B-cell antigen receptor expression and phosphatidylinositol 3-kinase signaling regulate genesis and maintenance of mouse chronic lymphocytic leukemia

Vera Kristin Schmid,[1] Ahmad Khadour,[1] Nabil Ahmed,[2] Carolin Brandl,[3] Lars Nitschke,[3] Klaus Rajewsky,[4] Hassan Jumaa[1] and Elias Hobeika[1]

[1]Institute of Immunology, University Medical Center Ulm, Ulm; [2]Uniklinik RWTH Aachen, Aachen; [3]Division of Genetics, Department of Biology, Friedrich Alexander University Erlangen-Nürnberg, Erlangen and [4]Max Delbrück Center for Molecular Medicine, Berlin, Germany

Correspondence:

Elias Hobeika
elias.hobeika@uni-ulm.de

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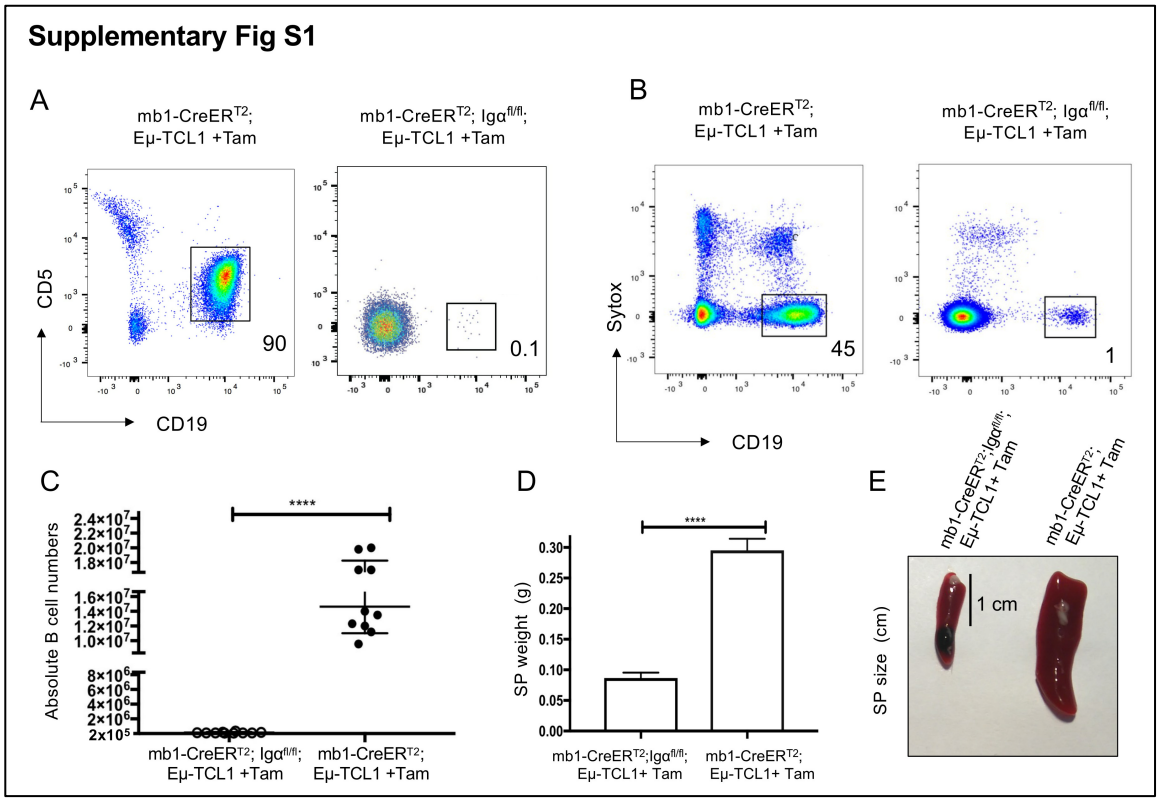
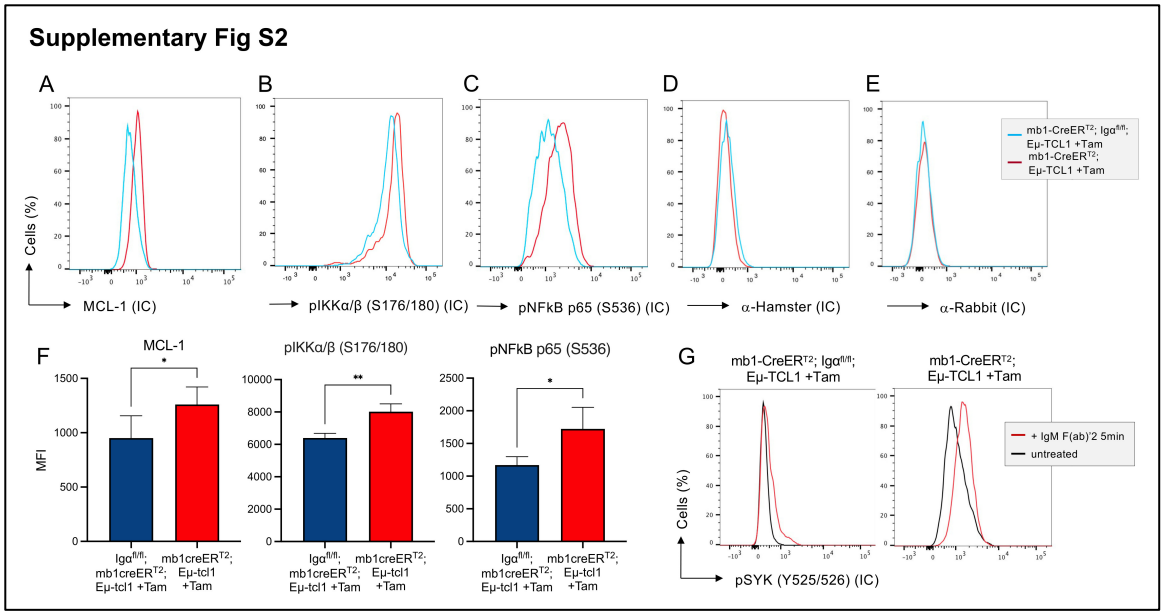
Supplementary Data

1) Supplementary Figures

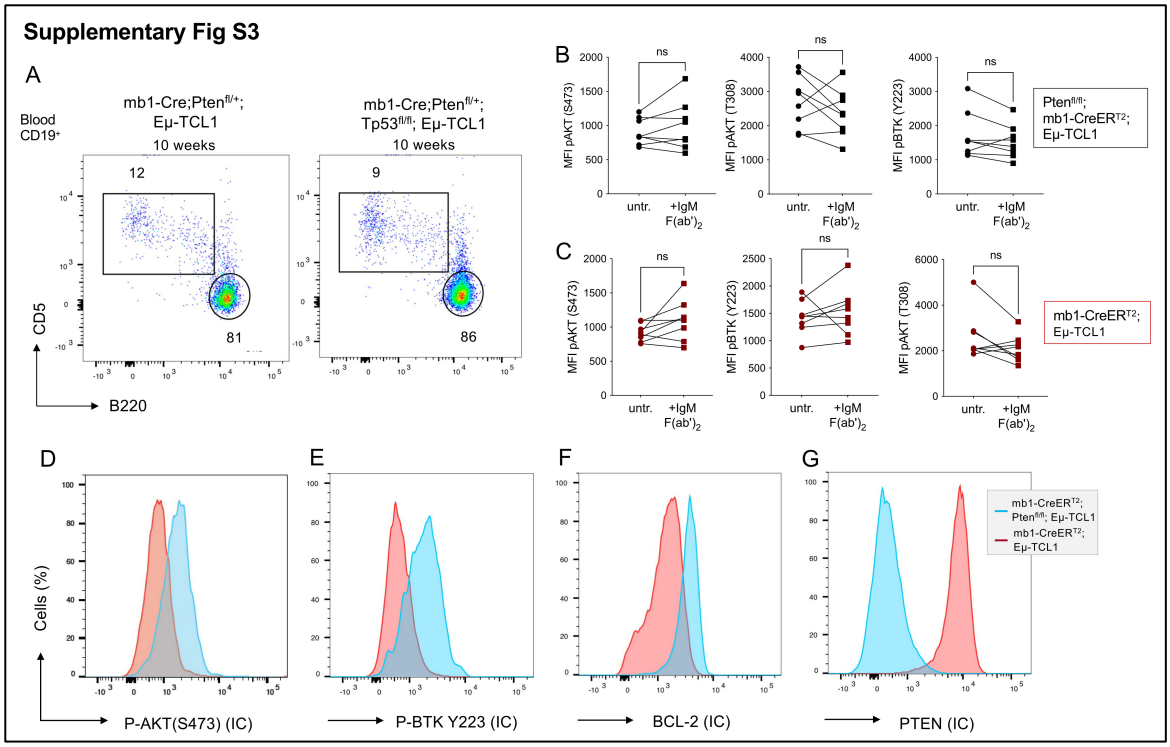


Supplementary Fig S1. Loss of CLL cells after inducible Igα-inactivation
Flow cytometric analysis of B cells 8 weeks after Tamoxifen-treatment from the peripheral blood (PBL) **(A)** and bone marrow (BM) **(B)** of mb1-ER^{T2};Eμ-TCL1 control mice (left) and mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 mice (right) after Tam-treatment as described in the Materials and Methods section. Shown are dot plots of the anti-CD19 vs anti-CD5 **(A)** or anti CD19 vs dead cells **(B)**. The numbers in the dot plots indicate the mean relative frequency of cells in the gate. **(C)** Statistical analysis of absolute cell numbers of CLL cells from the BM 8 weeks after Tam-treatment: empty circles indicating cells obtained from mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 mice (left) and filled circles from mb1-CreER^{T2};Eμ-TCL1 mice (right). Four asterisks (****) indicate $p < 0.0001$, P-values were obtained using two-tailed Student's t-test. Cell numbers of 10 mice per group are shown with each circle representing an individual animal. **(D)** Spleens of Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice at day 10 after transfer with mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 (left) and mb1-CreER^{T2};Eμ-TCL1 control (right) CLL cells +Tam. **(E)** Quantification of the SP sizes obtained from Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice transferred with mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 (left bar) or mb1-CreER^{T2};Eμ-TCL1 (right bar) mouse CLL-B cells +Tam. Graphs are presented as mean ± SEM. Four asterisks (****) indicate $p < 0.0001$, P-values were obtained using two-tailed Student's t-test. Cell numbers of five mice per group are shown.



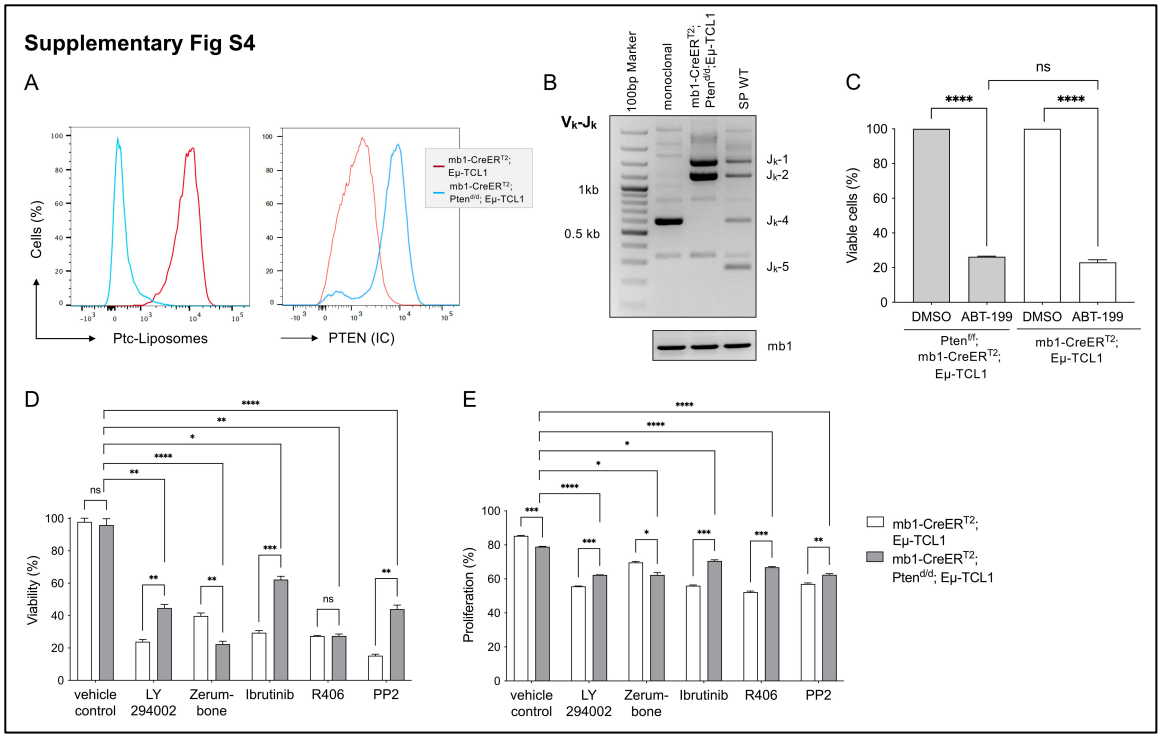
Supplementary Fig S2. NFκB signaling and MCL-1 expression is downregulated in CLL cells after induced Igα-deletion

(A-E) Flow cytometric analysis of splenic CLL cells from mb1-CreER^{T2};Eμ-TCL1 (red line) and mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 (blue line) mice 10 days after Tam-treatment. Shown are histograms indicating the fluorescence intensity of **(A)** MCL-1 expression, **(B)** phosphorylation of IKKα/β (S176/180), **(C)** phosphorylation of NFκB p65 (S536) and the secondary antibody **(D)** anti-hamster and **(E)** anti-rabbit. **(F)** Quantification of the intracellular mean fluorescence intensity (MFI) in splenic mouse CLL B cells from mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 (blue filled bars) and mb1-CreER^{T2};Eμ-TCL1 (red filled bars) mice. MFIs of MCL-1 (left), pIKKα/β (S176/180) (middle) and pNFκB p65 (S536) (right) are shown. Graphs are presented as mean ± SEM. P-values were obtained using the two-tailed Student's t-test (* $p < 0.05$; ** $p < 0.01$). Results from four mice per group are shown. **(G)** Flow cytometric analysis of splenic CLL cells from mb1-CreER^{T2};Eμ-TCL1 (right) and mb1-CreER^{T2};Igα$^{fl/fl}$;Eμ-TCL1 (left) untreated (black) or stimulated (red) with anti-IgM F(ab)'2 for 5 minutes. Shown are histograms indicating the fluorescence intensity of pSYK (Y525/526) before and after IgM stimulation.



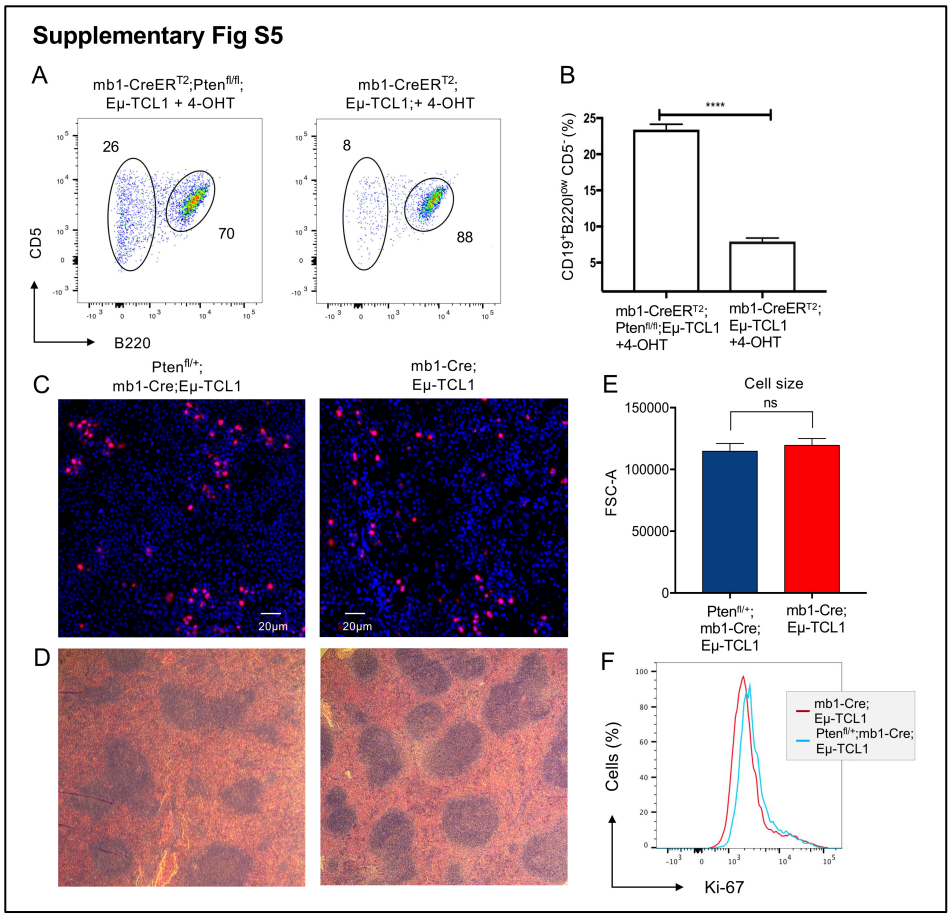
Supplementary Fig S3. AKT-Phosphorylation and BCL-2 expression are increased in PTEN-deficient CLL cells

(A) Flow cytometric analysis of B cells isolated from the blood of mb1-Cre;Pten$^{f/+}$;Eμ-TCL1 and mb1-Cre;Pten$^{f/+}$;Tp53$^{f/f}$;Eμ-TCL1 mice at the age of 10 weeks. The graph shows dot-plots of the anti-B220 vs anti-CD5 staining after gating on CD19^{+} B lymphocytes. The numbers in the dot plots indicate the mean relative frequency of cells in the gate. **(B-C)** Before-after plot of the mean fluorescence intensities of AKT-phosphorylation (S473), (T308) and BTK-phosphorylation (Y223) untreated and after stimulation of mb1-CreER^{T2};Pten$^{fl/fl}$;Eμ-TCL1 **(B)** and mb1-CreER^{T2};Eμ-TCL1 **(C)** splenic CLL cells with 10 μg/ml anti-IgM F(ab)'2 fragments. Statistical analysis was done using the two-tailed Student's t-test (ns = not significant). Analysis of 7 mice per group are shown with each circle/rectangle representing an individual animal. **(D-G)** Flow cytometric analysis of **(D)** AKT-phosphorylation at (S473), **(E)** BTK-phosphorylation at (Y223), **(F)** BCL-2 and **(G)** PTEN expression in splenic CLL cells (CD19^{+} CD93^{-} B220^{low} CD5^{+}) from Tam-treated mb1-CreER^{T2};Eμ-TCL1 (red lines) and mb1-CreER^{T2};Pten$^{fl/fl}$;Eμ-TCL1 mice (blue lines).



Supplementary Fig S4. Autonomously growing PTEN-deficient CLL-like cells in culture are oligoclonal and still dependent on BCR signaling

(A) Flow cytometric analysis of BCR binding Phosphatidylcholine (Ptc) (left) and PTEN expression (right) on splenic CLL culture cells (CD19^{+} B220^{low} CD5^{+}) from Tam-treated mb1-CreER^{T2};Pten$^{d/d}$;Eμ-TCL1 (blue line) and mb1-CreER^{T2};Eμ-TCL1 mice (red line). **(B)** Analysis of Vκ-Jκ at the Igκ locus in genomic DNA (gDNA) from the mb1-CreER^{T2};Pten$^{d/d}$;Eμ-TCL1 cells in comparison to gDNA isolated from a monoclonal B cell line and wt splenic B cells. PCR on the *mb1* gene shows the quantification of the DNA from the different samples. **(C)** Quantification of the relative cell count of (CD19^{+} B220^{low} CD5^{+}) splenic mouse B cells from mb1-CreER^{T2};Pten$^{f/f}$;Eμ-TCL1 (grey bars) and mb1-CreER^{T2}; Eμ-TCL1 (white bars) mice treated with 10 μM ABT-199 (venetoclax) or the vehicle control DMSO. DMSO-treated cells were set to be 100. Graphs are presented as mean ± SEM. Four asterisks (****) indicate $p < 0.0001$, (ns) indicates not significant. P-values were obtained using two-tailed Student's t-test. Results from 3 mice per group are shown. **(D)** Survival analysis of mb1-CreER^{T2};Pten$^{d/d}$;Eμ-TCL1 (grey bars) and mb1-CreER^{T2};Eμ-TCL1 (white bars) CLL-like culture cells 48h after treatment with the vehicle control (DMSO) or the inhibitors LY294002 (10μM), Zerumbone (10μM), Ibrutinib (1μM), R406 (5μM) and PP2 (10μM). Graphs are presented as mean ± SEM; bars indicate the percentage of survived cells normalized to vehicle control (100%). P-values were obtained using two-tailed Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Three technical replicates per group are shown. **(E)** Proliferation analysis of mb1-CreER^{T2};Pten$^{d/d}$;Eμ-TCL1 (grey bars) and mb1-CreER^{T2};Eμ-TCL1 (white bars) CLL-like culture cells 48h after treatment with the vehicle control (DMSO) or the inhibitors LY294002 (10μM), Zerumbone (10μM), Ibrutinib (1μM), R406 (5μM) and PP2 (10μM). Graphs are presented as mean ± SEM; bars indicate the percentage of proliferating cells. P-values were obtained using two-tailed Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Three technical replicates per group are shown.



Supplementary Fig S5. Heterozygous *Pten* deletion in mb1-Cre;Pten$^{f/+}$;Eμ-TCL1 mice does not lead to Richter's transformation

(A) Flow cytometric analysis of B220-purified, (not to include B1 cells which are B220 low) B cells from the spleens of mb1-CreER^{T2};Pten$^{fl/fl}$;Eμ-TCL1 (left) or mb1-CreER^{T2};Eμ-TCL1 (right) cultured *ex vivo* for 10 days after treatment with Tam. Shown are dot plots of the anti-B220 vs anti-CD5 staining after gating on CD19^{+} B cells. The gated regions in the dot plots correspond to CLL cells (CD19^{+} B220^{low} CD5^{+}) and healthy mature B cells (CD19^{+} B220^{+} CD5^{-}). The numbers in the dot-plots indicate the mean relative frequency of the cells in the gate. **(B)** Quantification of the relative numbers of (CD19^{+} CD93^{-} B220^{low} CD5^{+}) cells generated *ex vivo* from mb1-CreER^{T2};Pten$^{fl/fl}$;Eμ-TCL1 (left bar) or from mb1-CreER^{T2};Eμ-TCL1 (right bar), splenic B cells purified by CD19 beads and treated in culture with Tam. Graphs are presented as mean ± SEM. Four asterisks (****) indicate $p < 0.0001$, P-values were obtained using two-tailed Student's t-test. Results from five mice per group are shown. **(C)** Immunohistochemical analysis of diseased mb1-Cre;Pten$^{fl/+}$;Eμ-TCL1 and mb1-CreER^{T2}; Eμ-TCL1 splenic sections stained for phosphorylated histone H3 (PHH3) (red) and DAPI (blue). **(D)** Hematoxylin and eosin (H&E) staining of formalin-fixed and paraffin-embedded splenic sections of diseased mb1-Cre;Pten$^{fl/+}$;Eμ-TCL1 and mb1-Cre; Eμ-TCL1 mice. Pictures were captured using a light microscope with 5x magnification. **(E)** Quantification of the FSC-A flow cytometric analysis of mb1-Cre;Pten$^{fl/+}$;Eμ-TCL1 (blue) and mb1-Cre; Eμ-TCL1 (red) splenic CLL cells indicating the size of the cells. Graphs are presented as mean ± SEM. P-values were obtained using two-tailed Student's t-test (ns = not significant). Results from five mice per group are shown. **(F)** Flow cytometric analysis of mb1-Cre;Pten$^{fl/+}$;Eμ-TCL1 (blue) and mb1-Cre; Eμ-TCL1 (red) splenic CLL cells showing the fluorescence intensity of the proliferation marker Ki-67.

2) Supplemental Methods

Mouse analysis

To monitor malignant progression of CLL in Eμ-TCL1 mice, we used 10 to 12-months old mice and immunostained purified PBLs using fluorescent Abs against mouse CD19, B220, CD5, IgM, IgD. We analyzed $B220^{low}/CD5^{+}$ CLL cells on gated $CD19^{+}/IgM^{+}$ B-cell populations of Eμ-TCL1 mice, and confirmed that increased numbers of CLL cells were positively correlated with age of Eμ-TCL1 mice when compared to PBL B cells from control mice. To purify CLL cells, we sacrificed 10-month old mice with clear CLL presentation and purified CLL cells from spleens by staining with CD19 Micro Beads and performing positive selection using MACS columns. We consistently obtained a cell population containing 98% $CD5^{+}$ CLL cells.

Treatment of mice with anti-IL-7R Ab

The mb1-$CreER^{T2}$ and mb1-$CreER^{T2}$; $Ig\alpha^{fl/fl}$ mice were injected intraperitoneally (i.p.) with 1 mg of anti-IL-7R-blocking Ab (rat anti-mouse, clone A7R34, BXCell) 3× every 3rd day and then treated 5× every 3rd day with Tamoxifen-citrate (Tam) by oral gavage. The mice remained under anti-IL-7R treatment 1× with 1 mg every week till 8 weeks after Tam treatment, when lymphocyte subpopulations in the spleen, LN, and PC, PBL were harvested and analyzed by flow cytometry. mb1-$CreER^{T2}$;Eμ-TCL1 or mb1-$CreER^{T2}$;$Ig\alpha^{fl/fl}$;Eμ-TCL1 mice as well as transferred mice were only treatment with Tamoxifen with no additional anti-IL7R treatment not to interfere with T cells survival.

Tamoxifen treatment

Generally, mice were treated 5× every second day by gavage of 6 mg Tam (Ratiopharm) dissolved in 20% ClinOleic (Baxter) and sacrificed 8 weeks after the beginning of the treatment. Specifically, mb1-$CreER^{T2}$;$Ig\alpha^{fl/fl}$ mice were treated 5× with Tam every third day for 2 weeks.

mb1-$CreER^{T2}$;$Ig\alpha^{fl/fl}$;Eμ-TCL1 and mb1-$CreER^{T2}$;Eμ-TCL1 control mice were treated 5× with Tam every third day and then 1× every week. The mice were sacrificed 8 weeks after the beginning of the Tam-treatment.

mb1-$CreER^{T2}$;$Pten^{fl/fl}$;Eμ-TCL1 mice, mb1-$CreER^{T2}$;$Tp53^{fl/fl}$; Eμ-TCL1, and mb1-$CreER^{T2}$;Eμ-TCL1 control mice were treated at a young age (8 weeks) before the outbreak of mouse CLL 3× with Tam every third day.

For the intracellular flow cytometric detection of PTEN and p53 the mb1-$CreER^{T2}$;$Pten^{fl/fl}$;Eμ-TCL1 mice, mb1-$CreER^{T2}$;$Tp53^{fl/fl}$; Eμ-TCL1, and mb1-$CreER^{T2}$;Eμ-TCL1 control mice were treated with Tam 3× with Tam every second day and analyzed two weeks after the start of the treatment.

Transfer and propagation of CLL cells in Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice

Primary CLL cells were harvested from the spleens of mb1-CreER^{T2}; Ig$\alpha^{fl/fl}$;Eμ-TCL1 or mb1-CreER^{T2};Eμ-TCL1 mice with a high tumor burden, (95% CLL cells in PBL) and 1×10^7 cells were injected i.p. in Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice. After the development of the disease in PBL, recipient mice with a high tumor load (95% CLL cells in PBL) were sacrificed and the splenic CLL cells were retransferred again in Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice as mentioned above. For the survival curve splenic cells (1×10^7) from mb1-CreER^{T2};Eμ-TCL1 or mb1-CreER^{T2};Ig$\alpha^{fl/fl}$;Eμ-TCL1 mice were propagated (i.p.) sequentially 4× into Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice and retransferred into Rag2$^{-/-}$;$\gamma_c^{-/-}$ which were then treated with Tam 3×, every third day. Tumor load and survival was monitored weekly by analyzing PBL of recipient mice.

Flow cytometry: Extracellular cell staining

Flow cytometry (FACS) analysis was performed on 1×10^5-2×10^6 cells in FACS Buffer (PBS; 3% FCS (PAN); 0.05% NaN_3) on ice for 20 min. Before each staining, the cells were incubated with the anti-Fc receptor-blocking antibody (CD16/32, Clone 2.4G2) on ice for at least 5 min. For the staining, the following antibodies were used: PE- and AlexaFluor647-anti-CD19 (eBio1D3; eBioscience and 6D5; BioLegend, respectively), FITC- and PE-anti-IgD (11-26c.2a; BioLegend), AlexaFluor647, eFluor450- anti-IgM (eB121-15F9), PE- and APC-anti-CD93 (AA4.1), PerCP-Cy5.5-anti-CD5 (53-7.3) (all from eBioscience); Phosphatidylcholine (Ptc)-liposome Marina Blue-DHPE (1,2-dihexadecanoyl-sn-glyceo-3-phosphorzhanolamine) from (FormuMax Scientific). The residual unbound antibodies were washed out by adding 700 µl FACS buffer to the cells and subsequent centrifugation at 4°C, and 300×*g* for 5 min.

Flow cytometry: Intracellular (IC) cell staining

Intracellular cell staining was performed using the ADG Fix&Perm Kit (Dianova). Cells were pre-stained extracellular with CD19, B220, CD5, IgM or IgD, then fixed with paraformaldehyde-containing buffer A for 10 min at room temperature and washed with 1 ml PBS. All centrifugation steps were performed at 4°C and 300×*g* for 5 min. Antibodies for intracellular analysis were dissolved in saponin-containing Buffer B for 15 min at room temperature. Subsequently, the cells were washed with 0.5% saponin buffer (PBS; 0.5% saponin (Sigma); 0.2% BSA (PAA Laboratories); 0.02% NaN_3 (Sigma), centrifuged as described, then washed again with FACS buffer (PBS; 3% FCS (PAN); 0.05% NaN_3) before analysis. Antibodies used for intracellular analysis were Alexa Fluor647 anti-Igα (24C2.5; eBioscience; detects the cytoplasmic tail), anti-pAKT (S473) (D9E) Rabbit mAb # 4060, anti-pAKT (T308) (D25E6) rabbit mAb #13038, anti-pLYN (Y507) rabbit Ab #2731, anti-pBTK (Y223) Rabbit pAb # 5082, anti-pSYK (Y525/526) (C87C1) Rabbit mAb #2710, anti-p53 (1C12) Mouse mAb # 2524, anti-PTEN (138G6) Rabbit mAb #9559,

Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb #2697S were from (Cell Signaling). Moreover, we used anti-BCL-2 purified hamster anti-mouse (#554218; BD Pharmingen), Phospho-LYN (Y396) Rabbit pAb (MBS8543024; biozol), MCL-1 mAb Alexa Fluor 488 (LVUBKM, #53-9047-42; eBioscience), Ki-67 mAb eFluor 660 (SolA15; #50-5698-82; eBioscience) and Phospho-NFκB p65 (S536) mAb (T.849.2; #MA5-15160; thermos fisher). The secondary antibodies were anti-rabbit IgG DyLight 649/PE Clone: Poly4064 Cat: 406406 (Bio Legend), goat anti-mouse IgG PE, human ads Cat: 1030-09 (Southern Biotech) and anti-Armenian Hamster Alexa 647 (Jackson Immunoresearch). Fixable viability dye eFluor450 (eBioscience) was used in some cell culture experiments to exclude dead cells. FACS Canto II flow cytometer (BD Bioscience) was used for data acquisition. Analysis was performed using the FlowJo software (Tree Star).

Ca^{2+} influx measurement

Ca^{2+} influx in mb1-CreER^{T2};Pten$^{f/f}$;Eμ-TCL1 B cells was measured using the intracellular fluorescent dye Indo-1 (Invitrogen) . Cells ($0.5–1\times10^6$) were suspended in 1 ml plain Iscove's medium (Biochrom AG) supplemented with 1% FCS. The Indo-1 dye was prepared 5 min prior to incubation with the cell samples and consisted of 25 µl Indo-1 (dissolved in DMSO), 25 µl Pluronic F-127 (Invitrogen), and 113 µl FCS. Indo-1 aliquots (15 µl) were then added to each sample and cell samples were incubated for 45 min at 37°C protected from light. Cells were then centrifuged at 300×g and 4°C. For analysis, the cell pellets were resuspended in 500 µl of Iscove's medium supplemented with 1% FCS. Prior to the analysis, the cells were rested in Ca^{2+} -free medium at 37°C for 10 min. Samples were analyzed by LSR Fortessa flow cytometer (BD Biosciences) while the samples were kept at 37°C. The measurement was shortly interrupted after 35 seconds to add anti-IgM F(ab')$_2$ fragments (Jackson ImmunoResearch) (10µg/ml) for stimulation.

Magnetic activated cell sorting (MACS)

Splenic B cells were obtained by MACS-based positive selection using the magnetically labeled human CD19 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The cells were separated by the AutoMACS Pro Separator (Miltenyi Biotec). After cell sorting the B cells were analyzed for purity and viability using anti-human CD19 Ab (eBioscience, clone HIB19) and cytox (eBioscience), respectively.

Survival and Proliferation assay

Culture cells were labeled with Cell Proliferation Dye eFluor670 (Invitrogen) according to manufacturer's instructions. Afterwards, cells were transferred into a 96-well plates and cultured in complete medium (ISCOVE's, 10% FCS, 50 mg/ml gentamycin, 50 mM 2-mercapto-ethanol) containing inhibitors in the respective concentration: Venetoclax (ABT-

199; 10µM; Selleckchem), LY294002 (10µM; Cell Signaling), Zerumbone (10µM; Cayman Chemical), Ibrutinib (1µM; Selleckchem), R406 (5µM; Selleckchem) and PP2 (10µM; Cayman Chemical). Intensity of the Proliferation Dye and cell survival was monitored over 4 days using FACS Canto II flow cytometer (BD Biosciences) and fixable viability dye eFluor450 (eBioscience).

Immunohistochemistry

Spleens were embedded in OCT compound (SAKURA) and frozen at −80°C. 5 µm sections were prepared using a cryo-microtome (Reichert-Jung 2800 Frigocut) with a S35 knife (Feather) and fixed on SuperFrost Plus slides (Thermo Scientific) by treatment with pure acetone. For the staining of the spleens, sections were rehydrated with PBS + 2% BSA + 0.1% Na-azide and blocked with Fc-Block (α-CD16/32; BD Biosciences). Sections were stained with anti-phospho-Histone H3 (PHH3) antibody (Invitrogen) and mounted with fluoromount-G containing 4′,6′-diamidino-2-phenylindole (DAPI; Southern Biotech). Paraffin embedding of formalin-fixed splenic samples with subsequent hematoxylin and eosin (H&E) staining was kindly done by the department of pathology of Ulm University. Stained sections were analyzed using fluorescence microscope DMi8 (Leica).

CLL patient and HD sample isolation

The isolation of lymphocytes from human blood samples was performed using Ficoll-Paque PLUS (GE Healthcare, 17-440-03). The human blood samples were first diluted 1:1 with 1x PBS. Then 30 ml of this mixture was carefully layered upon 15 ml Ficoll-Paque PLUS prepared in a 50 ml tube. After centrifugation at 1400 rpm for 25 min at RT without break, the layer containing the mononuclear cells plus the Ficoll-layer was transferred into a fresh 50 ml tube. The cells were washed two times with up to 50 ml 1x PBS with subsequent centrifugation at 1400 rpm for 10 min at RT without break. In addition, erythrocytes were removed by adding 500µl of RBC lysis buffer to the cell pellet and 5 min incubation at RT. The suspension was filled up to 10 ml with PBS + 5% FCS, centrifuged at 1600 rpm for 5 min at 4°C and the cell pellet was resuspended in PBS + 5% FCS according to the counted cell number.

Quantitative RT-PCR

The isolated human lymphocytes were MACSed for CD19-positive B cells before we isolated total RNA using the TRIzol Reagent (Ambion, 15596026) according to manufacturer's instructions. Remaining DNA was removed by adding DNAse I (Promega, Z358A). 200ng of the DNA-free RNA samples were used to generate cDNA following the instructions of the SuperScript III First-Strand-Synthesis system for RT-PCR (Thermo Fisher, 18080051). Expression of *Pten* and *GAPDH* reporter gene was measured using a

TaqMan probe (*Pten* Assay ID Hs02621230_s1; *GAPDH* Assay ID Hs02758991_g; Thermo Fisher) according to the manufacturer's protocol. The samples were measured and analyzed by the StepOnePlus Real-Time PCR System (Applied Biosystems) performing the quantitative comparative C_T-test ($\Delta\Delta C_T$).

miRNA qRT-PCR

The miRNA was isolated from MACSed CD19-positive B cells using the *mir*Vana miRNA Isolation Kit (thermofisher, #AM1560) according to manufacturer's instruction. Reverse transcription and qRT-PCR of miR-29a, miR-29b, miR-29c, miR-21 was performed according to the manufacturer's protocol using the following TaqMan miRNA PCR Assays: hsa-miR-29a-3p Assay ID: 002112, hsa-miR-29b-3p Assay ID: 002112, hsa-miR-29c-3p, Assay ID: 000587, hsa-miR-21 Assay ID: 000397, RNU44 Assay ID: 001094. qRT-PCR reactions were run with Step One Plus Real-Time PCR System (BioRad), in triplicates. Relative expression was calculated as following: Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{target sample}} - \Delta Ct_{\text{reference sample}}$ ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$; Ct: threshold cycle). The small RNA RNU44 was used as reference.