SUPPLEMENTARY DATA

METHODS

IGHV gene analysis, FISH analyses, TP53, NOTCH1, and SF3B1 gene mutations

*IGHV* mutational status was assessed using cDNA as previously described. Sequences were aligned to the IMGT directory and analyzed using IMGT/VQUEST software.\(^1\)

Cytogenetic abnormalities involving deletions at chromosomal loci 11q22.3, 13q14.3, 17p13.1, and trisomy 12 were evaluated by FISH using the protocol provided by the manufacturer of the multicolor probes LSI D13S25/LSI 13q34, LSIp53/Cep17, LSI ATM/CEP11, and CEP12 (Abbot Park, IL, USA). A total of 200 interphase nuclei were analyzed for each probe set. The cut-off points (mean + 3 standard deviations) for positive values assessed on peripheral mononuclear cells from ten control subjects were 3.4%, 1.7%, 3.8% and 3.4% for +12, del(11q), del(13q), and del(17p), respectively\(^2\)

TP53 mutation status was investigated by next generation sequencing (NGS) using Roche Junior (Roche-454 Life Sciences, Penzberg, Germany) or by the Ion Torrent platforms (Thermo Fisher Scientific Carlsbad, CA) as previously described\(^3,4\) or by RT-PCR by direct sequence of DNA extracted from CD19+ purified B cells for exons 4-10 [primers have been synthesized by TIB Molbiol (Genoa, Italy)]. Primers and reactions conditions could be found at http://www-p53.iarc.fr. Mutations found were compared to available database of IACR TP53 or checked on Cosmic\(^5\).

The *NOTCH1*c.7541_7542delCT mutation was tested by NGS using Roche 454 technology or by an extremely sensitive ARMS (amplification refractory mutation system)-PCR approach as previously described\(^6,7\) [primers have been synthesized by TIB Molbiol (Genoa, Italy)]. *NOTCH1* 3'UTR mutational analysis was detected by NGS of a 309-bp fragment in the 3'UTR of *NOTCH1* gene within which noncoding mutations causing aberrant splicing events occur was performed on the Genome Sequencer Junior instrument (Roche-454 Life Sciences) as previously described. Variants were validated by Sanger sequencing of genomic DNA\(^7\).
SFB31 (exons 14, 15, and 16, including splice site RefSeq NM_012433.2) genes were analyzed by PCR amplification and Sanger sequencing of high molecular weight genomic DNA extracted from CD19+ purified B-cells (> 95%) as previously described⁸.

Peripheral blood mononuclear cell (PBMCs) from patients with CLL were isolated by Ficoll-Hypaque (Seromed, Biochrom) density gradient centrifugation and CD19-positive CLL cells were enriched by negative selection with the EasySep-Human B-cell Enrichment Kit without CD43 depletion (STEMCELL Technologies, Voden Medical Instruments S.p.A) or with B-CLL isolation Kit (Miltenyi Biotec Srl).

**MiRNA target prediction.**

For miRNA::mRNA target prediction we used a recently developed web tool named miRabel (http://bioinfo.univ-rouen.fr/mirabel)⁹. miRabel delivers a score based on the aggregation of the interaction ranks taken from other well-known prediction algorithms including computationally predicted human miRNA::mRNA interaction databases generated by miRanda¹⁰, PITA¹¹, SVMicrO¹² and TargetScan¹³. Each of these publicly available online algorithms uses different and complementary features of miRNA::mRNA interactions such as seed match, interspecies conservation, free energy, site accessibility and target-site abundance. Briefly, this method normalizes ranks with the maximal value of 1. The selected function (Mean, Default, Geometric mean, Median, Min, Stuart) is then used for lists aggregation. Finally, a probabilistic model is used to make the algorithm parameter free and robust to outliers, noise, and errors. Missing values are replaced by the maximum relative rank value. The new score resulting from the aggregation is used to re-rank each interaction and indicates the significance of the proposed rank as a miRabel score.

**Luciferase reporter assays**

For 3’UTR assays, HEK293 cells were seeded at 60-70,000 cells per well in a 24-well plate the day before transfection in RPMI growth medium supplemented with 10% FBS. The cells were transfected with 5 nmol/L miRNA mimic (either miR-146b-5p, mR-146a-5p, or miR-control)
(Ambion) using RNAiMAX (Invitrogen); 8 hours later, 400 ng of 3’UTR-IL-23R (Switchgear) or 800 ng IL-12Rβ1-3’UTR (Origene) reporter vectors were co-transfected using Lipofectamine LTX (Invitrogen). Data were normalized to firefly luciferase or Renilla luciferase activity, respectively. Forty-eight hours after transfection with the reporter plasmid, cells were lysed and Firefly and Renilla luciferase activities were measured consecutively using the Dual-Luciferase Report Assay System (Promega) according to the manufacturer’s protocol (Promega). Transfection efficiency was determined by co-transfection with a GFP plasmid and microscopy/FC. Experiments were performed at least in triplicate in three independent experiments. In preliminary tests, MEC-1 and OSU-CLL cell lines, that share more similarities with CLL cells because of their cell of origin, were used with the same transfection protocol described above. However, these cells lines showed a very poor transfection yield and therefore could not be employed further.

In another set of experiments, HEK293 cells were transfected with 25 nmol/L miR-146b-5p or miR-control using RNAiMAX; 8 hours later, 500 ng IL-12Rβ1-3’UTR reporter vector or 500 ng of the corresponding 3’UTR mutated reporter vector (IL-12Rβ1-MUT, Origene) were co-transfected with 5 ng of Renilla plasmid using Lipofectamine LTX. Forty-eight hours after the first transfection, cells were lysed and Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Report Assay System according to the manufacturer’s protocol (Promega). Luciferase activity was measured using a multilabel plate reader Mithras LB940 (Berthold Technologies); the experiments were performed at least in triplicate in four independent experiments.

To calculate the inhibitory effect of miRNA-146-5p on the target sequence (3’ UTR) the ratio of Firefly/Renilla luciferase signal was normalized on the ratio of Firefly/Renilla luciferase signal of the reporter vector transfected with the miR-CTR mimic, which was considered 100%.

**In vitro transfection of CLL cells with synthetic miRNAs**
MiRNA transfection was performed as previously described\textsuperscript{5,14}. MirVana\textsuperscript{TM} miRNA mimics or inhibitors (Ambion Inc., Thermo Fisher Scientific) were delivered to highly purified CLL cells with a Neon Transfection System (Invitrogen, Thermo Fisher Scientific) at the final concentration of 50 nM/2×10\textsuperscript{6} CLL cells. After transfection, cell suspensions were seeded in 24-well plates containing 500 µL of culture medium without antibiotics [RPMI-1640 with L-glutamine and 10% FBS (Gibco, Thermo Fisher Scientific), Na-pyruvate 0.1% (Euroclone)] at 37°C and incubated at a final concentration of 2×10\textsuperscript{6} CLL cells/mL/well in a 5% CO\textsubscript{2} atmosphere.

In the sets of experiments shown in Fig. 3E a total of 5×10\textsuperscript{6} CD19+/CD5+ CLL cells were electroporated with miRNA inhibitors or negative controls at a final concentration of 100 nM for 48 h using Nucleofector-4D Transfection System (Amaxa), with pulses FF120 in SF solution. In all transfection protocols, transfection efficiency was evaluated by flow cytometric analysis of CY3\textsuperscript{TM}-dye-labeled synthetic miRNA inhibitor (Invitrogen) transfection. Typical transfection efficiency in B-CLL ranged from 60 to 80%.

**Real time PCR**

MiR-146b-5p in purified CD19+ cells B cells was measured using Taqman Real Time PCR (RT-qPCR) assays (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA USA). Briefly, 10 ng of total RNA was retrotranscribed using the following miRNA-RT specific primers: miRNA-146b-5p (cod. 001097), control RNU44 (cod.001094) and U6 (cod.001973) according to TaqMan MicroRNA-assay (Applied Biosystems, Thermo Fisher Scientific). RT-qPCR was performed using the TaqMan Universal Master Mix II (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer’s instructions on the RotorGene 6000 (Qiagen, Hilden, Germany). All samples were run in duplicate and data were expressed as 2\textsuperscript{-ΔCt}. miR-146b-5p expression was normalized using RNU44 and U6 as controls. The increase or inhibition by transfection with miRNA mimics or inhibitors were expressed as:
\[
\% \text{ fold increase} = (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR-146b-5p of cells transfected with miR-146b-5p mimic}) - (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR-146b-5p of cells transfected with miR-CTR mimic posed = 1}) / (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR-146b-5p of cells transfected with miR-146b-5p mimic})*100
\]

\[
\% \text{ fold inhibition} = (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR-146b-5p of cells transfected with miR-CTR inhibitor posed = 1}) - (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR146b-5p of cells transfected with miR-146b-5p inhibitor}) / (\text{fold change } 2^{-\Delta\Delta CT} \text{ miR-146b-5p of cells transfected with miR-CTR inhibitor posed=1})*100.
\]

In another set of experiments, miR-146b-5p (483144_mir) and miR-146a-5p (478399_mir) were quantified using TaqMan advanced miRNA assay (Applied Biosystems/Thermo Fisher Scientific). The test was performed using fast advanced mastermix (Applied Biosystems) and miR-93-5p (478210_mir) as normal control. miR-146a-5p and miR-146b-5p expression were calculated as \(\Delta CT\) vs miR-93-5p.

IL-12Rβ1 mRNA expression was evaluated by quantitative real time PCR (q-RT PCR) using TaqMan Assays (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) specific for IL-12Rβ1 (cod. Hs01106578) normalized versus POLR2A (cod. Hs00172187). All samples were analyzed at different time points and run in triplicate (duplicate for the POL2RA gene). Data of single patients were exported from StepOne 3.2 software (Applied Biosystems) and collectively analyzed by using DataAssist software (Applied Biosystems)\(^{15}\).

**Flow-cytometry**

Surface IL-23R complex was detected by staining the cells with CD19 PE-CY7 (BD Biosciences, Inc, San Jose, CA), CD5 APC (BD Biosciences), anti-human IL-23R PE (R&D Systems Inc., Minneapolis, MN, USA), anti-human IL-12Rβ1 FITC (R&D Systems). The cells were analyzed using a FACSCanto flow-cytometer and DIVA 6 (BD Biosciences) or Flowlogic\(^{\text{TM}}\) software (Miltenyi Biotech). For data analysis CD5/CD19-double positive cells were gated within
the SSC/FSC lymphocyte gate and the percentage of IL-23R-positive and IL-12Rβ1-positive cells determined on the gated population.

Co-expression of the IL-23R complex with the Ki67 proliferation marker was evaluated by surface staining with IL-23R PE, and IL-12Rβ1 APC (R&D Systems), followed by cell fixation and permeabilization (Fix & Perm Invitrogen, ThermoFisher Scientific) and staining for Ki67 FITC (clone MIB-1, DakoCytomation S.r.L). CD80 expression was determined by single staining with CD80 PE (BD Biosciences). To determine the percentage of IRAK1 positive CLL cells, by flow cytometry, cells were fixed, permeabilized (Fix & Perm., Thermo Fisher Scientific) and stained with IRAK1 mouse antibody PE conjugated (F-4, cod 5288, Santa Cruz Biotechnology Inc) and analyzed by a FACSCanto flow-cytometer. Surface IL-21R was detected by staining with a mouse anti-human IL-21R PE conjugated antibody (FAB991P, R&D Systems Inc.)

**Cell viability assays**

Cultured cells were double stained with Annexin V-FITC conjugate (BD Biosciences Pharmingen, San Josè, CA, USA), and propidium iodide in isotonic solution, and then analyzed by flow cytometry. Viable cells were defined as double negative cells and apoptotic cells as Annexin V-positive cells.

**Western blotting**

SDS-PAGE and western blotting (WB) were performed according to standard protocols. Briefly cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EDTA] and anti-Protease PMFS (2mM, Sigma-Aldrich). Whole cells lysates (50 μg per lane) were separated using 10% Tris SDS-acrylamide gels (Biorad) with Laemmli Buffer system, electro-transferred to Hybond-C nitrocellulose (GE Healthcare) (10V for 40min), and immunoblotted with the mouse, anti-IL12Rβ1 (Santacruz, C-20, sc-658). After TBS-T washes and incubation with anti-mouse (HRP)-conjugated secondary antibody, the proteins were detected with LiteAblot plus Enhanced
Chemiluminescent Substrate (Euroclone). As a loading control, GAPDH expression levels were measured using an anti-GAPDH mouse mAb (AM4300; Life Technologies). Protein bands were analyzed using ImageJ Analysis Software version 1.48V (NIH, USA).

In another sets of experiments CLL cells were lysed in a buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM EDTA, 1 mM DTT, and protease inhibitors (0.5 mM PMSF, 1 mg/ml leupeptin, 2 mg/ml apro tinin, 1 mg/ml pepstatin A). Cell lysates were incubated for 30 min at 4°C and centrifuged at 14000 rpm at 4°C for 5 min. Supernatants were collected and protein concentration was determined using the BCA assay (ThermoFisher, Milano, Italy). 50 μg of total proteins were resolved on 4–15% Mini protean TGX precast gels (Bio-Rad, Milano, Italy) and transferred to nitrocellulose membranes by Trans-Blot Turbo Blotting System (Bio-Rad, Milano, Italy). Membranes were blocked with 2% non-fat dry milk in 0.1% Tween-20 in PBS for 1 h, then incubated for 1 h at room temperature or overnight at 4°C with the appropriate primary antibody. The following antibodies were employed: rabbit anti-human IL12Rβ1 (Santa Cruz, C-20, sc-658), mouse anti-human β-Actin (Sigma, A5316, clone AC-74), secondary anti-mouse IgG peroxidase conjugate (A9044, Sigma–Aldrich, Milano, Italy) and secondary anti-rabbit peroxidase conjugate (A9169, Sigma–Aldrich, Milano, Italy). Detection was carried out with ECL West PICO PLUS substrate (ECL-2001, Immunological Sciences, Roma, Italy). Chemiluminescence was analyzed by Alliance LD, UVITEC Cambridge (Cambridge, U.K.).

For the detection of TRAF6 protein purified CLL cells were transfected with miR-146b-5p or miR-146a-5p or miR-CTR mimics, seeded in RPMI medium for 6 h and subsequently cultured with CD40L-TC or Mock for another 48 h. After incubation, cells were detached by 2 mM EDTA solution in PBS, were lysed for 15 minutes in lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Brij97) containing 2 mM Na Orthovanadate and protease inhibitors (Complete Mini 04693124001, Roche Diagnostics, Mannheim, Germany).

Lysates were resolved under reducing conditions by 10% SDS-PAGE and analyzed by western blotting using the following antibodies: mouse anti human TRAF6 (D-10, cod. Sc-8409,
Santa Cruz Biotechnology Inc.) and anti-Tubulin-alpha mouse monoclonal clone B-5-1-2 (T6074, Sigma-Aldrich/Merck, Milan, Italy). HRP-conjugated anti-mouse (P0447) was from Dako/Agilent Technologies (Milan, Italy). Proteins were detected by ECL Prime (RPN2232, GE Healthcare, Milan, Italy) and visualized by a chemiluminescence gel documentation and analysis system (MINI HD, UVITEC, Cambridge, UK). Protein bands were analyzed using ImageJ Analysis Software version 1.48V (NIH, USA).

**CLL cells stimulation via BCR signaling**

CLL cells were cultured with Dynabeads M-450 Epoxy (Invitrogen, Thermo Fisher Scientific) coated with (10 μg/1×10^7 beads) Goat anti-Human IgM μ chain specific (Gαμ-Ab, Southern Biotechnology, Birmingham, AL) together with beads coated with Goat anti-Human IgD anti-δ-Ab (Gαδ-Ab; Southern Biotechnology) in the presence of IL-4 25 ng/mL (Gibco, Thermo Fisher Scientific) as described^{16}

**Xenogeneic mouse transplantation**

A total of 50×10^6 CLL cells were injected intravenously together with autologous T cells, which had been stimulated with Dynabeads Human T-activator CD3/CD28 (Gibco, Thermo Fisher Scientific, beads to T cells ratio 1:1) and recombinant IL-2 (Proleukin, Norvartis of 30 U/mL) for 48 hours, into six to eight week old female NOD/Shi-scid,γcnull (NSG) mice. Engraftment was verified by determining the proportion of human circulating CD45+CD19+CD5+ leukemic cells by flow cytometry starting at three weeks after inoculation.

To evaluate therapeutic effects of miRNA, mice were treated with miR-146b-5p or miR-CTR mimics after 4-6 weeks from CLL cell inoculum, at a time when all mice showed leukemia engraftment. Three doses (one every second day) of mirVana™ miRNA mimic, (In Vivo Ready formulation, Ambion Inc) complexed with Invivofectamine 2.0 (Thermo Fisher Scientific) were administered intraperitoneally at a final concentration of 0.7 mg/mL (200 μL/mouse). Mice were
sacrificed three days from the last injection in a saturated CO₂ chamber, and autopsies were performed. Blood, bone marrow flushing, and spleen from the mice were analyzed by flow cytometry, immunohistochemistry (IHC), and immunofluorescence staining (IF) in situ. Fresh spleen tissue samples were enzymatically digested using the Spleen Dissociation Kit (Miltenyi Biotech GmbH) and resuspended with gentleMACSTM Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

The cell suspensions from blood, bone marrow and spleen were stained with anti-human CD45 FITC, CD19 PECy7, CD5 APC (BD Biosciences) and analyzed with a FACSCanto (BD Biosciences) flow-cytometer.

Apoptotic cells present in the spleen suspensions were evaluated by Annexin V FITC, CD19 PE-Cy7, CD5 PE, CD45 APC staining.

Formalin-fixed and paraffin-embedded spleens were analyzed for the presence of human cells by IHC and IF. The primary antibodies used in the staining procedures were an anti-CD20 Mouse monoclonal antibody (clone L26) and a CD3 Rabbit Monoclonal Antibody (clone 2GV6). The binding of these antibodies to their cellular targets was revealed using the polymeric detection system, Ultraview Universal Red Detection Kit (Ventana Medical System). An appropriate positive tissue control was used for each staining run, while to obtain a negative control, the entire IHC procedure was carried out on adjacent sections in the absence of the primary antibody. All sections were counter-stained with Gill’s modified hematoxylin and then cover-slipped. The sections were evaluated by two observers with an Olympus light microscope using 4×, 10×, 40× objectives under a Leica DMD108 optical digital microscope (Leica Microsystems).

Tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH 6 or pH 9 in a thermostatic bath at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H₂O₂ and Fc blocking by a specific protein block (Novocastra), the samples were incubated with mouse anti-human Ki67
primary antibody (MIB-1, DakoCytomation, 1:75 pH6). The staining was revealed using rabbit anti-mouse IgG-Peroxidase antibody 1:200 (Sigma Aldrich) and DAB (3,3’- Diaminobenzidine, Novocastra) as substrate-chromogen.

Double staining of tissue sections for CD20 and Ki67 by IHC was performed as previously reported\textsuperscript{15}. Briefly, sections were incubated with a with a specific anti-human Ki67 antibody (MIB-1, DakoCytomation, dilution 1:25) at 37°C and followed by addition of the polymeric detection system Ultraview Universal DAB Detection Kit (Ventana Medical Systems, Roche). The automated program provides dispensing of the second antibody (anti-CD20, L26-Ventana Medical Systems, Roche), followed by addition of the polymeric detection system Ultraview Universal RED Detection Kit.

IF staining was performed as detailed above for IHC. The following primary and secondary antibodies were used: anti-IL-23 (Polyclonal, 1:100, pH 6, Abcam), IL-12Rβ1 (Polyclonal, 1:50, pH 9, Novus Biologicals NBP1-49875), CD20 (Clone L26, 1:100, pH 9, Novocastra, Leica Biosystems), primary antibody binding was revealed by secondary antibodies either conjugated with Alexa Fluor 488 or Alexa Fluor 568, after Fc-receptor blockade. The slides were counterstained with DAPI stain (all Invitrogen Molecular Probes, ThermoFisher Scientific). All the IF-stained sections were analyzed under a Zeiss Axioscope A1 optical microscope equipped with an IF module (Zeiss, Oberkochen, Germany) and microphotographs were collected using a Zeiss Axiocam 503 color digital camera (Zeiss).

**IHC index**

The IHC index is a measure of the spread of leukemia based on the average diameters of the follicular lesions. We assigned a numerical value of 1 to the follicles with diameters±SD of 102(±90) x 42(±7) µm; a value of 3 to follicles with a diameter 195(±80) x 138(±85) µm; a value of 6 to follicles between 399(±245) x 300(±39), and a value of 12 to follicles between 734(±461) x
540(±167). The IHC index is given by the sum of the number of follicles multiplied by the value assigned according to size as described previously⁵.
Supplementary Table 1. Biologic and molecular characteristics of CLL patients included in the experimental studies.

| ID    | IGHVᵃ | del(13)b (q14) | +12ᵇ | del(17)b (p13.1) | del(11)b (q22.3) | TP53 | NOTCH1 | SFB31 |
|-------|-------|----------------|------|------------------|------------------|------|--------|-------|
| PF0024| UM    | + (38%)        |      |                  |                  | WT   | WT     | WT    |
| HG0135| UM    | +(44%)         |      |                  |                  | WT   | WT     | p.Lys700Glu |
| SR0112| UM    | -              |      |                  |                  | WT   | WT     | WT    |
| CA0058| UM    | -              |      |                  |                  | WT   | WT     | WT    |
| DF0319| UM    | -              | + (93%) |                  |                  | WT   | WT     | WT    |
| RD0468| UM    | -              | + (100%) |                  |                  | WT   | WT     | WT    |
| MA0151| M     | + (82%)        |      |                  |                  | WT   | WT     | WT    |
| AR0090| M     | + (60%)        |      |                  |                  | WT   | WT     | WT    |
| PD0164| M     | + (100%)       |      |                  |                  | WT   | WT     | WT    |
| LG0337| M     | + (100%)       |      |                  |                  | WT   | WT     | WT    |
| VF0384| UM    | -              |      |                  |                  | WT   | WT     | WT    |
| MG0482| UM    | -              |      |                  |                  | WT   | WT     | WT    |
| GC0015| UM    | -              | + (94%) |                  |                  | p.Asn235Serᶜ | WT   | WT    |
| CM18  | UM    | +              |      |                  |                  | WT   | WT     | WT    |
| SR1-ME1077| UM | -              |      | + (18%)          |                  | WT   | WT     | WT    |
| GE1-AG114| M | + (90%)        |      |                  |                  | WT   | WT     | WT    |
| GE1-GA191| UM | -              |      |                  |                  | WT   | WT     | WT    |
| GE1-CC190| M | + (71%)        |      |                  |                  | WT   | WT     | WT    |
| GE1-PM129| M | -              |      |                  |                  | WT   | WT     | WT    |
| GE1-RO148| M | -              |      |                  |                  | WT   | WT     | WT    |
| GE1-DM210| UM | + (59%)        |      |                  |                  | WT   | WT     | WT    |
| SV1-SA4078| UM | -              |      | + (39%)          |                  | WT   |WT     | WT    |
| SV1-SA4078| UM | -              |      | + (39%)          |                  | WT   |WT     | WT    |
| SV1-SA4078| UM | -              |      | + (39%)          |                  | WT   |WT     | WT    |
| NT0628| UM    | -              |      | + (40.7%)        |                  | WT   | ND     | ND    |
| CS142  | M     | -              |      |                  |                  | WT   | WT     | WT    |
| CS152  | M     | -              |      |                  |                  | WT   | WT     | WT    |
| SV1-BL726| M | + (84%)        |      |                  |                  | WT   | WT     | WT    |

ᵃUM, unmutated; M, mutated. ᵇ% of positive nuclei on CD19+ cells is indicated for each lesion. ᵇ TP53 germline variant. ᵇ NOTCH1 3’ UTR variant 136495700T>C (10.83%)
| ID         | IGHV<sup>a</sup> | miRNome values<sup>b</sup> (quartile) | RT-qPCR<sup>c</sup> |
|------------|-------------------|----------------------------------------|-----------------------|
| PF0024     | UM                | 9.98 (1<sup>st</sup>)                  | 0.081                 |
| HG0135     | UM                | 11.61 (1<sup>st</sup>)                 |                       |
| SR0112     | UM                | 14.16 (1<sup>st</sup>)                 | 0.008                 |
| CA0058     | UM                | 17.15 (1<sup>st</sup>)                 |                       |
| DF0319     | UM                | 13.30 (1<sup>st</sup>)                 | 0.07                  |
| RD0468     | UM                | ND                                     | 0.41                  |
| MA0151     | M                 | 115.94 (4<sup>th</sup>)                | 0.62                  |
| AR0090     | M                 | 160.82 (4<sup>th</sup>)                |                       |
| PD0164     | M                 | 125.73 (4<sup>th</sup>)                |                       |
| LG0337     | M                 | 87.20 (4<sup>th</sup>)                 |                       |
| VF0384     | UM                | ND                                     | ND                    |
| MG0482     | UM                | ND                                     | ND                    |
| GC0015     | UM                | 24.29 (2<sup>nd</sup>)                 | 0.36                  |
| CM18       | UM                | ND                                     | 0.044                 |
| SR1-ME1077 | UM                | ND                                     | 0.10                  |
| GE1-AG114  | M                 | ND                                     | 0.31                  |
| GE1-GA191  | UM                | ND                                     | 0.016                 |
| GE1-CC190  | M                 | ND                                     | 1.4                   |
| GE1-PM129  | M                 | ND                                     | 1.2                   |
| GE1-RO148  | M                 | ND                                     | 0.26                  |
| GE1-DM210  | UM                | ND                                     | 0.26                  |
| SV1-SA4078 | UM                | ND                                     | 1.5                   |
| NT0628     | UM                | ND                                     | 0.09                  |
| CS142      | M                 | ND                                     | ND                    |
| CS152      | M                 | ND                                     | 0.35                  |
| SV1-BL726  | M                 | ND                                     | 0.12                  |

<sup>a</sup> UM, unmutated; M, mutated.  
<sup>b</sup> miRNome values in natural scale (ref).  
<sup>c</sup> Data are expressed as $2^{-(dCT)}$ and miR-93 was used as reference gene. ND not determined. The RT-qPCR was performed in duplicate.
Supplementary Table 3. Univariate Cox Regression Analysis.

| Variable                                      | HR   | 95% CI   | P     |
|-----------------------------------------------|------|----------|-------|
| Rai Stage (I-II versus 0)                     | 1.8  | 1.2-2.7  | 0.009 |
| β2-M, mg/dL (≥5 versus <5)                   | 2.2  | 1.5-3.1  | <0.0001 |
| Absolute lymphocytosis (CLL versus MBL)       | 1.8  | 1.0-3.2  | 0.048 |
| ZAP-70 expression (≥30% versus <30%)         | 2.9  | 1.9-4.4  | <0.0001 |
| CD38 expression (≥30% versus <30%)           | 2.7  | 1.7-4.1  | <0.0001 |
| IGHV mutational status (UM versus M)          | 4.7  | 3.1-7.2  | <0.0001 |
| NOTCH1 mutation (M versus WT)                 | 2.1  | 1.3-3.5  | 0.004 |
| Fish analysis [neither versus del(17p) or del(11q)] | 6.4  | 3.8-10.8 | <0.0001 |

Supplementary Table 4. Multivariate Cox Regression Analysis models.

| Variable                                      | model 1 (all cases) |
|-----------------------------------------------|---------------------|
|                                               | HR   | 95% CI   | P     |
| Rai Stage (I-II versus 0)                     | 1.7  | 1.0-3.0  | 0.042 |
| β2-M, mg/dL (≥5 versus <5)                   | 1.9  | 1.2-3.1  | 0.006 |
| Absolute lymphocytosis (CLL versus MBL)       | 1.3  | 0.7-2.6  | 0.4   |
| ZAP-70 expression (≥30% versus <30%)         | 1.1  | 0.6-2.1  | 0.7   |
| CD38 expression (≥30% versus <30%)           | 1.0  | 0.6-1.8  | 0.9   |
| IGHV mutational status (UM versus M)          | 2.4  | 1.2-5.0  | 0.015 |
| NOTCH1 mutation (M versus WT)                 | 1.8  | 0.9-3.6  | 0.08  |
| FISH analysis [neither versus del(17p) or del(11q)] | 3.3  | 1.5-7.2  | 0.002 |
| miR-146b-5p (1st quartile versus 2nd-4th quartiles) | 1.5  | 0.9-2.4  | 0.13  |
Supplementary Table 5. Analysis of somatic mutations in miR-146b genomic region: metadata from a dataset of 551 CLL patients by ICGC Data portal.

|                        | GRCh37/hg19 position           | # mutations |
|------------------------|--------------------------------|-------------|
| miR-146b               | chr10:104196269-104196341      | 0           |
| putative promoter locus (3kb upstream TSS) | chr10:104193000-104196269 | 0           |

GeneHancer predictions:

| GeneHancer predictions: | GRCh37/hg19 position           | # mutations |
|------------------------|--------------------------------|-------------|
| GH10J102438_Promoter   | chr10:104197957-104198158      | 0           |
| GH10J102462_Promoter/Enhancer | chr10:104222401-104224127    | 0           |
| GH10J102595_Enhancer   | chr10:104354850-104356316     | 0           |
| GH10J102478_Enhancer   | chr10:104237959-104239557      | 0           |
| GH10J102467_Enhancer   | chr10:104227305-104228450      | 0           |
| GH10J102384_Enhancer   | chr10:104143801-104146157      | 0           |
| GH10J102475_Enhancer   | chr10:104234758-104236316      | 0           |
| GH10J102469_Enhancer   | chr10:104229557-104229758      | 0           |
| GH10J102390_Enhancer   | chr10:104105558-104152356      | 0           |
| GH10J102473_Enhancer   | chr10:104232759-104233957      | 0           |
| GH10J102484_Enhancer   | chr10:104244061-104246062      | 0           |
Supplementary Table 6. Copy number alterations (CNA) analysis in miR-146b locus: metadata from a dataset of 551 CLL patients by ICGC Data portal.

| ICGC donor ID | mutation type | chr | chr start | chr end | donor sex | donor vital status | donor age at diagnosis | disease status | last followup | donor relapse interval (days) | donor tumour stage at diagnosis | donor survival time (days) |
|---------------|---------------|-----|-----------|---------|-----------|-------------------|------------------------|---------------|--------------|----------------------------|-------------------------------|-----------------------------|
| DO223381      | loss          | 10  | 97599696  | 107053503| female    | deceased          | 76                     | progression    |              | 3555                       | BINET_A                       | 3612                         |
| DO223422      | loss          | 10  | 81326034  | 112503404| female    | alive            | 64                     | complete remission|              | 1216                       | BINET_A                       | 1707                         |
| DO223555      | loss          | 10  | 1.01E+08  | 105840348| female    | alive            | 58                     | complete remission|              | 1470                       | BINET_A                       | 1702                         |
| DO52705       | loss          | 10  | 1.03E+08  | 135449667| male      | deceased          | 75                     | partial remission |              | 1943                       | BINET_A                       | 2227                         |
| DO6370        | loss          | 10  | 1.03E+08  | 105060989| male      | alive            | 59                     | partial remission |              | 2396                       | BINET_A                       | 6965                         |
| DO6420        | loss          | 10  | 1.03E+08  | 106419703| female    | alive            | 59                     | complete remission|              | 3312                       | BINET_A                       | 4591                         |
| DO7112        | loss          | 10  | 1.04E+08  | 104620409| male      | alive            | 78                     | stable          |              | 2961                       | BINET_A                       | 3150                         |
### Supplementary Table 7. Inhibition of CLL cell growth in NSG mice by miR-146b-5p mimic treatment.

| ID       | miRNAs treatment | NSG mice ID | CD45+CD19+CD5+ CLL cells % PBL (FC) | CD45+CD19+CD5+ T cells % PBL (FC) | CD45+CD19+CD5+ CLL cells% BM (FC) | CD45+CD19+CD5+ T cells % BM (FC) | CD45+CD19+CD5+ CLL cells% Spleen (FC) | CD45+CD19+CD5+ T cells % Spleen (FC) | IHC index Spleen |
|----------|------------------|-------------|------------------------------------|-----------------------------------|----------------------------------|---------------------------------|------------------------------------|-----------------------------------|-----------------|
| GE1-PM129 | miR-CTR mimic    | NSG 2       | 14.90                              | 80.31                             | 7.46                             | 90.10                           | 22.26                              | 77.45                            | 69              |
|          |                  | NSG 4       | 26.12                              | 64.71                             | 8.46                             | 88.49                           | 26.75                              | 72.47                            | 43              |
|          |                  | NSG 6       | 23.34                              | 75.71                             | 9.26                             | 86.63                           | 31.39                              | 68.27                            | 152             |
|          |                  | NSG 13      | 22.90                              | 75.26                             | 16.51                            | 79.18                           | 38.18                              | 58.80                            | 153             |
|          |                  | NSG 14      | 26.12                              | 72.00                             | 6.14                             | 90.28                           | 23.07                              | 76.36                            | 140             |
|          | miR-146b-5p mimic| NSG 0       | 10.49                              | 85.07                             | 2.83                             | 92.99                           | 9.18                               | 87.48                            | 10              |
|          |                  | NSG 1       | 14.69                              | 88.81                             | 2.20                             | 89.40                           | 11.92                              | 87.22                            | 5               |
|          |                  | NSG 3       | 11.55                              | 85.72                             | 2.48                             | 87.96                           | 9.54                               | 90.07                            | 12              |
|          |                  | NSG 5       | 9.93                               | 88.77                             | 2.00                             | 91.11                           | 10.15                              | 83.05                            | 22              |
|          |                  | NSG 16      | 9.38                               | 90.16                             | 1.12                             | 94.14                           | 8.51                               | 90.40                            | 33              |
| GE1-RO148 | miR-CTR mimic    | NSG 5       | 30.29                              | 68.90                             | 31.83                            | 48.63                           | 46.87                              | 44.19                            | 156             |
|          |                  | NSG 6       | 41.04                              | 57.76                             | 39.80                            | 47.33                           | 43.24                              | 34.58                            | 180             |
|          | miR-146b-5p mimic| NSG 3       | 21.48                              | 75.59                             | 12.79                            | 71.25                           | 18.60                              | 62.08                            | 81              |
|          |                  | NSG 4       | 17.04                              | 76.16                             | 12.36                            | 70.37                           | 23.94                              | 54.34                            | 75              |

PBL = peripheral blood lymphocytes, BM = bone marrow flushing, FC = flow cytometry; IHC index = immunohistochemical index
Supplementary Fig 1: miR-146b-5p correlation with *IGHV* mutational status. Graphical representation of the miR-146b-5p values calculated by RT-qPCR displayed in Supplementary Table 2. Comparison of miR-146b-5p values in the CLL cases grouped by *IGHV* mutational status. Data are expressed as $2^{-\text{dCT}}$ and miR-93 was used as reference gene. The RT-qPCR was performed in duplicate. Mean ± SD and *P*-values of the difference of miR-146b-5p expression between CLL cells from *IGHV*-M and *IGHV*-UM CLL cases are indicated (Mann-Whitney test). Asterisk indicates statistically significant *P* value (*P*<0.05).
Supplementary Fig 2: Comparison between miR-146a-5p and miR-146b-5p expression levels in CLL samples. (A) Differential expression of miR-146b-5p and miR-146a-5p in IGHV-M (n=144) and IGHV-UM (n=80) CLL groups. Data expressed are by miRNome values on natural scale. For each group mean ± SD and P-values of the difference in miRNAs expression between CLL cells from IGHV-M and IGHV-UM CLL cases are indicated (Mann-Whitney test). Asterisk indicates statistically significant P values (P<0.05). (B) Association between miR-146a-5p levels and IGHV mutational status in 224 CLL cases. The numbers of CLL cases are indicated in each bar. (C) miRnome values of miR-146b-5p or miR-146a-5p in the quartiles (left panels) and graphical representation of the mean values and SD (right panels). (D) Graphical representation of the correlation between miR-146b-5p and miR-146a-5p miRNome values in the same 224 CLL cases.
**Supplementary Fig 3:** Kaplan-Meier curves comparing TTFT by computing the quartiles of miR-146b-5p miRNome values per group of *IGHV*-UM and *IGHV*-M CLL cases. Statistical significance of associations between individual variables and survival was calculated using the log-rank test (lower panels). The consistent survival association was observed only within the *IGHV*-UM group.
Supplementary Fig 4: Expression of miR-146b-5p does not change in CLL cells of patients who experienced disease progression. Comparison of miR-146b-5p values in 21 pairs of CLL samples purified from PBMC of patients taken at disease onset and at disease progression. Data expressed are by miRNome values in natural scale. For each group mean ± SD and P-values of the difference of miR-146b-5p expression between CLL cells from CLL patients at disease onset and the same cases at disease progression (Wilcoxon test) or between CLL cells from *IGHV*-M and *IGHV*-UM CLL cases at disease onset are indicated (Mann-Whitney test). Asterisk indicates statistically significant P values (P<0.05).
Supplementary Fig 5: Methylation levels of miR-146b locus in CLL and normal B cells. (A) Six CLL samples were analyzed by WGBS in the Blueprint epigenome data analysis portal. Mean (solid line), minimum and maximum methylation levels (shadow area) in the region encompassing the miR-146b locus are indicated. Genomic coordinates of 500 bp upstream and downstream region to miR-146b single exon/transcript are shown in the figure. (B) The same analysis as in A was performed in three samples from circulating Naïve B cells and in two samples from Memory B cells subsets.
Supplementary Fig 6: Correlation analysis between IL-23R expression and miR-146b-5p. CLL cells from 93 CLL patients were analyzed for the surface expression of both IL-23R and IL-12Rβ1 side chains of the IL-23R complex by flow cytometry as described in the Methods section. Only the IL-23R side chain was expressed in circulating CLL cells as previously described by our group\textsuperscript{15}. The correlation analysis between miR-146b-5p miRNome values and IL-23R percentage positive cells is shown in the figure.
Supplementary Fig 7: Gating strategy to analyze IL-23R complex in CLL cells. (A) CLL cells from a representative case (SR1-ME-1077) were purified by negative selection and analyzed for IL-23R complex expression. 2 CLL cell subpopulations could be distinguished based upon their SSC-A/FSC-A features. Viable cells (live cells) were double negative for Annexin V/PI staining (lower panels). The expression of IL-23R and IL-12Rβ1 side chains of the IL-23R complex was determined within the gate of viable cells (CD19+CD5+cells only after the purification procedure). This gating strategy was applied to all the experiments of cell
transfection with miRNA mimic and inhibitors. (B) The panels show the gating strategy by quadrants to calculate the percentages of cells positive for the IL-23R and/or the IL-12Rβ1 side chains of the IL-23R complex in the same time course experiment shown in Fig 3A (CLL GE1-AG114). The position of the quadrants is based on the background values determined on cells stained with the isotype control antibodies (CTR-FITC and CTR-PE).
Supplementary Fig 8: Relationship between the BCR signaling pathway and miR-146b-5p expression. Purified CLL cell suspensions from three different CLL cases (SR1-ME1077; SV1-BL726; GE1-DM210) were cultured with anti-μ and anti-δ Ig-chain-coated beads, in the presence of IL-4 for the indicated times and investigated for the expression of miR-146b-5p by RT-qPCR. Data are expressed as $2^{-}\Delta\text{dCT}$ and miR-93-5p was used as reference gene No significant differences in the expression of miR-146b-5p were observed during time. Statistical analysis was performed by using the Brown-Forsythe and Welch ANOVA test.
Supplementary Fig 9: Inhibition of IL-23R complex expression but not of IL-21R by enforced expression miR-146b-5p mimic.

(A) Flow cytometric analysis of IL-23 receptor complex expression by CLL cells treated with the indicated miR-mimics and cultured for 72 h with CD40L-TC cells in a representative case (DF0319). (B) Flow cytometric analysis of IL-21 receptor expression by CLL cells treated as in A case DF0319 (C) Comparison of IL-23R complex or IL-21R expression by CLL cells from 6 cases (PF0024, HG0135, SR0112, DF0319, RD0468, PD0164) treated with the indicated miR-mimics and cultured with CD40L-TC cells. Data are expressed as a percentage of positive cells (mean ± SD).
Supplementary Fig 10: Inhibition of TRAF6 and IRAK1 by enforced expression of both miR-146a-5p and miR-146b-5p mimics. Purified CLL cell were transfected with miR-146a-5p or miR-146b-5p or miR-CTR mimics and stimulated in vitro by CD40L-expressing murine NIH-3T3 cell line (CD40L-TC) or with control cells stably transfected with the pIRES vector alone (Mock). (A) Immunoblotting analysis of TRAF6 expression before (T0) and after miRNA transfection and 48 h of culture in the presence of Mock or CD40-L-TC (CLL case GC0015). (B) Percentage inhibition of TRAF6 expression caused by pretreatment with the indicated miRNA mimics. Protein bands from immunoblotting in A were analyzed using ImageJ Analysis Software. Data are presented as TRAF6/Tubulin. (C) Flow cytometric analysis of IRAK1 expression of the same CLL case treated as in A and stimulated for different times. The panels show IRAK1 expression after 72h stimulation, relative fluorescence intensity (RFI) or
percentage of positive CLL cells are indicated. (D) The entire time course experiment displayed in C.
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