

LINC00152 is a lncRNA modulated by microenvironmental stimuli that has pathogenetic and clinical relevance in CLL

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Abstract

Chronic lymphocytic leukemia (CLL) is a common lymphoid neoplasm with heterogeneous biological features and clinical behavior, influenced by acquired genetic alterations and microenvironment interactions. We explored non-coding RNAs expression to understand their role in CLL pathogenesis and identify new clinically relevant biological biomarkers that could improve CLL prognosis and management. We initially profiled coding and non-coding RNA families in different CLL cohorts (N = 21) to identify those modulated by a combination of agonist microenvironment stimuli. Then we focused on lncRNAs that were overexpressed in response to the agonist stimuli, even in the presence of Ibrutinib, and whose baseline expression was associated with a shorter time-to-treatment in an independent CLL series (N = 266). Three lncRNAs (*LINC00152*, *LINC00158* and *RP11-161H23.5*) were selected for functional validations in MEC-1 CLL cell line, where they demonstrated to regulate genes and pathways activated by microenvironmental stimuli, including induction of miR-155, a key oncomiR also enhanced by agonist treatment. *LINC00152* expression was associated with proxies of microenvironment stimulation *in vivo*, and its silencing *ex vivo* reduced cell growth under combined microenvironmental stimulation and Ibrutinib in a subset of Ibrutinib-relapsed primary CLL samples, underscoring its potential relevance for further studies to improve CLL therapy.

Introduction

Non-coding RNAs are regulators of protein coding gene expression that have been increasingly described as key players in normal and pathological conditions, most remarkably in cancer.^{1,2} In B-cell lymphoid neoplasms, we and others have previously shown the deregulation of several non-coding miRNAs and lncRNAs associated with the control of cell proliferation and other pathogenetic relevant cellular processes.^{3–6} Among these neoplasms, chronic lymphocytic leukemia (CLL) could benefit from further studies on such transcripts. CLL is the most prevalent type of leukemia in adults, but disease course and manifestation are heterogeneous, ranging from asymptomatic cases not requiring treatment, to aggressive forms developing resistance to therapy.⁷ There are several molecular mechanisms/pathways deregulated in CLL, including B cell receptor (BCR) signaling, mitogen-activated protein kinase (MAPK/ERK), phosphatidylinositol 3-kinase (PI3K/AKT), *TP53* tumor suppressor pathway, and nuclear factor kappa B (NF- κ B), that are also clinically and pathogenetically relevant,⁸ and some of them have been targeted by the most recently developed drugs.^{9,10} Nevertheless, the appearance of treatment resistances and toxicities are hindering their potential clinical benefits.¹¹ These relevant pathways in CLL are activated by driver mutations and also stimulated by microenvironment factors produced by different reactive cells, mainly in the proliferation centers of the lymph nodes.^{12–14} Indeed, CLL cells actively recirculate between peripheral blood (PB) and lymph nodes (LN). In PB, the reciprocal expression of CD5 and CXCR4 define distinct CLL subpopulations involved in this dynamic: a proliferative fraction (CXCR4dim/CD5bright, PF), consisting of recently divided cells that have egressed from the LN, and a resting fraction (CXCR4bright/CD5dim, RF) composed of older, quiescent cells.¹⁵ The current model supports a linear transition from the PF to the RF, which subsequently re-enters the lymph node niche upon CXCL12/SDF-1 gradient to undergo further cycles of proliferation.¹⁶ In the context of Bruton's Tyrosine Kinase Inhibitors (BTKi) treatment, the PF is reconstituted upon clinical progression on ibrutinib treatment and enriched in *BTK/PLCG2* mutations, suggesting the presence of active clonal selection dynamics taking place within the lymph node microenvironment that enable tumor cells to overcome the therapeutic effects of the BTKi.

Non-coding RNAs are regulators of the expression of genes in CLL,¹⁷ some of which have been previously demonstrated to be relevant for CLL outcome as mediators of microenvironment effects on CLL cells.¹² Therefore, such RNAs may represent promising therapeutic to counteract the emergence of microenvironment-dependent BTKi resistance, particularly if they control genes upregulated in the PF. In this work, we performed a comprehensive screening of lncRNAs and miRNAs upregulated by microenvironment factors and identified candidates for *in vitro* testing of their regulatory

roles in pathogenetic pathways modulated by those factors. We also explored the association between microenvironment-modulated lncRNAs identified in ex vivo CLL cells and gene signatures and PB cell subpopulations linked to microenvironment stimulation. This approach allowed us to evaluate and further validate their potential as targets to indirectly overcome long-term BTKi resistance in CLL.

Results

CLL cohort stimulation

To find non-coding RNAs modulated ex vivo by microenvironment factors, different CLL cohorts with specific genetic aberrations previously related to Ibrutinib sensitivity¹⁸ were selected (Supplementary Table 1). We studied 6 cases with *TP53* disruptions (mutations and/or deletion) vs. 15 cases devoid of *TP53* disruptions, specifically 3 cases with Trisomy 12, and 12 cases wild type (WT) for both *TP53* and Trisomy 12 (Supplementary Table 1). Based on phosphoflow analysis all samples showed a responsive BCR signaling when stimulated with anti-IgM beads respect to stimulation with beads coated with NGS (Supplementary Fig. 1). For each case, agonist mix stimulations (using CpG-ODN2006, CD40L and IL10) were compared to the corresponding controls either in the presence or absence of Ibrutinib, as reported in the experimental design summary (see methods and Supplementary Fig. 2). To verify the correct stimulation process, we measured the protein expression levels of MCL1 and CD69 by flow cytometry as indicators of microenvironment-mediated B-cell activation.¹⁹ In WT and Trisomy 12 CLL, the agonist mix stimulation demonstrated the effective upregulation of both markers (condition A versus C), as previously described,¹⁹ even when Ibrutinib was added to the agonist mix (condition B versus D) (Fig. 1a/1b). Conversely, in *TP53* disrupted cases, MCL1 and CD69 expression levels did not change (Fig. 1c). Apoptosis measured by cleaved-PARP expression levels were only slightly reduced by the stimulation, as previously reported (Supplementary Fig. 3).¹⁹

Non-coding RNAs modulation by microenvironment factors ex vivo

Transcriptomic analyses of the different conditions were performed with dedicated microarray platforms to measure gene expression of coding and different non-coding gene families, including mature microRNAs. Specifically, our approach allowed us to identify for each CLL cohort a specific set of DE transcripts modulated by the agonist mix stimulation (Table 1). The number of significant DE non-coding genes found was higher than the coding ones for each cohort, as summarized in Table 1. Among the DE coding genes *EZH2* was included among the significantly upregulated genes (Supplementary Table 3, and Supplementary Fig. 4a), concordantly with its key role on microenvironment stimulation of CLL cells by factors included in our experimental system.²⁰ We also found a number of DE microRNAs that have been previously identified as targets of microenvironment factors as well as having pathogenetic and prognostic value in CLL (e.g. *miR-17* or *miR-155*) (Supplementary Table 4).^{21–23} The pathway enrichment analysis on coding genes modulated by the agonist mix also captured significant enrichments of known relevant pathogenetic pathways in CLL (Supplementary Table 5a-5e, and Supplementary Fig. 4b). Many of those pathways are related to microenvironment stimuli and have been concordantly found upregulated in LN versus PB CLL samples,²⁴ further supporting the validity of our experimental system and the potential functional involvement of the DE non-coding RNAs found on pathways stimulated in CLL cells by the microenvironment.

Table 1

Number of differentially expressed transcripts modulated by agonist mix treatment (with and without Ibrutinib)

Overexpressed	LncRNAs	Pseudogene	Small_RNA	miR precursors	Ribosomal	Other	Total non coding	Total coding
TP53altered CLL cohort	851	176	25	14	2	291	1359	237
Trisomy12 CLL cohort	1273	108	29	43	4	514	1971	671
WT CLL cohort (Barcelona)	1622	344	67	38	11	565	2647	1326
WT CLL cohort (Aviano)	1039	203	30	12	2	370	1656	697
Underexpressed	LncRNAs	Pseudogene	Small_RNA	miR precursors	Ribosomal	Other	Total non coding	Total coding
TP53altered CLL cohort	784	47	65	47	3	707	1653	540
Trisomy12 CLL cohort	3031	76	39	76	39	1189	4450	253
WT CLL cohort (Barcelona)	2350	66	13	66	13	1640	4148	599
WT CLL cohort (Aviano)	1551	50	10	50	10	1245	2916	853

Selection of clinically relevant ncRNA candidates

To select lncRNA candidates of clinical interest we took advantage of already available transcriptomic data in 266 CLL patients of the ICGC project.²⁵ In this cohort we found 1171 lncRNAs whose expression levels were significantly associated with shorter TTT (Supplementary Table 6). We found 163 lncRNA of this list that were also upregulated in our experiments with microenvironment stimuli in at least one of the comparisons between experimental conditions (Supplementary Table 7). As we aimed to further functional validations, we focused only on 83 lncRNA that were also expressed in the MEC-1 CLL cell line according to the RNAseq data obtained from ICGC (Fig. 2). Among these 83 lncRNAs, we further selected 8 of them due to their high expression levels in MEC-1 (top 10th percentile, range 150.9–3.75 RPKM) (Fig. 2, and Supplementary Table 8). Finally, we focused on the top three candidates according to the feasibility for later validations (qPCR amplicon design and amplification efficiency), as well as novelty and potential interest. Accordingly, we selected *RP11-161H23.5* (ENSG00000258232), an antisense RNA poorly known in relation to cancer (Fig. 2). *LINC00152* (ENSG00000222041), also known as *CYTOR*, is an intergenic lncRNA upregulated in several cancers,²⁶ as well as by microenvironment factors in CLL (Fig. 2).²⁷ Noticeably, we found the expression of this lncRNA significantly associated with a shorter TTT in the ICGC cohort that in a multivariate analysis was independent of the Binet stage, IGHV mutational analysis and epigenetic subtypes (Supplementary Figs. 5 and 6). Finally, *LINC00158* (ENSG00000185433) was selected among candidates shared by CLL cases with/without *TP53* alterations as overexpression of this intergenic lncRNA has been previously observed in transformed germinal center cells (BL-2 cell line) stimulated by CD40, IgM, LPS or IL21.²⁸

Functional impact of candidate lncRNAs in CLL

To investigate the functional impact of the three selected lncRNAs we analyzed the transcriptomic impact of their expression silencing with different Gapmers targeting relevant transcript variants in CLL patients. Particularly, for *LINC00152* we selected the transcript variant 207 as a target because it had a significant impact on TTT even though it was not the most highly expressed variant (Supplementary Figs. 7 and 8). For *LINC00158* we designed a Gapmer to target the variant 203, which also had significant clinical impact (Supplementary Figs. 7 and 8). Another Gapmer targeting a common exon between 203 and the most highly expressed transcript variant 201 was also included because of the sequence limitations for optimal design of an exclusive Gapmer for this last variant (Supplementary Fig. 3) (Table 2).

Effective silencing was obtained with all Gapmers achieving a diminished expression ranging from 46% to 90% (Supplementary Fig. 9a). PCA analysis of the transcriptomic data of these experiments in MEC-1 cell line showed a clear segregation of the silenced samples from the corresponding paired controls (Supplementary Fig. 9b). Noticeably, among the DE genes found upon lncRNA silencing we observed the downregulation of several relevant genes in CLL, as the antiapoptotic protein *MCL1* (for *LINC00158* and *RP11-161H23.5*) and the activation marker *CD69* (for *RP11-161H23.5*), already known to be upregulated by microenvironment factors (Fig. 3).¹⁹ Moreover, all three lncRNAs regulated the expression of *MIR155HG*, the precursor of *miR-155*, which expression is linked to poor prognosis and sensitivity to BCR pathway inhibition and reactivated in relapsing CLL cases.²³ These results are concordant with the fact that *miR-155* was always detected as significantly upregulated after stimulation with our agonist stimuli in both WT and *TP53* altered CLL cases (Supplementary Table 4), and also with the downregulation of *MIR155HG* (precursor of *miR-155*) observed in our silencing experiments (Fig. 3).

Pathway enrichment analyses on all significant DE genes in these experiments also showed that *RP11-161H23.5* silencing had an impact on some relevant pathways related to CLL microenvironment stimuli (Fig. 3 and Supplementary Table 9a). Finally, *LINC00158* silencing experiments showed its involvement in the regulation of many pathogenetic and microenvironment CLL related pathways (Fig. 3 and Supplementary Table 9b). Surprisingly, in the case of *LINC00152* we identified deregulated pathways previously related to Ibrutinib resistance (as fatty acid oxidation) and toxicity (cardiac fibrosis, integrins and extracellular matrix-collagen) (Fig. 3 and Supplementary Table 9c).^{29–31}

LINC00152 and LINC00158 expression is related with proxies of cell proliferation driven by the microenvironment in the lymph nodes even in CLL primary samples after ibrutinib treatment

CLL proliferative drive (CLL-PD) is a multiomic signature identified as a key determinant of disease outcome in CLL independently of other described molecular markers of patient prognosis.³² Furthermore, CLL-PD captures a complex biological axis that is positively related to cell proliferation, including a higher proliferative induction *in vitro* by CpG-ODN. Therefore, we assessed the possible correlation of our lncRNA candidates with a CLL-PD score calculated in a regularized linear model from 84 gene signatures in 49 cases belonging to the ICGC cohort analyzed and also included in the Multiomics study describing CLL-PD.³² In this way we found that only *LINC00152* and *LINC00158* were significantly positively correlated with the CLL-PD score (Fig. 4A).

To further investigate evidence of the relationship of both lncRNAs with *in vivo* stimulation from the microenvironment in CLL patients in the context of Ibrutinib treatment, we reanalyzed previously published RNAseq data of Pozzo *et al.*³³ to compare the expression of both lncRNAs between the proliferative fraction (CXCR4dim/CD5bright, PF), recently divided and egressed from the lymph node, and a resting fraction (CXCR4bright/CD5dim, RF) of older, quiescent cells. We could observe the significant increase in the expression of *LINC00152* and *LINC00158* in the PF vs RF, either previously ($P = 0.021$ and $P = 0.039$, respectively) as at relapse ($P = 0.00024$ and $P = 0.0005$, respectively) to Ibrutinib treatment in CLL patients (Fig. 4B). Because of this consistent increased expression associated with cell proliferation seen in samples

from patients either both prior to Ibrutinib treatment and at relapse, we considered both lncRNAs as interesting candidate for proof-of-concept studies aiming to assess the impact of their silencing in the context of Ibrutinib treatment

LINC00152 and LINC00158 expression impact in cell growth of ex vivo CLL primary samples

We evaluated the impact of the *LINC00152* or *LINC00158* silencing in primary purified CLL samples from three CLL patients that were refractory to Ibrutinib treatment and without *BTK* and *PLCG2* mutations. Samples were assessed ex vivo growing in a culture media supplemented with 1 μ M Ibrutinib together with agonist mix (as previously described). RT-qPCR demonstrated effective downregulation of both lncRNA in all samples (Supplementary Fig. 10).

We observed a significant decrease in cell growth upon *LINC00152* silencing in two of the three CLL samples (CLL#1 $P = 0.013$ and CLL#3 $P = 0.009$) (Fig. 5), but no significant changes were found related with *LINC00158* silencing (Supplementary Fig. 11).

Taken together, these findings support the role of *LINC00152* in the regulation of proliferation/viability processes induced by microenvironment stimuli even in some samples derived from patients with relapsed disease following BTKi treatment.

Discussion

The influence of the microenvironment stimulation on CLL pathogenesis and patient treatment response is well established, sparking significant interest in blocking this pro-tumoral influence to enhance existing therapies.^{34,35} In this context, ncRNAs, a large group of transcripts that regulate coding gene expression through different molecular mechanisms,³⁶ represent a promising field for discovering new key targets in molecular pathways control and new biomarkers to improve the clinical management of patients.² In CLL, we have previously identified several lncRNAs whose expression levels have prognostic value and are related to microenvironment factors.^{6,37} Building on this, we aimed to identify additional lncRNAs as potential biomarkers and explore their therapeutic relevance in comparison with established treatments such as Ibrutinib.

Our initial screening included CLL patients with distinct genetic profiles, focusing on *TP53* alterations and trisomy 12, both of which are known to modulate Ibrutinib sensitivity.¹⁸ Among the three validated lncRNAs regulating microenvironment-driven pathways, two were exclusively modulated in cases lacking these genetic alterations, while *LINC00158* was upregulated upon agonist stimulation in cases without *TP53* alterations. Notably, its heterogeneous modulation suggests variability in microenvironmental responsiveness, potentially influencing disease aggressiveness and treatment outcomes.

Functional validation confirmed that selected lncRNAs regulate genes involved in CLL proliferation and survival, including MCL1 (regulated by *LINC00158*, and *RP11-161H23.5*) and CD69 (regulated by *RP11-161H23.5*). *LINC00152* (*CYTOR*) was associated with pathways linked to Ibrutinib resistance, such as beta-oxidative lipid metabolism,²⁹ or secondary toxicity events,^{30,31} but not with microenvironment-related signaling in contrast to the other lncRNA candidates. Although our approach focused on transcriptomic effects, RNA-protein interactions remain a possible alternative mechanism by which *LINC00152* could influence microenvironment-related signaling pathways. Importantly, we found that *LINC00152* expression correlated with shorter time-to-treatment, in contrast to previous reports suggesting no clinical impact.²⁷ Our analysis of a large CLL cohort confirmed its poor prognostic value independent of Binet stage and other clinical or biological factors, underscoring its functional relevance.

We further demonstrated that *LINC00152* and *LINC00158* are upregulated in PF vs RF from peripheral blood, both before and after Ibrutinib relapse. These samples were previously analyzed by Pozzo *et al.* and correspond to CLLs lacking *BTK*

or *PLCG2* mutations that could otherwise explain their resistance to treatment. Such cases are typically characterized by an increased PF/RF ratio and, therefore, enhanced microenvironment stimulation. This *in-vivo* association of these lncRNAs with the cells recently egressed from the lymph nodes¹⁵ was also supported by the observed correlation between the expression of both lncRNAs and the PD-score, a marker of the degree of proliferation in the lymph nodes³². Further experiments exploring the impact of lncRNA silencing in *ex vivo* cultures of CLL samples after relapse to Ibrutinib, revealed a tendency for *LINC00152* downregulation to induce a reduction of cell growth even in the presence agonist mix mimicking the microenvironment stimulation in the lymph nodes, as well as Ibrutinib, to which these patients have developed resistance. Altogether, these findings highlight *LINC00152* as a paradigmatic example of a broader group of lncRNA still to be fully uncovered and characterized that could be involved in the regulation of microenvironment-driven processes influencing tumor behavior and response to therapy.

In a more general view on the role of lncRNAs in microenvironment-dependent stimulation of CLL cell growth, we also highlight our finding that *miR-155* was consistently upregulated *in vitro* upon agonist mix stimulation. This observation is in line with our identification of the *miR-155* precursor as a predicted target of all our selected lncRNA candidates, suggesting a potential direct interaction between lncRNA and microRNA regulation. This is noteworthy because increased expression of *miR-155* is essential for the optimal proliferation and survival of B-cells in the germinal centers of lymph nodes.³⁸ Moreover, *mir-155* overexpression appears to be pro-oncogenic, as demonstrated in a pre-B lymphoma model mouse model, where its downregulation led to a regression of the lymphadenopathy.³⁹ Notably, *miR-155* overexpression has also been associated to increased CLL aggressiveness and enhanced BCR responsiveness, with its expression induced by microenvironment crosstalk.⁴⁰ At the molecular level, *miR-155* has been proposed to participate in a positive feed-back loop with NFAT1,⁴¹ a key transcription factor associated with lymphocyte activation. *NFAT* expression is upregulated by microenvironmental stimuli and is elevated in cases with high CLL-PD score.³² Interestingly, higher levels of *miR-155* have also been associated with a shorter time to relapse during Ibrutinib treatment in CLL.²³ Altogether, these findings suggest that *miR-155* may be an interesting target in CLL therapeutics. In this regard, the *miR-155* inhibitor Cobomarsen (MRG-106), a LNA-modified oligonucleotide, has showed encouraging results in phase 1/2 clinical trials across several lymphoma types, although CLL was not included in these trials.^{42,43} Nevertheless, the evidence here reported suggests that this inhibitor could also be beneficial in CLL. However, the oncogenic mechanisms of the three lncRNAs identified in this study appeared to be more complex than *mir-155* regulation alone, according to the differences set of genes they regulate.

In conclusion, the evidences reported in this study supports the biological and clinical relevance of some ncRNAs in CLL, particularly *LINC00152*, highlighting the regulatory role that lncRNAs play in controlling both coding and non-coding targets within pathways essential for the proliferation and survival of CLL cells, especially under microenvironmental stimulation. Moreover, our results suggest that targeting specific non-coding RNAs could be a promising approach, warranting further research to enhance current treatment options for CLL.

Patients, material and methods

CLL Cohorts

Cryopreserved CLL samples were obtained from the Hematopathology Biobank of Hospital Clínic de Barcelona-IDIBAPS (Spain) and the clinical experimental-oncohematology unit of Aviano (Italy) (Supplementary Table 1). Cases were selected by genetic status: *TP53*-disrupted (n = 6, Aviano), trisomy 12 (n = 3, Barcelona), and wild-type for both (n = 12; 6 Barcelona, 6 Aviano), as these variants influence *ex vivo* Ibrutinib sensitivity.¹⁸ For lncRNA expression in proliferative/resting fractions, we reanalyzed samples from Pozzo *et al.*,³³ including 9 pre-Ibrutinib and 14 post-relapse cases without *BTK/PLCG2* mutations (8 of them paired). Three additional relapsed cases without *BTK/PLCG2* mutations were included

for functional validation. All samples complied with the Declaration of Helsinki and were approved by the Hospital Clínic IRB. Patients provided written informed consent.

Ex Vivo CLL Experimental System

Cryopreserved samples were thawed and assessed by flow cytometry for ≥ 85 –90% tumor purity. Samples with a lower percentage were enriched with EasySep™ Human B Cell Enrichment Kit II (StemCell Technologies). Protein G Magnetic SureBeads (Bio-Rad) were coated with anti-IgM (SouthernBiotech) or normal goat serum (NGS; Invitrogen). To confirm BCR responsiveness, cells were stimulated with NGS or anti-IgM beads, and phosphoflow cytometry verified p-BTK (Btk pY233/Itk pY180; BD Biosciences) and p-ERK (Erk1/2 pT202/pY204; BD Biosciences) induction.

Cell culture and agonist mix followed Jayappa *et al.* [19]: CpG-ODN20006 (InvivoGen), Mega-CD40L (Enzo Biosciences), and IL-10 (PeproTech) were used. Four conditions were tested at 24 h: (A) DMSO + NGS beads; (B) Ibrutinib 1 μ M + anti-IgM beads; (C) agonist mix + NGS beads; (D) agonist mix + Ibrutinib + anti-IgM beads (Supplementary Fig. 2). Analyses compared C vs A and B vs D.

Agonist effects were assessed by flow cytometry using CD19-BV510 and CD5-PE-Cy7 (BD Biosciences) for CLL B-cell gating, Fixable Violet Dead Cell Stain (ThermoFisher) for viability, CD69-Alexa Fluor 488 (BioLegend), MCL1-PE (Cell Signaling) for stimulation, and Cleaved PARP-Alexa Fluor 647 (Cell Signaling) for apoptosis. Labeling followed Jayappa *et al.*,¹⁹ Median Fluorescence Intensity (MFI) was used for comparisons.

RNA Extraction and Transcriptomic Profiling

Total RNA was extracted using miRNeasy kit (Qiagen) with DNase treatment. Quantity and quality were assessed by Nanodrop 1000 (ThermoScientific) and TapeStation 2400 (Agilent); all samples had RIN > 6. Microarray platforms included Clariom D WT pico (10 ng RNA input; ThermoFisher) and Agilent microRNA expression kit (100 ng RNA input; Agilent).

Bioinformatic and Statistical Analyses

Principal Component Analysis (PCA) was used to identify outliers according to expression profiles obtained with Clariom D arrays (Supplementary Fig. 12). PCA of basal expression revealed differences between WT series from both institutions, likely due to Barcelona's purification step and, therefore analyses to find differentially expressed (DE) genes were stratified by origin. DE analysis was performed using Transcriptome Analysis Console 4.0.2 (Applied Biosystems) for Clariom D arrays and GeneSpring v14.9.1 for miRNA arrays. Clariom D data were normalized with Signal Space Transformation–Robust RMA. DE genes/miRNAs were defined as $|\log_2FC| \geq 1.5$ and $p < 0.05$. Pathway enrichment was assessed using Metascape (www.metascape.org),⁶

Associations between lncRNA expression and time-to-treatment (TTT) or overall survival (OS) from International Cancer Genome Consortium (ICGC) CLL patients were evaluated in R using Cox regression TTT considered competing risks; cut-offs were optimized using Optimal Cutpoints and Maxstat algorithms.⁶ For both OS and TTT association analyses, false discovery rate (Benjamini-Hochberg) correction was applied. Additional analyses and plots were generated in GraphPad Prism 8.0.1. and statistical significance was assessed by unpaired Student's t-test with Welch's correction when necessary.

LncRNA Candidate Silencing in MEC-1 Cells

Two Locked Nucleic Acid (LNA) Gapmers (Exiqon) targeting selected transcript variants of lncRNA candidates were designed (Supplementary Table 2; Supplementary Fig. 8). For *RP11-161H23.5*, only exon 1 allowed design; for others, variants were chosen based on TTT impact (*LINC00152* ENST00000414584; *LINC00158* ENST00000440205) or high expression (*LINC00158* ENST00000332587). Silencing was performed with 1 μ M Gapmer in 50,000 MEC-1 cells/well (alone or combined) in triplicates, with scrambled controls, for 3 days. MEC-1 cell line was purchased from DSMZ (Braunschweig, Germany). Transcriptomic changes were assessed using Clariom D WT pico arrays (ThermoFisher) on 10 ng RNA.

Silencing efficiency was validated by qPCR using variant-specific primers and U6 as endogenous control (Supplementary Table 2). For *LINC00152*, primers were designed to avoid concurrent amplification of homologous sequences in *MIR4435-2HG* by introducing a mismatch at the reverse primer's 3' end.⁴⁴

RT was performed with High-Capacity RT kit (ThermoFisher) on 50 ng RNA; qPCR used SybrPower polymerase (ThermoFisher) in quadruplicates, with melting curves for checking specificity. As *RP11-161H23.5* could not be reliably amplified, its silencing was estimated from microarray data.

LncRNA Silencing in Primary CLL Cells

Purified CLL cells (50,000/well, triplicates) were treated for three days with scrambled or combos of the two specific Gapmers already designed (Supplementary Table 2) against *LINC00152* or *LINC00158*. Ibrutinib (1 μ M) plus agonist mix (same concentrations as screening) were added the last 48h. RNA was extracted using miRNeasy kit (Qiagen) with DNase treatment; RT was performed with Superscript VILO IV (ThermoFisher). *LINC00158* expression was quantified by qPCR using TaqMan assays (Supplementary Table 2) and TaqMan Fast Advanced polymerase (Thermo Fisher) to improve sensitivity, as primary samples expressed lower levels than MEC-1 cells. *TBP* and *HPRT1* genes were used as endogenous controls.

Alamar Blue assay

Cell proliferation/viability of primary CLL samples in the *LINC00158* silencing experiments were performed using Alamar Blue HS along 4h incubation, and absorbance measures were finally used as a readout following the supplier protocol (ThermoFisher S.L.).

Declarations

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Authors' contributions

Conceived and designed the Study: L.H. and V.G. Collection and curation of cryopreserved material and clinical databases of CLL from ICGC/Hospital Clinic de Barcelona: D. C., J. A. P., S.B., J.D. and E.C.; Final sample selection: R.O. and L.H. RNAseq/microarrays preparation and bioinformatic analysis for lncRNA: P.F-G., B. D., F.N. and L.H. RNAseq/microarrays preparation and bioinformatic analysis for microRNA: R.P. and F.V. Ex vivo CLL experiments including Cell cytometry and RNA purification: A.D., B. D., C.M-M., R.B. and T.B. qRT-PCR design L.H. and D.R. qRT-PCR performance D.R., C. M-M. and R. S. Wrote the manuscript: L.H., R.B., E.C. and V.G. Co-First authorship T.B. and R.B. (equal contribution). L.H and V.G are co-corresponding authors. All authors read and approved the final manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Microarray data is available at GEO (GSE289506, GSE288926, and GSE292247). These are available at the following URLs, respectively: <https://identifiers.org/geo:GSE289506> (review token access: wnufcegwffslzgh); <https://identifiers.org/geo:GSE288926> (review token access: krohskyuzlavjuz) and <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE292247> (it has no review token assigned as it can be accessed directly from the included link).

Competing interest disclosures

E.C. has been a consultant for Takeda, NanoString, AbbVie and Illumina; has received honoraria from Janssen, EUSPharma and Roche for speaking at educational activities and research funding from AstraZeneca and is an inventor on 2 patents filed by the National Institutes of Health, National Cancer Institute: “Methods for selecting and treating lymphoma types,” licensed to NanoString Technologies, and “Evaluation of mantle cell lymphoma and methods related thereof”, not related to this project. F.N. has received honoraria from AbbVie, AstraZeneca, Janssen, and Sophia Genetics for speaking at educational activities. FN and EC have licensed to Diagnostica Longwood and Sophia Genetics the IgCaller algorithm. D.C. has received honoraria from AbbVie, Sophia Genetics, Thermofisher and AstraZeneca for speaking at educational activities. The remaining authors declare no competing financial interests.

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Ethics approval and consent to participate

Samples from CLL patients were obtained from the Hematopathology collection of the Biobank of the Hospital Clínic de Barcelona-IDIBAPS (Spain). RNAseq data and full clinical annotations from CLL patients were obtained from the Spanish ICGC consortium, which had an approval of the Institutional Review Board of Hospital Clínic de Barcelona, in accordance with national regulations and the Declaration of Helsinki. All patients provided written informed consent.

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Figures

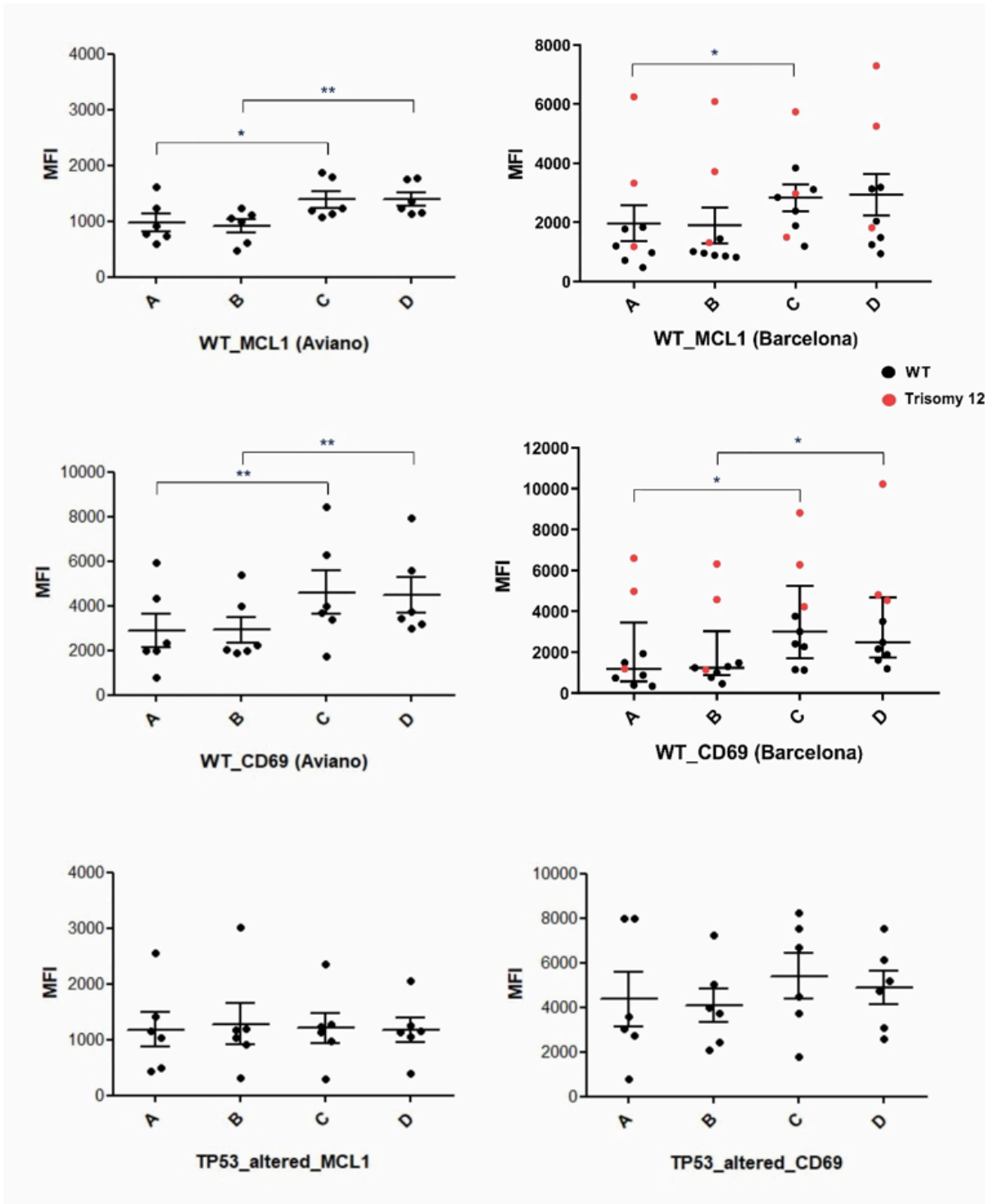


Figure 1

Ex vivo treatment of several cohorts of primary CLL samples with a mix of agonists mimicking microenvironment stimulation upregulate known key proteins to CLL B-cell activation by the microenvironment, even in presence of Ibrutinib. Cell cytometry quantification of MCL1 and CD69 expression mean fluorescence intensity (MFI) in CLL cases with *TP53* alterations, Trisomy 12 or without these alterations (WT). Comparisons were made between condition A (no stimulation) and condition C (stimulation) in the presence of control beads coated with normal goat serum (NGS beads), and between condition B (no stimulation) and condition D (stimulation) in the presence of beads coated with anti-IgM and

Ibrutinib 1uM. In Barcelona cohort reported P-values in red refer to Trisomy 12 cases and black ones to WT cases. Data is shown as mean with standard error of the mean (SEM).

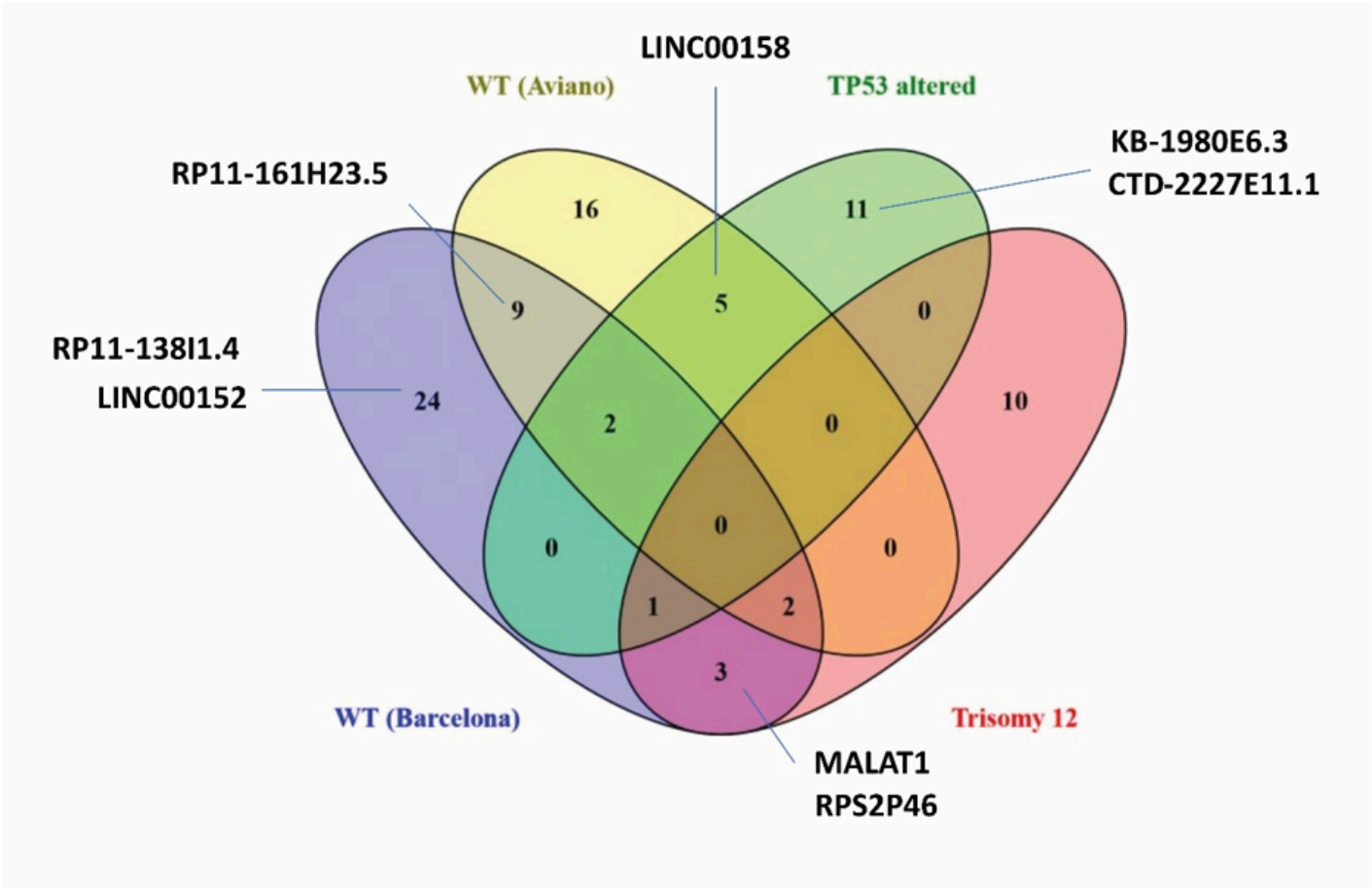
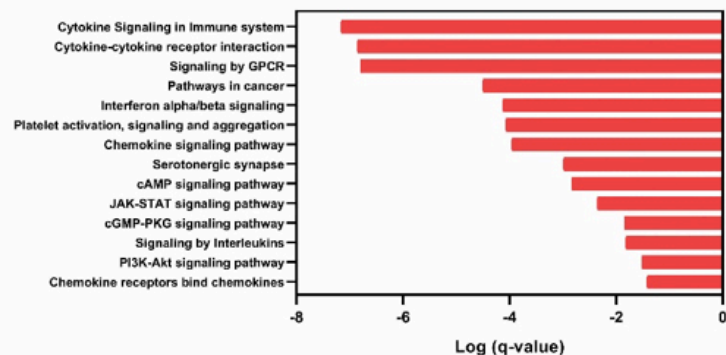
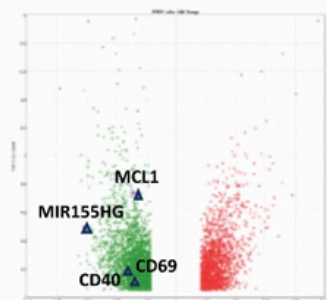


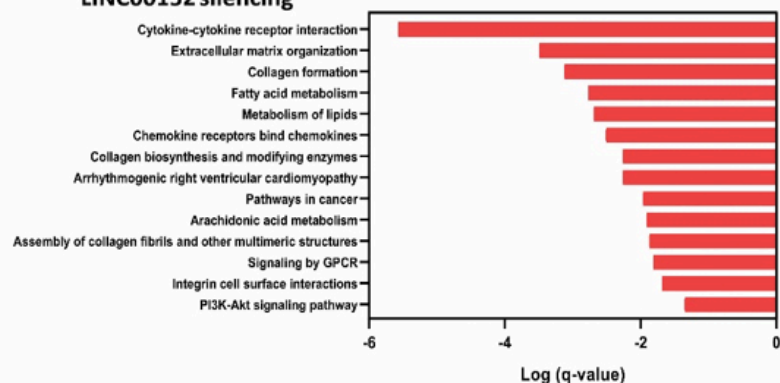
Figure 2

Venn diagrams showing the distribution among the different patient cohorts of non-coding RNAs found significantly modulated by the agonist mix in *ex vivo* experiments (A vs C and B vs D comparisons). Numbers refers to DE lncRNAs associated with shorter time-to-first treatment (TTT) in the ICGC CLL series. Additionally, 8 lncRNA candidates with high levels of expression in MEC-1 CLL cell line are also depicted.

RP11-161H23.5 silencing



LINC00152 silencing



LINC00158 silencing

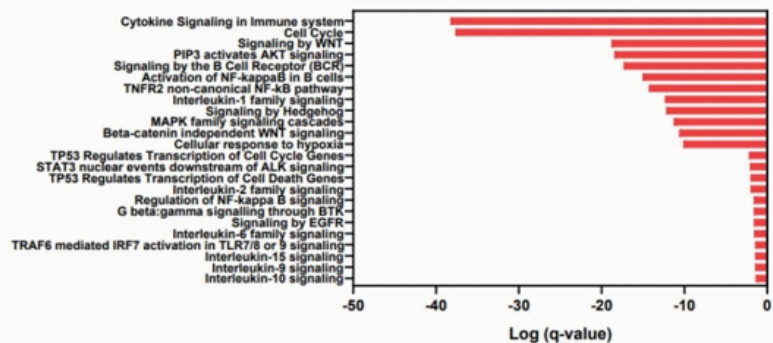
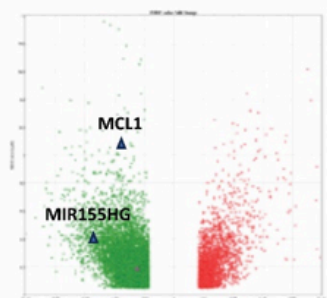


Figure 3

Silencing of lncRNA candidates on the MEC-1 CLL cell line revealed the functional involvement of such transcripts in the regulation of relevant processes of CLL pathogenesis also related with microenvironment stimuli or Ibrutinib effects. Left panels: Volcano plots of DE coding genes found in the silencing experiments of the indicated lncRNAs compared to controls treated with scrambled Gapmers. Right panels: Selection of relevant pathways found significantly enriched among the genes downregulated upon silencing of the indicated lncRNA candidates.

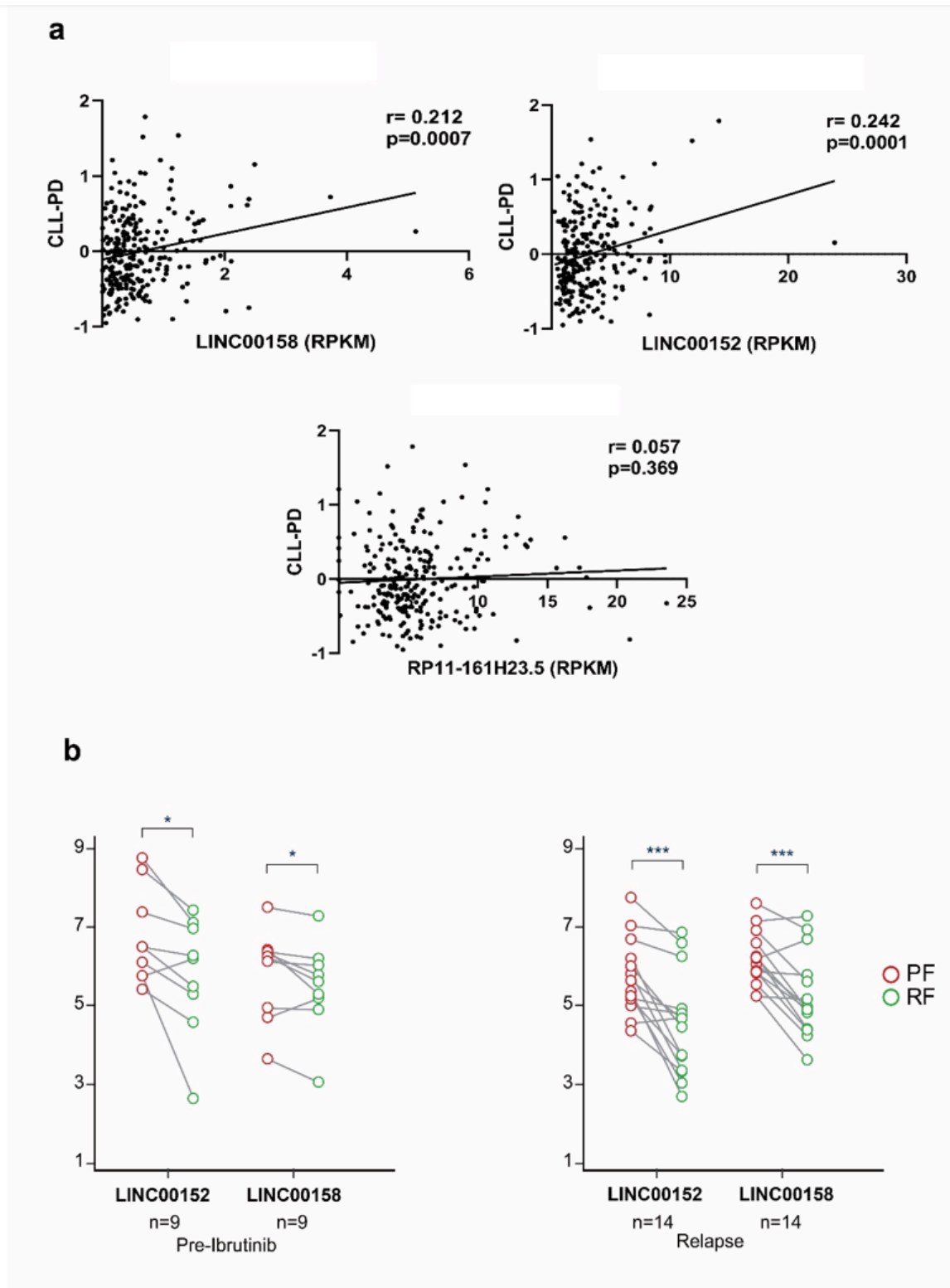


Figure 4

Relationship of the lncRNA candidates with markers measured in peripheral blood related to microenvironment-induced proliferation in the lymph nodes. a Linear correlation analysis of the Proliferative Drive score (CLL-PD) gene signature score with the expression levels of the lncRNA candidates in a series of 49 cases from ICGC project. *LINC00158* and *LINC00152* showed a significant correlation with PD score previously related with increased proliferation rate, particularly upon CpG-ODN stimulation. **b** Paired analysis of *LINC00152* and *LINC00158* expression in proliferative fraction (PF) vs

resting fraction (RF) cells of CLL patients before or after relapsing form Ibrutinib treatment. A significant increase in expression of both lncRNAs was found in PF vs RF in both sample types.

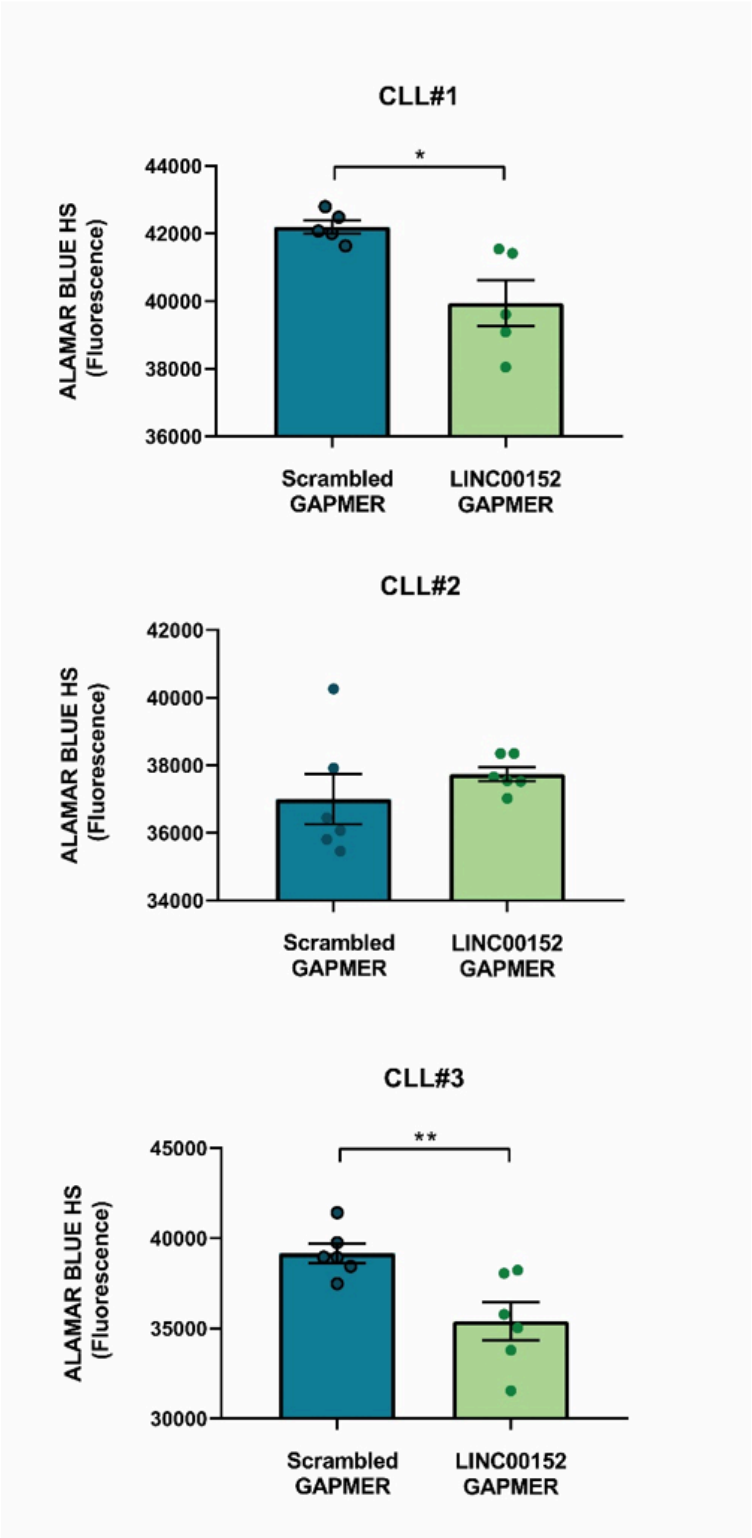


Figure 5

LINC00152 silencing was associated with reduced cell growth in several primary CLL samples of patients relapsed from Ibrutinib treatment. Quantification of fluorescence from AlamarBlue HS assay showed the significant reduction of cell

growth in two out of three samples from CLL cases relapsed from Ibrutinib treatment that were assayed *ex vivo* in the presence of Ibrutinib together with microenvironment-related agonist factors. Significance level (* $p < 0.05$, ** $p < 0.01$).

Supplementary Files

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