NOTCH1-mutated chronic lymphocytic leukemia cells are characterized by a *MYC*-related overexpression of nucleophosmin 1 and ribosome-associated components

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In chronic lymphocytic leukemia (CLL), the mechanisms controlling cell growth and proliferation in the presence of *NOTCH1* mutations remain largely unexplored. By performing a gene expression profile of *NOTCH1*-mutated (*NOTCH1*-mut) versus *NOTCH1* wild-type CLL, we identified a gene signature of *NOTCH1*-mut CLL characterized by the upregulation of genes related to ribosome biogenesis, such as nucleophosmin 1 (*NPM1*) and ribosomal proteins (*RNP*s). Activation of NOTCH1 signaling by ethylenediaminetetraacetic acid or by coculture with JAGGED1-expressing stromal cells increased *NPM1* expression, and inhibition of NOTCH1 signaling by either NOTCH1-specific small interfering RNA (siRNA) or γ-secretase inhibitor reduced *NPM1* expression. Bioinformatic analyses and *in vitro* activation/inhibition of NOTCH1 signaling suggested a role of *MYC* as a mediator of NOTCH1 effects over NPM1 and RNP expression in *NOTCH1*-mut CLL. Chromatin immunoprecipitation experiments performed on NOTCH1 intracellular domain (NICD)-transfected CLL-like cells showed the direct binding of NOTCH1 to the *MYC* promoter, and transfection with *MYC*-specific siRNA reduced *NPM1* expression. In turn, NPM1 determined a proliferation advantage of CLL-like cells, as demonstrated by *NPM1*-specific siRNA transfection. In conclusion, *NOTCH1* mutations in CLL are associated with the overexpression of *MYC* and *MYC*-related genes involved in protein biosynthesis including *NPM1*, which are allegedly responsible for cell growth and/or proliferation advantages of *NOTCH1*-mut CLL.

INTRODUCTION

Mutations of *NOTCH1* gene occur at diagnosis in ~10% of chronic lymphocytic leukemia (CLL) cases, and are enriched in CLL patient subgroups carrying trisomy 12 and/or an unmutated *IGHV* gene status.[1–9] In CLL, all the described *NOTCH1* mutations localize either in the coding or in the 3′-untranslated non-coding regions (3′-UTR), and cause impaired degradation and accumulation of the NOTCH1 intracytoplasmic domain (NICD).[3–7,9]

NOTCH1 encodes for a transmembrane receptor acting as a ligand-activated transcription factor.[10–12] In particular, NOTCH1 signaling initiates when the ligand, from either the JAGGED or DELTA families, binds to the receptor and induces successive proteolytic cleavages, resulting in the release and nuclear translocation of the NICD. In the nucleus, the NICD becomes part of a transcriptional activation complex along with the transcription factor RBPJ, which leads to the derepression/activation of specific target genes, including *HES1* and *MYC*.[10,11,13–21] At variance with normal B cell, CLL cells constitutively express the NOTCH1 receptor as well as its ligands JAGGED1 and JAGGED2, suggesting autocrine/paracrine loops for NOTCH1 signaling activation.[22,23] Moreover, in a mouse model, NOTCH1 signaling has been shown to be critical for CLL development *in vivo*.[23]

In CLL, the sustained activation of the NOTCH1 pathway due to the presence of *NOTCH1* mutations[3,4,24,25] has clinical implications in the prognosis and response to immunochemotherapeutic regimens of *NOTCH1*-mutated (*NOTCH1*-mut) CLL patients.[6,26–28] Nevertheless, the mechanisms controlling cell growth, proliferation and apoptosis downstream of *NOTCH1* in *NOTCH1*-mut CLL remain largely unexplored.

In the present study, we identify a gene expression signature of *NOTCH1*-mut CLL characterized by the overexpression of genes related to ribosome biogenesis, such as nucleophosmin 1 (*NPM1*) and genes codifying for ribosomal proteins (*RNP*s). We also provide evidence that NOTCH1 signaling is capable of modulating *NPM1* and *RNP* expression via *MYC* transcription. The suggested link between NOTCH1 signaling activation and enhanced ribosome biogenesis machinery might eventually provide proliferative advantages to *NOTCH1*-mut CLL.

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MATERIALS AND METHODS

Primary CLL cells

The study is part of a comprehensive CLL characterization approved by the Internal Review Board of the Aviano Centro di Riferimento Oncologico (Approval no. IRB-05-2010, no. IRB-05-2015) upon informed consent in accordance with the Declaration of Helsinki, and included peripheral blood (PB) samples from 188 patients with CLL (Supplementary Table S1).[29] The *NOTCH1* mutational characterization of this cohort was included in a previously published study of 692 CLL cases.[28] According to the availability of biological material, 76 out of 87 *NOTCH1*-mut cases were included in the present study cohort, overall accounting for 80 *NOTCH1* mutations (66 c.7541-7542delCT mutation, 11 *NOTCH1* mutations within the coding region other than the c.7541-7542delCT, 3 *NOTCH1* mutations in the 3′-UTR). Two co-occurring *NOTCH1* mutations were detected in four cases (Supplementary Table S2).[3,4,7,30] A randomly selected subset of 112 *NOTCH1* wild-type (*NOTCH1*-wt) cases was also included in this study as a comparison group for *in vitro* validations and functional studies (Supplementary Table S1).

Primary CLL cells were obtained from PB samples by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and used either directly or cryopreserved until use. All studies were performed on highly purified cells (>95% pure), as results of negative selection by immunomagnetic beads when required.[28] CLL cases were characterized for *IGHV* mutational status, the main cytogenetic abnormalities, and CD38, CD49d and ZAP70 expression, as described.[30–32]

NOTCH1 mutational status

NOTCH1 mutational status was assessed by next-generation sequencing (NGS) with an amplicon-based strategy to cover with at least a ×2000 coverage the whole *NOTCH1* exon 34 and part of 3′-UTR, according to Puente *et al.*[5] PCR products were generated using a high-fidelity *Taq* polymerase (Phusion High-Fidelity DNA Polymerase; ThermoFisher Scientific, Waltham, MA, USA) and subjected to NGS on a MiSeq sequencer (Illumina, San Diego, CA, USA). Data were analyzed with MiSeq reporter (Illumina) and IGV software (software.broadinstitute.org/software/igv/)[33] against human genome assembly hg19. Results were expressed as the percentage of mutated DNA. *NOTCH1* NGS primer list is available upon request.

Flow cytometry and cell sorting experiments

Primary CLL cells were evaluated by flow cytometry using a LSRFortessa Cell Analyzer (BD Biosciences, Milan, Italy) for NPM1 expression (0412; Santa Cruz Biotechnology, Dallas, TX, USA), total NOTCH1 expression (Ab27526, anti-NOTCH1 antibody chromatin immunoprecipitation (ChIP) grade; Abcam, Cambridge, UK), surface NOTCH1 expression (MHN1-519, phycoerythrin-conjugated; BioLegend, San Diego, CA, USA), JAGGED1 expression (HMJ1-29, phycoerythrin-conjugated; BioLegend). Intranuclear staining was performed with Fix&Perm Kit (Caltag Medsystems, Buckingham, UK) with methanol modification and subsequently saturated with blocking buffer (1% bovine serum albumin, 2% fetal bovine serum in phosphate-buffered saline) for 30 min. Incubations with primary antibodies were performed overnight at 4 °C, and then with appropriate secondary antibodies for 30 min at room temperature. Aqua Live/Dead Fixable Stain (ThermoFisher Scientific) was used as viability stain. Anti-CD3 and anti-CD14 antibodies (BD Biosciences) were used, when necessary, for negative gating of B cells. Irrelevant isotype-matched antibodies were used to determine background fluorescence. Experiments were performed upon instrument calibration with CS&T beads (BD Biosciences) and analyzed with FacsDiva software (BD Biosciences). Quantitative flow cytometry was performed upon instrument calibration with PE Phycoerythrin Fluorescence Quantitation Kit (BD Biosciences). Cell size was estimated using the forward scatter values.

Primary CLL cells from selected *NOTCH1*-mut cases were sorted according to NPM1 expression. Sorting was performed utilizing a FACSAriaIII cell sorter (BD Biosciences), as described.[28] The $NPM1^{low}$ and $NPM1^{high}$ fractions were selected below the 25th percentile or above the 75th percentile of NPM1 expression, respectively.

In vitro treatment with chemical compounds

Purified primary CLL cells (2×10^6 cells per ml) were cultured as reported.[28] To modulate NOTCH1 signaling, purified primary CLL cells were treated with γ-secretase inhibitor (GSI L-685 458 (Sigma-Aldrich, St Louis, MO, USA), 10 μM) or with ethylenediaminetetraacetic acid (EDTA, 1 mM) for up to 48 h. In control conditions, equal volume of the appropriate solvent compound was added.

To induce MYC expression by Toll-like receptor 9 triggering, purified primary CLL cells (2×10^6 cells per ml) were treated for up to 48 h, with 7.5 μg/ml complete phosphorothioate CpG oligonucleotide 2006 (5-TCGTCGTTTTGTCGTTTTGTCGTT-3; Microsynth, Balgach, Switzerland) in the presence of 100 U/ml interleukin-2 (IL-2; R&D Systems, Minneapolis, MN, USA), as reported previously.[34–38]

Coculture with M2-10B4 stromal cells

M2-10B4 stromal cells, maintained in RPMI 10% fetal bovine serum, were seeded and left to adhere for 18 h, to obtain a layer of JAGGED1-expressing stromal cells.[39] CLL cells were then seeded on the culture at 0.37×10^6 for 1 cm^2.[40]

Proliferation experiments

Proliferation was evaluated in NICD-transfected MEC-1 cells by using CellTrace Proliferation Kit (for flow cytometry; ThermoFisher Scientific), as reported previously.[41]

Further details regarding the materials and methods used are provided as Supplementary Information.

RESULTS

GEP of *NOTCH1*-mut CLL cells

To investigate the influence of *NOTCH1* mutations on NOTCH1 signaling in CLL cells, a gene expression profiling (GEP) was performed by comparing constitutive CLL cell samples of five *NOTCH1*-mut and five *NOTCH1*-wt cases in the context of the homogeneous subgroup of cases with an unmutated *IGHV* status (*IGHV* UM cases). The five *NOTCH1*-mut cases were selected among cases with the highest mutational load (i.e. >20% of *NOTCH1*-mut DNA). Three hundred and six probes (213 upregulated and 93 downregulated), corresponding to 275 genes (185 upregulated and 90 downregulated), were found differentially expressed in the *NOTCH1*-mut category (Supplementary Figure S1 and Supplementary Table S3).

A gene set enrichment analysis,[42] focused on the C.2 (curated) gene sets, selected the 'RIBOSOMAL_PROTEINS' and 'hsa03010_RIBOSOME' gene sets as having the lowest nominal *P*-value ($P = 0$, false discovery rate *q*-value = 0, for both the gene sets; Supplementary Figure S2a), among those significantly enriched in upregulated genes in *NOTCH1*-mut cases. Moreover, in the same gene set enrichment analysis, gene sets referring to the nuclear factor-κB pathway ('TNFA_NFKB_DEP_UP', $P = 0.0037$, $q = 0.0315$) and the AKT pathway ('AKTPATHWAY', $P = 0.0261$, $q = 0.1225$), both known to be directly related to NOTCH1 signaling, turned out to be significantly enriched in upregulated genes in the *NOTCH1*-mut category, in keeping with previous studies (Supplementary Figure S2b).[18,43] Consistently, a gene-ontology (GO) tree machine analysis[44] of the 306 differentially expressed probes revealed an over-representation of gene-ontology categories related to RNA processing (Supplementary Table S4).[18] Altogether, these bioinformatic analyses along with previously published data obtained in the T-cell acute lymphoblastic leukemia model[18] prompted us to focus on genes upregulated in *NOTCH1*-mut CLL and related to ribosome biosynthesis (Figure 1a). In particular, we focused on the *NPM1* gene, found significantly upregulated in *NOTCH1*-mut CLL, given its main multifunctional chaperone role in ribosome biosynthesis as well as its key role in several hematological malignancies.[45–47]

Correlation between NPM1 expression and *NOTCH1* mutational status in CLL

Results from GEP were validated by quantitative real-time PCR (QRT-PCR). Transcript levels of *NPM1,* as evaluated in 188 cases (76 *NOTCH1*-mut), were significantly higher in *NOTCH1*-mut compared with that in *NOTCH1*-wt cases ($P = 0.0046$; Figure 1b). In the same cohort, also transcript levels of *RPL7A*, *RPL18* and *RPS6* were

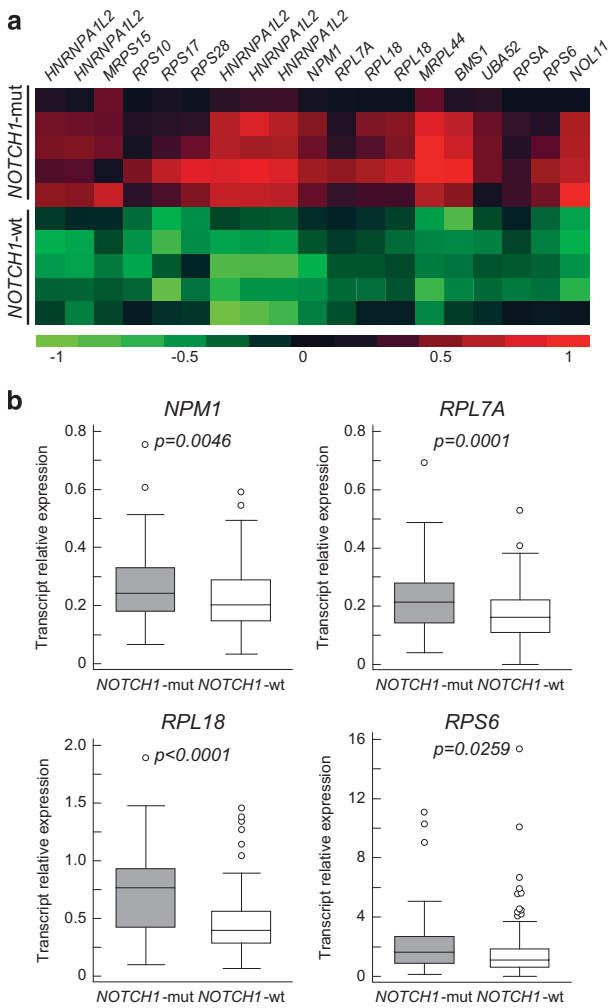


Figure 1. GEP of *NOTCH1*-mut and *NOTCH1*-wt cases. (**a**) Heat-map generated with 19 probes corresponding to 14 genes related to ribosome biosynthesis, found to be differentially expressed between *NOTCH1*-mut and *NOTCH1*-wt CLL samples. Color codes for gene expression values refer to mean centered log-ratio values. (**b**) Validation of GEP results. Box-and-whiskers plot representing transcript expression levels of *NPM1* and of genes codifying for RNPs, *RPL7A*, *RPL18* and *RPS6*, evaluated by QRT-PCR in a series of 76 *NOTCH1*-mut and 112 *NOTCH1*-wt CLL cases. *P*-value (Mann–Whitney *U*-test) for each gene is shown.

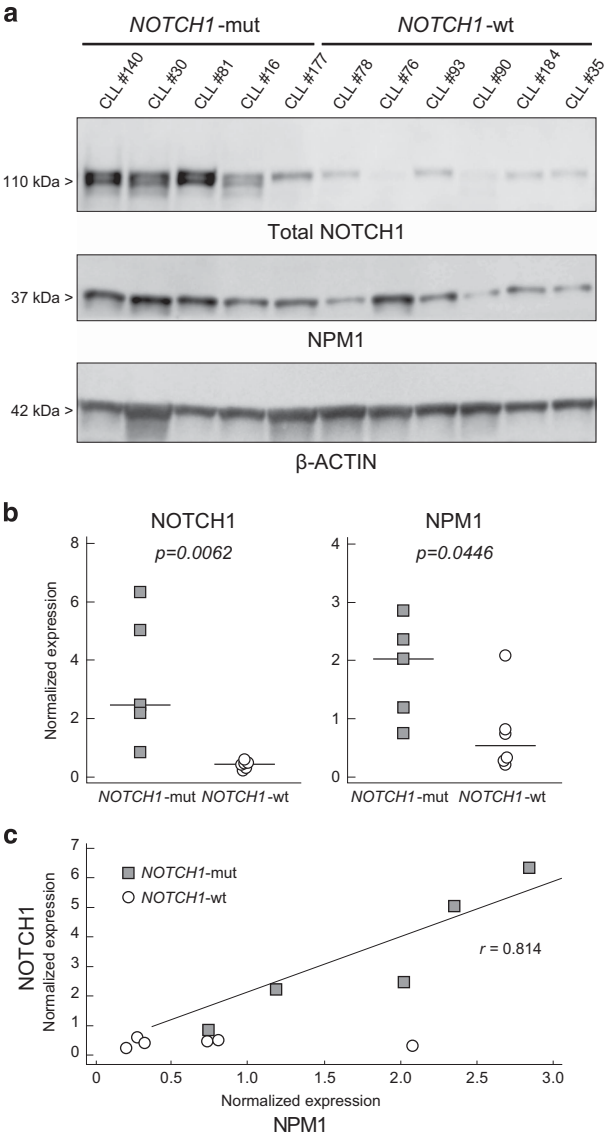


Figure 2. NPM1 protein expression in *NOTCH1*-mut and *NOTCH1*-wt CLL cases. (**a**) NPM1 and NOTCH1 protein expression in five *NOTCH1*-mut (carrying the c.7541-7542delCT) and six *NOTCH1*-wt CLL cases, as evaluated by WB. β-Actin was used as a loading control. (**b**) Dot plots representing NOTCH1 (left panel) and NPM1 (right panel) protein expression levels by densitometric analysis of the WB, in *NOTCH1*-mut and *NOTCH1*-wt cases. The corresponding *P*-value (Mann–Whitney *U*-test) is reported. (**c**) Correlation between NPM1 and NOTCH1 protein expression, as evaluated by WB, in *NOTCH1*-mut and *NOTCH1*-wt cases; *r* = Pearson's correlation coefficient.

upregulated in the *NOTCH1*-mut category ($P = 0.0001$, $P < 0.0001$, $P = 0.0259$, respectively; Figure 1b).

In CLL, NPM1 expression was previously found higher in *IGHV* UM cases compared with cases with mutated *IGHV* gene status (*IGHV* M cases), although information regarding *NOTCH1* mutations were not available back then.[48] In our series, no significant difference in *NPM1* transcript expression was found by comparing *IGHV* UM and *IGHV* M cases (Supplementary Figure S3a). Moreover, when *IGHV* UM cases and *IGHV* M cases were considered separately, *NPM1* transcript expression was confirmed significantly higher in *NOTCH1*-mut compared with that in *NOTCH1*-wt cases in the *IGHV* UM subgroup ($P = 0.0113$; Supplementary Figure S3b) and showed the same trend also in the *IGHV* M subgroup, although without reaching the statistical significance allegedly due to the low number ($n = 16$) of *NOTCH1*-mut cases in the context of *IGHV* M CLL (Supplementary Figure S3b).

We then assessed the protein expression of NPM1 by western blot (WB). NOTCH1 protein expression was evaluated by WB in five *NOTCH1*-mut cases and, for comparison, in six *NOTCH1*-wt cases. In keeping with the presence of mutations that impair NICD degradation,[10] *NOTCH1*-mut cases showed significantly higher NOTCH1 protein levels than *NOTCH1*-wt cases by using antibodies against total NOTCH1 ($P = 0.0062$; Figures 2a and b) and the NICD fragment (Supplementary Figure S4). In agreement with GEP and QRT-PCR data, NPM1 protein expression was significantly higher in *NOTCH1*-mut compared with that in *NOTCH1*-wt cases ($P = 0.0446$; Figures 2a and b), with a direct correlation between NPM1 and NOTCH1 expression ($r = 0.814$; Figure 2c).

To corroborate the association between NPM1 expression and *NOTCH1* mutations, we performed cell sorting experiments to isolate the extreme $NPM1^{high}$ and $NPM1^{low}$ sub-populations in six *NOTCH1*-mut cases with different *NOTCH1* mutational load. When stained for flow cytometry, NPM1 yielded the expected dot-like

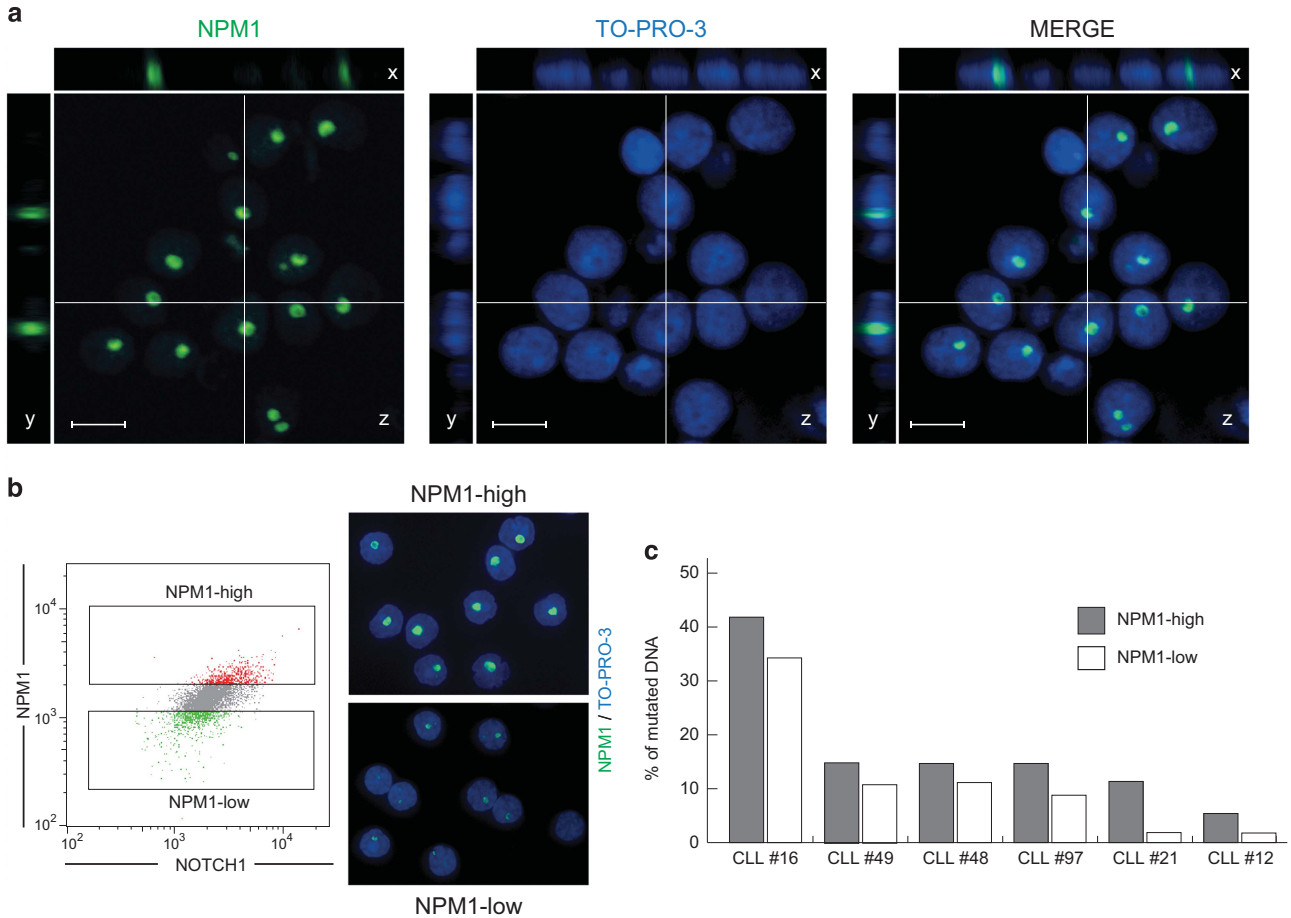


Figure 3. NPM1 staining and cell sorting of *NOTCH1*-mut CLL cases. (**a**) Confocal microscopy imaging of NPM1 staining for flow cytometry analysis of a *NOTCH1*-mut case. The expected pattern of nuclear NPM1 is recognizable. Green pseudocolor, NPM1; blue pseudocolor, TO-PRO-3 nuclear staining. x, y and z letters indicate longitudinal, transversal and horizontal sections, respectively. Perpendicular lines indicate the section planes. Scale bar: 10 μm. (**b**) Left panel: Gating strategy of cell sorting experiments according to NPM1 expression in six *NOTCH1*-mut CLL cases. The NPM1^{high} or NPM1^{low} CLL cell fractions were selected above the 75th percentile or below the 25th percentile of NPM1 expression, respectively. Right panel: Fluorescence microscopy of sorted CLL cell populations. Green pseudocolor, NPM1; blue pseudocolor, TO-PRO-3 nuclear staining. (**c**) Bar graphs showing *NOTCH1* mutational load in the NPM1^{high} or NPM1^{low} CLL cell fractions, as determined by NGS and expressed in the percentage of *NOTCH1*-mut DNA.

nuclear staining, as clearly showed by confocal microscopy preparations of the same samples analyzed for the flow cytometry (Figure 3a).[47] In the six *NOTCH1*-mut cases, the percentage of *NOTCH1*-mut DNA was 41% (CLL no. 16), 14% (CLL no. 49), 13% (CLL no. 48), 11% (CLL no. 97), 6% (CLL no. 21) and 3% (CLL no. 12) of total DNA. As shown by resequencing of the separated subpopulations, NPM1^{high}-sorted cells always had a relative enrichment in the *NOTCH1* mutational burden when compared with the NPM1^{low} cell counterpart, that is, 42% vs 34% (CLL no. 16), 15% vs 11% (CLL no. 49), 15% vs 11% (CLL no. 48), 15% vs 9% (CLL no. 97), 11% vs 2% (CLL no. 21), 5% vs 2% (CLL no. 12; Figures 3b and c).

NOTCH1 signaling and NPM1 expression in CLL

To evaluate if *NOTCH1* signaling could influence NPM1 expression in primary CLL cases, CLL cells from six *NOTCH1*-mut and six *NOTCH1*-wt cases were treated at different time points with EDTA to activate NOTCH1 signaling.[49] Upon EDTA treatment, NOTCH1 signaling resulted activated, as demonstrated by the increase of *HES1* and *DTX1* transcript levels,[16,50] after a 3 h exposure (Figure 4a). At 48 h, NPM1 protein and transcript levels were significantly augmented by EDTA treatment (NPM1 protein, *NOTCH1*-mut cases, median mean fluorescence intensity (MFI) untreated 2637 vs median MFI EDTA treated 3287; *NOTCH1*-wt cases, median MFI untreated 2773 vs median MFI EDTA treated 3096; $P = 0.0313$ for all the paired comparisons; Figures 4b and c). Moreover, transcript levels of *RPL7A*, *RPL18* and *RPS6* were also significantly increased by 48 h EDTA treatment ($P = 0.0313$ for all the paired comparisons; Figure 4c). The influence of NOTCH1 signaling on NPM1 expression was further confirmed by performing coculture of CLL cells from six *NOTCH1*-mut and six *NOTCH1*-wt cases with M2-10B4 stromal cells, expressing JAGGED1,[39,40] as evaluated by WB and confocal microscopy (Supplementary Figures S5a and b). In 48 h coculture with M2-10B4 stromal cells, NOTCH1 signaling was activated, as defined by *HES1* and *DTX1* transcript increases (Supplementary Figure S5c). Moreover, both NPM1 transcript and protein level were significantly augmented (NPM1 protein, *NOTCH1*-mut cases, median MFI untreated 8139 vs median MFI cocultured 10 857; *NOTCH1*-wt cases, median MFI untreated 6385 vs median MFI cocultured 7282; $P = 0.0313$ for all the paired comparisons; Supplementary Figure S5d). Of note, *NPM1* transcript levels were consistently decreased when CLL cell samples were pre-treated with GSI,[22,28] and, after 3 h, coculture with M2-10B4 stromal cells was performed (three *NOTCH1*-mut and three *NOTCH1*-wt cases, $P = 0.0313$; Supplementary Figure S5e).

To further evaluate the association between NOTCH1 signaling and NPM1 expression, CLL cells from six *NOTCH1*-mut and six

NOTCH1-wt cases were transiently transfected with siRNA for *NOTCH1*. *NOTCH1* silencing effectively reduced *NOTCH1* expression at both transcript and protein levels. Consistently, *NPM1* transcript expression was significantly reduced at 24 h ($P = 0.0313$ for all the paired comparisons; Supplementary Figure S6a). Moreover, upon treatment with GSI, NPM1 expression was significantly reduced at 48 h at both transcript and protein level (*NOTCH1*-mut, median MFI untreated cases 2637 vs median MFI GSI treated cases 2340; *NOTCH1*-wt, median MFI untreated cases 2746 vs median MFI GSI treated cases 2094, $P = 0.0313$ for all the paired comparisons; Supplementary Figure S6b).

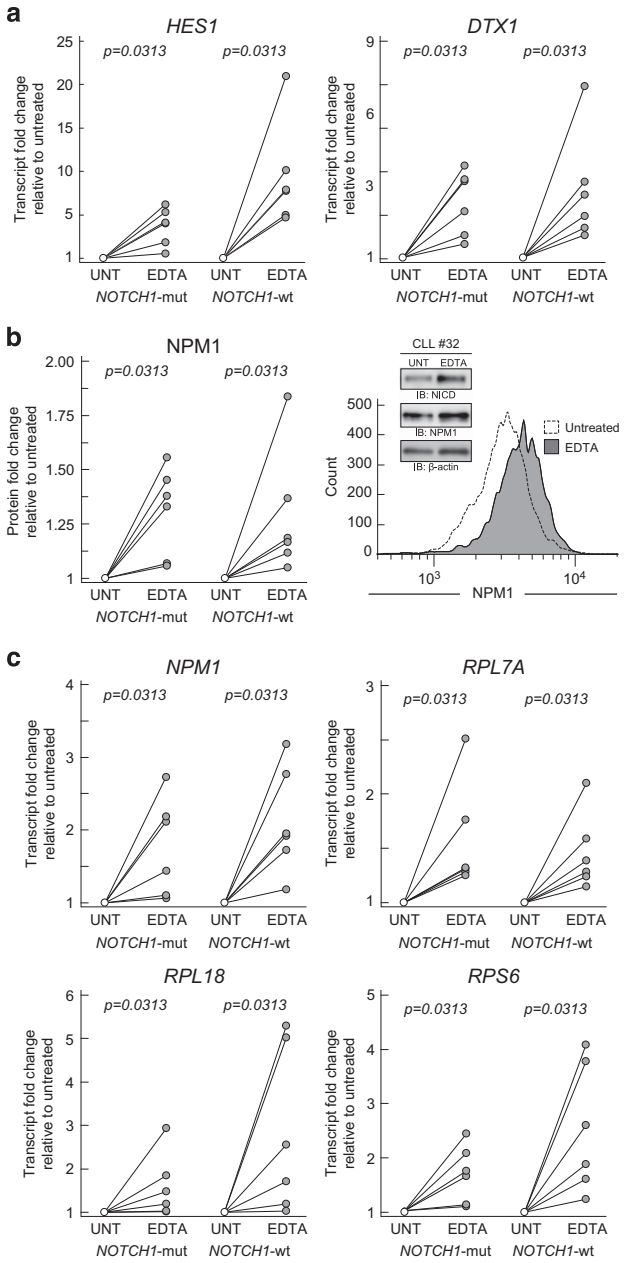


Figure 4. Induction of NPM1 and RNP expression by NOTCH1 signaling activation using EDTA treatment. (**a**) Dot-and-line plots showing *HES1* (left panel) and *DTX1* (right panel) transcript fold-change increases between untreated (UNT) CLL samples and CLL samples treated with EDTA (EDTA) for 3 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. (**b**) Left panel: Dot-and-line plot showing NPM1 protein fold-change increases between untreated (UNT) CLL samples and CLL samples treated with EDTA (EDTA) for 48 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by flow cytometry. Right panel: Representative overlay histogram of NPM1 expression by flow cytometry and representative WB (inset) of CLL samples left untreated or EDTA treated of a *NOTCH1*-mut case. (**c**) Dot-and-line plots showing fold-change increases of transcript expression levels of *NPM1*, *RPL7A*, *RPL18* and *RPS6*, between untreated (UNT) CLL samples and CLL samples treated with EDTA for 48 h of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. The *P*-value (Wilcoxon's signed-rank test) is reported above each comparison.

MYC as a potential mediator of NOTCH1-dependent NPM1 upregulation

Previous studies identified the proto-oncogene *MYC* as a direct transcriptional target of the NOTCH1 activation complex,[14,18–20,51] which, in turn, operates as transcriptional activator for both NPM1 and RNPs.[52]

To investigate whether NOTCH1 directly regulates the transcription of *MYC* in CLL, we took advantage of an *in vitro* model of NICD-transfected cells (NICD cells) of the CLL-like MEC-1 cell line.[28] NICD cells showed higher NOTCH1 protein levels than null cells as well as higher *HES1* transcript levels (Supplementary Figure S7a and b).[28] Moreover, NICD cells showed higher *NPM1*, *RPL7A*, *RPL18* and *RPS6* transcript levels than null cells (Supplementary Figure S7c).[28] ChIP assay for NOTCH1 performed on nuclear lysates from NICD transfectants showed higher levels of DNA corresponding to the *HES1* promoter in NICD cells compared with null cells (Figure 5a). The same ChIP assay demonstrated direct NOTCH1 binding to the *MYC* promoter. Of note, higher levels of DNA corresponding to the *MYC* promoter were found in NOTCH1 chromatin immunoprecipitates from NICD cells compared with null cells (Figure 5a). Consistently, NICD cells showed higher *MYC* transcript and protein levels than null cells (Figures 5b and c).

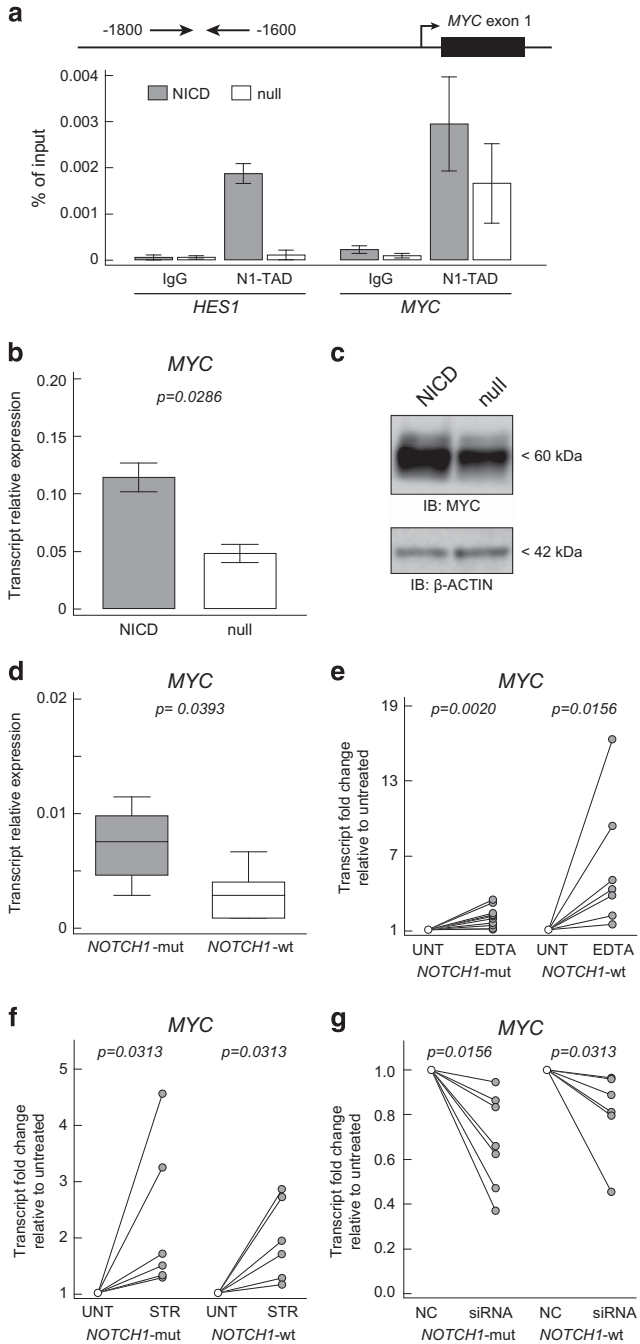
When cultured in complete medium for 48 h, CLL cells from both *NOTCH1*-mut and *NOTCH1*-wt cases expressed NOTCH1 and JAGGED1 protein (Supplementary Figure S8a). Moreover, both *NOTCH1*-mut and *NOTCH1*-wt cases expressed *HES1* and *DTX1* transcripts, with higher levels in *NOTCH1*-mut cases (Supplementary Figure S8b). Consistently, at 48 h, *NOTCH1*-mut cases showed NICD accumulation by WB (Supplementary Figure S8c).[11,53] In this condition, *NOTCH1*-mut cases showed higher *MYC* transcript levels than *NOTCH1*-wt cases (10 *NOTCH1*-mut cases, 7 *NOTCH1*-wt cases; $P = 0.0393$; Figure 5d). Moreover, activation of NOTCH1 signaling by EDTA or coculture with M2-10B4 stromal cells was able to significantly increase the transcription levels of *MYC* in *NOTCH1*-mut and *NOTCH1*-wt cases (EDTA at 48 h, 10 *NOTCH1*-mut cases, $P = 0.0020$, 7 *NOTCH1*-wt cases, $P = 0.0156$; coculture with M2-10B4 cells at 48 h, 6 *NOTCH1*-mut cases, $P = 0.0313$, 6 *NOTCH1*-wt cases, $P = 0.0313$; Figures 5e and f). Finally, NOTCH1 signaling inhibition by transfection with siRNA for *NOTCH1* decreased the transcription levels of *MYC* in *NOTCH1*-mut and *NOTCH1*-wt cases (at 24 h, 7 *NOTCH1*-mut cases, $P = 0.0156$, 6 *NOTCH1*-wt cases, $P = 0.0313$; Figure 5g).[14,18–20]

In keeping with the previously published gene expression signature of *NOTCH1*-mut CLL,[4] there was no significant difference in *MYC* transcript levels between PB CLL cell samples of *NOTCH1*-mut and *NOTCH1*-wt categories (Supplementary Figure S1, Supplementary Figure S9a and Supplementary Table S3), consistently with the well-known rapid mRNA and protein turnover of MYC.[54–56] On the other hand, gene set enrichment analysis of the present GEP data identified a gene set related to upregulated MYC targets as significantly enriched in upregulated genes in the *NOTCH1*-mut cases ($P = 0$, false discovery rate q-value $= 0.0201$; Supplementary Figure S9b), in accordance with a model in which MYC exerts its role of transcription factor downstream of the NOTCH1 pathway.

To confirm the association between MYC and NPM1 expression, NICD transfectants were transiently transfected with siRNA for *MYC*. At 48 h, *MYC* silencing effectively reduced *MYC* transcript and

protein expression (Figure 6a). Consistently, at 48 h, *NPM1* expression was significantly reduced at both transcript (*NICD* cells, $P = 0.0348$; null cells, $P = 0.0084$; Figure 6b) and protein (*NICD* cells, $P = 0.0005$; null cells, $P = 0.0078$; Figure 6b) levels.

The role of MYC in NPM1 regulation was further investigated by treating CLL cell samples with CpG-ODN/IL-2 to trigger Toll-like receptor 9. As expected,[34–37] CpG-ODN/IL-2-treated samples showed increased MYC transcript and protein expression levels at 48 h (Supplementary Figures S10a and b). Consistently, at the same time-point, CpG-ODN/IL-2 treatment also increased NPM1 expression at both the transcript and protein level (*NOTCH1*-mut cases, median MFI untreated 7560 vs median MFI CpG-ODN/IL-2 treated 9382; *NOTCH1*-wt cases, median MFI untreated 6011 vs median MFI CpG-ODN/IL-2 treated 7766, $P = 0.0313$ for all the paired comparisons; Figure 6c).



NOTCH1 signaling-dependent proliferation and cell growth

To investigate the contribution of NPM1 expression on proliferation in NICD transfectants, we perfomed a CellTrace assay with the simultaneous NPM1 silencing by transfection of siRNA for *NPM1*. NPM1 expression was effectively reduced by *NPM1*-specific siRNA transfection, and proliferation rates were consistently reduced in both NICD cells (at day 1, $P < 0.05$) and null cells (at day 1, $P < 0.001$; Supplementary Figures S11a and b). Moreover, in keeping with a more general role of MYC in cell proliferation,[52] transfection with siRNA for *MYC* was associated with a stronger reduction of proliferation rates in NICD cells and null cells (Supplementary Figures S11a and b and Figure 6a).

To further test the hypothesis that NOTCH1 directly controls genes regulating cell growth/protein biosynthesis,[18] forward scatter values were evaluated to investigate difference in cell size upon activation of NOTCH1 signaling by EDTA treatment and by coculture with JAGGED1-expressing M2-10B4 stromal cells, or inhibition of NOTCH1 signaling by GSI treatment. Activation of NOTCH1 signaling induced a significant increase of forward scatter values (EDTA treatment, 6 *NOTCH1*-mut cases, median MFI untreated 61 599 vs median MFI EDTA treated 72 420, 6 *NOTCH1*-wt cases, median MFI untreated 63 525 vs median MFI EDTA treated 70 475; coculture with M2-10B4 stromal cells, 6 *NOTCH1*-mut cases, median MFI untreated 93 959 vs median MFI cocultured 106 582, 6 *NOTCH1*-wt cases, median MFI untreated 91 437 vs median MFI cocultured 98 631, $P = 0.0313$ for all the paired comparisons; Supplementary Figures S11c and d).[18] On the other hand, inhibition of NOTCH1 signaling by GSI treatment induced a significant reduction of forward scatter values (6 *NOTCH1*-mut cases, median MFI untreated cases 124 612 vs median MFI GSI treated cases 120 092; 6 *NOTCH1*-wt cases, median MFI untreated cases 110 801 vs median MFI GSI-treated cases 107 750, $P = 0.0313$ for both the paired comparisons; Supplementary Figure S11e).[18]

DISCUSSION

By performing a gene expression profile of *NOTCH1*-mut versus *NOTCH1*-wt CLL cases, we showed that *NOTCH1*-mut CLL have a

Figure 5. *NOTCH1*-dependent *MYC* transcription. (**a**) ChIP assays of NOTCH1 binding to *MYC* promoter sequences. Bar graphs showing QRT-PCR analysis of *HES1* (left panel) and *MYC* (right panel) promoter sequences. Values were normalized to levels of an unrelated intragenic fragment, as reported in the material and methods section. Bar graphs represent mean values, and error bars represent s.e.m. Chromatin immunoprecipitates were performed with NOTCH1 antibody (N1-TAD) and rabbit immunoglobulin G (IgG) as isotype control. (**b**) Bar graphs showing *MYC* transcript expression levels in NICD cells and null cells, as evaluated by QRT-PCR. Bar graphs represent mean values, error bars represent standard error of the mean (s.e.m.). The *P*-value (Mann–Whitney *U*-test) is reported. (**c**) WB showing MYC protein expression in NICD cells and null cells. β-Actin was used as a loading control. (**d**) Box-and-whiskers plots showing *MYC* transcript expression in *NOTCH1*-mut and *NOTCH1*-wt CLL cases after 48 h culture in complete medium, as evaluated by QRT-PCR. *P*-value (Mann–Whitney *U*-test) is reported. (**e**) Dot-and-line plots showing *MYC* transcript fold-change increases between untreated (UNT) CLL samples and CLL samples treated with EDTA for 48 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. The *P*-value (Wilcoxon's signed-rank test) is reported. (**f**) Dot-and-line plots showing *MYC* transcript fold-change increases between untreated (UNT) CLL cell samples and CLL cell samples cocultured with M2-10B4 stromal cells (STR) for 48 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. The *P*-value (Wilcoxon's signed-rank test) is reported. (**g**) Dot-and-line plots showing *MYC* transcript fold-change increases between CLL samples upon transfection with negative control (NC) and with siRNA for *NOTCH1* (siRNA) for 24 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. The *P*-value (Wilcoxon's signed-rank test) is reported.

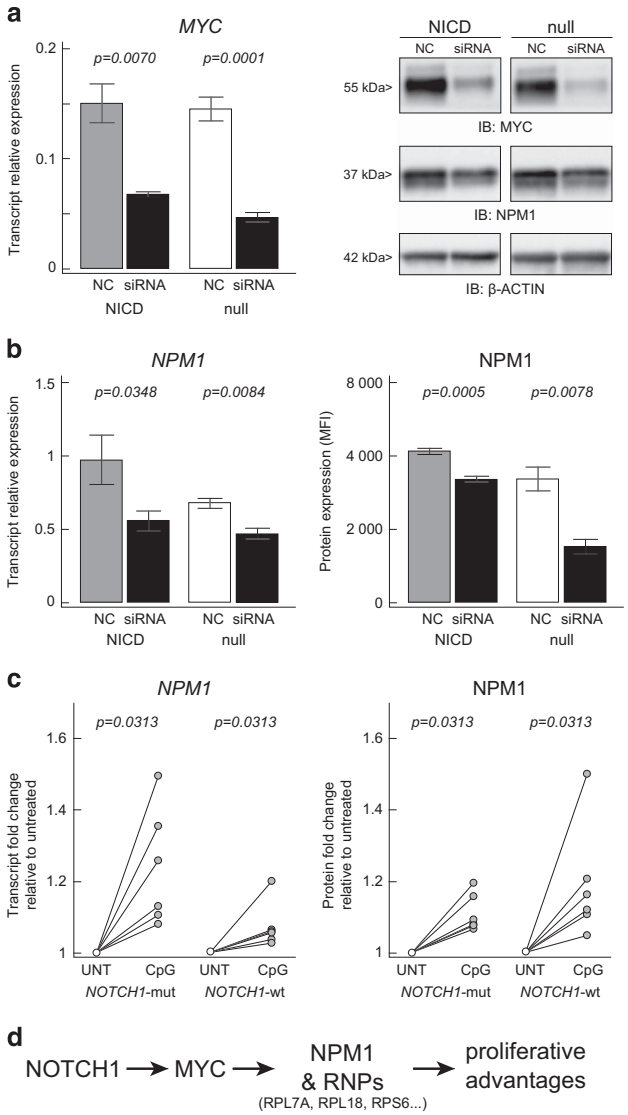


Figure 6. Induction of *NPM1* expression by modulation of *MYC* expression levels. (**a**) Left panel: Bar graphs showing *MYC* transcript expression levels of NICD and null transfectants upon transfection with siRNA for *MYC* for 48 h, as evaluated by QRT-PCR. Bar graphs represent mean values, and error bars represent s.e.m. The *P*-value (*t*-test) is reported for each comparison. Right panel: WB showing MYC and NPM1 protein expression in representative cell sample replicates of NICD and null transfectants. (**b**) Bar graphs showing *NPM1* transcript (left panel) and protein expression (right panel) of NICD and null transfectants upon transfection with siRNA for *MYC* for 48 h, as evaluated by QRT-PCR or flow cytometry, respectively. NC, negative control; siRNA, siRNA for *MYC*. Bar graphs represent mean values, and error bars represent s.e.m. The *P*-value (*t*-test) is reported for each comparison. (**c**) Dot-and-line plots showing *NPM1* transcript (left panel) and protein (right panel) fold-change increases between untreated (UNT) CLL cell samples and CLL cell samples treated with CpG-ODN/IL-2 (CpG) for 48 h, of *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by QRT-PCR. The *P*-value (Wilcoxon's signed-rank test) is reported. (**d**) Schematic drawn of *NOTCH1*, *MYC* and *NPM1/RNP* relation.

gene signature heavily characterized by the overexpression of the *NPM1* gene and of genes encoding for several RNPs. Consistently, gene set enrichment analysis identified gene sets related to the ribosomal machinery for having a significant enrichment of upregulated genes in the *NOTCH1*-mut category. Moreover, a gene-ontology tree machine analysis identified gene-ontology categories related to the RNA processing as significantly represented in the gene expression signature of the *NOTCH1*-mut CLL.[44]

Although GEP was performed in a quite limited case cohort, the validation by QRT-PCR in a wider CLL cohort confirmed the initial results. The association between NPM1 overexpression and the presence of *NOTCH1* mutations in CLL was further confirmed by cell sorting experiments of CLL samples with different burden of *NOTCH1* mutation, in which higher percentages of *NOTCH1*-mut DNA were found in the sorted NPM1^{high} component compared with the NPM1^{low} counterpart. A higher NPM1 expression was previously found in *IGHV* UM cases compared with that in *IGHV* M cases.[48] Here, we were not able to find the difference in *NPM1* transcript levels between *IGHV* UM and *IGHV* M cases.[48] On the other hand, we found higher *NPM1* expression levels in *NOTCH1*-mut compared with that in *NOTCH1*-wt cases also by considering the *IGHV* UM CLL subset only. These findings suggest that the higher *NPM1* expression in *NOTCH1*-mut cases is independent of *IGHV* mutational status. The higher NPM1 protein expression previously observed in *IGHV* UM cases[48] could be, at least in part, explained by an enrichment of *NOTCH1*-mut cases in the context of *IGHV* UM CLL.[3–7,9]

In the present study, we also demonstrated that activation of NOTCH1 signaling, by *in vitro* treatment with EDTA or coculture with JAGGED1-expressing M2-10B4 stromal cells,[21,25,40,49] is able to significantly increase NPM1 expression at transcript and protein level in both *NOTCH1*-wt and *NOTCH1*-mut CLL cells. The effectiveness of transfection with siRNA for NOTCH1 or of GSI treatment in decreasing NPM1 expression clearly confirmed this association.

These results can be considered in keeping with what was previously reported for T-cell acute lymphoblastic leukemia cells, in which the inhibition of NOTCH1 signaling defined a gene expression signature dominated by downregulated biosynthetic pathway genes.[18] In particular, the upregulation of genes of the biosynthetic pathway in *NOTCH1*-mut CLL, in the presence of a constitutive NOTCH1 protein accumulation, could be considered as the opposite of what happens when NOTCH1 signaling is inhibited by GSI exposure, as in Palomero *et al.*[18]

The previously published gene expression signature of *NOTCH1*-mut CLL reported in Puente *et al.*[4] did not identify NPM1 as overexpressed, although genes belonging to metabolic pathways were found differentially expressed also in that context.[4] Several discrepancies between this study[4] and the present study could be ascribed to the frequent subclonal nature of *NOTCH1* mutations in CLL.[3–5,7,28] In particular, the different *NOTCH1* mutational load of *NOTCH1*-mut cases used for GEP experiments could, in principle, have at least, in part, influenced the final results. In an attempt to reduce this effect, we selected *NOTCH1*-mut cases with the highest mutational load (i.e. >20% of *NOTCH1*-mut DNA) and performed the GEP in the context of the homogeneous subgroup of cases with an *IGHV* UM status.

Results of ChIP experiments shown in the present study by taking advantage of stably transfected CLL-like NICD cells strongly suggest that *MYC* is a transcriptional target of the NOTCH1 activation complex in CLL.[14,18–21,51] We also showed that modulation of NOTCH1 signaling directly influences *MYC* transcript levels, in keeping with the hypothesis that a *NOTCH1* mutation-dependent perduration of NOTCH1 signaling activation, as in *NOTCH1*-mut CLL, can be, in turn, responsible for a higher MYC-dependent transcription of *NPM1* and *RNP*s (Figure 6d).[18,21] On the other hand, the fact that we were not able to observe a differential expression of *MYC* between PB samples of *NOTCH1*-mut and *NOTCH1*-wt CLL, in accordance with what was reported previously,[4] could be ascribed to the rapid mRNA and protein turnover of MYC,[54–56] as well as to the absence of a sustained stimulation of NOTCH1 pathway in circulating cells.[25]

MYC can induce global protein synthesis by stimulating ribosome biogenesis through the upregulation of multiple components including NPM1 and RNPs.[52,57,58] Here, we provided evidence that *MYC* inhibition by siRNA transfection decreases *NPM1* expression in CLL-like NICD cells. Moreover, Toll-like receptor 9 triggering by CpG-ODN treatment, to increase *MYC* expression by a proliferative stimulus other than the NOTCH1 signaling activation,[34–37] was also able to increase NPM1 expression in primary CLL cells. These results suggest a direct role of MYC in the regulation of NPM1 in CLL cells.

NPM1 is a major nucleolar protein that modulates multiple steps of ribosome biogenesis.[47,59] Here, we showed a reduced proliferation by *NPM1* silencing that suggests a role for NPM1 in proliferation of CLL cells. Thus, *NPM1* overexpression and overexpression of *RNPs* might reflect an enhanced biosyntetic pathway, contributing to cell proliferation and growth in *NOTCH1*-mut CLL. Moreover, the well-known constitutive activation of NOTCH1 signaling also outside the *NOTCH1*-mut CLL subset may represent a broader mechanism regulating CLL cell proliferation and growth.[22,23]

In conclusion, *NOTCH1* mutations in CLL are associated with a MYC-related overexpression of *NPM1* and genes codifying for RNPs, which might reflect proliferative advantages concurring to explain the worse clinical behavior of *NOTCH1*-mut CLL. Clinically, this observation might suggest the possibility to investigate the role of NPM1 inhibitors in the therapy of *NOTCH1*-mut CLL.[60]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

FP contributed to writing the manuscript, analyzed the data and performed the research, TB performed the research, EV, RB, PB, FMR, AZ, FS, DB, ET and MD contributed to perform the research, GDA, FDR, FZ, GP, GDP, DR, GG provided well-characterized biological samples and contributed to writing the manuscript, VG and MDB designed the study, interpreted data and wrote the manuscript.

REFERENCES

1 Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H *et al.* Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute—Working Group 1996 guidelines. *Blood* 2008; **111**: 5446–5456.
2 Hallek M. Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. *Am J Hematol* 2013; **88**: 803–816.
3 Fabbri G, Rasi S, Rossi D, Trifonov V, Khiabanian H, Ma J *et al.* Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *J Exp Med* 2011; **208**: 1389–1401.
4 Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011; **475**: 101–105.
5 Puente XS, Bea S, Valdes-Mas R, Villamor N, Gutierrez-Abril J, Martin-Subero JI *et al.* Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2015; **526**: 519–524.
6 Rossi D, Rasi S, Fabbri G, Spina V, Fangazio M, Forconi F *et al.* Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. *Blood* 2012; **119**: 521–529.
7 Rossi D, Rasi S, Spina V, Bruscaggin A, Monti S, Ciardullo C *et al.* Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood* 2013; **121**: 1403–1412.
8 Wang L, Lawrence MS, Wan Y, Stojanov P, Sougnez C, Stevenson K *et al.* SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med* 2011; **365**: 2497–2506.
9 Del Giudice I, Rossi D, Chiaretti S, Marinelli M, Tavolaro S, Gabrielli S *et al.* NOTCH1 mutations in +12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of +12 CLL. *Haematologica* 2012; **97**: 437–441.
10 Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006; **7**: 678–689.
11 Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol* 2016; **17**: 722–735.
12 Yuan JS, Kousis PC, Suliman S, Visan I, Guidos CJ. Functions of notch signaling in the immune system: consensus and controversies. *Annu Rev Immunol* 2010; **28**: 343–365.
13 Castel D, Mourikis P, Bartels SJ, Brinkman AB, Tajbakhsh S, Stunnenberg HG. Dynamic binding of RBPJ is determined by Notch signaling status. *Genes Dev* 2013; **27**: 1059–1071.
14 Herranz D, Ambesi-Impiombato A, Palomero T, Schnell SA, Belver L, Wendorff AA *et al.* A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. *Nat Med* 2014; **20**: 1130–1137.
15 Hsieh JJ, Hayward SD. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* 1995; **268**: 560–563.
16 Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* 2003; **194**: 237–255.
17 Klinakis A, Szabolcs M, Politi K, Kiaris H, rtavanis-Tsakonas S, Efstratiadis A. Myc is a Notch1 transcriptional target and a requisite for Notch1-induced mammary tumorigenesis in mice. *Proc Natl Acad Sci USA* 2006; **103**: 9262–9267.
18 Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A *et al.* NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci USA* 2006; **103**: 18261–18266.
19 Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C *et al.* c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* 2006; **20**: 2096–2109.
20 Yashiro-Ohtani Y, Wang H, Zang C, Arnett KL, Bailis W, Ho Y *et al.* Long-range enhancer activity determines Myc sensitivity to Notch inhibitors in T cell leukemia. *Proc Natl Acad Sci USA* 2014; **111**: E4946–E4953.
21 Jitschin R, Braun M, Qorraj M, Saul D, Le BK, Zenz T *et al.* Stromal cell-mediated glycolytic switch in CLL cells involves Notch-c-Myc signaling. *Blood* 2015; **125**: 3432–3436.
22 Rosati E, Sabatini R, Rampino G, Tabilio A, Di IM, Fettucciari K *et al.* Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood* 2009; **113**: 856–865.
23 Shukla V, Shukla A, Joshi SS, Lu R. Interferon regulatory factor 4 attenuates Notch signaling to suppress the development of chronic lymphocytic leukemia. *Oncotarget* 2016; **7**: 41081–41094.
24 Sportoletti P, Baldoni S, Cavalli L, Del PB, Bonifacio E, Ciurnelli R *et al.* NOTCH1 PEST domain mutation is an adverse prognostic factor in B-CLL. *Br J Haematol* 2010; **151**: 404–406.
25 Arruga F, Gizdic B, Serra S, Vaisitti T, Ciardullo C, Coscia M *et al.* Functional impact of NOTCH1 mutations in chronic lymphocytic leukemia. *Leukemia* 2014; **28**: 1060–1070.
26 Bo MD, Del Principe MI, Pozzo F, Ragusa D, Bulian P, Rossi D *et al.* NOTCH1 mutations identify a chronic lymphocytic leukemia patient subset with worse prognosis in the setting of a rituximab-based induction and consolidation treatment. *Ann Hematol* 2014; **93**: 1765–1774.
27 Stilgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Dohner K *et al.* Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood* 2014; **123**: 3247–3254.
28 Pozzo F, Bittolo T, Arruga F, Bulian P, Macor P, Tissino E *et al.* NOTCH1 mutations associate with low CD20 level in chronic lymphocytic leukemia: evidence for a NOTCH1 mutation-driven epigenetic dysregulation. *Leukemia* 2016; **30**: 182–189.
29 Matutes E, Owusu-Ankomah K, Morilla R, Garcia MJ, Houlihan A, Que TH *et al.* The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* 1994; **8**: 1640–1645.
30 Dal BoM, Bulian P, Bomben R, Zucchetto A, Rossi FM, Pozzo F *et al.* CD49d prevails over the novel recurrent mutations as independent prognosticator of overall survival in chronic lymphocytic leukemia. *Leukemia* 2016; **30**: 2011–2018.
31 Gattei V, Bulian P, Del Principe MI, Zucchetto A, Maurillo L, Buccisano F *et al.* Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. *Blood* 2008; **111**: 865–873.

32 Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000; **343**: 1910–1916.

33 Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 2013; **14**: 178–192.

34 Arunkumar N, Liu C, Hang H, Song W. Toll-like receptor agonists induce apoptosis in mouse B-cell lymphoma cells by altering NF-kappaB activation. *Cell Mol Immunol* 2013; **10**: 360–372.

35 Bomben R, Gobessi S, Dal BM, Volinia S, Marconi D, Tissino E *et al.* The miR-17 approximately 92 family regulates the response to Toll-like receptor 9 triggering of CLL cells with unmutated IGHV genes. *Leukemia* 2012; **26**: 1584–1593.

36 Decker T, Schneller F, Kronschnabl M, Dechow T, Lipford GB, Wagner H *et al.* Immunostimulatory CpG-oligonucleotides induce functional high affinity IL-2 receptors on B-CLL cells: costimulation with IL-2 results in a highly immunogenic phenotype. *Exp Hematol* 2000; **28**: 558–568.

37 Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 2000; **164**: 944–953.

38 Longo PG, Laurenti L, Gobessi S, Sica S, Leone G, Efremov DG. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. *Blood* 2008; **111**: 846–855.

39 Shimizu K, Chiba S, Kumano K, Hosoya N, Takahashi T, Kanda Y *et al.* Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. *J Biol Chem* 1999; **274**: 32961–32969.

40 Kim WK, Meliton V, Tetradis S, Weinmaster G, Hahn TJ, Carlson M *et al.* Osteogenic oxysterol, 20(*S*)-hydroxycholesterol, induces notch target gene expression in bone marrow stromal cells. *J Bone Miner Res* 2010; **25**: 782–795.

41 Hutterer E, Asslaber D, Caldana C, Krenn PW, Zucchetto A, Gattei V *et al.* CD18 (ITGB2) expression in chronic lymphocytic leukaemia is regulated by DNA methylation-dependent and -independent mechanisms. *Br J Haematol* 2015; **169**: 286–289.

42 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; **102**: 15545–15550.

43 Xu ZS, Zhang JS, Zhang JY, Wu SQ, Xiong DL, Chen HJ *et al.* Constitutive activation of NF-kappaB signaling by NOTCH1 mutations in chronic lymphocytic leukemia. *Oncol Rep* 2015; **33**: 1609–1614.

44 Zhang B, Schmoyer D, Kirov S, Snoddy J. GOTree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies. *BMC Bioinform* 2004; **5**: 16.

45 Cazzaniga G, Dell'Oro MG, Mecucci C, Giarin E, Masetti R, Rossi V *et al.* Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. *Blood* 2005; **106**: 1419–1422.

46 Falini B, Martelli MP, Bolli N, Sportoletti P, Liso A, Tiacci E *et al.* Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity? *Blood* 2011; **117**: 1109–1120.

47 Lindstrom MS. NPM1/B23: a multifunctional chaperone in ribosome biogenesis and chromatin remodeling. *Biochem Res Int* 2011; **2011**: 195209.

48 Rees-Unwin KS, Faragher R, Unwin RD, Adams J, Brown PJ, Buckle AM *et al.* Ribosome-associated nucleophosmin 1: increased expression and shuttling activity distinguishes prognostic subtypes in chronic lymphocytic leukaemia. *Br J Haematol* 2010; **148**: 534–543.

49 Rand MD, Grimm LM, rtavanis-Tsakonas S, Patriub V, Blacklow SC, Sklar J *et al.* Calcium depletion dissociates and activates heterodimeric notch receptors. *Mol Cell Biol* 2000; **20**: 1825–1835.

50 Matsuno K, Eastman D, Mitsiades T, Quinn AM, Carcanciu ML, Ordentlich P *et al.* Human deltex is a conserved regulator of Notch signalling. *Nat Genet* 1998; **19**: 74–78.

51 Wang H, Zou J, Zhao B, Johannsen E, Ashworth T, Wong H *et al.* Genome-wide analysis reveals conserved and divergent features of Notch1/RBPJ binding in human and murine T-lymphoblastic leukemia cells. *Proc Natl Acad Sci USA* 2011; **108**: 14908–14913.

52 van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer* 2010; **10**: 301–309.

53 Sprinzak D, Lakhanpal A, Lebon L, Santat LA, Fontes ME, Anderson GA *et al. Cis*-interactions between Notch and Delta generate mutually exclusive signalling states. *Nature* 2010; **465**: 86–90.

54 Blanchard JM, Piechaczyk M, Dani C, Chambard JC, Franchi A, Pouyssegur J *et al.* C-myc gene is transcribed at high rate in G0-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. *Nature* 1985; **317**: 443–445.

55 Jones TR, Cole MD. Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. *Mol Cell Biol* 1987; **7**: 4513–4521.

56 Meyer N, Penn LZ. Reflecting on 25 years with MYC. *Nat Rev Cancer* 2008; **8**: 976–990.

57 Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN *et al.* Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci USA* 2000; **97**: 3260–3265.

58 Thoma C, Fraterman S, Gentzel M, Wilm M, Hentze MW. Translation initiation by the c-myc mRNA internal ribosome entry sequence and the poly(A) tail. *RNA* 2008; **14**: 1579–1589.

59 Murano K, Okuwaki M, Hisaoka M, Nagata K. Transcription regulation of the rRNA gene by a multifunctional nucleolar protein, B23/nucleophosmin, through its histone chaperone activity. *Mol Cell Biol* 2008; **28**: 3114–3126.

60 Martelli MP, Gionfriddo I, Mezzasoma F, Milano F, Pierangeli S, Mulas F *et al.* Arsenic trioxide and all-trans retinoic acid target NPM1 mutant oncoprotein levels and induce apoptosis in NPM1-mutated AML cells. *Blood* 2015; **125**: 3455–3465.