SUPPLEMENTAL METHODS

Cell purification, cultures and stimuli

$CD19^+$ B cells were negatively selected from whole blood using the RosetteSep B-cell enrichment kit (StemCell Technologies) following the manufacturer's instructions. The purity of all samples always exceeded 95% for $CD19^+$ cells as revealed by flow cytometry analysis.

The purified leukemic B cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 15 mg/ml gentamicin (Sigma-Aldrich) at 37^oC in a humidified atmosphere containing 5% CO_2. Cells were cultured at a density of 3×10^6 cells/ml in the presence or absence of specific ligands for certain time points, depending on the assay. The ligands used in the cell cultures included: goat F(ab') anti-human IgM (20 mg/ml) (ThermoFisher Scientific) for BcR; soluble CD40L (100 ng/ml) plus enhancer (1 μg/ml) for improved stability and enhanced immune activation (Alexis Biochemicals) of CD40; and, CpG oligonucleotides (2.5 mg/ml) (ODN2006, stimulatory CpG-ODN type B, human specific; Invivogen) for TLR9.

Quantification of EZH2 mRNA expression

Total cellular RNA was isolated from purified B cells cultured in the presence or absence of ligands for the BcR, TLR9 and CD40 with the QIAamp RNA Blood Mini Kit (QIAGEN), including a DNAse incubation step. Quantification of EZH2 mRNA levels was achieved by RQ-PCR, using the specific RT^2 qPCR Primer Assay for the EZH2 human gene (QIAGEN), according to the manufacturer's instructions. Briefly, one microgram of RNA was reverse transcribed to cDNA and a 1:50 aliquot of the RT product was used as the template for RQ-PCR. The ABL gene was used as reference (housekeeping gene). For RQ-PCR experiments all samples were run in triplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ algorithm.

Western blotting

Whole-protein extracts from cells at the basal level as well as treated and untreated cultured cells were run on 10% NuPAGE Bis-Tris gel (Invitrogen) and transferred to PVDF membranes (Invitrogen). The antibodies for p-PLCγ2, EZH2 and H3K27me3 were from Cell Signaling Technology (Leiden, The Netherlands), BD Biosciences (San Jose) and Millipore

(Massachusetts), respectively. To ensure equal loading, membranes were stripped and reprobed with anti-actin and anti-histone H3 antibodies (Sigma-Aldrich and AbCam, respectively). Immunoreactivity was revealed by incubation with goat anti-mouse or rabbit Ig (ThermoFisher Scientific) conjugated with horseradish peroxidase and followed by enhanced chemiluminescence reaction (Pierce). Ratios of each protein band intensity relative to the respective housekeeping gene band intensity were calculated for each sample using the Image J software.

Treatment of CLL cells with signaling and EZH2 pharmacologic inhibitors

Purified $CD19^+$ B cells were cultured as described above in the presence of EZH2 inhibitors, GSK126, GSK343 or signaling inhibitors Ibrutinib (IB) and Idelalisib (IDE) (Selleckchem) or BCL-2 inhibitor, Venetoclax (Biovision). In more detail, cells were treated with DMSO or increasing concentrations of IB or GSK126 for 3 and 6 days for the selection of effective concentrations[1,2]. Also, for selected cases, cells were pre-stimulated as described above in the presence of CpG for 24 hours and then exposed to either single treatment with EZH2 inhibitors (10μM GSK343, 10μM GSK126) or signaling inhibitors (5μM IB, 10μM IDE), or BCL-2 inhibitor (5nM Venetoclax) or combined inhibition with IB/GSK343 or IB/GSK126 or IDE/GSK343 or IB/Venetoclax or GSK126/Venetoclax. Cells were then collected and processed for either immunoblotting or flow cytometry analysis for viability and/or H3K27me3 levels after certain time points. Cell viability assays were also performed using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega Corporation) and measured in a Mithras luminometer, as described in more detail in the **'Cell Viability Assay and Combination Index Calculation'** section in **Supplemental Methods**.

Flow cytometry studies

Intracellular calcium flux was measured by the fluorogenic probe Fluo-3, AM (Invitrogen) as reported previously[3,4]. Patients were classified as responders when the percentage of cells responding to anti-IgM stimulation was >5%, as previously described[5]. Cell viability was measured using 7-Aminoactinomycin D (7-AAD) (Beckman Coulter) or double staining for Annexin V and Propidium Iodide (PI) using the FITC Annexin V Apoptosis Kit (Thermo Scientific).

For concomitant analysis of viability and H3K27me3 levels, cells were washed with PBS and stained with BD Horizon Fixable viability stain 660 (BD Biosciences) for 10 min. The excess stain was washed off with PBS and cells were fixed with formaldehyde of a final concentration of 4%, for 10 minutes at 37^{o}C. Samples were cooled off on ice for one minute, washed with PBS and incubated again on ice with methanol for 30 minutes. Cells were washed twice with incubation buffer containing 0.5% BSA in PBS prior to the addition of the conjugated antibodies, Alexa Fluor® 488 Rabbit H3K27me3 and Alexa Fluor®488 Rabbit IgG XP® Isotype Control (Cell Signaling). The same procedure was followed also for the analysis of Bcl-2, Bcl-xl, Mcl-1, cleaved PARP and cleaved Caspase 3, using respective PE-conjugated antibodies (Cell Signaling). For all experiments data was acquired on a BD FACSCalibur flow cytometer (BD Bioscience). Data analysis was performed using Flow Jo Software (Tree Star) and Kaluza® Analysis software (Beckman Coulter).

Cell Viability Assay and Combination Index Calculation

Cell viability assays were performed in order to evaluate the effects of either single *ex vivo* treatment with IB or GSK126 in increased concentrations of 1, 5 and 10μM or combined treatment of these drugs at the above doses in a 1:1 ratio; all analyses were conducted after 72 hours of incubation. Viability of CLL cells was measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega Corporation), according to the manufacturer's instructions. This method determines the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Cells were seeded in a 96-well plate suitable for luminescence measurements, at a density of 3 x 10^6 cells/ml. Seventy-two hours after seeding, equal volume of cultured cells in medium was mixed with CellTiter-Glo® Buffer and incubated in the dark and RT for 10 min. A Mithras luminometer was used for the measurement of luminesence.

The values obtained from the luminometer were used for the determination of potential drug synergism using the method of Chou and Talalay implemented in the software package CompuSyn (Biosoft, Cambridge, UK)[6]. A Combination Index (CI) of less than one (CI<1) indicates synergy.

The same procedure was also performed for treatments with GSK126 and Venetoclax.

Co-culture conditions

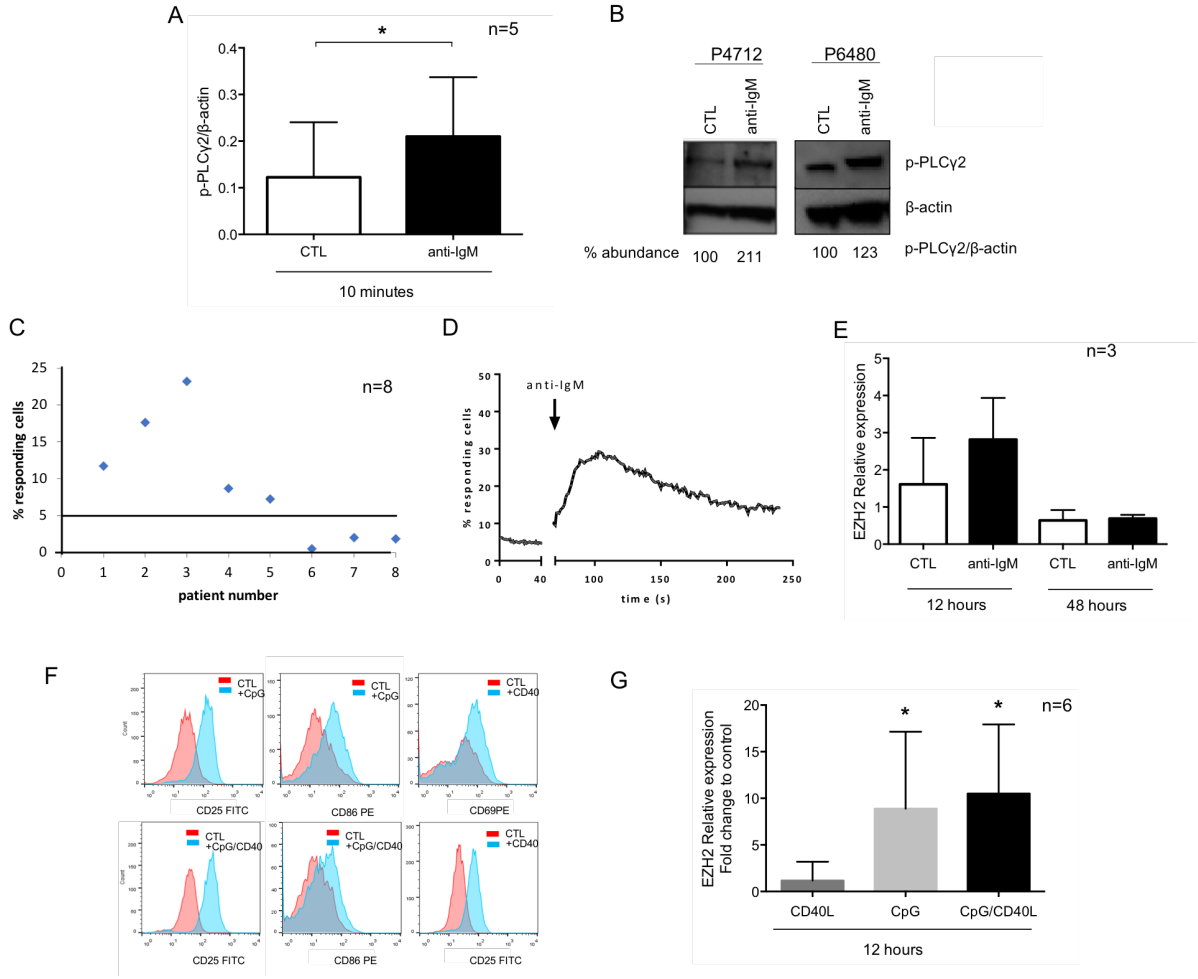
Human bone marrow stromal HS-5 cells were seeded at a concentration of 200 x 10^3 cells/per well in 12-well plates and incubated for 24 hours to allow cells to adhere. Purified CLL cells were then added to the culture, on confluent layers of stromal cells at a ratio of 15:1 and treated with DMSO (control-treated) or the EZH2 inhibitor GSK126 (10μM) for 3 days. CLL cells were harvested by gentle pipetting, leaving the adherent stromal cell layer intact. Flow cytometry analysis was then performed for assessing viability, EZH2 and H3K27me3 levels.

Statistical analysis

Descriptive statistics for quantitative variables included statistical measures like mean and SD. Significance of bivariate relationships between factors and variables normally distributed was assessed with the use of Student t test or Mann Whitney test. For all comparisons, a significance level of $p \leq 0.05$ was set. All statistical analyses were performed with the use of the GraphPad Prism 5 software (La Jolla).

SUPPLEMENTAL FIGURES AND LEGENDS

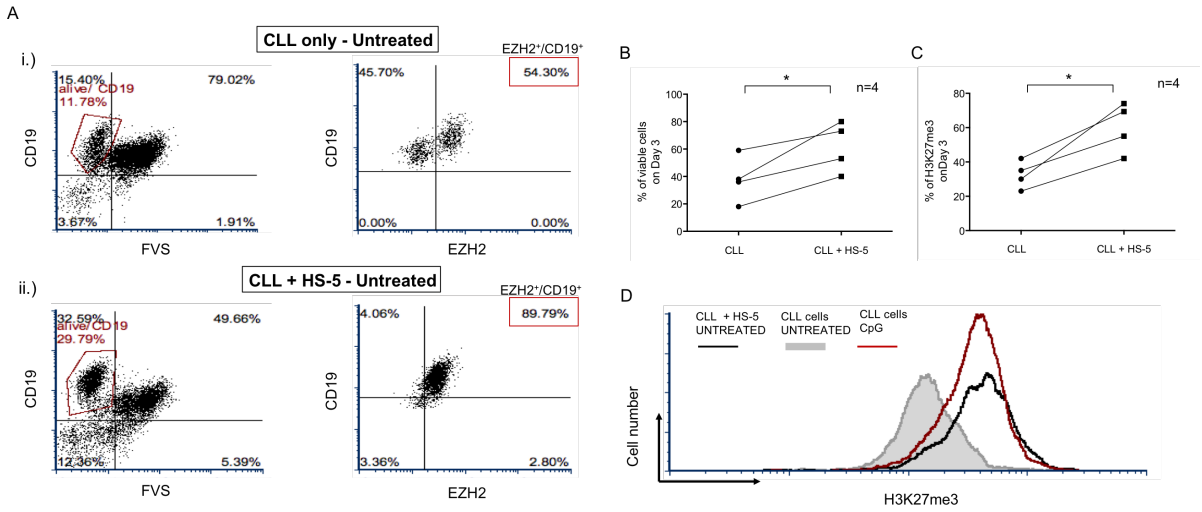
Supplemental Figure 1



Supplemental Figure 1: EZH2 expression and external stimuli in CLL. CLL cases that were capable of responding to activation with anti-IgM *in vitro* (10/13 cases tested; 9 with unmutated and 1 with mutated IGHV genes) were identified by analysis of phosphorylation status of PLCγ2 by western blot and intracellular Ca^{2+} release by flow cytometry. **A**. Increase of PLCγ2 phosphorylation status (n=5, fold change (FC)=1.7, $p<0.05$) upon stimulation with anti-IgM for 10 minutes. Each bar depicts the mean values with Standard Deviation (SD) of p-PLCγ2 normalized to β-actin. **B**. Western blotting analysis of p-PLCγ2 of two representative responsive cases. **C-D.** Leukemic cells from 8 CLL patients were labelled with the calcium-sensitive dye Fluo-3–AM and analyzed by flow cytometry before and after BcR stimulation with anti-IgM for 10 minutes. **C.** The graph shows individual data points for each case, and the cut off value for positivity (dotted horizontal line, 5%). **D.** Analysis of flow cytometry

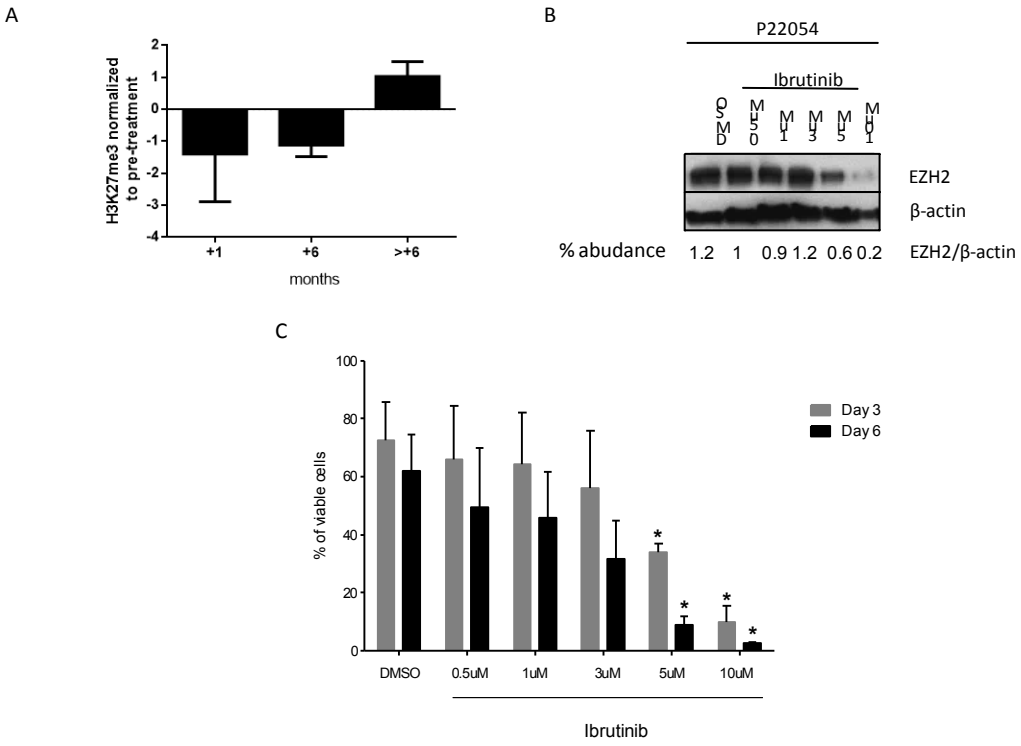
data for one representative responsive case. **E.** Kinetics analysis of EZH2 mRNA expression after BcR stimulation, using RQ-PCR. The bars show the mean values with SD of EZH2 mRNA levels from 3 CLL cases stimulated or not through the BcR for 12 and 48 hours. EZH2 gene expression was upregulated (FC=1.8) after 12 hours; the observed effect was dampened at 48 hours. No significant upregulation of EZH2 mRNA levels was detected, thus we focused our attention on the 12-hour timepoint. **F.** Flow cytometric analysis of B cell activation markers (CD25, CD86, CD69) in cells stimulated with CpG, CD40 or CpG/CD40 for 24 hours in one representative CLL case. **G.** Each bar in the graph shows the mean values of FC of EZH2 relative expression (as analyzed by RQ-PCR) in cells stimulated with CD40L and/or CpG for 12 hours, normalized to unstimulated-control cells. Asterisks indicate significant differences compared to the unstimulated control (n=6). *$p<0.05$

Supplemental Figure 2



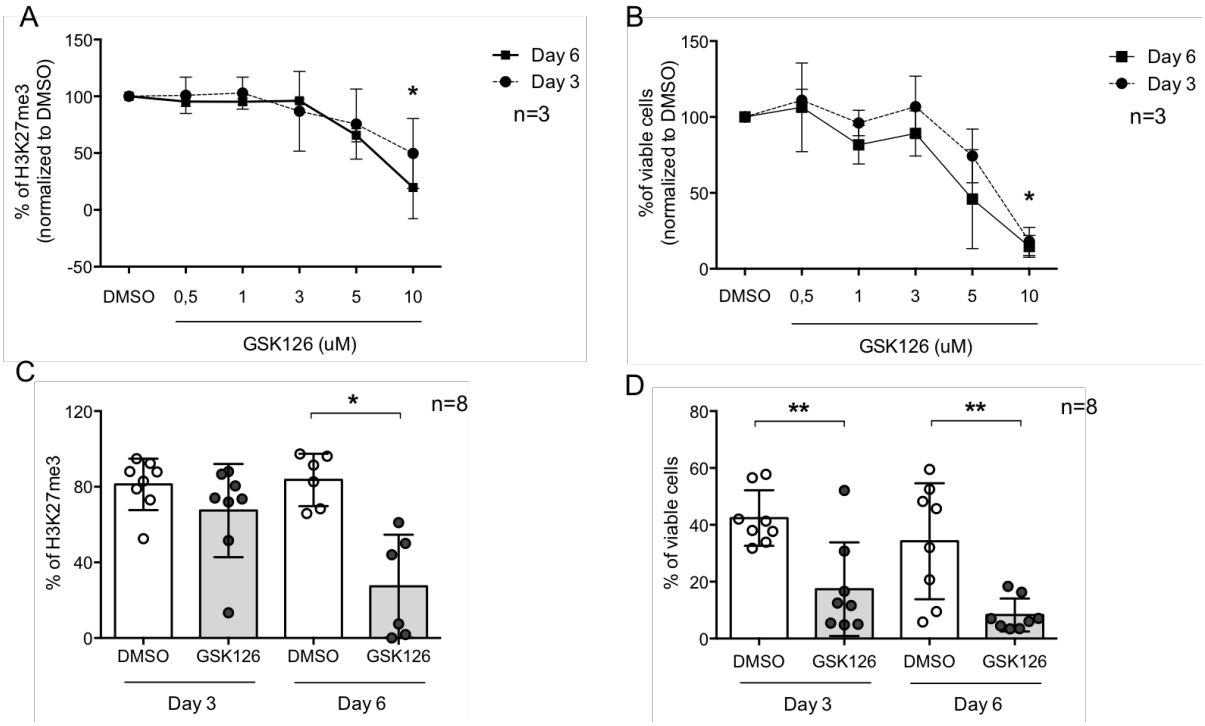
Supplemental Figure 2: **EZH2 expression and function is increased in a stromal co-culture system.** Effects of incubation of CLL cells alone or co-cultured with the stromal HS-5 cells for 3 days on **A.** EZH2 expression, **B.** CLL cell viability and **C.** H3K27me3 levels. **A.** Dot plot representing in the left panel the alive/CD19^{+} gated cells and in the right panel the positive fraction for EZH2/CD19 cells for 3-day cultures of (i) untreated CLL cells; and, (ii) untreated CLL + HS-5 cells in one representative case, using flow cytometry. **B, C.** Cell viability analysis (**B**) and H3K27me3 levels (**C**) at day 3, using flow cytometry. In the graph, connected points represent the percentage of viable cells or H3K27me3 levels in CLL cells alone and CLL + HS-5. **D.** Single parameter histogram showing H3K27me3 levels of the alive/CD19^{+} cells cultured in the following conditions: CLL cells - untreated, CLL cells stimulated with CpG, CLL + HS-5 cells - untreated, in one representative case, using flow cytometry. *p<0.05

Supplemental Figure 3



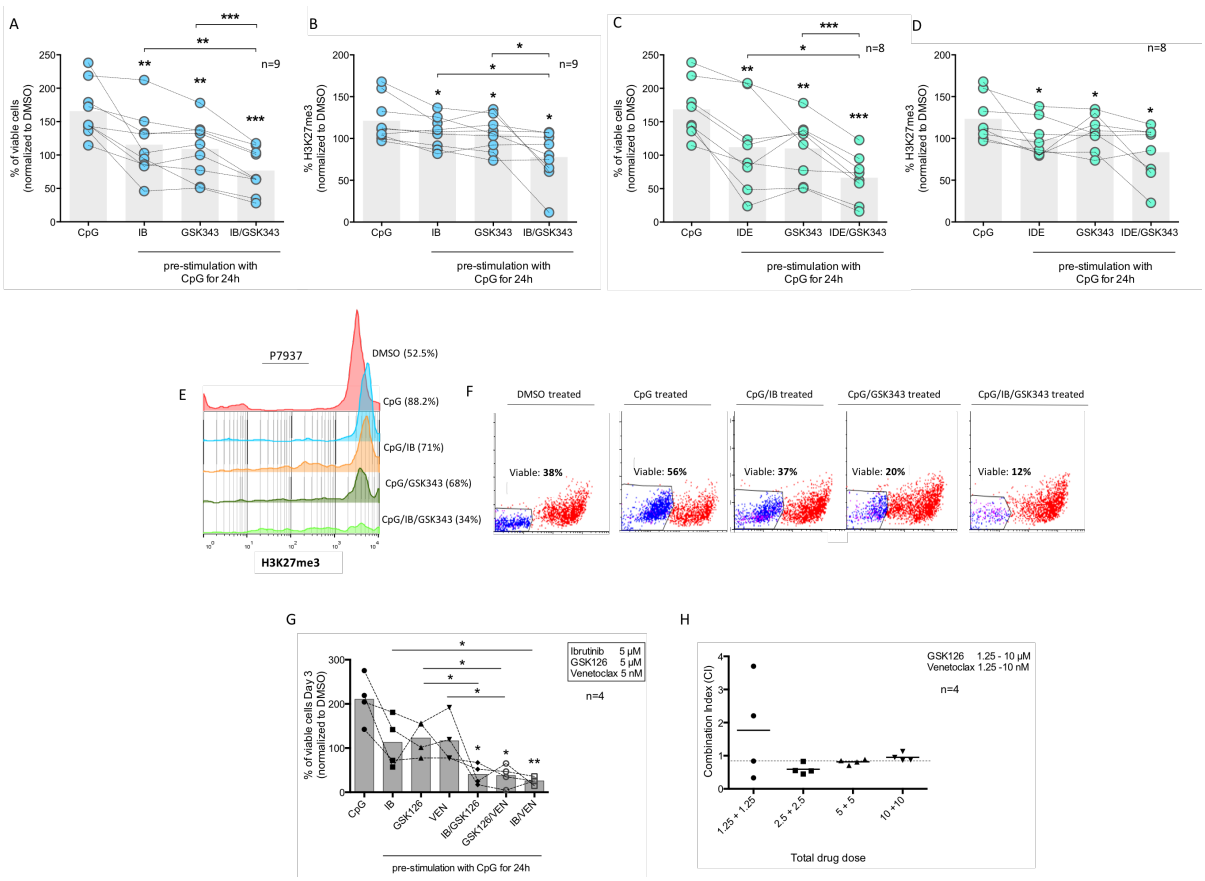
Supplemental Figure 3: **Ibrutinib treatment modulate EZH2 function and selection of effective concentration of Ibrutinib in primary CLL cells. A.** CD19^{+} cells from patients before treatment initiation with ibrutinib (IB) and at +1, +6 and >+6 months under treatment were lysed for western blotting analysis of H3K27me3 levels**.** Each bar in the graph represents the median values with standard deviation (SD) of H3K27me3/H3 levels normalized to the pre treatment sample (n=9; p=0.07). **B, C.** We treated cells from 3 CLL cases with increasing concentrations of IB for 3 days and by analyzing EZH2 protein expression and cell viability we selected 5uM as the most effective concentration for IB to be used in following experiments. **B.** Western blotting analysis for EZH2 protein expression after treatment with increasing concentrations of IB in one representative case. **C.** The bars in the graph show the mean values with SD of the percentage of viable cells from three cases, treated with increasing concentrations of IB normalized to control cells (DMSO-treated) after 3 and 6 days in culture, using flow cytometry. *p<0.05

Supplemental Figure 4



Supplemental Figure 4: **Pharmacological inhibition of EZH2 catalytic activity with GSK126.** Cells from 3 CLL cases were treated with increasing concentrations of the pharmacological inhibitor of EZH2, GSK126, for 3 and 6 days and by analyzing H3K27me3 levels and cell viability we selected 10 µM, as the most effective concentration to be used in our following experiments. **A, B**. Mean percentage of H3K27me3 levels **(A)** and cell viability (**B**) of CLL cells from 3 cases treated with increasing concentrations (0.5, 1, 3, 5 and 10 µM) of GSK126 or control cells (DMSO-treated), after 3 and 6 days in culture, using flow cytometry. **C-D.** Effects of treatment with 10 µM GSK126 on CLL cells from 8 cases after 3 and 6 days. In the graphs the scatter plot shows the percentage of H3K27me3 levels (**C**) or viable cells (**D**) and the bars correspond to the mean values. *$p < 0.05$, **$p < 0.01$

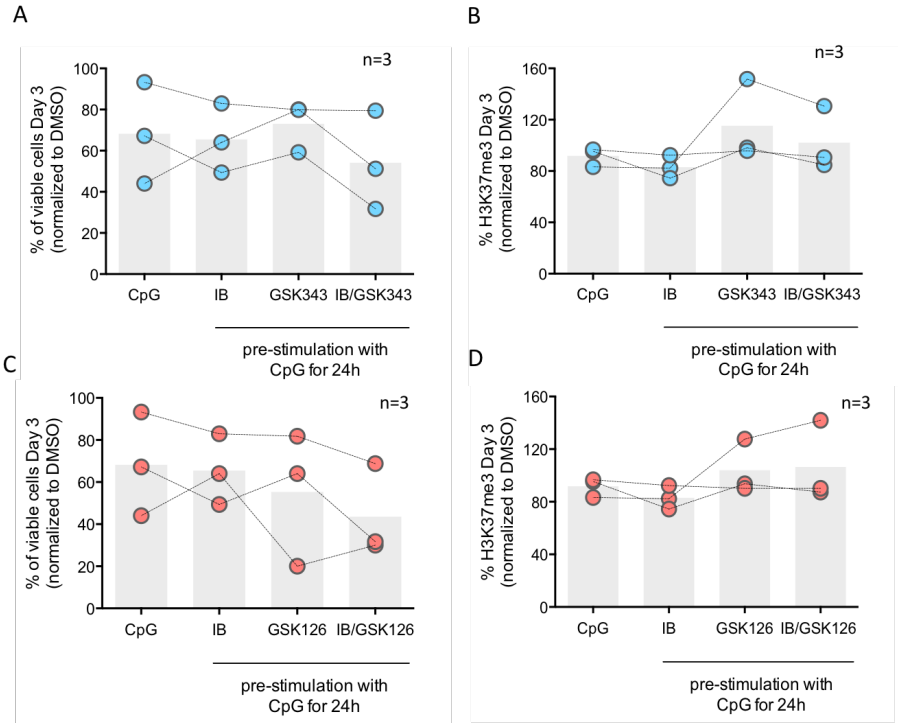
Supplemental Figure 5



Supplemental Figure 5: **Combined treatment of signaling and EZH2 inhibitors in primary CLL cells. A, C.** Similar effects with IB vs IB/GSK126 treatment, were also observed in incubation with CpG for 24 hours following single or combined treatment of 5 µM IB or 10µM Idelalisib (IDE) with 10 µM GSK343 for 3 days on CLL cell viability (FC=1.5, $p<0.01$; n=9 for IB vs IB/GSK343 and FC=1.7, $p<0.05$; n=8 for IDE vs IDE/GSK343). **B, D.** Concordant effects were also observed on H3K27me3 levels, using flow cytometry. In the graphs connected points represent the percentage of viable cells or H3K27me3 levels for each case in all conditions described, normalized to control cells (DMSO-treated). The bars in the graphs show the mean values. Asterisks above bars indicate significant differences compared to CpG-treated cells. **E**. Single parameter histogram showing H3K27me3 levels for one representative case after the indicated treatments for 3 days. **F**. Dot plot representing CLL cells stained with fixable viability dye (FVS) for one representative case. The negative fraction of cells for FVS represent the percentage of viable cells, using flow cytometry. **G.** Effects of incubation with CpG for 24 hours following single or combined treatment of 5 µM

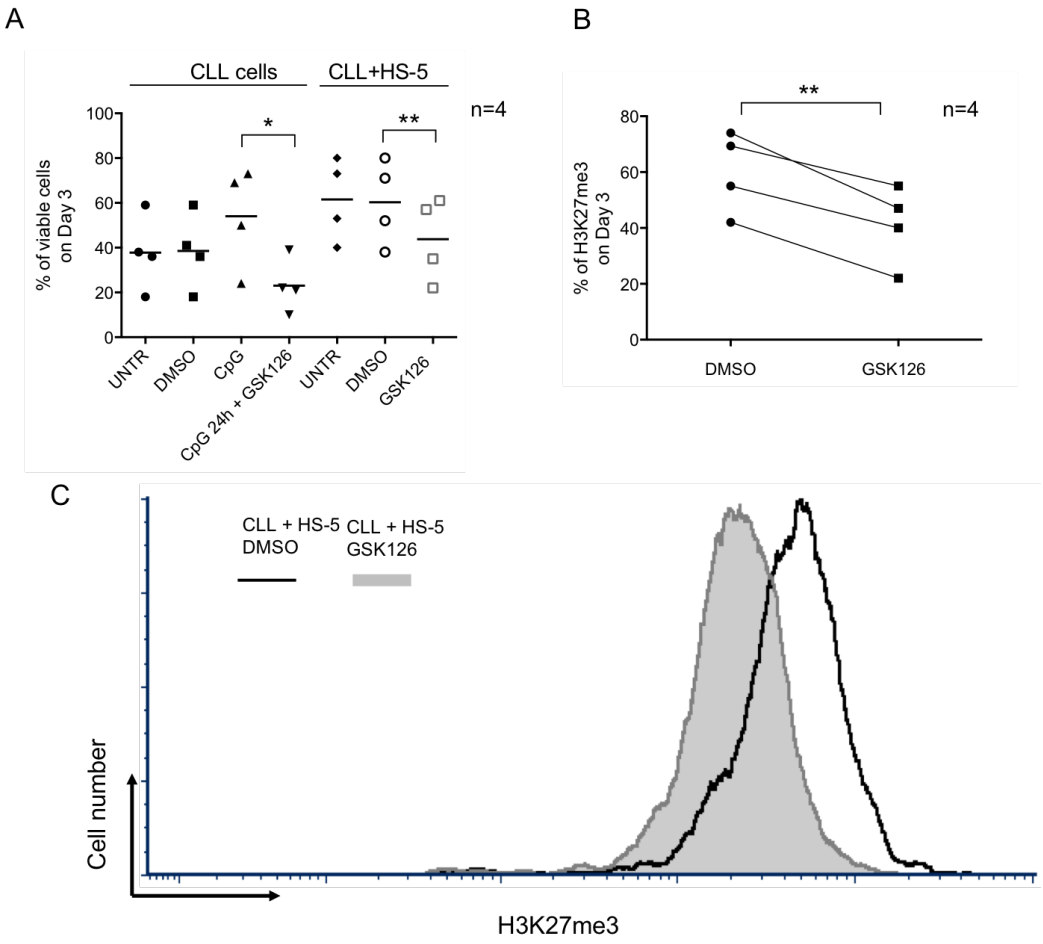
GSK126 and/or 5 nM Venetoclax and/or 5 μM IB for 3 days on CLL cell viability as measured by a Mithras luminometer. In the graphs connected points represent the percentage of viable cells for each case in all conditions described, normalized to control cells (DMSO-treated). The bars in the graphs show the mean values. The asterisks above bars indicate significant differences compared to CpG-treated cells. **H.** Cells from 4 CLL cases were assessed for cell viability after pre-stimulation with CpG for 24 hours, followed by single GSK126 (1.25, 2.5, 5 and 10 μM) or Venetoclax (1.25, 2.5, 5 and 10 nM) treatment or combined treatment with these drugs at a 1:1 ratio at the aforementioned doses for a total of 72 hours, as measured by a Mithras luminometer. The interaction between GSK126 and Venetoclax was synergistic (combination index, CI values <1) in primary CLL cells for the combination of 2.5 μM + 2.5 nM, 5 μM + 5 nM and 10 μM + 10 nM (GSK126 + Venetoclax). In the graph the black line corresponds to the mean values of CI of 4 CLL cases (n=4). *p<0.05, **p<0.01 and ***p<0.001**.**

Supplemental Figure 6



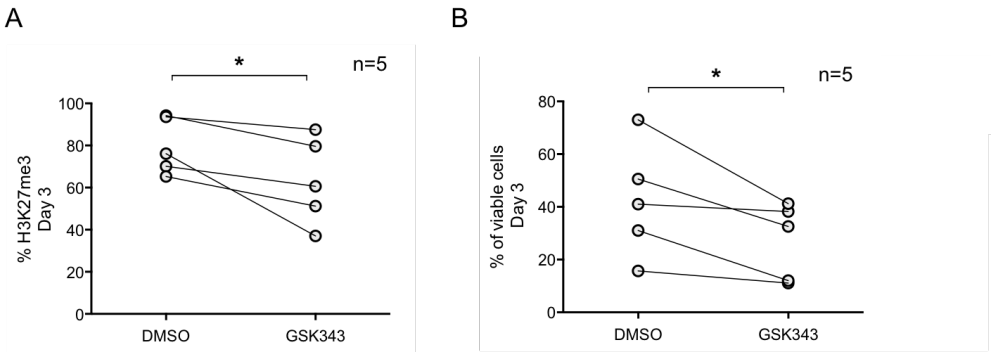
Supplemental Figure 6: **Combined treatment of immune signaling and EZH2 inhibitors in EZH2^{low} and unresponsive to TLR9 stimulation cases.** Effects of incubation with CpG for 24 hours following single or combined treatment of 5 µM Ibrutinib (IB) with EZH2 inhibitors, 10 µM GSK343 or 10 µM GSK126 for 3 days on CLL cell viability **(A, C)** and on levels of H3K27me3 **(B, D)** using flow cytometry. In the graphs connected points represent the percentage of viable cells or H3K27me3 levels in all conditions described, normalized to control cells (DMSO-treated). The bars in the graphs show the mean values of viable cells or the H3K27me3 levels normalized to control cells (DMSO-treated) (n=3).

Supplemental Figure 7



Supplemental Figure 7: **EZH2 inhibition with GSK126 in CLL cell monocultures after pre-stimulation with CpG and in a stromal co-culture system.** Effects of incubation of either CLL cells cultured alone as previously described, with CpG for 24 hours following treatment with 10 µM GSK126, or CLL cells co-cultured with stromal HS-5 in the presence or absence of 10 µM GSK126 on **A.** CLL cell viability after 3 days, analyzed with annexin V/PI double staining (FC=2.3, $p<0.05$; n=4 for CLL cells alone and FC=1.5, $p<0.01$; n=4 for CLL cells + HS-5) and in **B.** H3K27me3 levels, using flow cytometry. In the graph, connected points represent the percentage of H3K27me3 levels in two conditions, GSK126-treated cells and control cells (DMSO-treated) (FC=1.5, $p<0.01$; n=4) **C.** Single parameter histogram showing H3K27me3 levels of the live cells in the co-culture system after 3 days in the indicated conditions for one representative case, using flow cytometry. *$p<0.05$, **$p<0.0$

Supplemental Figure 8



Supplemental Figure 8: Effects of incubation with 10 µM of the EZH2 inhibitor, GSK343 for 3 days on levels of H3K27me3 **(A)** and on CLL cell viability **(B)** using flow cytometry. In the graphs connected points represent the percentage of H3K27me3 or viable cells treated with GSK126 or DMSO-treated control cells (n=5). *$p < 0.05$

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