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Supplemental Information

Protein Kinase C-β-Dependent Activation of NF-κB in Stromal Cells Is Indispensable for the Survival of Chronic Lymphocytic Leukemia B Cells In Vivo

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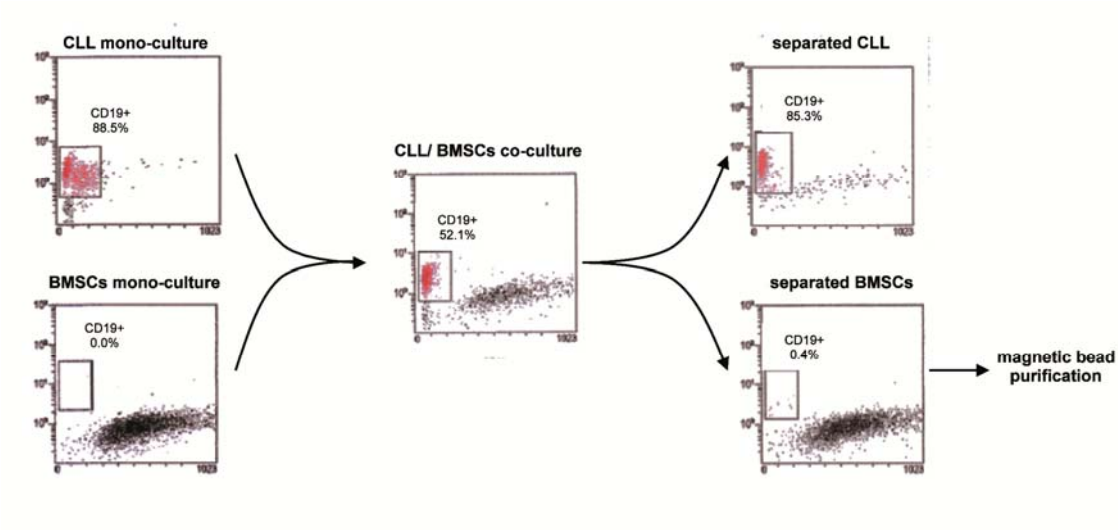
Inventory of Supplemental Information

Supplemental Data

A



B

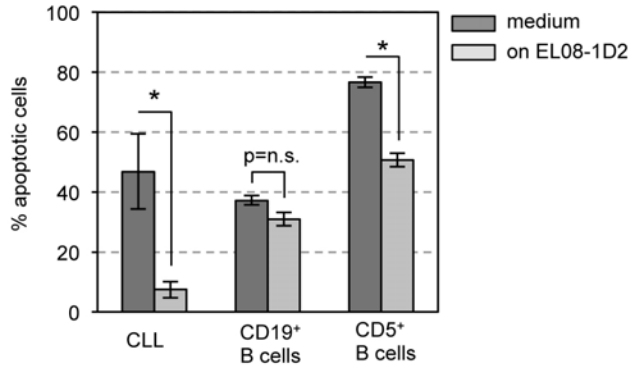


Figure S1, related to Figure 1.

(A) Purified CLL cells ($CD19^+$) were cultured on BMSCs or EL08-1D2 cells for 5 days, CLL cells were then physically removed from stromal cells by repeated washing. Stromal cells were further purified using anti-CD19-magnetic beads. For flow cytometry analysis, cells were stained with an anti-CD19 antibody (y-axis, side scatter on x-axis).

(B) CLL cells or CD19-purified or CD5-purified B cells from the peripheral blood of healthy donors were co-cultured on EL08-1D2 cells. After 5 days apoptotic cells were analyzed by Annexin-V/PI staining (CLL: n=5; $CD19^+$: n=3 and $CD5^+$: n=3, error bars show mean ± SEM).

Table S1, related to Figure 1. Genes induced by CLL contact in EL08-1D2 cells. Provided as an Excel file.

Table S2, related to Figure 1. Genes suppressed by CLL contact in EL08-1D2 cells. Provided as an Excel file.

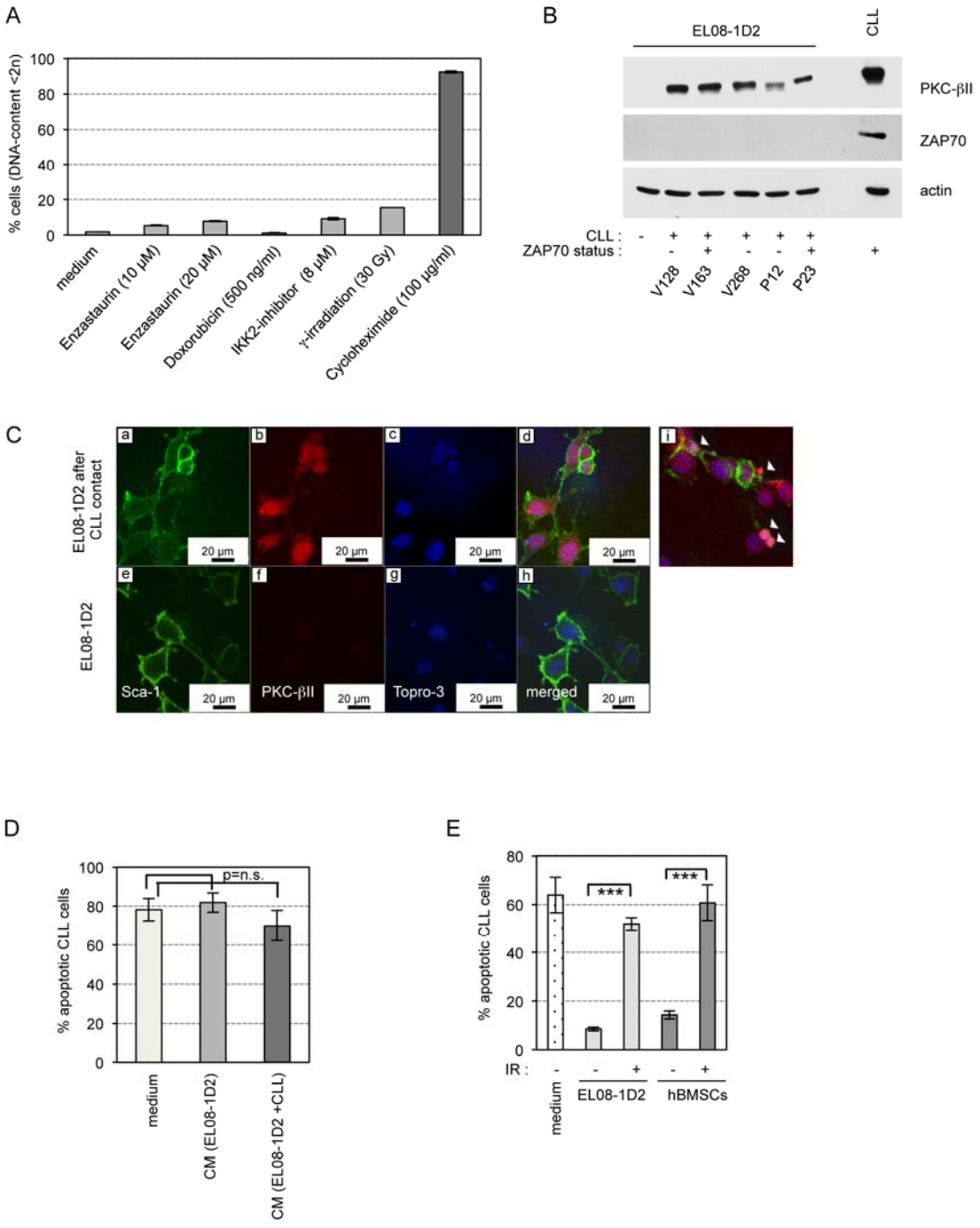


Figure S2, related to Figure 2.

(A) EL08-1D2 cells were irradiated or cultured in the presence of indicated compounds, for the same length of time as in the corresponding experiments. Apoptotic cells were detected by flow cytometry of fixed cells, stained with PI (sub-G1-fraction). Cycloheximide was used as a positive control. Error bars show mean ± SEM.

(B) Primary CLL cells from ZAP70 positive and negative patient samples (indicated by V-, and P-numbers) were co-cultured on EL08-1D2 cells for 5 days before the expression of PKC-βII was analyzed in stromal cells by immunoblotting.

(C) PKC-βII expression in EL08-1D2 cells (Sca-1^{+}) was assessed by immunofluorescence after 5 days of culture with (a-d) or without CLL cells (e-h). To visualize CLL cells they were incompletely removed at the end of the experiment (white arrows in (i)). (The red channel was focused so as to capture the perinuclear PKC-βII).

(D) Primary CLL cells were cultured in medium or medium derived from EL08-1D2 mono-cultures (CM(EL08-1D2)) or from 5 day-EL08-1D2/CLL co-cultures (CM(EL08-1D2+CLL)). After 5 days, apoptotic CLL cells were analyzed by Annexin-V/PI staining. (n=6, error bars show mean ± SEM).

(E) Confluent monolayers of EL08-1D2 cells or hBMSCs were γ–irradiated (30Gy and 15Gy respectively) before co-culturing with CLL cells. Percentage of apoptotic CLL cells was analyzed after 5 days. (n=6, error bars show mean ± SEM).

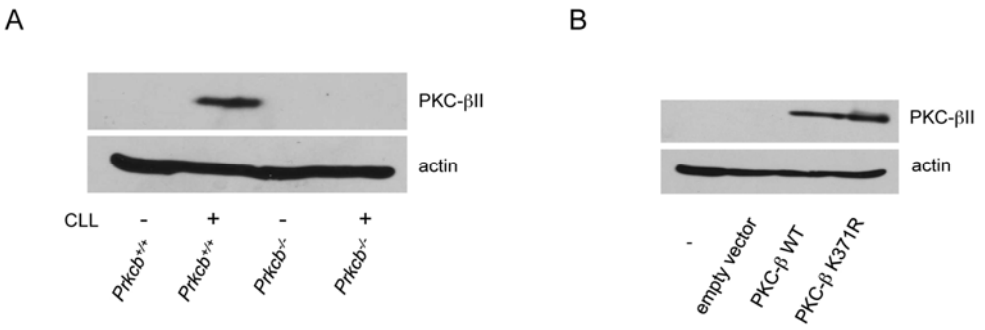


Figure S3, related to Figure 3.

(A) CLL cells were cultured on BMSCs from wild-type or *Prkcb*$^{-/-}$ animals according to the experiment depicted in Figure 3D. PKC-βII expression in BMSCs was assessed by immunobloting after 5 days.

(B) The expression of PKC-βII was analyzed by western blotting in control *Prkcb*$^{-/-}$ BMSCs (-) or in *Prkcb*$^{-/-}$ BMSCs transduced with empty vector virus or virus expressing PKC-βII wild-type or mutant (K371R) cDNA.

Table S3, related to Figure 3. Clinical and pathological features of primary CLL samples.

patient ID	IGHV status	ZAP70|	FISH cytogenetic abnormalities	*TP53, NOTCH1, MYD88* mutations (*)	%- Δ Annexin-V (❖)
P12	unmutated	negative	del(11q), del(13q)	-	31.9
P21	n.a.	n.a.	normal	-	11.3
P26	unmutated	n.a.	del(17p), del(13q) homozygous	p53 (p.Arg248Leu)	61.6
V121	n.a.	positive	normal	-	20.7
V128	unmutated	negative	del(11q), del(13q)	-	33.2
V131	unmutated	positive	del(11q), del(13q)	-	31.3
V132	mutated	negative	del(13q) homozygous	-	15.1
V147	unmutated	positive	del(11q), del(13q)	-	12.3
V163	mutated	positive	del(13q)	p53 (p.Tyr234Cys)	29.9
V164	n.a.	n.a.	normal	-	13
V172	unmutated	positive	del(11q), del(13q)	-	18
V180	mutated	negative	del(13q)	-	10.7
V190	no amplification	negative	del(13q)	-	0.1
V194	unmutated	positive	del(11q), trisomy(12)	-	13.1
V211	mutated	negative	del(13q)	-	27.8
V231	unmutated	positive	del(13q)	Notch1 (p.Pro2222Gln)	13.4
V250	unmutated	positive	del(13q)	-	13.1
V253	n.a.	positive	normal	-	41.6
V260	n.a.	negative	normal	-	34.2
V261	no amplification	negative	trisomy(12)	-	67.2
V270	n.a.	negative	del(13q)	-	29.6

| ZAP70 positivity in case >20% of CLL cells were positive by flow cytometry analysis
* Genomic DNA was sequenced for *Notch1* (exons 25-34), *MyD88* (exons 4-5) and *TP53* (exons 4-11) mutations.
❖ %Annexin-V positive CLL cells (cultured on PKC-β deficient stromal cells – cultured on PKC-β wild type stroma)
n.a.= data not available

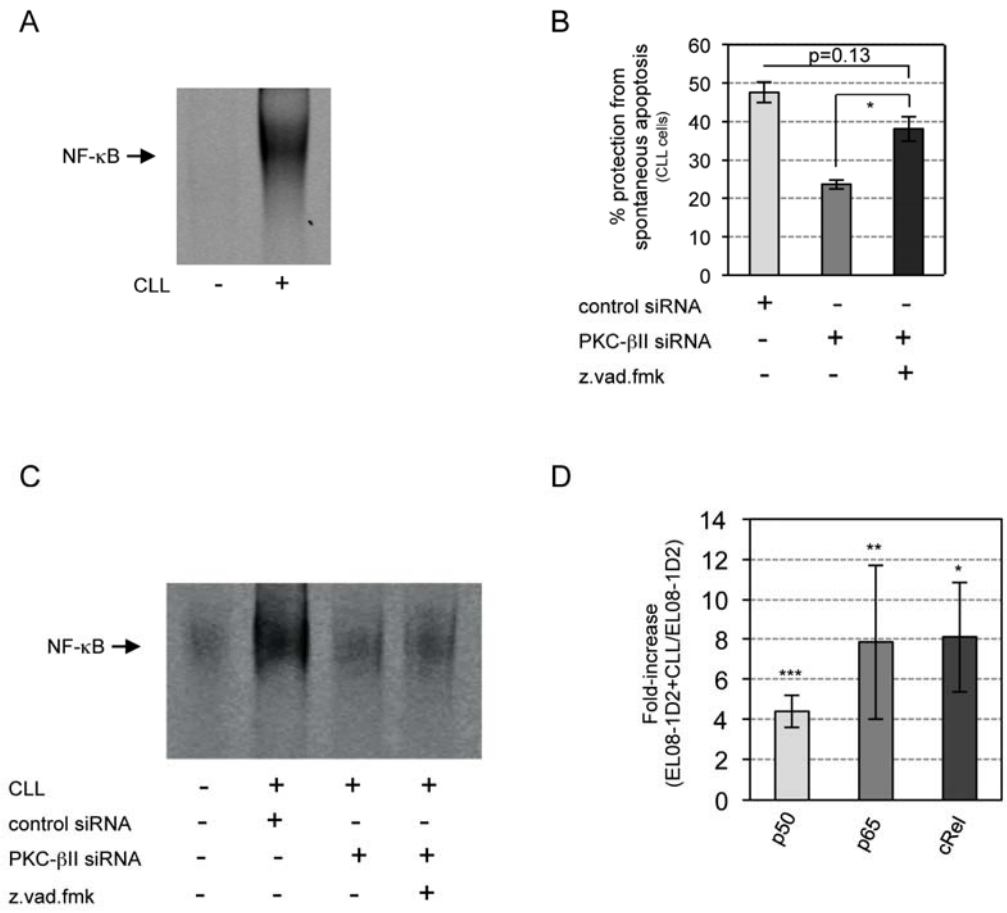


Figure S4, related to Figure 4.

(A) CLL cells were cultured on hBMSCs for 5 days, activation of NF-κB in hBMSCs was analyzed by EMSA performed on nuclear proteins (n=3).

(B) Primary CLL cells were cultured on EL08-1D2 cells transfected with a siRNA directed against PKC-βII or a control siRNA. Z.vad.fmk (100 μM) was added to the co-culture as indicated. After 5 days, apoptotic CLL cells were analyzed by Annexin-V/PI staining. Bars represent the percentage of apoptosis protection compared to cells cultured in medium (n=3, error bars show mean ± SEM).

(C) NF-κB activation was analyzed by EMSA from EL08-1D2/CLL co-cultures depicted in (B).

(D) Western blots depicted in Figure 4C were analyzed by densitometry using the ImageJ® software. Fold-increase indicates the amount of nuclear p50, p65 and cRel after CLL contact relative to EL08-1D2 cells not co-cultured with CLL cells. Error bars show mean ± SEM.

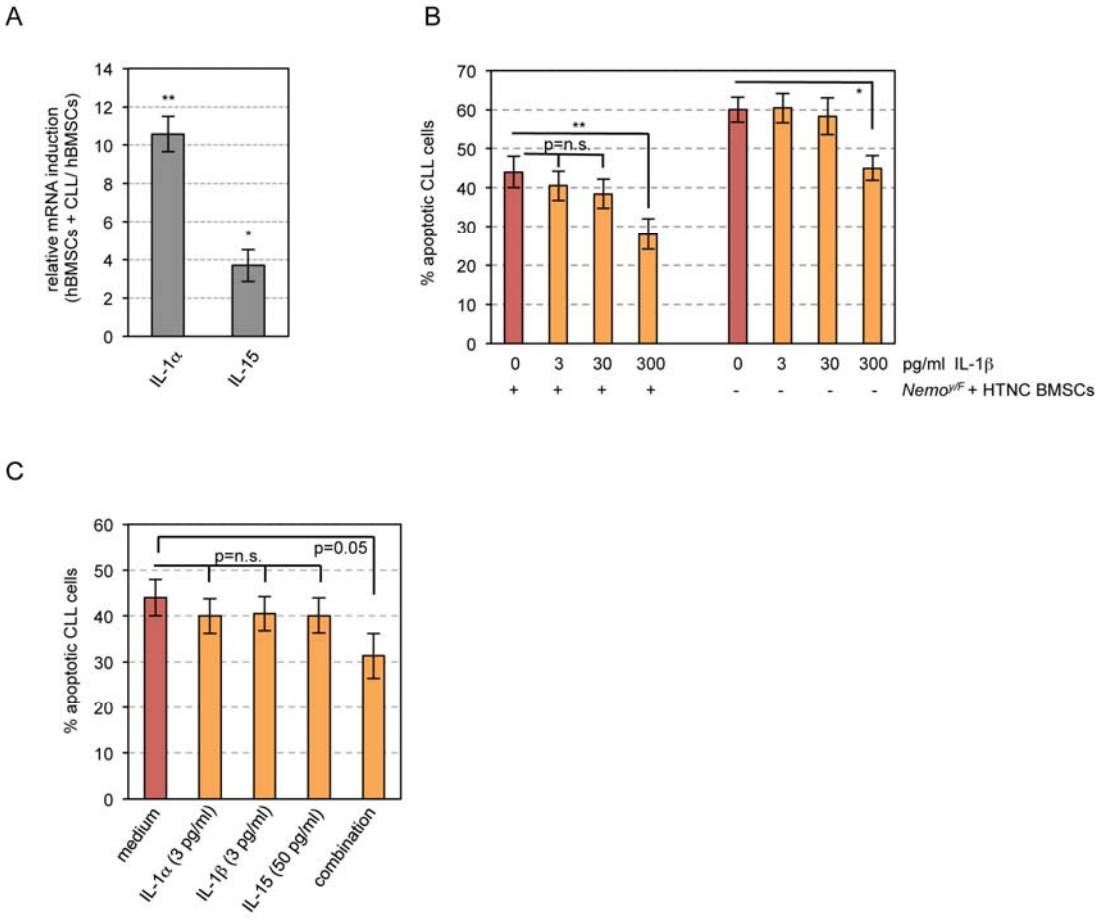


Figure S5, related to Figure 5.

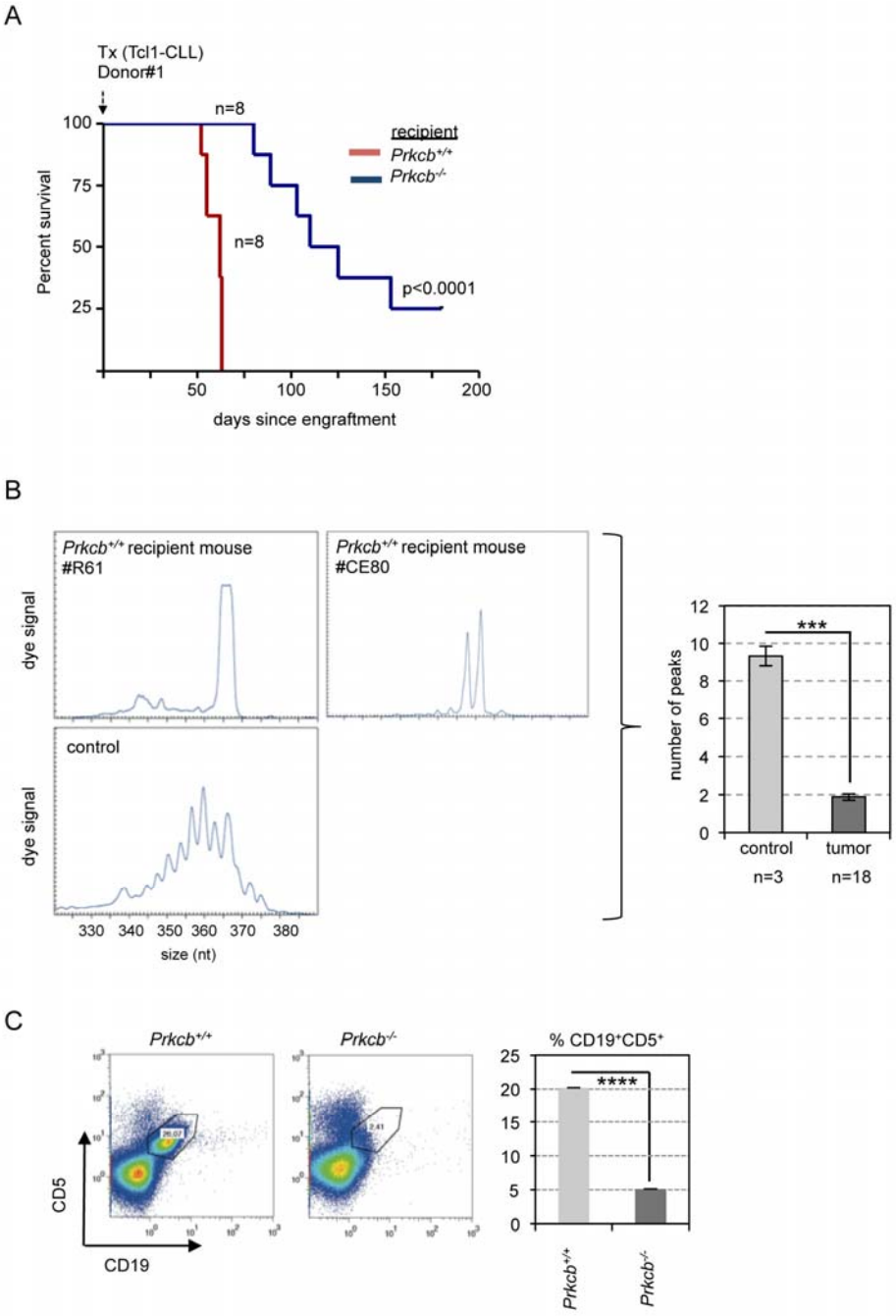
(A) CLL cells were cultured on monolayers of hBMSCs for 5 days. After purification of hBMSCs the amount of IL-1α and IL-15 mRNA relative to untreated hBMSCs was assessed using quantitative RT-PCR (n=8).

(B) Increasing doses of IL-1β were supplemented to the media of CLL mono-cultures or CLL/NEMO-deficient BMSCs co-cultures. After 5 days the rate of apoptotic CLL cells was analyzed by Annexin-V/PI staining (n=8).

(C) CLL cells were cultured on HTNC treated *Nemo*$^{Y/F}$ BMSCs for 5 days. IL-1α, IL-1β and IL-15 were added individually or in combination. After 5 days the amount of apoptotic CLL cells was assessed by Annexin-V/PI staining (n=8).
Error bars in the entire figure show mean ± SEM.

Table S4, related to Figure 5. Nemo induced genes. Provided as an Excel file.

Table S5, related to Figure 5. Nemo suppressed genes. Provided as an Excel file.


A
Tx (Tcl1-CLL)
Donor#1
n=8
recipient
Prkcb+/+
Prkcb-/-
n=8
p<0.0001
Percent survival
days since engraftment
B
Prkcb+/+ recipient mouse
#R61
Prkcb+/+ recipient mouse
#CE80
control
dye signal
size (nt)
number of peaks
***
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CD5
CD19
% CD19+CD5+
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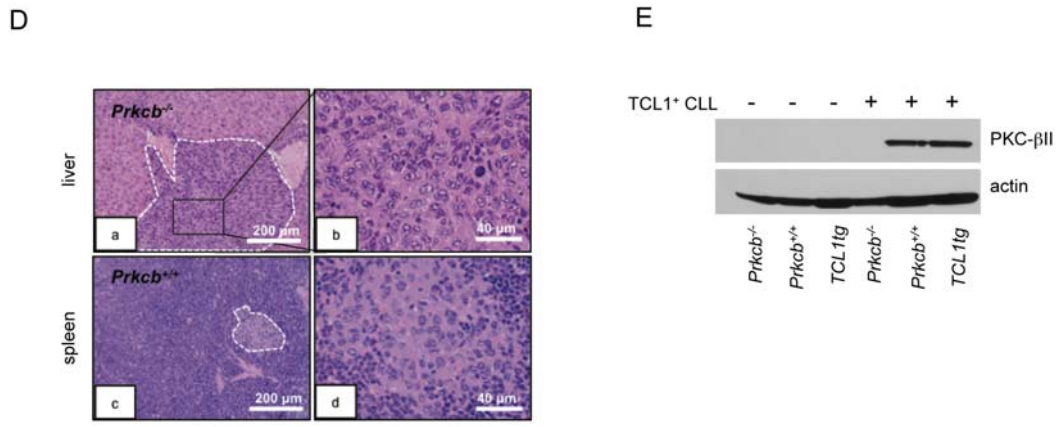

Figure S6, related to Figure 6.

(A) 2x10^7 splenic PBMCs from a diseased *TCL1tg* mouse (donor#1) were transplanted into syngeneic wild-type or *Prkcb*$^{-/-}$ mice. Mice in both cohorts were followed for signs of illness and killed when moribund.

(B) Rearranged immunoglobulin VDJ sequences from tumor samples (CD5-sorted B cells) or wild-type B cells were PCR amplified and analyzed for fragment length variability to determine clonality. The upper two graphs representatively shows VDJ rearrangements from two individual tumors transplanted into wild-type recipient mice. On average 1.8 clones were detected in recipient mice, error bars show mean ± SEM. A representative control sample from wild-type splenocytes is shown in the lower graph(n=3).

(C) Single cell suspensions from spleens of wild-type and *Prkcb*$^{-/-}$ recipient mice were analyzed for the percentage of CD19$^+$CD5$^+$ cells at the time of sacrifice (n=13 for wild-type- and n=5 for *Prkcb*$^{-/-}$ recipient mice, error bars show mean ± SEM).

(D) Representative histological images of necrobiopsy of mice transplanted with tumor#1. White dotted lines outline histiocytic soft tissue sarcomas in *Prkcb*$^{-/-}$ (a+b) and in *Prkcb*$^{+/+}$ (c+d) recipient mice. Right panels show high magnification of the histiocytic sarcomas in the left panels.

(E) CD19$^+$CD5$^+$ B cells derived from moribund *TCL1tg* mice were cultured on mBMSCs from *TCL1tg*, wild type or *Prkcb*$^{-/-}$ mice for 5 days. PKC-βII expression in purified BMSCs was assessed by immunoblotting.

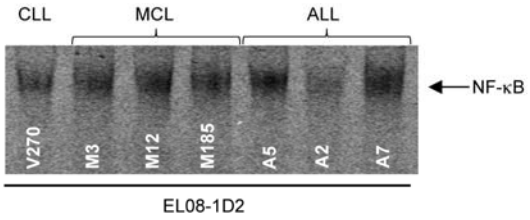

Figure S7, related to Figure 7.

Primary leukemic cells from three MCL and three ALL patients were cultured on EL08-1D2 cells for 5 days according to the experiment depicted in Figures 7A and 7B. NF-κB activity in EL08-1D2 cells was assessed by EMSA. Patients´ ID is encrypted by V-, M- and A-numbers.

Supplemental Experimental Procedures

Reagents and Antibodies

Polyclonal antibodies specific for PKC-βII (clone C-18), PKC-βI (clone C-16), NF-κB p50 (NLS, sc-114), NF-κB p65 (clone C-20), cRel (sc-71) and Bcl10 (C78F1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibodies ZAP70 (clone 29) were purchased from BD Transduction Laboratories (Heidelberg, Germany) and PKC-α (2056) was obtained from New England Biolabs (Frankfurt am Main, Germany). NEMO and actin were immunoblotted with mouse IKKγ (clone 72C627) (Millipore, CA, USA) and β-actin (clone AC-15, Sigma Deisenhofen, Germany). Immunofluorescence analyses were performed using anti-RhoA (67B9) (New England Biolabs Frankfurt am Main, Germany), anti-αSMA (ab5694) (Abcam, Cambridge, UK), anti-Phalloidin-FITC (Invitrogen, Oregon, USA), anti-Sca1 (clone D7) (eBioscience, Frankfurt, Germany) and To-Pro3 (Invitrogen, Darmstadt, Germany). For immunohistochemistry (Figure 6D), the following antibodies were used: b220 (clone RA3-6B2) and CD5 (clone 53-7.3) from Pharmingen (Schwerte, Germany), CD3 (clone sp7) from Innovative Diagnostic System (Hamburg, Germany) and mib-1 (clone sp6) from Thermo scientific (Schwerte, Germany).

The cytotoxic agents doxorubicin and cyclohexamide were purchased from Sigma (Deisenhofen, Germany). Enzastaurin is provided by LC Labs, (Woburn, MA, USA). The IKK2 inhibitor was a kind gift from Karl Ziegelbauer (Bayer Schering Pharma AG).

Cells and culturing conditions

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by centrifugation over a Ficoll-Hypaque layer (Biochrom, Berlin, Germany) of 1.077 g/ml density. For separation of CLL B cells, PBMC were incubated with anti-CD2 and anti-CD14 magnetic beads (Dynabeads M450, Dynal, Oslo, Norway) according to the manufacturer's instructions. After separation B cells from CLL patients were >98% pure as assessed by direct immunofluorescence. Cells were harvested under ice-cold conditions and were cultured in RPMI 1640 (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS) penicillin/streptomycin 50 U/ml, Na-pyruvate 1mM, L-glutamine 2 mM, L-asparagine 20 mg/ml, 2-mercaptoethanol 0.05mM, HEPES 10mM and MEM non-essential amino acids (Gibco).

For separation of peripheral blood B cells from healthy donors, PBMNCs were incubated with anti-CD19 magnetic beads and isolated B cells were released from CD19 beads by using CD19 DETACHaBEAD (Invitrogene, Karlsruhr, Germany) according to the manufacturer's instructions.

For isolation of $CD5^+$ B cells from the peripheral blood of healthy donors, PBMC were isolated by Ficoll density gradient centrifugation (Amersham, Freiburg, Germany) and subsequently, $CD19^+$ B cells were negatively enriched by the EasySep® Human B Cell Enrichment Kit 19054 (Stemcell, Cologne, Germany). $CD5^+$ B cells were then enriched by anti-CD5-APC (UCHT2) and anti-APC MicroBeads (Miltenyi) to a purity of >70%. Antibodies were purchased from BD Biosciences (Becton Dickinson, Heidelberg, Germany.

The murine embryonic liver cells EL08-1D2 and all primary mouse bone marrow stromal cell cultures were cultured in MEM Alpha + GlutaMAX medium (Invitrogen) supplemented with 10% fetal calf serum (PAA, Cölbe, Germany), 10% horse Serum (StemCell technologies, Grenoble, France) 10 µM 2-mercaptoethanol and 1% penicillin/ streptomycin (Gibco). These cells were grown on 0.1% gelatine coated cell culture plates. RPMI medium containing 10% fetal bovine serum, 10% horse serum, 10 µM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids, 2 mM L-glutamine, 10 mM HEPES, 10 mg/ml L-asparagine, 100 U/ml penicillin/streptomycin and 10^{-6} M hydrocortisone was used for the primary human stromal cells. Human Umbilical Vein Endothelial Cells (HUVEC) were maintained in EBM-2 (+Bullet-Kit) medium (LONZA, MD, USA), the murine osteoplastic cell line MC3T3 in α-MEM medium supplemented with 10% FCS and the human primary osteoblasts (hOB) in Osteoblast Growth medium + SupplementMix (Promocell, Heidelberg, Germany). All cells were cultured at 37°C and 5% CO2 in a fully humidified atmosphere.

Cytokine array

Supernatants from EL08-D1 cells and co-cultures were centrifuged to discharge cells and cell debris. These supernatants were analyzed concerning their content of cytokines using a RayBio Mouse Cytokine Antibody Array (RayBiotech, Inc., Georgia, USA). After blocking the provided membranes

overnight, they were incubated with the samples at 4°C overnight. The following washing steps, biotin-conjugated-antibody incubation and detection with HRP-conjugated streptavidin were prepared according to the manufacturer's instructions.

Microarray profiling and data analysis

Primary CLL cells were separated from co-cultures with stromal cells by repeated washing steps with PBS (see Figure S1A). Flow-cytometry analysis indicated that more than 99% $CD5^{+}CD19^{+}$ cells were successfully isolated from CLL-stroma-co-cultures. Subsequently, single cell suspensions were treated with magnetic anti-CD19 beads to allow removal of the remaining CLL cells. Total RNA was extracted from stromal cells (EL08-1D2 or mBMSCs), which had been co-cultured with CLL cells for 5 days or cultured without CLL, using the miRNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instruction. To remove remaining genomic DNA, RNA was treated with RNase-free DNase Set (Quiagen).

For microarray analyses, we used the Affymetrix GeneChip platform employing the Express Kit protocol for sample preparation and microarray hybridization. Total RNA (200ng) was converted into biotinylated cRNA, purified, fragmented and hybridized to MG-430_2.0 microarrays (Affymetrix). The arrays were washed and stained according to the manufacturer's recommendation and finally scanned in a GeneChip scanner 3000 (Affymetrix). Arrays were processed in two batches consisting of one control and two or four co-cultured samples. Both batches were analyzed separately.

Array images were processed to determine signals and detection calls (Present, Absent, Marginal) for each probeset using the Affymetrix GCOS1.4 software (MAS 5.0 statistical algorithm). Arrays were scaled across all probesets to an average intensity of 1000 to compensate for variations in the amount and quality of the cRNA samples and other experimental variables of non-biological origin. Pairwise comparisons of co-cultured *versus* control samples were carried out with GCOS1.4, which calculates for each probeset the significance (change p-value) of each change in gene expression based on a Wilcoxon ranking test. By comparing the change p-value against thresholds empirically determined by Affymetrix, a Change Call (I, MI, NC, MD or D) is generated, indicating "Increased", "Marginally Increased", "No Change", "Marginal Decreased" or "Decreased" expression in the experimental samples. To limit the number of false positives, we restricted further target identification to those probesets, which received at least one present detection call in the treated/control pair. Only probesets exhibiting a significant increase or decrease in the comparison analysis and at least one Present detection call per pair were considered as potentially regulated targets. Probesets identified as consistently up- or down-regulated in ≥ 5 of the 6 (83%-Figure 1D) or 7 of 7 (100%-Figure 5A) pair-wise comparisons of co-cultured versus control cultures were considered as consistent targets.

Immunofluorescence of EL08-1D2 cells

EL08-1D2 cells were plated on 6cm dishes with glass cover slips, previously coated with 0.1% gelatin and Poly-L-Lysine. After 24 hr primary CLL cells were cultured on stromal cells. After 5 days CLL cells were carefully removed, stromal cells washed with PBS and cover slips were transferred to 6-well culture plates, rinsed again 3 times with PBS, and fixed with 4% PFA (0.5% PFA in case of phalloidin staining) for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 and blocked with 10% FCS in PBS for 1h. Rinsing cells three times with PBS terminated permiabilization. Primary antibodies diluted in blocking buffer (10% FCS, 0.1% Triton X-100 in PBS) were added and incubated overnight at 4°C in a humid chamber. Samples were rinsed three times with PBS prior to the addition of the secondary antibody, applied in blocking buffer, for 2 hr at room temperature. Nuclei were counterstained with 100 µM To-Pro3 for 1h at room temperature. After rinsing with PBS cover slips were mounted on microscope slides in the Slow Fade Gold antifade reagent (Invitrogen) and kept dry at -20°C. Confocal laser scanning microscopy was carried out using a LSM510 confocal laser microscope (Zeiss).

Primer sequences for quantitative RT-PCR

name	species	sequence 5`-3`
PKC-β reverse	Mus musculus	TGCAGCACTTTCTACATGCC
PKC-β forward	Mus musculus	GCCGAGTTACATCCGTGTTT
PKC-α reverse	Mus musculus	GAAGCGGTTGGCCACGTCCTG
PKC-α forward	Mus musculus	ACGTTTACCCGGCCAACGACT
actin reverse	Mus musculus	GAAAGGGTGTAAAACGCAGC
actin forward	Mus musculus	CAGCAAGCAGGAGTACGATG
actin reverse	Homo sapiens	AGTGGGGTGGCTTTTAGGAT
actin forward	Homo sapiens	CCGAGGACTTTGATTGCACA
IL-1α reverse	Homo sapiens	GGCTGGCAGCTTAAGCC
IL-1α forward	Homo sapiens	TGTAGCCACGTAGCCAC
IL-15 reverse	Homo sapiens	TAGGAAGCCCTGCACTGAAACAGC
IL-15 forward	Homo sapiens	ACCCAGTTGGCCCAAAGCACCT

Transfection of EL08-1D2 cells
$1x10^5$ EL08-1D2-cells were seeded on coated 6cm dishes and transfected at the next day with a PKC-βII siRNA-mix (5`-CCGGUAUAUUGA-3` and 5`-CAAGUUUAAGAU-3`) or non-binding control siRNA (On-Target plus Control Pool, Thermo Scientific Dharmacon) using Lipofectamin 2000 (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. 200 pM of PKC-βII or control siRNA was added to serum- and antibiotic free OptiMEM medium. After 24 hours EL08-1D2 cells were washed in PBS and re-cultured in RPMI. Primary CLL cells were co-cultured on these EL08-1D2 cells for 5 days.

Reintroduction of PKC-β into murine BMSCs
Constructs were obtained by cloning PKC-βII cDNA into an IRES GFP containing MSCV-based vector (pMIG). Kinase dead mutation (K371R) was generated with Site directed mutagenesis Kit (Promega) using the forward primer GAGCTCTATGCTGTGAGGATCCTGAAGAAGGACG and the reverse primer CGTCCTTCTTCAGGATCCTCACAGCATAGAGCTC*.* Retroviral supernatant was produced by transiently transfecting the Phoenix ecotropic producer line with the pMIG control vector, pMIG-PKC-β-wild-type or pMIG-PKC-β-K371R constructs. Medium was changed 8 hr after transfection and collected three times. After 15 hours, after 9 hours and again after 15 hr, the medium was harvested and filtered through a 45-µm syringe filter to remove floating cells. Stromal cells were infected in 6 cm plates in the presence of 4 µg/ml polybrene three times directly after collecting the supernatants.

Electrophoretic mobility shift assay
Nuclear extracts were prepared as mentioned elsewhere (Ferch et al., 2007). The binding of NF-κB was determined by electrophoretic mobility shift assay (EMSA). Nuclear protein extracts (10µg) were incubated at room temperature in 2 µl binding buffer (100 mM Tris, pH 7.5, 500 mM KCl,10 mM dithiothreitol and 1 µg poly (dI-dC)) with IRDye700-labeled, double-stranded oligonucleotide probes (NF-κB, 5` -AGTTGAGGGGACTTTCCCAGGC -3`) for 20 min in the dark. Upon electrophoretic separation on a 5% polyacrylamide gel in TBE buffer, the gels were analyzed using Odyssey Infrared Imaging System.

Cre-Transduction into mouse bone marrow cells
Primary BMSCs were isolated from femora of *Nemo*$^{F/F}$ and *Nemo*$^{y/F}$-mice as describe under experimental procedures and cultured until they reached 90% confluence. Cells were incubated overnight in a 1:1 mixture of medium (DMEM w/o FCS) and PBS containing 1 µM Tat-Cre protein (HTNC). NEMO-deficient BMSCs were then cultured at least 8 days after transduction in DMEM supplemented with PS and 10% FCS before co-culture experiments were performed. BMSCs from *R26R-EYFP* (YFPr) reporter mice carrying an insertion of sequences encoding enhanced yellow fluorescent protein (eYFP) preceded by a loxP-flanked STOP cassette, were used as control for successful delivery and function of Cre-recombinase.

Immunofluorescence on human bone marrow trephine biopsies
For triple immunofluorescence the following antibodies were used: CD34 mouse monoclonal antibody (Novocastra (1:50)), PKC-βII (clone C-18) (1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA Cruz) and CD79a mouse monoclonal antibody (Thermo Scientific, Fremont, CA (1:20)). After deparaffinization and rehydration the slides were placed in a pressure cooker in 0.01 M citrate buffer,

ph 6.0 and were heated for 7 min. Antibodies were incubated overnight at 4°C or for 1h at room temperature. After incubation of primary mouse anti-CD79a, species was changed by incubation with goat anti mouse FAB fragment (Jackson ImmunoResearch, Baltimore; PA) to rule out cross-reactivity during detection of mouse anti-CD34 and mouse anti-CD79a. Primary antibody or goat anti mouse FAB fragment was detected by incubation with the following secondary antibodies: donkey anti rabbit conjugated with Dylight 488 (Jackson ImmunoResearch), donkey anti goat conjugated with Dylight 549 (Jackson ImmunoResearch) and donkey anti mouse conjugated with Cy5 (Jackson ImmunoResearch). After incubation of slides with conjugated secondary antibody (1:100 for 30 min), they were counterstained and mounted with mounting medium (Vectashield, Vector laboratories; Bulingame, CA).

Immunoglobulin heavy chain CDR3 spectratyping
Tumor samples were collected from spleens of sacrificed mice and sorted to >95% purity using CD5 magnetic beads (Miltenyi, Bergisch Gladbach). Whole splenocytes were prepared from wild-type mice as controls. Total RNA was extracted using High Pure RNA Isolation Kit (Roche, Basel). First strand cDNA was generated (BioRad, California) and subjected to 40 cycles of PCR using a degenerate primer set (MWG, Germany) of VH specific forward primers (MH1 to MH7: MH1, 5'-SARGTNMAGCTGSAGSAGTC-3'; MH-2, 5'-SARGTNMAGCTGSAGSAGTCWGG-3'; MH-3, 5'-CAGGTTACTCTGAAAGWGTSTG- 3'; MH-4, 5'-GAGGTCCARCTGCAACARTC- 3'; MH-5, 5'-CAGGTCCAACTVCAGCARCC-3'; MH-6, 5'-GAGGTGAASSTGGTGGAATC-3'; MH-7, 5'-GATGTGAACTTGGAAGTGTC- 3';) and an Alexa-647 conjugated JH specific reverse primer (JHR, 5'-CCTGMRGAGACDGTGASHRDRGTBCCTKKRCCCC- 3') as previously described (Wang et al., 2000).

The PCR products were run on an Beckman Coulter CEQ™ 8000 Genetic Analysis System using a 400 base size standard (GenomeLab, Beckman Coulter, California) and analyzed using CEQ 8000 software (Version 8.0).

Supplemental References

Ferch, U., zum Buschenfelde, C. M., Gewies, A., Wegener, E., Rauser, S., Peschel, C., Krappmann, D., and Ruland, J. (2007). MALT1 directs B cell receptor-induced canonical nuclear factor-kappaB signaling selectively to the c-Rel subunit. Nat Immunol *8*, 984-991.

Wang, Z., Raifu, M., Howard, M., Smith, L., Hansen, D., Goldsby, R., and Ratner, D. (2000). Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3' to 5' exonuclease activity. J Immunol Methods *233*, 167-177.