Supplemental Data

Other Experimental Procedures:

RNA isolation and quantitative RT-PCR

Total RNA isolation and quantitative (q) RT-PCR were performed as previously reported [1]. The primers sets used for the study are indicated below.

Primer sets for qRT-PCR

GAPDH (5′-GAGTCAACGGATTTGGTCGT-3′, 5′-GACAAGCTTCCCGTTCTCAG-3′)

ORF73/LANA (5′-CCTGGAAGTCCCACAGTGTT-3′,5′-AGACACAGGATGGGATGGAG-3′);

vCyclin (5′-GCCGCGCTTTTTAACTTCTGAC-3′,5′-AAATAGGCGTGAGGCTTCTGAG-3′);

vFLIP (5′-GGATGCCCTAATGTCAATGC-3′,5′-GGCGATAGTGTTGGGAGTGT-3′);

Kaposin (5′-AGGCTTAACGGTGTTTGTGG-3′,5′-GTTGCAACTCGTGTCCTGAA-3′);

ORF50/RTA (5′-CAAGGTGTGCCGTGTAGAGA-3′,5′-TCCCAAAGAGGTACCAGGTG-3′);

K8/K-bZip (5′-GGCCCTAGAGGCCGTCTCCC-3′,5′-GGGAGGTCACGGGACGCTCT-3′);

ORF45 (5′-CATGGGATGGGTTAGTCAGG-3′,5′-GGGTCGCTGTATGGTGAACT-3′);

ORF21/vTK(5′-ACGCCGTGTGCGGGATCTTG-3′,5′-GACGCACCAAGTGAGTGCCCC-3′);

ORF36/vPK (5′-CCCCCGGTGTGCCCTGAAAC-3′,5′-ATCCTGGTGCGTGCACTGCC-3′);

ORF39 (5′-GCCCGATATACGGCCACCGC-3′,5′-CAGCGAAGCACCTCACCCCG-3′);

ORF49 (5′-CGAGAAGGCCCCTTAAAGAT-3′,5′-GGTACGTGGCAGTCTGGATT-3′);

ORF74/vGPCR(5′-TGTGTGGTGAGGAGGACAAA-3′,5′-GTTACTGCCAGACCCACGTT-3′);

K8.1 (5′-CACCACAGAACTGACCGATG-3′,5′-TGGCACACGGTTACTAGCAC-3′);

ORF8/gB (5′-CTGGGGACTGTCATCCTGTT-3′,5′-ATGCTTCCTCACCAGGTTTG-3′);

ORF39/gM (5′-GCCCGATATACGGCCACCGC-3′,5′-CAGCGAAGCACCTCACCCCG-3′);

EBNA1 (5′-GGTTCCAGCCAGAAATTTGA-3′,5′-TGGAATAGCAAGGCCAATTC-3′);

LMP1 (5′-TGAACACCACCACGATGACT-3′,5′-GTGCGCCTAGGTTTTGAGAG-3’);

BRLF1(5′-CTCGGGGGTTACTGCGGGGG-3′,5′-ACCGGTCGGATCCCTAACGCC-3′);

BXLF1 (5′-GTGGGCACCTGGTAGAGGCC-3′,5′-CCAGGAAAGGAGGCCGGCCC-3′);

BGLF4 (5′-CCTGAAAGCCCCGCACCAGG-3′,5′-CCCCCTCGAGAGACCCAGGC-3′);

BZLF2 (5′-GGAGGATCCACCGGCCAGACC-3′,5′-AGGAGGAGGGCGGGTGGCA-3′);

spXBP1(5’-CTGAGTCCGCAGCAGGTGCA-3’,5’-GGTCCAAGTTGTCCAGAATGCCCAA-3’);

p21 (5′-GGCGAGGCCGGGATGAGTTG -3′,5′-CTGCCGCCGTTTTCGACCCT -3′);

p53 (5′- CTGCCCTCAACAAGATGTTT -3′,5′- CTCCGTCATGTGCTGTGACT -3′);

c-Myc (5′-CAGATCAGCAACAACCGAAA -3′,5′-GGCCTTTTCATTGTTTTCCA-3′);

MDM2 (696-857) (5’- GGTGGGAGTGATCAAAAGGA -3’, 5’-GTGGCGTTTTCTTTGTCGTT -3’)

Immunoprecipitation and Western blot analyses

Immunoprecipitation was carried out using Pierce Co-Immunoprecipitation (IP) Kit (Thermo Scientific)in accordance to manufacture’s protocol. Briefly, 15μg of MDM2 or p53 specific antibody, or non-specific IgG, were coupled to 50μL of AminoLink plus amine-reactive resin (aldehyde-activated beaded agarose) prior to the addition of 400 μg of crude lysate to the

resin beads. The suspension was rotated at 4C for 16 hours, followed by washing with 200μL of IP lysis, and eluted with 60 μL of Elution Buffer. The supernatant (or flow through) from washes was discarded or saved for later testing to measure free (unbound) protein. The elutes containing the IP target proteins were neutralized with 5 μL of 1M TRIS pH 8.0 prior to denatured and loaded onto a SDS-PAGE gel for analysis.

For Western-blots whole cell extracts, prepared by lysing $2x10^6$ cells were immunoblotted with specified antibodies using the methods described previously [2].

CD30 cell surface staining

For cell surface CD30 staining, $0.1x10^6$ UM-PEL-1 cells obtained from peritoneal effusions of mice 7d after inoculation were washed with phosphate-buffered saline (PBS), resuspended in cold staining buffer [Hanks balanced salt solution with 2% FBS, and 1μg/ml blocker (BD Bioscience)] and incubated for 15min. FITC conjugated anti-CD30 antibody or isotype control were added for 30min followed by 3 washes and resuspended in cold staining buffer. Cells were analyzed on a BD LSR Analyzer (BD Biosciences).

Proliferation, cell cycle and apoptosis studies

Proliferation was assessed with CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega) following manufacturer's instructions. For cell cycle studies, $1x10^6$ UM-PEL-1c cells were incubated for 24h with PBS, Btz, SAHA or Btz/SAHA at indicated concentrations, collected, washed with PBS and fixed with 70% ethanol at 4˚C. Cells were then washed with PBS and incubated with PI (50μg/ml) and 0.1mg/ml RNAse (Invitrogen) for 30-45 min. Cell cycle analysis was performed on a BD LSR analyzer (BD Biosciences). Apoptosis studies were performed using YO-PRO-1 (or FITC conjugated annexin V) and PI as reported previously [1].

Relative quantification of K8.1 expressing cells

MetaMorph 7.7 was used to quantify K8.1/Cy3+ cells and normalize to DAPI+ nuclei using a pixel based approach. Images were acquired using Zeiss Axiovision 4.8.2 with a Hamamatsu ORCA-R2 CCD camera and Zeiss Axiovert 200M inverted fluorescence microscope, saved in Zeiss .ZVI file format, and batch exported to single channels monochrome 16-bit TIFF format using Axiovision File - Export - Batch command. Cy3 series and DAPI TIFF series were opened as respective stacks in MetaMorph. DAPI stack was thresholded, and the positive values were used to generate a binary mask stack. This mask was applied to the Cy3 stack to zero out non-cellular areas. MetaMorph Region Measurements command was used to quantify the number of Cy3+ thresholded pixels in the masked Cy3 stack vs. the number of pixels in the entire mask. Treated cells were normalized to untreated control. Error bars represent the variation in K8.1 expression between 12 fields at 10x magnification.

Virion production assays and viral DNA quantification

For testing effects on virion production in vitro, we used an infectivity read out. Briefly, $1x10^6$ UM-PEL-1c cells were stimulated for 72h with 10nM Btz, 0.75μM SAHA or 10nM Btz/0.75μM SAHA. Supernatant was harvested, spun at 3000rpm for 10min and filtered through a 0.45uM filter (ThermoScientific). Polybrene (8μg/mL) was added to $1x10^6$ uninfected HEK293 cells for 30min prior to the addition of the cleared supernatant. At 48h after infection, cells were fixed and stained for LANA as previously described[3]. LANA positive cells in 10 random fields at 20x magnification were counted and averaged. For data presentation, one LANA+ cell was considered as one infectious unit.

For quantifying in vivo effects on virion production, ascites fluid was removed from mice treated with either single dose of Btz (0.3mg/kg), SAHA (60mg/kg) or the combination of Btz/SAHA for 72h and then spun at 3,200xg for 15min at 4°C to remove cells and cellular debris. Polyethelene glycol (Abcam) was added to the cleared supernatant and incubated overnight at 4°C to precipitate the virus as per manufacturer's instructions. Precipitated virus was pelleted and DNAse treated to eliminate non-encapsidated viral DNA. Encapsidated viral DNA was then isolated using the DNeasy Blood and Tissue kit (Qiagen), with the modification of adding 3μg of carrier DNA as per manufacturer recommendations for samples with less than 5ng DNA (<10,000 copies), and quantified with qRT-PCR using primers to viral ORF73/LANA. To quantify, a KSHV DNA standard curve was generated by diluting a bacterial artificial chromosome containing the full KSHV genome ENREF_38 and amplifying the same region of LANA as was used for the experimental samples. Ct values for the BAC36 were converted into DNA copy number using the derivation of DNA mass formula: where n= DNA size in base pairs, m= mass, Avogadro's number= 6.023e23 molecules/mole, average MW of a double-stranded DNA molecule= 660g/mole, as per ABI protocol. An ABI PRIZM 7300 sequence detector was used to run the reactions and amplification plots were analyzed using the SDS software. Non-template controls were run to verify absence of reagent contamination. All error bars represent SEM.

To quantify intracellular KSHV DNA, total cellular DNA was harvested with the AllPrep RNA, DNA, and protein kit (Qiagen) and quantified using a Nanodrop spectrophotometer. 10ng/well was loaded in quintuplicate and viral DNA copy number was quantified as described above.

NF-κB electrophoretic mobility shift assay (EMSA)

UM-PEL-1 cells (25×10^6) isolated from ascites of tumor bearing mice were resuspended in 200 μL ascites fluid and injected i.p. into NOD/SCID mice. At day 7 post tumor cells injection, mice were treated i.p. with DMSO (50μl), Btz (0.3mg/kg), SAHA (60mg/kg), Btz/SAHA (0.3mg/kg/60mg/kg) and sacrificed 24h after treatment. UM-PEL-1 cells harvested from the peritoneal effusions were used to prepare nuclear extracts for EMSA. NF-κB EMSA was performed as previously reported[1].

Supplemental References

1. Sarosiek KA, Cavallin LE, Bhatt S, et al. Efficacy of bortezomib in a direct xenograft model of primary effusion lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(29):13069-13074.
2. Jiang X, Lu X, McNamara G, et al. HGAL, a germinal center specific protein, decreases lymphoma cell motility by modulation of the RhoA signaling pathway. *Blood*. 2010;116(24):5217-5227.
3. Mutlu AD, Cavallin LE, Vincent L, et al. In vivo-restricted and reversible malignancy induced by human herpesvirus-8 KSHV: a cell and animal model of virally induced Kaposi's sarcoma. *Cancer cell*. 2007;11(3):245-258.

Supplemental Figures

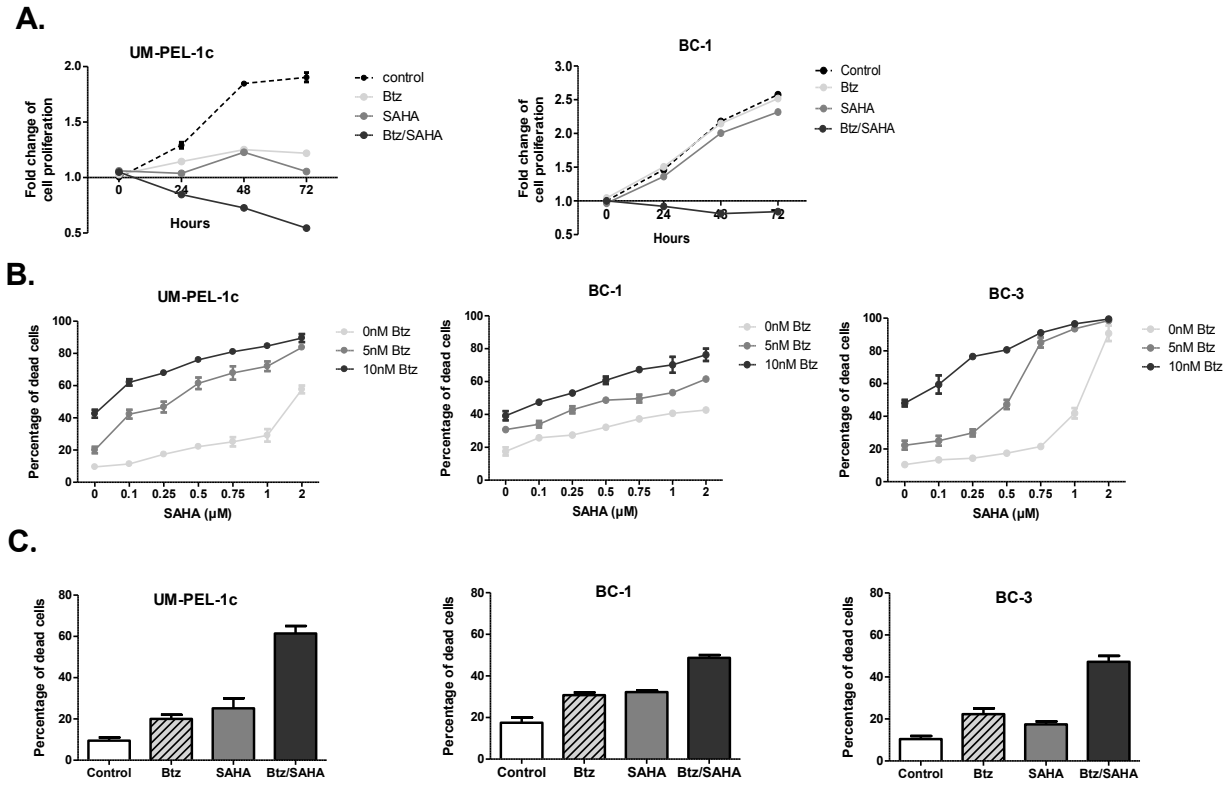

Figure S1. Combination of Btz/SAHA induces proliferative arrest and apoptosis in PEL cell lines in vitro. For panels A-B, in vitro cultured UM-PEL-1c, BC-1 and BC-3 cells were treated with 5nM Btz, 0.5μM SAHA or 5nM Btz/0.5μM SAHA. (A) Cell proliferation was determined at indicated time points by MTT assay. (B) UM-PEL-1c, BC-1 and BC-3 cells were treated with concentrations of SAHA (ranging from 0 to 2μM) with or without of 5nM or 10nM Btz for 24h then stained for YO-PRO-1-/PI to quantify apoptosis by flow cytometry. (C) Apoptosis was quantified by YO-PRO-1/ PI staining at 24h after treatment. Experiments were repeated at least three times and error bars indicate SEM.

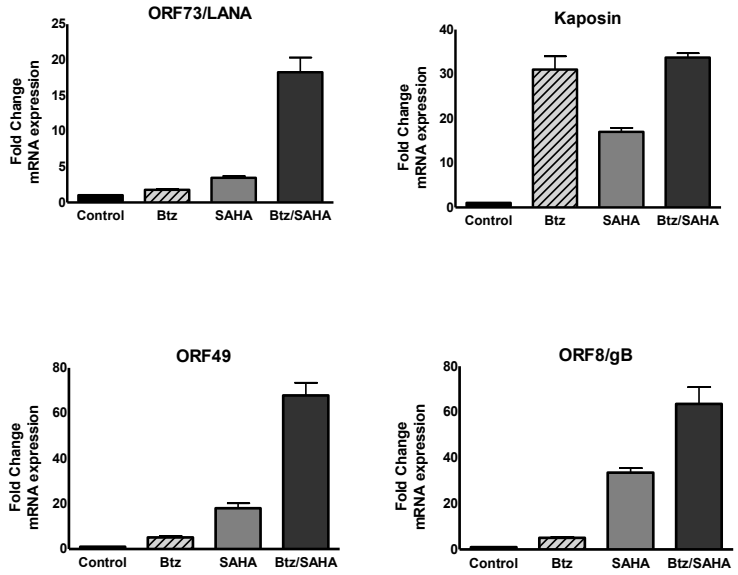

Figure S2. Additional KSHV genes analyzed in UM-PEL-1c. In vitro cultured UM-PEL-1c were treated with 0.5μM SAHA, 10nM Btz or combination of 0.5μM SAHA/10nM Btz, and total RNA was harvested for qRT-PCR analysis of latent (OEF73/LANA), early lytic (ORF49), delayed early lytic (Kaposin), and late (ORF8) viral mRNA at 24h after treatment. Results are representative of 3 individual experiments. Error bars represent the SEM.

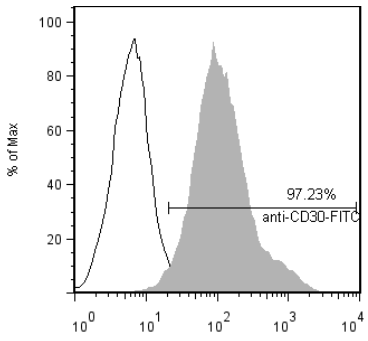

Figure S3: UM-PEL-1 xenograft mice have classical CD30+ PEL phenotype. (A) Flow cytometry histogram indicates positive staining for CD30 in UM-PEL-1 cells harvested from mice 7d after inoculation. Grey histogram represents staining with CD30 and white histogram represents isotype control.

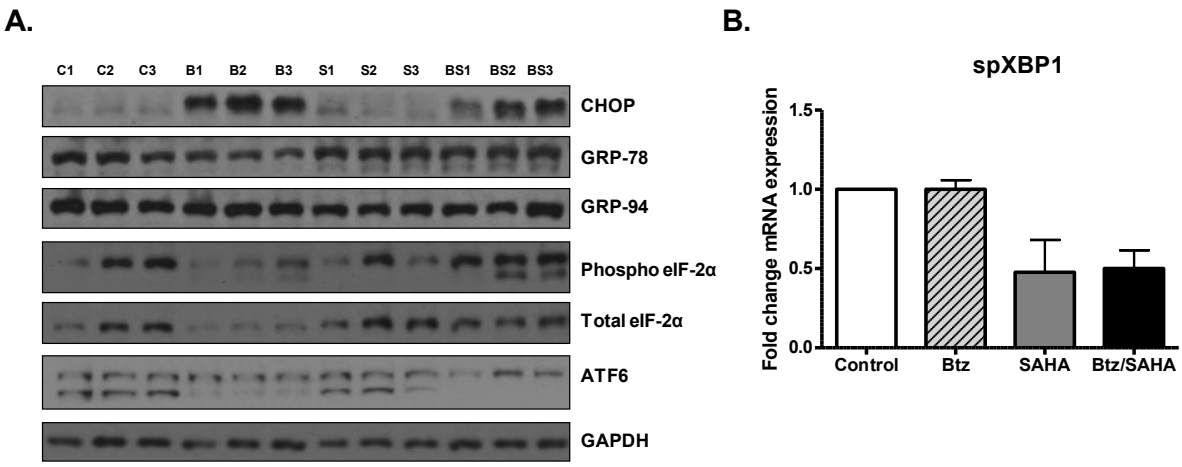

Figure S4. Effect of Btz/SAHA combination on terminal UPR pathway in UM-PEL-1 xenografts. Tumor bearing mice (n=3 mice/group) were treated with a single dose of DMSO (control), SAHA, Btz or combination (Btz/SAHA) (C=DMSO control, B=Btz, S=SAHA, and BS=Btz/SAHA). (A) At 24h after treatment, whole cell lysates were prepared from peritoneal effusions and used for immunoblotting with the indicated antibodies. GAPDH served as a loading control. Results are representative of two independent experiments. (B) qRT-PCR analysis of spXBP1. Error bars represent SEM between triplicate samples.

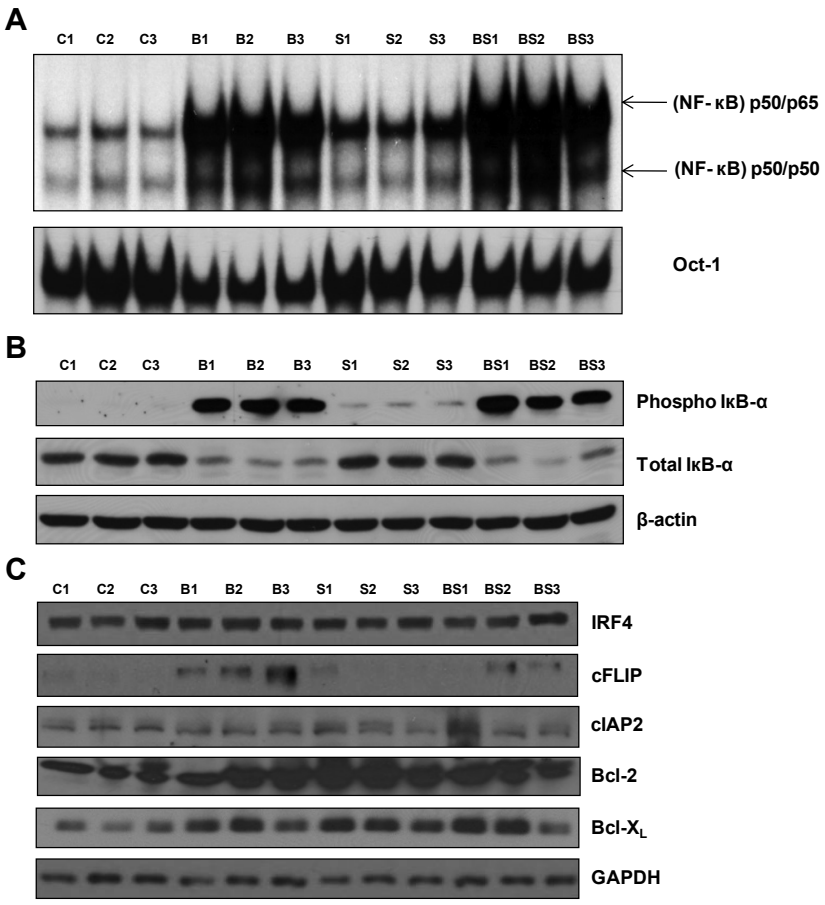

Figure S5. Btz and SAHA induce NF-κB activity in vivo in PEL xenografts, while expressions of NF-κB target genes, except cFLIP, remain unaltered. UM-PEL-1-bearing mice (n=3) were treated with a single dose of Btz, SAHA, or combination of Btz/SAHA (C=DMSO control, B= Btz, S=SAHA, and BS=Btz/SAHA). Peritoneal cells from ascites were harvested at 24h. (A) Nuclear extracts prepared from ascites of tumor bearing mice were subjected to EMSAs using NF-κB–specific consensus oligonucleotides probes to capture NF-κB complexes. Oct-1 was the normalizing control. (B and C) Western blots of specified proteins from whole cell lysate of UM-PEL-1 ascites collected at 24h after specified treatments. β-actin served as a loading control. Results are representative of a minimum of 2 independent experiments.

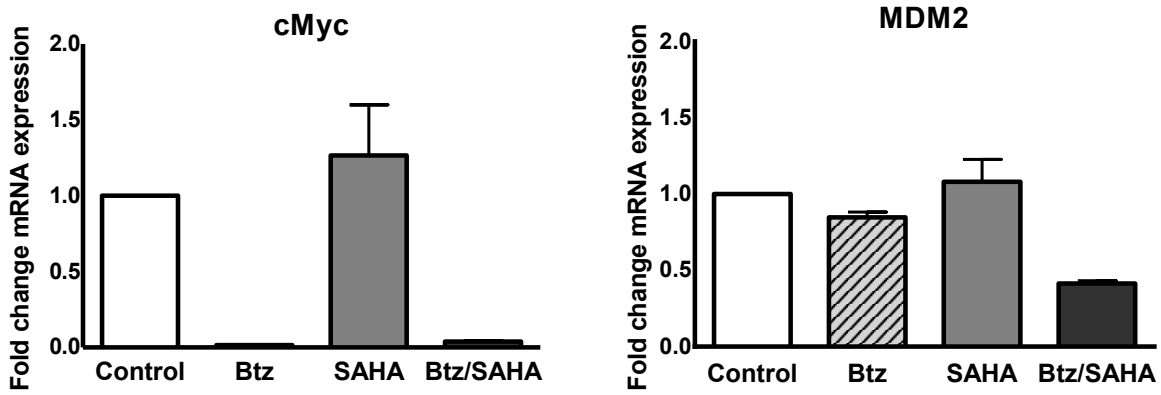

Figure S6. Effect of Btz and SAHA on transcript levels of cMyc and MDM2 in PEL xenografts in vivo. UM-PEL-1-bearing mice were treated with a single dose of Btz, SAHA, or combination of Btz/SAHA. Tumor cells from ascites were harvested at 24h. Total RNA was isolated and qRT-PCR was performed for gene expression. Results are representative of 2 independent experiments performed in triplicate reactions. Error bars represent SEM.

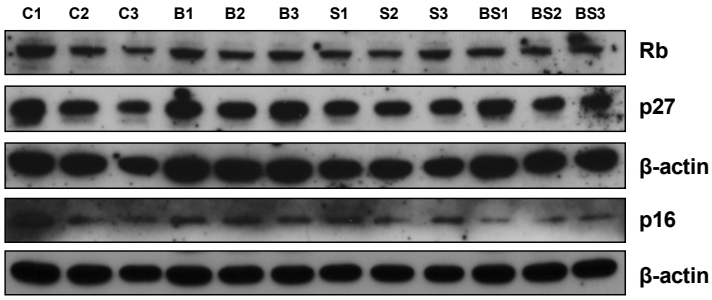

Figure S7. Btz/SAHA combination does not affect expression of p27, p16 and Rb in PEL xenograft mice. Tumor bearing mice (n=3 mice/group) were treated with a single dose of DMSO (control), SAHA, Btz or combination (Btz/SAHA) (C=DMSO control, B=Btz, S=SAHA, and BS=Btz/SAHA). After 24h of treatment, whole cell tumor lysates were prepared from peritoneal effusions and subjected for immunoblotting using the indicated antibodies. β-actin served as loading control. Results are representative of two independent experiments.

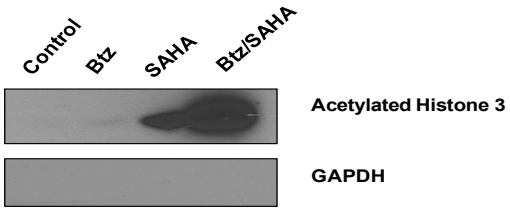

Figure S8. Btz/SAHA combination increases acetylation of histone 3 subunit in PEL xenograft cells treated ex-vivo. Whole cell lysate obtained from UM-PEL-1c treated in culture for 24h with 10nM Btz, 0.75μM SAHA or 10nM Btz/0.75μM SAHA were utilized for protein extraction and subsequent western-blot analysis. GAPDH was used as a loading control. Results are representative of 2 independent experiments.

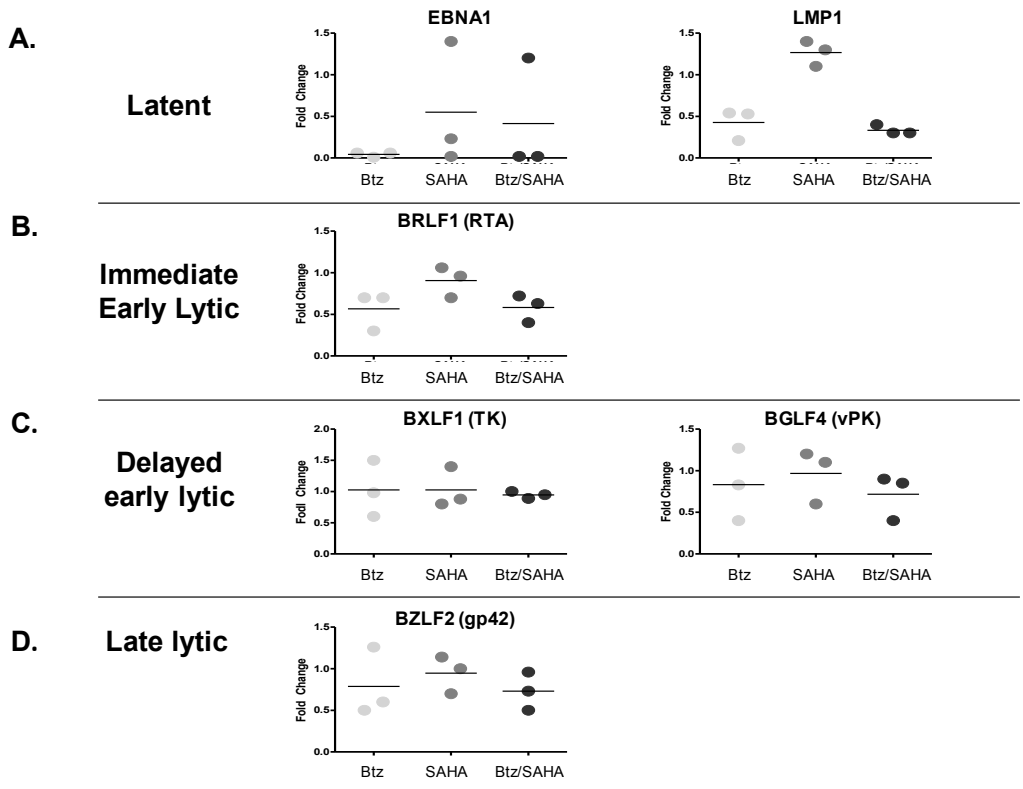

Figure S9. qRT-PCR analysis of representative EBV gene expression in PEL xenografts treated with Btz and/or SAHA. For all panels, UM-PEL-1 tumor bearing mice (n=3 mice/group) were treated with a single dose of Btz, SAHA, or the combination of Btz/SAHA. At 24h after treatment, peritoneal effusions were harvested, total RNA was isolated and qRT-PCR was performed. (A-D) mRNA expression of latent, immediate early, delayed early lytic and late lytic EBV genes. The line at 1 on the y-axis represents DMSO treated control mice to which the experimental mice were normalized. Each circle represents one mouse with the line drawn at the mean fold induction. Results are representative of 2 independent experiments.