib: ibrutinib.



Translation initiation factors such as eIF4G, eIF4A, and eIF3b were induced

24 h after stimulation, with *NOTCH1*-M samples showing a slightly but consistently more robust effect (Fig. 4b and Supplementary Fig. 3A). In line with previous data [20], cross-linking of BCR downregulated expression of PDCD4, a negative regulator of eIF4A [30] (Fig. 4b). At variance with MYC, levels of eIFs were unmodulated at earlier time points (Supplementary Fig. 3B-C), corroborating the hypothesis of a direct MYC-dependent regulation of the translation machinery.

Nucleophosmin1 (NPM1), a nucleolar protein regulating ribosome biogenesis and therefore taking part to translation [31], was recently pointed out as a mediator of proliferation and survival in CLL, being overexpressed in *NOTCH1*-M samples in a MYC-dependent way [32]. In keeping with these observations, BCR engagement strongly induced *NPM1* transcription selectively in *NOTCH1*-M samples with DAPT significantly inhibiting this effect (Fig. 4c).

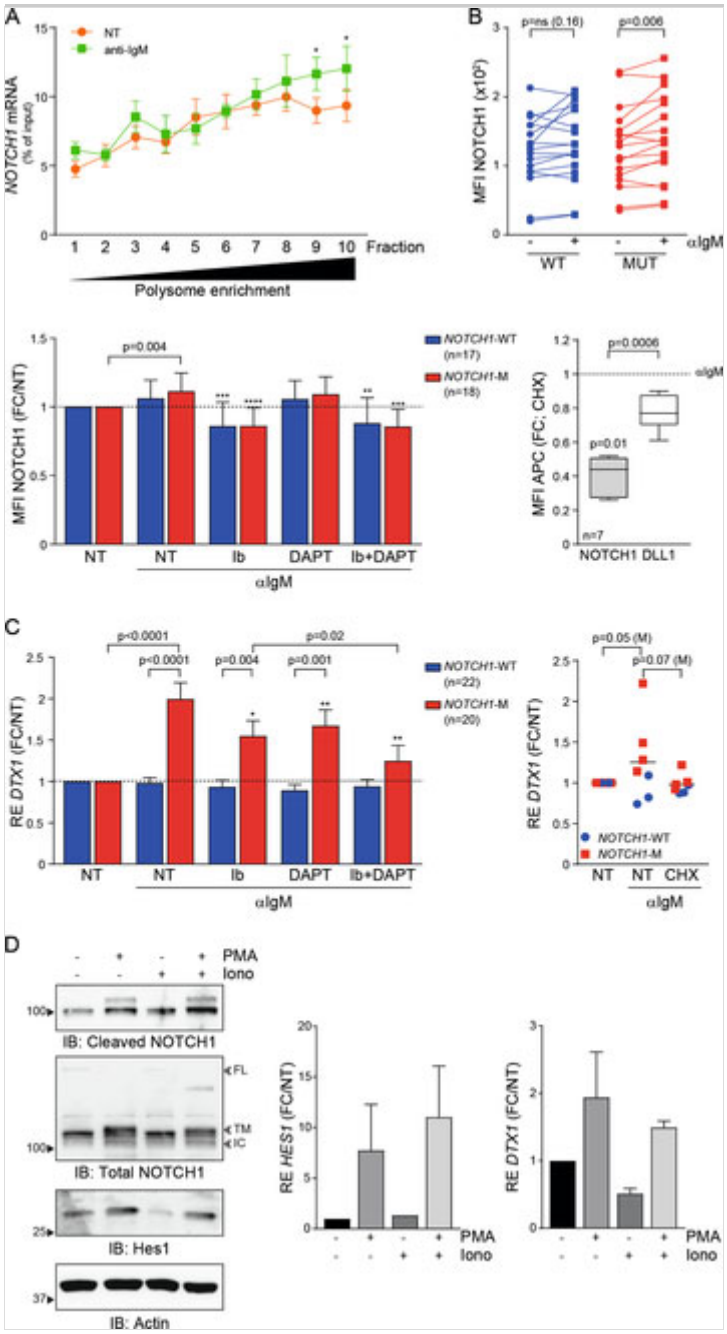
Triggering of BCR signaling increases NOTCH1 surface levels and promotes its activation

We showed that NOTCH1 activation impacts on BCR signaling particularly in the presence of PEST domain mutations. To explore bidirectional functional interactions, we asked if BCR-induced translation involved NOTCH1 itself or its ligands. Polysome profiling confirmed enrichment of *NOTCH1* mRNA in the latest fractions, indicating active translation (Fig. 5a). In contrast, no enrichment of *DLL1* mRNA was observed, suggesting a NOTCH1-restricted effect (Supplementary Fig. 4A). Consistently, we observed variation in surface NOTCH1 levels upon BCR cross-linking, but not in DLL1 (Fig. 5b and Supplementary Fig. 4C, D). Specifically, while *NOTCH1*-WT samples showed variable modulation of NOTCH1 expression at the membrane level, with samples increasing NOTCH1 MFI and others remaining unmodulated, -M cells were more consistently responsive (Fig. 5b). Ibrutinib significantly downregulated surface NOTCH1 in both -M and -WT samples, while treatment with DAPT did not, supporting a BCR-mediated effect (Fig. 5b, bottom left). Modulation of NOTCH1 occurred specifically at the protein level, as mRNA expression was modestly increased, more in the -M subset, but treatment with inhibitors did not result in major alterations of gene expression, suggesting a post-transcriptional regulation (Supplementary Fig. 4B). Consistently, stimulation of BCR signaling in the presence of cycloheximide (CHX), to block protein translation, significantly reduced NOTCH1 expression, whereas it had no effects on DLL1 surface levels

(Fig. 5b bottom right and Supplementary Fig. 4C, D). Lastly, downmodulation of NOTCH1 expression by CHX was not due to perturbation of cell homeostasis or viability, as *NOTCH1* mRNA levels were increased upon CHX treatment, suggesting either accumulation of untranslated mRNA or even enhanced transcription to overcome impaired translation (Supplementary Fig. 4E).

Fig. 5

NOTCH1 expression and activation are induced upon BCR ligation. **a** Polysome fractionation profiling show enrichment of *NOTCH1* mRNA in fractions 9 and 10, marking more active translation, upon plate-bound α-IgM stimulation. **b** NOTCH1 MFI is consistently increased in *NOTCH1*-M samples at variance with -WT cells. Cumulative data and effects of treatments are shown in the bottom left panel. The presence of CHX significantly impairs α-IgM-mediated surface NOTCH1 increase but has no effect on surface DLL1 (bottom right panel, CHX: cycloheximide). **c** *DTX1* levels are markedly increased upon BCR ligation specifically in *NOTCH1*-M samples. Treatment with ibrutinib (Ib) and DAPT, either alone or in combination, significantly reduces *DTX1* induction with drug combination showing the greatest inhibition. Consistent with the effect on NOTCH1 surface levels, CHX strongly prevents *DTX1* induction. *P* values for M samples are shown. **d** Stimulation of *NOTCH1*-M CLL cells with PMA and Ionomycin (Iono) for 6 h induces NICD cleavage and *HES1* and *DTX1* expression. Star marks above each bar indicate significance referred to corresponding (paired) α-IgM NT condition. Results are expressed as fold change over unstimulated cells (FC/NT). RE: relative expression.



Upregulation of NOTCH1 expression was accompanied by activation of the signaling pathway, specifically in the *NOTCH1*-M subset. *DTX1* and *HES1* levels were strongly upregulated upon BCR ligation in -M samples, with both ibrutinib and DAPT showing efficient inhibition as single agents and enhanced effects when used in combination (Fig. 5c and Supplementary Fig. 5). Interestingly, CHX also inhibited *DTX1* induction similarly to NOTCH1 overexpression (Fig. 5c, right panel). In line with NOTCH1 regulating a BCR gene signature, we observed significant upregulation of *BTK*, *LYN* and *BLNK* in *NOTCH1*-M samples induced by α-IgM stimulation. This effect was downmodulated both by ibrutinib and DAPT and was prevented by CHX, just as *DTX1* (Supplementary Fig. 6A–D). As a control,

neither *CCL3* nor *MYC* levels were downregulated by CHX, being rather upregulated possibly as a result of mRNA accumulation, thus limiting the effect to a strictly NOTCH1-dependent gene signature (Supplementary Fig. 6E).

These results indicate that not only NOTCH1 surface levels are increased upon BCR signaling, but also that the pathway is activated, suggesting a ligand-independent NOTCH1 activation, likely involving protein kinase C (PKC), as recently demonstrated [33]. To test this hypothesis, we stimulated *NOTCH1*-M CLL cells with PMA and ionomycin, both alone and in combination, to activate PKC and Ca^{2+} release from intracellular stores, respectively. We observed that PMA alone efficiently triggered NOTCH1 activation, evaluated both as NICD cleavage and *HES1* and *DTX1* upregulation, and that addition of ionomycin potentiated the effect (Fig. 5d). In contrast, ionomycin as a single agent did not result in major modifications of NOTCH1 activity, suggesting that PKC-mediated activation of ADAM and γ-secretase is the predominant mechanism and that intracellular calcium mobilization may further contribute to NOTCH1 activation.

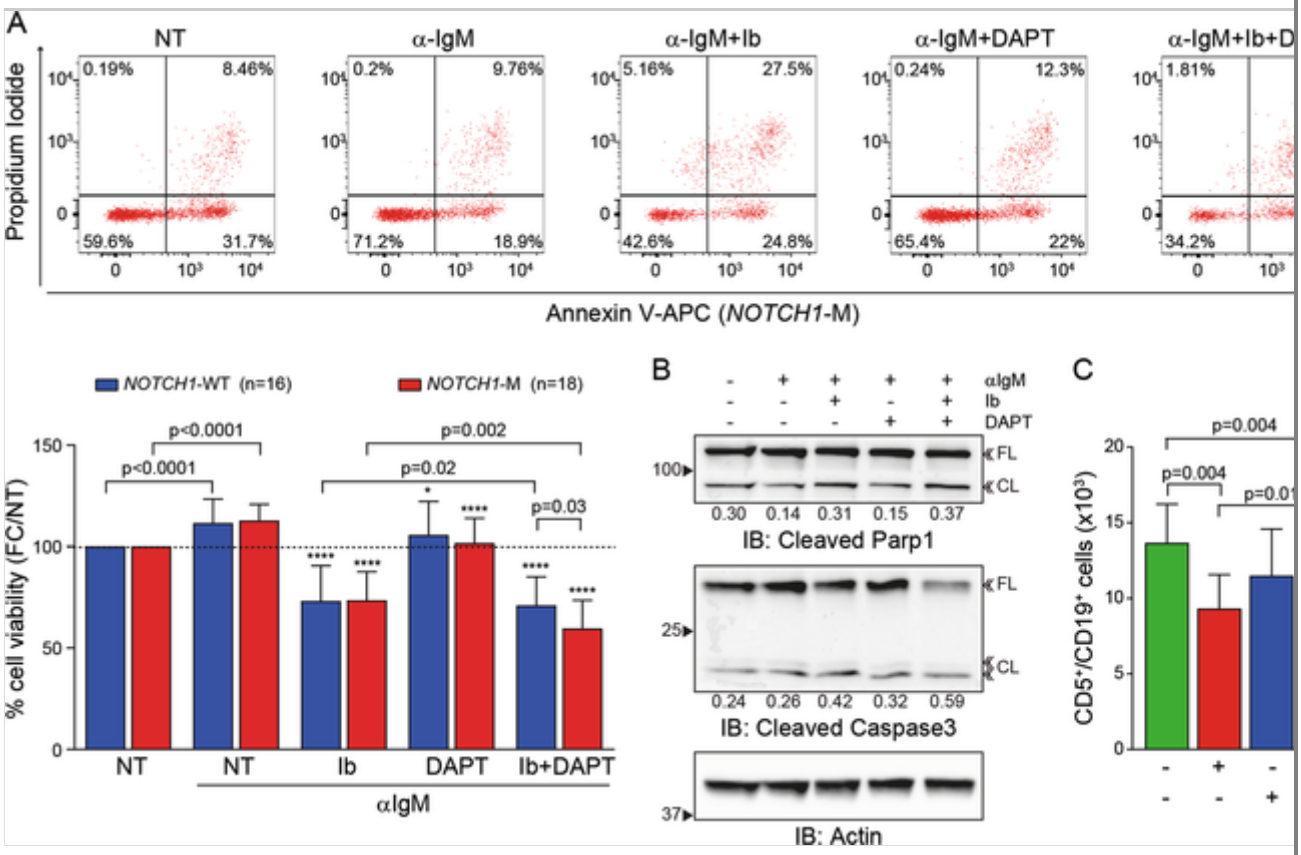
BCR and NOTCH1 pathways cooperate in sustaining CLL cell survival

We then asked whether interrupting the crosstalk between BCR and NOTCH1 pathways would affect CLL cell homeostasis. Stimulation of CLL cells with immobilized α-IgM promoted leukemic cell survival by rescuing them from spontaneous apoptosis occurring ~~in vitro~~*in vitro*, without differences between *NOTCH1*-WT and -M samples. As expected, ibrutinib led to a prominent apoptotic response independently of *NOTCH1* mutations, while DAPT completely abrogated the benefits of BCR triggering without major toxicity for CLL cells, suggesting that NOTCH1 pathway partly mediates BCR-driven pro-survival signals. Accordingly, addition of DAPT potentiated ibrutinib effects, independently of *NOTCH1* mutations. Notably, cell viability in *NOTCH1*-M samples was significantly more affected by the drug combination compared ~~with~~to -WT cells, supporting the hypothesis of an interplay between the two pathways that is further sustained in the presence of PEST domain mutations (Fig. 6a). Drug combination analyses revealed a trend toward synergism between ibrutinib and DAPT in *NOTCH1*-M samples (CI = 0.87) at variance with WT cells where responses to dual targeting were similar to the expected additive effect (CI = 1). In line with flow cytometry data, activation of PARP and Caspase 3 was maximal in cells treated with drug combination

(Fig. 6b).

Fig. 6

BCR and NOTCH1 pathways cooperate in sustaining CLL cell survival. **a** Representative dot plot of an Annexin V/ Propidium iodide assay to evaluate apoptosis of CLL cells in response to α-IgM stimulation in the presence or absence of the indicated treatment conditions. Cumulative data are shown as histograms in the bottom panel and expressed as fold change of cell viability compared with unstimulated/untreated cells (FC/NT). **b** WB of PARP1 and Caspase 3 cleavages in the indicated conditions. Ratio of full length (FL) and cleaved (CL) protein band intensities is shown below each lane. Star marks above each bar indicate significance referred to corresponding (paired) α-IgM NT condition. **c** Flow cytometry analysis of $CD5^{+}/CD19^{+}$ cells in the spleen of NSG mice injected with primary CLL cells ($n = 3$ samples) and treated with the indicated drugs. Each spleen was divided in three parts and each fragment dismantled and analyzed independently, resulting in three measurements for each mouse. Cell numbers are normalized over 100,000 acquired events. Ib: ibrutinib.



To confirm ~~in vitro~~*in vitro* findings, we explored dual targeting approaches in short-term CLL-PDX models. Primary cells collected from 3 *NOTCH1*-M

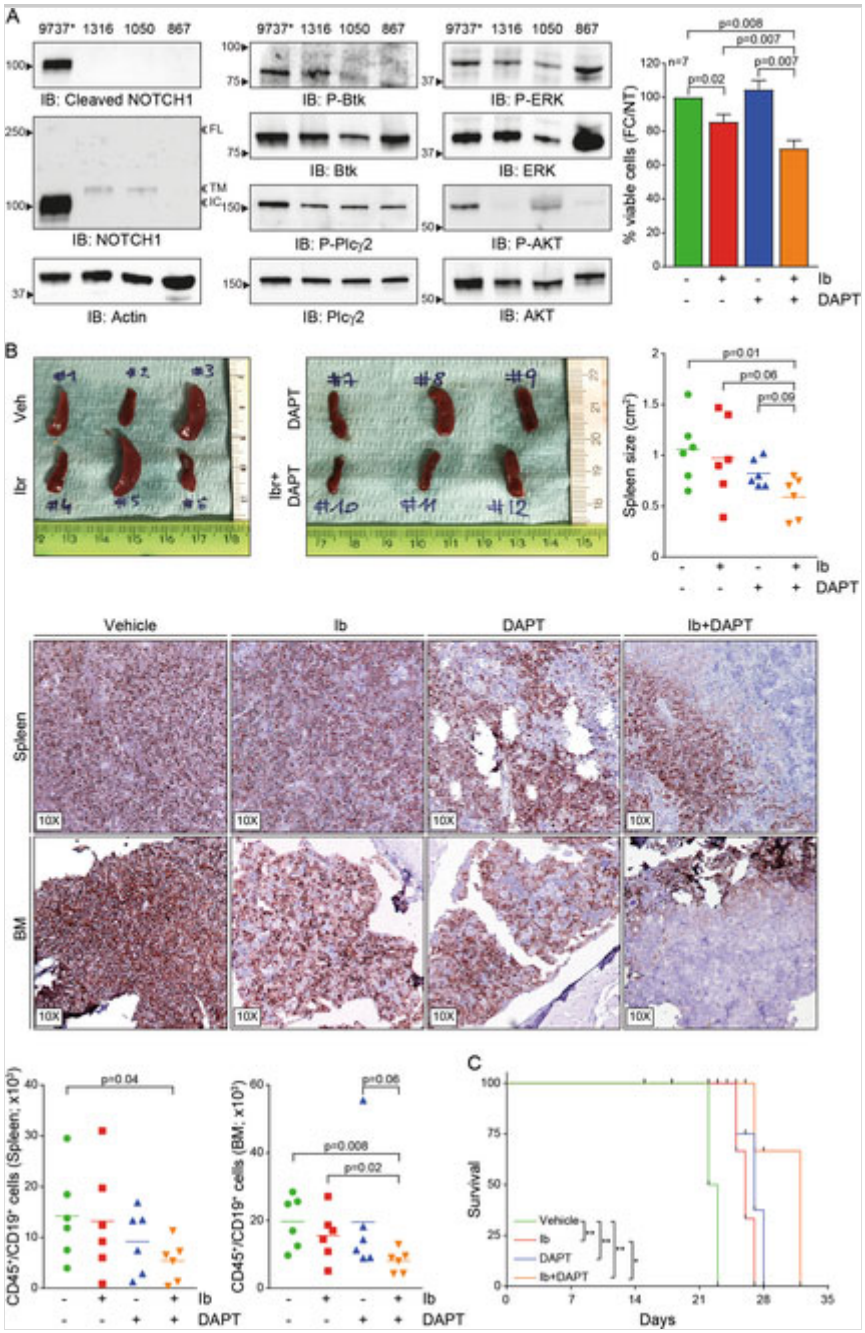
CLL patients of our cohort were i.v. injected in busulfan-primed NSG mice. Treatment with ibrutinib and DAPT, either as single agents or in combination, was initiated at day −1 before injection and continued for the following 3 days. After this schedule, mice were euthanized to analyze and compare CLL cells engraftment in the different treatment arms. Ibrutinib used as single agent significantly reduced CLL spleen colonization, whereas DAPT alone led to a modest decrease of CLL cells number. In line with ~~in vitro~~*in vitro* results, drug combination further potentiated ibrutinib effects with a trend toward synergism (CI = 0.81; Fig. 6c).

Dual inhibition of BCR signaling and NOTCH1 pathway is effective in RS models

Lastly, we asked whether dual inhibition of BCR signaling and NOTCH1 pathway could represent an opportunity for novel treatment strategies in RS, a fatal complication of CLL which remains drug-orphan. To this aim, we took advantage of four RS-PDX models recently established by our group [34]. Relevant molecular features of these models are recapitulated in Supplementary Table 1, full genetic details were previously described [34]. We focused our attention on RS9737 that was the only sample carrying the c.7541–7542_delCT mutation in ~~NOTCH1~~*NOTCH1* PEST domain and showing activation of both NOTCH1 and BCR pathways at higher levels than the other models (Fig. 7a). ~~In vitro~~ *In vitro* treatment of RS9737 cells revealed only partial responses to ibrutinib and DAPT as single agents, whereas synergism of drug combination significantly increased apoptosis (CI = 0.35; Fig. 7a right panel). Cells were then i.v. injected in NSG mice to evaluate the effects of treatments on disease dissemination and on survival. Cells were allowed to engraft for 10 days before daily administration of ibrutinib and DAPT, either alone or combined (Supplementary Fig. 7A). At the end of the treatment schedule, mice were either euthanized to compare engraftment in spleen and bone marrow (BM) or monitored to generate a survival curve. Compared with vehicle or to ibrutinib and DAPT used as single agents, drug combination significantly reduced spleen and BM colonization, as documented macroscopically by measuring spleen size and microscopically by immunohistochemical analyses or leukemic cells count in both districts (CI = 0.64 in the spleen, CI = 0.35 in the BM; Fig. 7b). Similar results were obtained in liver, lung, kidney, brain, and PB (Supplementary Fig. 7B). Importantly, drug combination also significantly prolonged mice survival (Fig. 7c). Treatment with ibrutinib or DAPT alone only partially limited RS growth and spread, with variable responses (Fig. 7b, c).

Fig. 7

Dual targeting of BCR signaling and NOTCH1 pathway opens the way to novel treatment options in RS models. **a** (left panels) WB analysis of expression and activation of NOTCH1 pathway and BCR signaling in four RS samples grown in PDX models. RS cells were lysed immediately after collection from PDX to benefit from previous ~~in vivo~~*in vivo* pathways activation. RS9737 was the only sample carrying the c.7541-7542DelCT mutation in *NOTCH1* and showing high activity of both pathways. RS9737 also harbored a partial deletion of chromosome 9, thus making mutated *NOTCH1* the only allele expressed. For this reason, experiments were carried out mostly on this sample (asterisk). (Right panel) RS9737 cell viability upon ~~in vitro~~ *in vitro* treatment with ibrutinib (Ib) and DAPT either alone or in combination. **b** Mice treated with the combination of ibrutinib and DAPT show significantly reduced spleen size as documented by pictures (top left) and recapitulated by measures (top right) (Veh vehicle-treated mice). IHC sections of spleens and breastbones (representative of BM), collected from mice in the different treatment arms, show decreased anti-CD20 staining in double-treated animals (middle panels). Reduced spleen and BM colonization is confirmed by flow cytometry analyses of $CD45^+/CD19^+$ cells (bottom panels). Cell numbers are normalized over 100,000 acquired events. **c** Efficacy of dual inhibition of BCR signaling and NOTCH1 is documented also by prolonged animals' survival (mean survival with the combination 29.75 ± 1.3 days vs. 26.25 ± 0.5 days with ibrutinib vs. 26.75 ± 0.6 days with DAPT vs. 22.5 ± 0.3 days with vehicle)



Discussion

Since the first approval of ibrutinib by the Food and Drug Administration in February 2012, the therapy of CLL has undergone a revolution, with patients moving toward chemotherapy-free regimens [35]. The novel drugs interrupt constitutive BCR signaling, such as Btk and PI3K inhibitors [36] or induce apoptosis, such as bcl2 inhibitors [37, 38]. The development of resistance in a minority, but still relevant proportion of patients, has prompted novel efforts into studying drug alternatives or combinations that may fully eradicate the disease [39, 40]. In order to do so, it is important to know the hierarchy of signaling pathways within the CLL cell. This work was undertaken with the

aim of understanding whether and how BCR and NOTCH1 signaling pathways interact. Preliminary evidence indicates that genes encoding elements of BCR signaling are part of a signature directly regulated by NOTCH1 [24, 41]. Furthermore, evidence in different tumor types suggests the existence of a NOTCH/PI3K axis that may represent a connection between NOTCH1 and BCR pathways in B-cell malignancies [42, 43, 44, 45].

For this work, we used only primary patient cells that were carefully selected in order to avoid confounding factors that may impact on BCR signaling. While this approach is certainly a limitation as genetic manipulation of primary cells remains limited, it offers indirect proof of the validity of the results in a “real-life” situation. We focused on *IGHV*-UM CLLs for two main reasons. The first is that the UM subset is more responsive to BCR ligation as witnessed also by a more aggressive disease with a worse clinical prognosis [17]. The second is that *NOTCH1* mutations are more frequent in ~~IGHV~~*IGHV*-UM samples.

Results demonstrate the existence of a functional loop linking NOTCH1 and the BCR in CLL cells. We decided to start studying the loop by artificially activating NOTCH1 using EDTA, which is believed to cause a 3D conformational modification of the extracellular portion of the molecule, exposing the cleavage site for ADAM proteases [23]. Under stringent conditions, i.e., a transient 15 min incubation with EDTA followed by 2–6 h recovery, we can detect transcriptional activation of targets selectively in *NOTCH1*-M samples. Interestingly, these patients also show increased sIgM expression and transcription of genes that code for integral components of the BCR pathway, including *LYN*, *BTK,* and *BLNK*. If we open the loop at the other end, i.e., if we activate the BCR using antibodies, we detect increased NOTCH1 expression and signaling. Considered together, these two findings can be explained by hypothesizing (i) that BCR directly controls translation of NOTCH1 and (ii) that somehow the BCR can also control NOTCH1 activation in a ligand-independent, possibly PKC-dependent, way. The OPP translation assay confirmed globally increased translation following BCR activation. While this finding is not novel, the relevant observation is that there is a markedly higher translation in *NOTCH1*-M compared with -WT cases. The finding of mRNA enrichment in polysome fractions marking active translation confirms that NOTCH1 is among the proteins subjected to this regulation. Furthermore, the finding that the NOTCH1 ligand DLL1 is not regulated in this way confirms that the signal is specific and that there are

likely ligand-independent NOTCH1 activation mechanisms. If no ligand is bound, NOTCH receptors are internalized through endosome vesicles and either driven to lysosome-mediated degradation or alternatively maintained into late endosomes and possibly recycled to the membrane, an event facilitated by DTX1 [46]. It was recently demonstrated that in endosomes from T lymphocytes, NOTCH1 can be activated upon TCR triggering through a ligand-independent PKC-dependent mechanism that promotes ADAM- and γ-secretase-mediated NOTCH1 cleavage [33]. In our context, these observations imply that activation of the BCR signaling pathway, with concomitant induction of PKC activation and calcium mobilization, would be enough to drive NOTCH1 pathway activation, underlining the strong functional connection between the two pathways (Supplementary Fig. 8). In this scenario, mutations stabilizing active NOTCH1 might confer an advantage to leukemic cells, as weak stimuli would be sufficient to trigger relatively big effects. Mechanistically, one possible explanation is that BCR-driven PKC-dependent activation of ADAM and γ-secretase results in a modest NICD cleavage, which is rapidly degraded in the absence of stabilizing mutations. In contrast, PEST-mutated NICD accumulates because of inefficient dismantling, amplifying NOTCH1 signaling activation upon BCR engagement. Consistently, we could appreciate considerable NOTCH1 activation upon triggering of BCR only in samples harboring *NOTCH1* mutations.

Our results support the view of a feed-forward loop of functional cooperation between NOTCH1 and the BCR, where the former has direct effects on IgM signaling and the latter can induce ligand-independent NOTCH1 activation further enhancing ~~anti~~α-IgM responses, remarkably in the presence of *NOTCH1* PEST mutations.

These findings provide the rationale for co-targeting BCR and NOTCH1. In line with our hypothesis and with recent observations [47], CLL cells ~~in vitro~~*in vitro* die at higher rates in the presence of NOTCH1 and BCR inhibitors. Notably, the effects of dual inhibition are more prominent in *NOTCH1*-M cells likely because the functional cooperation between the BCR and NOTCH1 is more pronounced in this subset. However, the CLL field is very crowded, with many novel, effective, and highly safe drugs, that already offers therapeutic alternatives to ibrutinib-resistant patients, before exploiting combination strategies. This is not the case for RS, a rare but very often fatal complication of CLL, with limited therapeutic options beside the CHOP/R-

CHOP regimens [48]. We recently established four PDX models of RS and showed that one of them harbors *NOTCH1* PEST domain mutation [34]. These cells have a constitutively hyperactive NOTCH1 pathway, with a prominent band corresponding to the NICD. Intriguingly, they also have the highest levels of Btk and PI3K activation, suggesting that the BCR-NOTCH1 circuit could be fully functional in these cells. For these reasons, this model was selected to perform pilot experiments co-targeting the two pathways. After excluding Btk mutations, we selected ibrutinib and the γ-secretase inhibitor DAPT to treat these animals. In experiments where all animals were treated every day for two weeks before evaluating disease spread, mice treated with the combination of ibrutinib and DAPT had significantly less tumor burden in every compartment considered. Accordingly, these mice lived longer than vehicle-treated mice.

In conclusion, our data highlight that NOTCH1 and BCR pathways are connected in a functional loop, with *NOTCH1*-M CLL being more responsive to BCR signaling. Future studies will tell whether this knowledge can be exploited therapeutically, expanding to nonmutated NOTCH1 activated cases and particularly in the context of RS, which remains an incurable condition with limited therapeutic options.

Supplementary information

The online version of this article (https://doi.org/10.1038/s41375-019-0571-0) contains supplementary material, which is available to authorized users.

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Author contribution

FA designed the study, performed experiments, analyzed, and interpreted data and together with SD wrote the paper; VB, NV, KG, and AY performed experiments; TV established RS-PDX models and performed experiments; MC, GD'A, JNA, RRF, and GG: provided patient samples and relevant clinical information and contributed to data interpretation; GP and FF: discussed results and contributed to data interpretation; SD: designed the study, interpreted data and together with FA wrote the paper.

Compliance with ethical standards

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

Supplementary information

Supplemental methods and figures

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