SUPPLEMENTAL INFORMATION

Supplemental Material and Methods

Rates of protein synthesis

To monitor protein synthesis, MEFs were exposed in culture to silvestrol or HHT for 1 h and labeled during the last 20 min with [^{35}S]Easy Tag Express Protein Labeling mix (1175 Ci/mmol) (Perkin Elmer, Waltham, MA, USA) essentially as described.[1] Cells were lysed in RIPA buffer, proteins precipitated with TCA and ^{35}S incorporation into proteins measured via scintillation counting (HIDEX 300SL; Hidex Oy, Turku, Finland). Scintillation counts were standardized to total protein content, which was determined using the Micro BCA protein assay (Pierce, Scoresby, Australia) and set relative to control (DMSO).

Cell cycle analysis

MEFs deficient in both Bax and Bak were treated with silvestrol, HHT, or nocodazole for the indicated times. PBS washes, trypsin and medium were all collected, centrifuged and the resultant cell pellets fixed in 70% ethanol for at least 30 min on ice, washed in PBS and digested with 1 mg/mL RNAse A/PBS for 30 min/37^{o}C. Propidium iodide (10 μg/mL) was then added and samples analyzed by flow cytometry as previously described.[2]

Western blotting

Wild-type MEFs were treated in culture with translation inhibitors with or without the pan-caspase inhibitor Q-VD-OPh. At the indicated time points, samples were lysed in 20 mM Tris pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, and 1% Triton X-100 supplemented with a protease inhibitor cocktail (complete mini EDTA-free protease inhibitor cocktail tablet; Roche). Human leukemia lines were also lysed in the same buffer.

Samples were cleared of cellular membranes and debris, then resolved on 10% NU-PAGE gels, and transferred onto nitrocellulose using the iBlot system (Invitrogen). Protein levels were examined using antibodies to murine Bcl-x_L (44; BD Biosciences), human Bcl-x_L (9C9; WEHI), murine Bcl-2 (3F11; WEHI), human Bcl-2 (Bcl-2-100; WEHI), Mcl-1 (19C4; WEHI), Bak (Sigma), murine Bax (21C10; WEHI), human Bax (2D2;Sigma), β-actin (loading control; AC-40; Sigma) and HSP70 (loading control; N6; gift from W Welch).

Supplemental References

1. Bordeleau ME, Robert F, Gerard B, Lindqvist L, Chen SM, Wendel HG *et al.* Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model. *J Clin Invest* 2008; **118**(7)**:** 2651-60.

2. Crissman HA, Steinkamp JA. Rapid, one step staining procedures for analysis of cellular DNA and protein by single and dual laser flow cytometry. *Cytometry* 1982; **3**(2)**:** 84-90.

Table S1.

EC_{50} (nM) ± SEM of PI-exclusion after 24 h treatment with translation inhibitor

n = 3-6	**Silvestrol**		**HHT**	
	wt	***Bax*$^{-/-}$*Bak*$^{-/-}$**	**wt**	***Bax*$^{-/-}$*Bak*$^{-/-}$**
Pro-pre B cells	2.59 ± 0.30	> 750	7.67 ± 0.87	> 750
Immature B cells	2.92 ± 0.53	> 750	13.7 ± 1.22	> 750
Transitional B cells	10.7 ± 2.16	> 750	29.4 ± 4.43	> 750
Recirculating B cells	7.15 ± 1.11	> 750	20.7 ± 5.67	> 750
T1 B cells	10.5 ± 1.75	> 750	33.7 ± 3.49	> 750
T2 B cells	7.72 ± 0.89	> 750	19.7 ± 2.44	> 750
Follicular B cells	9.09 ± 1.61	> 750	24.0 ± 2.87	> 750
Marginal zone B cells	12.2 ± 2.56	> 750	22.7 ± 2.47	> 750
$CD8^+$ T cells	33.7 ± 10.4	251 ± 33.2	12.7 ± 4.38	170 ± 24.0
$CD4^+$ T cells	11.0 ± 2.3	349 ± 62.3	6.71 ± 1.34	302 ± 38.8
***Eμ-Myc* lymphomas**	20.1 ± 6.35	N/A	26.4 ± 3.77	N/A

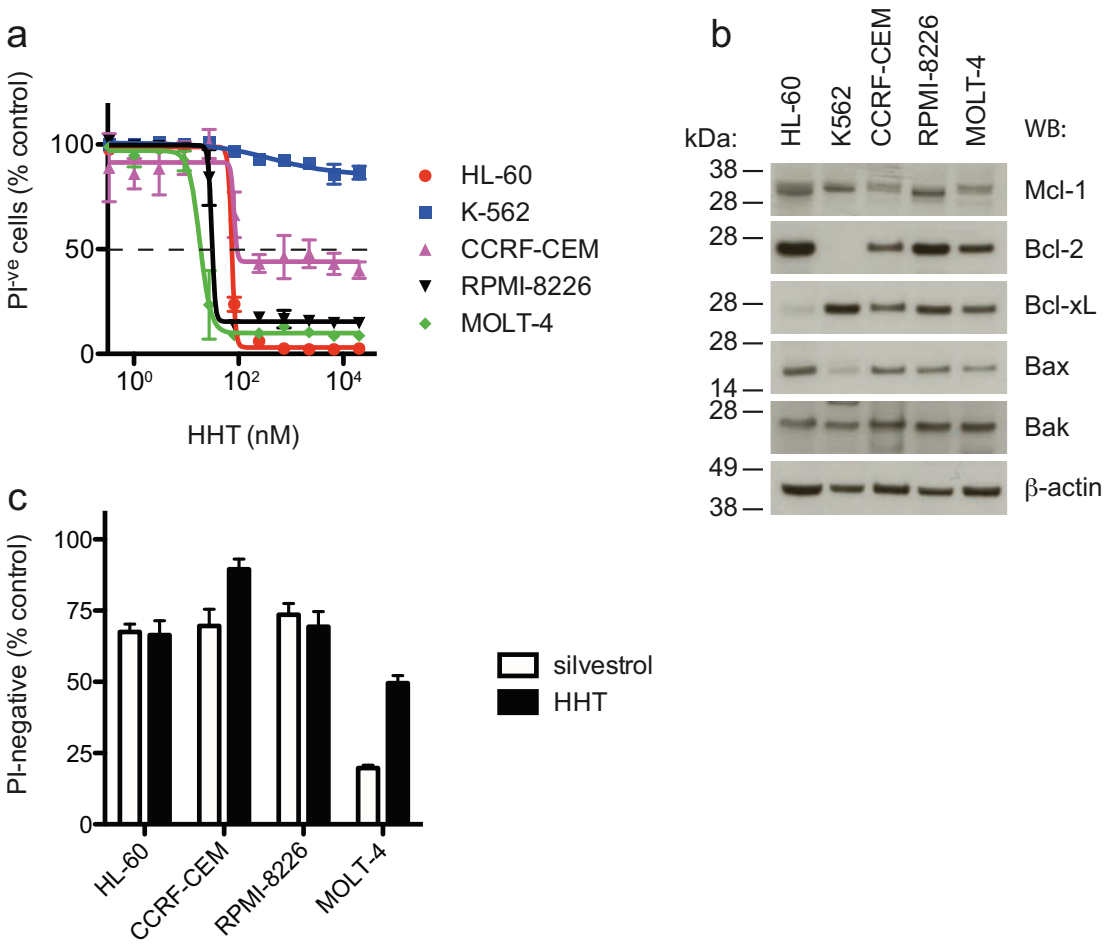

Figure S1 (**a**) Survival (assessed by PI exclusion and flow cytometric analysis) of a panel of leukemia and lymphoma derived cell lines 72 h after exposure to HHT. Data represent the mean (relative to control cells treated with the vehicle, DMSO) of 2 independent experiments performed in duplicate; error bars represent the standard deviation.

(**b**) Western blot illustrating the relative protein levels of the Bcl-2 family members in the indicated leukemia-derived cell lines.

(**c**) Survival of leukemia-derived lines following a 24 h exposure to 1 μM silvestrol or HHT. Data represent the mean of 3 independent experiments and the standard error of the mean.

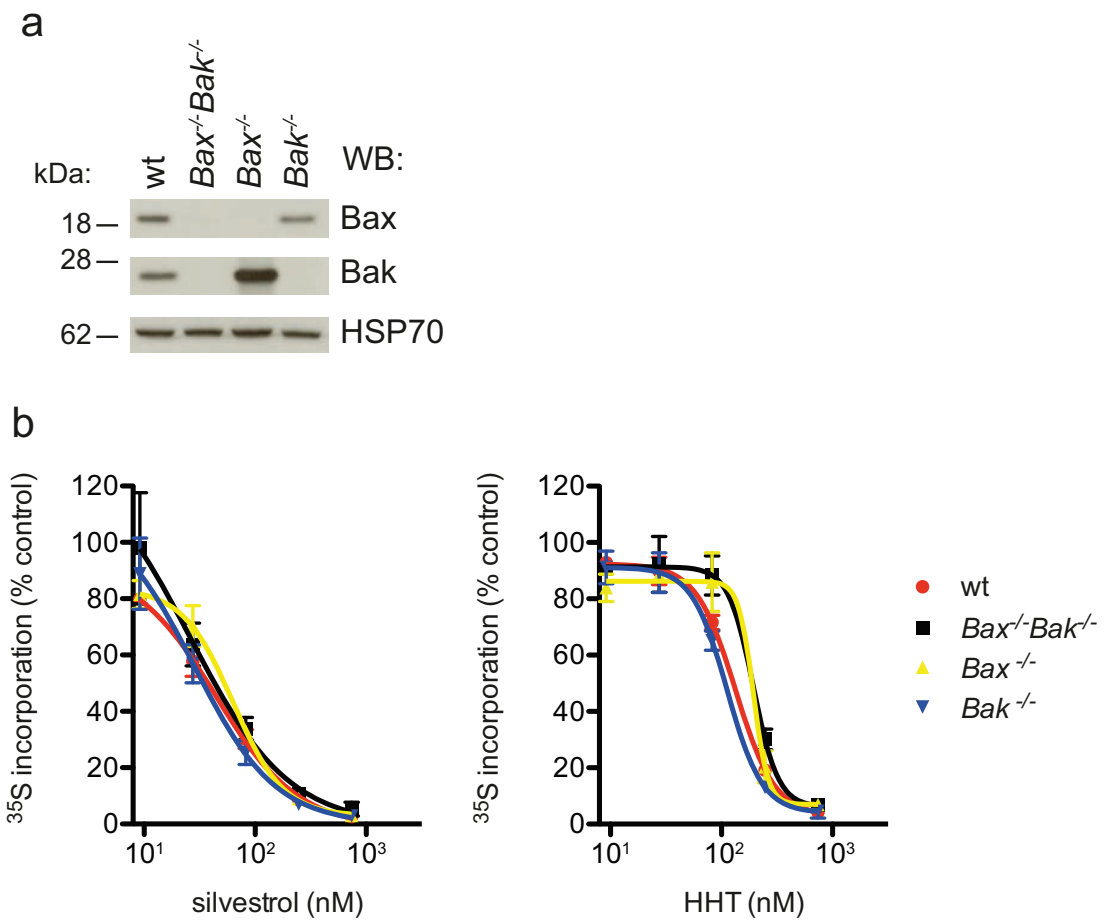

Figure S2 (**a**) Representative Western blot analysis of lysates from wildtype (wt) MEFs or those lacking pro-apoptotic Bcl-2 family members. Probing for HSP70 was used as a loading control.

(**b**) Rates of translation in MEFs after treatment with silvestrol or HHT. The mean [^{35}S]-methionine/cysteine incorporation was normalized to total protein content and represented as a percentage relative to DMSO-treated controls. The mean of 2 independent experiments with experimental quadruplicates is shown. Error bars represent the standard deviation.

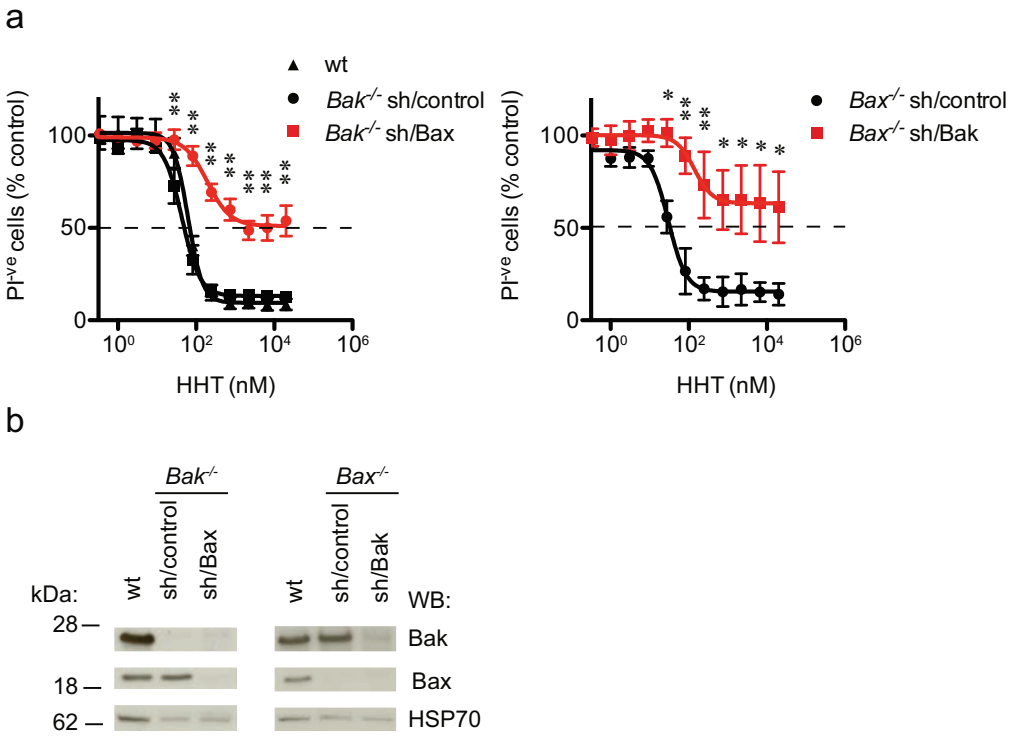

Figure S3 (**a**) Bax and Bak are important for HHT-induced killing of *Eμ-myc* lymphoma cells. The survival (PI^{-ve}) of (left) wt *Eμ-myc* lymphoma cells, ones lacking Bak (*Eμ-myc;Bak*$^{-/-}$) or sub-clones also expressing an shRNA to mouse *Bax* (or an irrelevant control hairpin) was determined after 8 h of treatment with HHT. Alternatively (right), *Eμ-myc* lymphoma cells lacking Bax (*Eμ-myc; Bax*$^{-/-}$) or sub-clones of them that are also stably expressing an shRNA to mouse *Bak* (or an irrelevant control hairpin) were examined under identical conditions. Two independent lines of each genotype were studied and each experiment repeated at least 5 times. Statistical analysis was performed using two-way ANOVA (*$p<0.05$, **$p<0.01$). Cell survival was normalized to vehicle treated controls; error bars represent the standard errors of the mean (SEM).

(**b**) Representative Western blot analysis of the lymphoma derived cell lines used in (a) and Figure 2c. Probing for Hsp70 was used as a loading control.

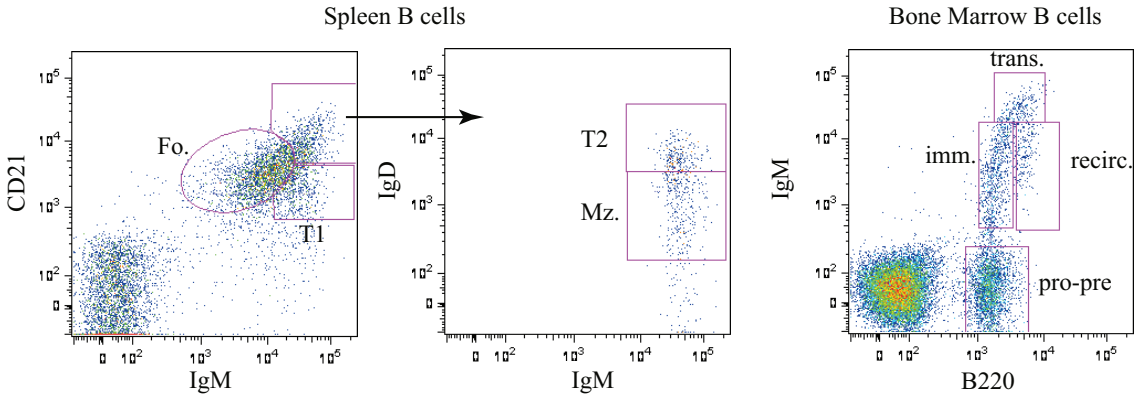

Figure S4 Representative flow cytometry analysis of control samples from spleen and bone marrow demonstrating definitions used for the different B cell subsets. Abbreviations: Fo, follicular; Mz, marginal zone; imm, immature; trans, transitional; recirc, recirculating.

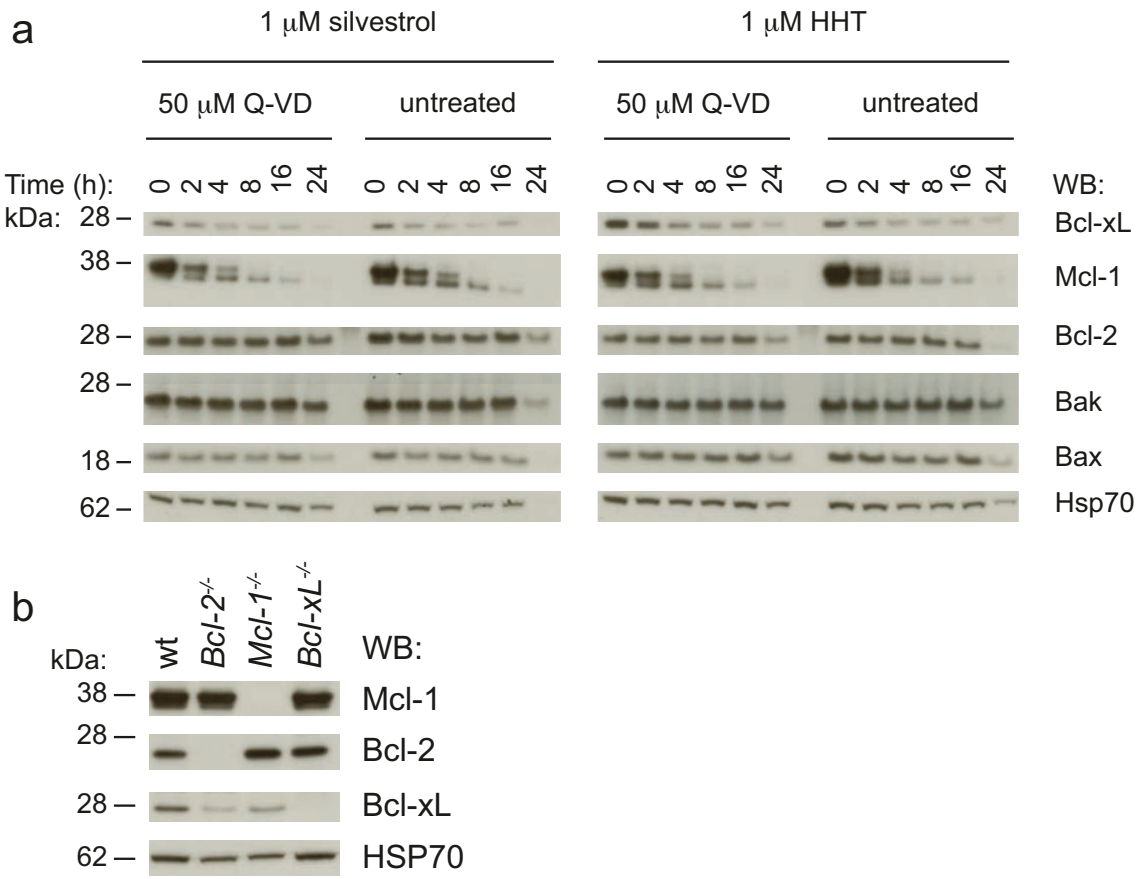

Figure S5 (**a**) Western blot analysis to examine the levels of select Bcl-2 family members in wild-type MEFs after treatment for the indicated times with translation inhibitors, in the presence or absence of the broad spectrum caspase inhibitor Q-VD-OPh.

(**b**) A representative Western Blot of MEFs lacking a pro-survival Bcl-2 member. Hsp70 was used as a loading control.

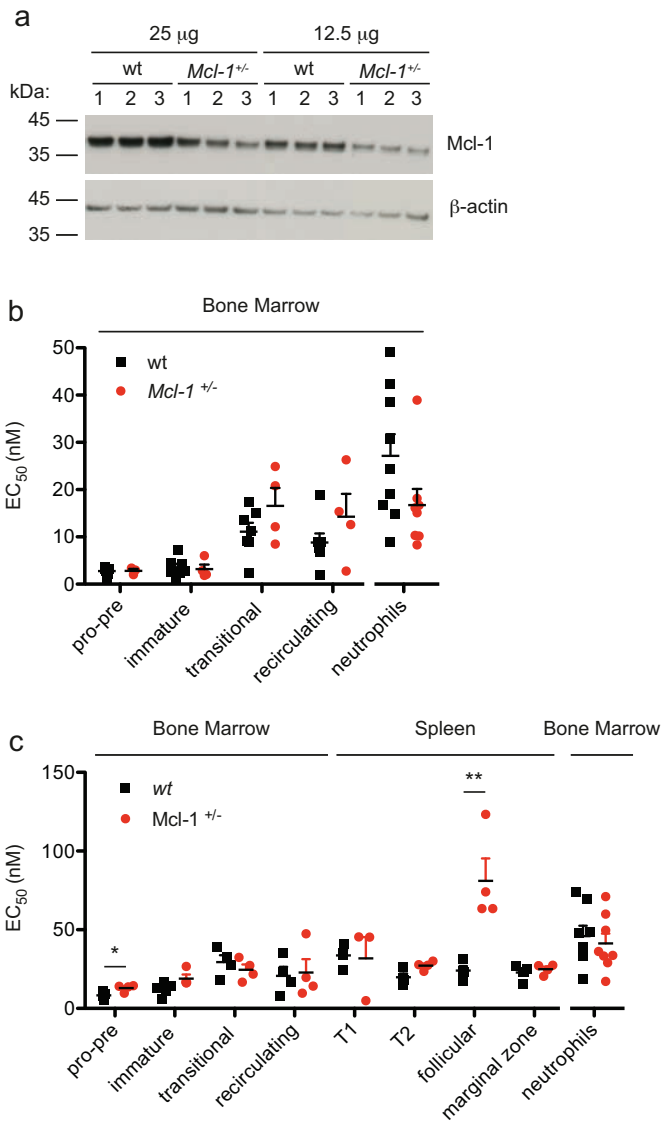

Figure S6 (**a**) Western blot showing the Mcl-1 protein level in 3 independent mouse spleens per indicated genotype.

(**b-c**) The survival of wt (*Mcl-1*$^{+/+}$) or *Mcl-1*$^{+/-}$ B cells and neutrophils was assessed after 24 h of treatment in culture with (**b**) silvestrol or (**c**) HHT by PI staining and flow cytometric analysis (n=4-8 mice per genotype). P-values (two-tailed t-test) are depicted as follows: * p <0.05, ** p < 0.01. Cell survival was normalized to vehicle (DMSO) treated controls; error bars represent the standard errors of the means (SEM).

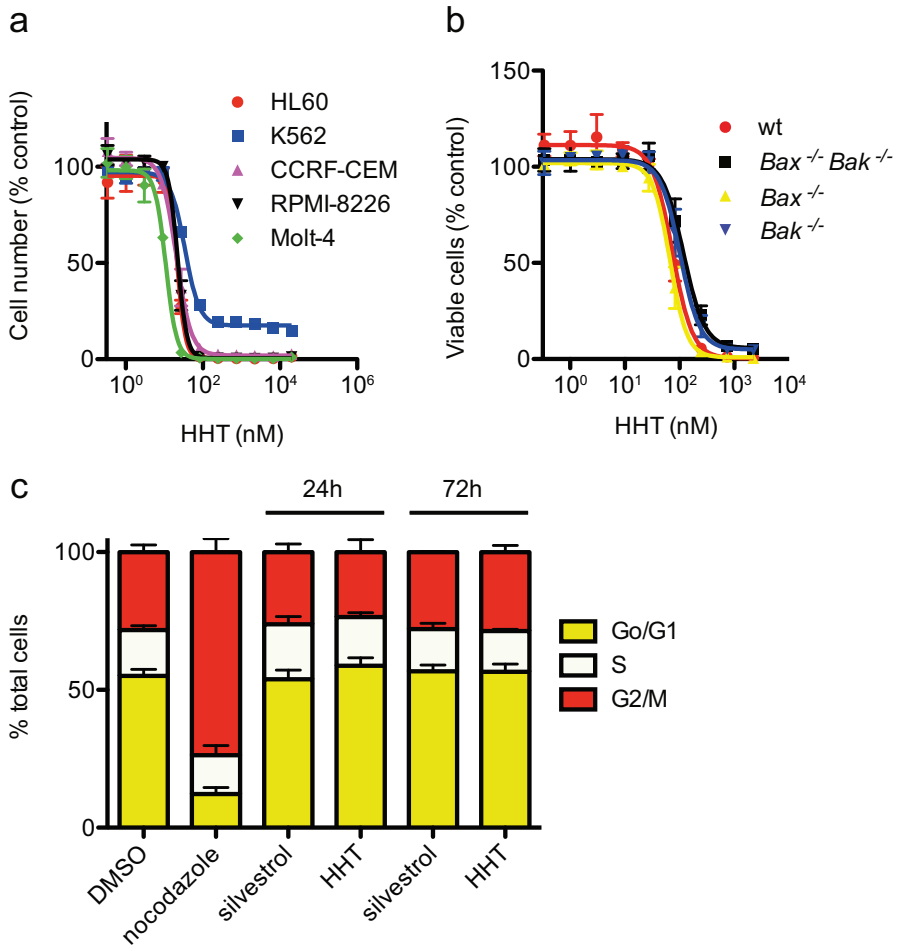

Figure S7 (**a**) The mean number of viable (metabolically active) leukemic cells were determined after 72 h of treatment with HHT using the CellTiter-Glo assay (n = 3).

(**b**) The mean numbers of viable MEFs were determined after 72 h treatment with HHT via CellTiter-Glo and data represented as a percentage of controls (n = 3 independent experiments).

(**c**) Cell cycle analysis. To separate induction of cell death from cell cycle arrest, *Bax*$^{-/-}$ *Bak*$^{-/-}$ MEFs were treated for 24 or 72 h with silvestrol (740 nM) HHT (740 nM), or for 16 h with nocodazole (330 nM), fixed in ethanol and their cell cycle distribution determined by flow cytometric analysis after PI-staining (n = 3-4).

Error bars represent the SEM in all graphs.

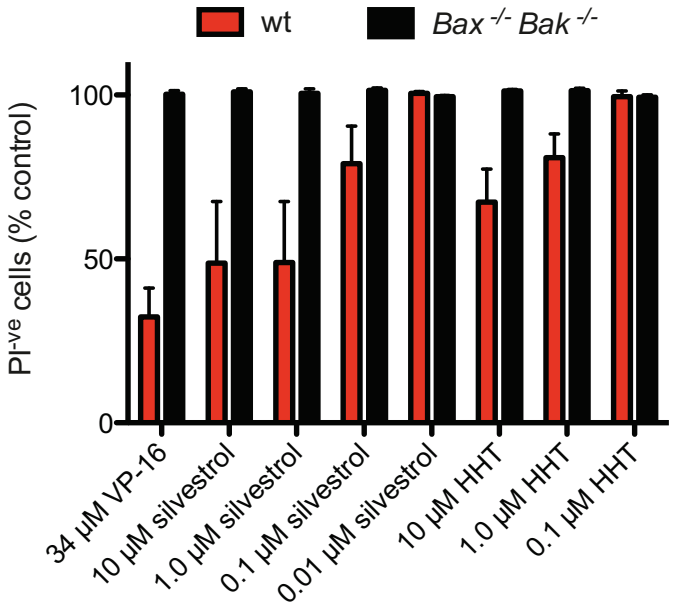

Figure S8 The survival (PI^{-ve}) of wild-type (wt) MEFs and ones lacking both Bak and Bax was determined after 14 h exposure to indicated concentrations of etoposide (VP-16), silvestrol or HHT. Data represent the mean from two cell lines of each genotype, n = 3 independent experiments; error bars represent the SEM.