Inhibition of Polo-like kinase 1 (PLK1) to facilitate the reactivation and elimination of latent gamma-herpesviruses

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Key words

KSHV, EBV, latency, reactivation, viral reservoir, PLK1, cell survival.

Abstract

Both Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) can establish the persistent, life-long infection primarily at the latent status, and contribute to certain types of tumors, including B cell lymphomas, especially in immuno-compromised individuals, such as people living with HIV (PLWH). Lytic reactivation of these viruses can be employed to kill tumor cells harboring latently infected viral episomes, through the viral cytopathic effects and

the subsequent antiviral immune responses. In this study, we identified that expression of Polo-like kinase 1 (PLK1) in B cells is elevated in the context of HIV infection and by HIV Nef protein. We further demonstrated that PLK1 depletion or inhibition can promote KSHV reactivation and cell death of KSHV-reactivated tumor cells. Mechanistically, PLK1 regulates Myc protein that is critical for both maintenance of KSHV latency and support of cell survival, and affects the level of H3K27me3 suppressive mark both globally and at certain loci of KSHV viral episomes. Lastly, we recognized that PLK1 inhibition can synergize with STAT3 inhibition to induce efficient KSHV reactivation. PLK1 depletion or inhibition yielded the similar effect on promoting EBV reactivation and cell death of EBV-reactivated tumor cells. Our findings illustrated that PLK1 is a novel host target that can be inhibited to benefit the viral oncolysis to eliminate KSHV/EBV-infected tumor cells.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), is an etiological agent of Kaposi's sarcoma (KS) – a common AIDS-associated malignancy[1], as well as two lymphoproliferative diseases, namely primary effusion lymphoma (PEL) and multicentric Castleman's disease (CAD)[2,3]. As similar to other herpesviruses, KSHV infection also includes two phases of replication: latent and lytic cycles [4,5]. Following acute infection, KSHV establishes latency mostly in immune-compromised patients [5]. In latency, KSHV can evade the host immune surveillance and facilitate the establishment of persistent live-time infection. KSHV latently infected cells constitutes a viral reservoir for chronic infection. KSHV has a diverse range of *in vivo* and *in vitro* cell tropism, but CD19+ B cells appear to be the primary target for latent infection [6,7]. Epstein-barr virus (EBV) belongs to the same human γ-herpesvirus family as KSHV. It is interesting to note that nearly 70%

of PEL cell lines are co-infected with EBV. Studies have demonstrated that EBV co-infected PEL cell lines are more tumorigenic compared to the EBV negative ones [8,9].

Oncolytic viruses have been recently engineered as novel anticancer agents and shown to increase the therapeutic promise[10,11]. Similarly, lytic reactivation of intrinsic latent viruses in tumor cells can also lead to oncolysis and be applied as an anticancer therapy. Although these findings are promising, such approaches are still at the infant stage and have received only limited investigation so far. Therefore, we are interested in understanding the common cell survival mechanisms associated with reactivation of latent KSHV and EBV, which can be employed as a therapeutic strategy to eliminate the tumor cells harboring these viruses.

Polo-like kinase 1 (PLK1) plays an important role especially in cell survival by stabilizing MYC protein in B-cell lymphoma[12] and in tumor cells[13]. Recently we have reported that PLK1 can be elevated during HIV-1 reactivation from latency or *de novo* infection[14], which plays a critical role in survival of HIV-infected $CD4^+$ T cells. Although it has been reported that PLK1 expression is also elevated in various human cancers, and abnormally elevated expression of PLK1 is often linked to tumor aggressiveness and poor clinical prognosis, there are scanty information about its role in supporting KSHV or EBV associated cancer progression. As PLK1 inhibition can successfully compromise tumor cell survival without affecting non-tumor cells[15,16], it was really worthy of investigating the effect of PLK1 inhibition on KSHV/ EBV infection.

In this study, we identified that PLK1 plays a critical role in maintaining the latent infection of KSHV/EBV in B lymphoma cells and supporting their cell survival status, which has never been previously reported. Specific inhibition of PLK1 can facilitate the viral lytic reactivation and subsequently facilitate the elimination of B lymphoma cells harboring latent KSHV/EBV

reservoirs, which can be used as a future therapeutic approach with great impact to treat KSHV/EBV-positive B lymphomas.

Materials and Methods

Cells. Jurkat Clone E6-1 (Cat. #177) were received from the NIH AIDS reagent program. TREx-BCBL1-RTA cells[17] were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% Pen-Strep, 20 mg/ ml hygromycin B and 2 mM L-glutamine (Invitrogen). KSHV-positive cell lines (BCBL-1 WT) were grown in RPMI 1640 supplemented with 20% heat-inactivated FBS and 2 mM l-glutamine[17]. BJAB cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% Pen-Strep. iSLK.r219 cells[18] were maintained in DMEM medium (Corning) containing 10% FBS, 1% Pen-Strep, 10 µg/ml puromycin (Corning), 250 mg/ml Geneticin (Corning), and 400 mg/ml hygromycin B (Corning). iSLK-BAC16 cells were maintained in the presence of 1 µg/ml puromycin, 250 µg/ml G418, and 1,200 µg/ml hygromycin B[19]. Akata/BX cells were maintained in RPMI 1640 supplemented with 10% FBS. EBV-negative Akata cell line was clonally selected for the loss of viral episome from the original EBV-positive Akata Burkitt's Lymphoma cell line[20], which was subsequently re-infected with EBV BX strain and selected with neomycin[21].

Isolation of primary B cells. Peripheral blood mononuclear cells (PBMCs) from HIV-infected individuals were acquired from Vitrologic Biological source (Charleston, SC). PBMCs from healthy donors were purchased from STEMCELL™ Technologies (Cambridge, MA). PBMCs from HIV+/- donors were subjected to B-cell isolation . For B-cell isolation, cells were captured by using anti-CD19 antibody conjugated to colloidal paramagnetic microbeads (B-cell isolation

kit; Miltenyi Biotec, Bergisch-Gladbach, Germany) and passed through a magnetic separation column (LS; Miltenyi Biotec). The purity of isolated B cells was over 95% as assessed by flow cytometric analysis of PE-CD19+ cells (Miltenyi Biotec) [22,23].

Viruses. HIV-1 IIIB wild-type virus was kindly provided by the NIH AIDS reagent program[24,25]. Jurkat cells were used for HIV-1 IIIB infection at multiplicity of infection (MOI) = 1 for 5 days[1425].

Antibodies. ChIP-grade mouse anti-PLK1 (Cat. # SAB1404220) and mouse IgG (Cat. #sc2025) antibodies were obtained from Sigma Aldrich. Anti-FLAG (Cat. #2368) antibody was purchased from Cell Signaling Technology. Mouse Anti-V5 (Cat. # R960-25), anti-HA (Cat. # 26183), HRP-conjugated goat anti-Mouse IgG (H+L) secondary antibody (Cat # 31430), HRP-conjugated goat anti-Rabbit IgG (H+L) secondary antibody (Cat # 31460) antibodies were purchased from Thermo Fisher Scientific. Antibodis against c-Myc (Cat. # 9E10: sc-40), HHV-8 K8.1A/B (Cat. # sc-65446), EBV ZEBRA (Cat. # sc-53904), GAPDH (Cat. # sc-32233) were purchased from Santa Cruz Biotechnology.

siRNAs and shRNAs. For siRNA knockdown assays, PLK1 siRNA (s449) or non-targeting control (NT) siRNA (AM4636) were purchased from Thermo Fisher Scientific. TREx-BCBL1-RTA cells were transiently reverse-transfected with siPLK1 (50nM) or siNT (50nM) using RNAiMAX reagents[26] according to the manufacturer's instruction and incubated for 72 hour. These cells were treated with doxycycline (2μg/ml) to induce KSHV reactivation. For shRNA assays, endogenous PLK1 was knocked down in AKATA/Bx cells by using its shRNAs expressed from the pINDUCER10 Dox-inducible lentiviral vector, according to the reported protocol[27,14]. Briefly, shRNAs targeting PLK1 (5′-GTT CTT TAC TTC TGG CTA TAT-3′) and the control shRNA targeting firefly luciferase (shNT: 5′-CAC AAA CGC TCT CAT CGA CAA G-3′) were

transiently transfected in AKATA/bx cells through electroporation and incubated for 72 hour. These cells were treated with Doxycycline to induce shRNA expression and human IgG (2µg/ml) to induce EBV reactivation for 48 hours.

Compounds. DMSO was purchased from Fisher Scientific. SBE 13 HCl were purchased from Selleck Chemicals. Recombinant HIV-1 Nef protein (rNEF) were provided by NIH AIDS reagent program. The UMB-158 PLK1-BET dual inhibitor was synthesized according to an earlier publication[28]. The STAT3 inhibitor, Cryptotansinone and Stattic, were purchased from Sigma-Aldrich.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was conducted according to the manufacturer protocol (Millipore Sigma, cat # 17-395) as described previously[29,30]. Briefly, Cells were cross-linked by using 0.5% formaldehyde, followed by treating with 1X glycine to quench the reaction. After washing with cold 1× PBS, nuclei were isolated using nuclei isolation buffer with rigorous vortexing. Nuclear lysates were sonicated for 2 min to fragment genomic DNAs. All extraction procedure was carried out in presence of protease inhibitor cocktail. 1% input were separated before the next step. The lysates were incubated with pre-washed Magna ChIP A/G beads along with specific antibodies or control mouse IgG or rabbit IgG overnight at 4°C. After subsequent washes samples were eluted through magnetic separator. Every samples were treated with proteinase K. To reverse the cross-linking, the eluted samples were incubated at 65°C for 2 hours and then 95°C for 15 mins. Next, final magnetic separation was done to elute the samples. KSHV lytic gene promoter expression were quantified by real time RT-PCR. Input (1%) was used for qPCR analysis. PCR primers: ORF50 (F:5′- ATGAAGATGTGGTAGAGCCA -3′; R: 5′-TAGCGCCATCTCTGCCCCCC -3′), OriLyt (F: 5′- CCTACATGGGCAGCTTGTCC -3′; R: 5′-

TGCTGCCGGGGCTCCTCGTT -3′), ORF59 (F:5′- CACACTTCCACCTCCCCTAA -3′; R: 5′-CGCACAGAGAAATCACAGGA -3′)[31].

Fluorescence imaging. Fluorescence was measured on a BioTeK plate reader by using its GFP and RFP channels. Expression of GFP and RFP proteins from KSHV.r219 viral strain are respectively driven by the eIF1a promoter and KSHV lytic PAN promoter[32].

Flow cytometry. Isolated B-cells from HIV+/- subjects were immuno-stained with PLK1 and CD19 antibodies. Immediately after isolation, portion of B-cells were incubated with CD19 antibody (1/200 of stock, Milteny) for 30 mins to check the purity using BD Accuri C6 Plus with corresponding optical filters. Other set of B-cells were washed and fixed with 4% paraformaldehyde at RT for 20 min. Pelleted cells were washed and permeabilized with saponin-containing 1× Perm/Wash buffer (BD Biosciences) as described[14]. Cells were then incubated with anti-PLK1 antibody (200μg/ml) diluted in 1× Perm/Wash buffer overnight at 4°C, followed by the incubation with fluorophore-conjugated secondary antibodies for 1 hour at RT in the dark. The staining buffer [1× D-PBS with 2% bovine serum albumin (BSA)] was added to resuspend cells, followed by flow cytometry analysis using the BD Accuri C6 Plus with corresponding optical filters. For co-culture assays of Jurkat and BJAB cells, dual-color immuno-staining was performed. BJAB cells were labeled separately with CFSE (Thermo Fisher) for 30min according to instruction and then incubated with HIV-1 IIIB infected Jurkat cells to determine the B-cell specific PLK1 expression. The MFI and the percentage of fluorescence-positive cells were determined by using the FlowJo V10 software.

Cell viability assay. The cytotoxicity of compounds was determined by using the ATP- based CellTiter-Glo Luminescent Cell Viability Assay (Promega) and analyzed by the Cytation 5 multimode reader (luminescent mode). Death of virus-infected or compound-treated cells was

determined by using the LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Invitrogen) and analyzed by the BD Accuri C6 Plus (flow cytometry)[14].

Apoptosis Assay Kit. The Vybrant Apoptosis Assay Kit (Thermo Fisher, cat # V13243) were used to detect the apoptosis. YO-PRO-1 and propidium iodide nucleic acid stains were used as per instructions. YO-PRO-1 stain selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence. Necrotic cells are stained red-fluorescent with propidium iodide[33].

Genomic DNA isolation. Extraction of genomic DNA was performed by using the DNeasy Blood & Tissue Kit (Qiagen, cat # 69504) according to the manufacture's instruction.

Real-time qPCR. Total RNAs were extracted from the assayed cells by using the RNeasy kit (Qiagen), and 0.2-1 µg of RNA was reversely transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time PCR assay was conducted using the SYBR Premix ExTaq II (Bio-Rad) and gene-specific primers. The PCR reactions were performed on a Bio-Rad CFX connect qPCR machine under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative percentage of gene expression was normalized to the GAPDH control, and was calculated using the formula: $2^{(\Delta \text{ C T of gene} - \Delta \text{ CT of GAPDH})}$. RT-qPCR primers: PLK1 (F : 5'- CCA ACA CCA CGA ACA CGA-3'), R:5'- GCC TCT TGT CTC TTA GAT TTG GTC-3'), GAPDH (F:5'- GCC TCT TGT CTC TTA GAT TTG GTC -3'; R: 5'- TAG CAC TCA CCA TGT AGT TGA GGT -3'). All the other KSHV and EBV lytic gene primers are described in Table S1.

Immunoblotting assays. Immunoblotting was carried out using the existing protocol[14,25]. In brief, cell pellets were homogenized in ice with 1X RIPA containing protease inhibitor cocktail. The cell lysate was cleared by centrifugation at 12,000rpm for 10 min. The protein concentration was measured by BCA kit (Thermo Fisher Scientific). Using same amount of protein samples were

boiled in 2X SDS loading buffer, and analyzed by immunoblotting. Protein samples were separated by SDS-PAGE, and transferred to PVDF. Blots were blocked with 5% skimmed milk in PBS and probed with specific antibodies followed by anti-mouse HRP-conjugated secondary antibodies. Protein bands were visualized with ECL Plus chemiluminescence reagent.

Results

HIV Nef protein induces PLK1 upregulation in B cells.

As the incidence of B-cell lymphoma among HIV-infected subjects greatly exceeds that of the general population[34], we first compared the PLK1 expression in B cells between HIV-negative and positive subjects. B cells were isolated from PBMCs of these donors (**Fig 1A**), and PLK1 mRNA and protein were measured by qPCR and immunostaining respectively. Increased PLK1 mRNA level (**Fig 1B**) was consistent across HIV+ subjects compared to healthy donors. Increased PLK1 protein level was also observed in HIV+ subjects (**Fig 1C,D**). We further determined the impact of HIV infection on PLK1 expression in B cells *in vitro*. Jurkat cells were subjected to HIV-1 IIIB infection, verified by immunoblotting of HIV-1 Gag p24 protein (**Fig S1A**). HIV-infected Jurkat cells were co-cultured with CFSE pre-stained BJAB cells, which led to the moderate increase of PLK1 protein level measured by immunostaining once compared to mock-treated BJAB cells (**Fig 1E**). We previously showed that HIV Nef protein contributes to the upregulation of PLK1 in HIV-infected T cells. We expected that Nef plays a similar role in B cells as well. BJAB cells were incubated with recombinant Nef (rNef) protein at the increased dose, which indeed resulted in the upregulation of PLK1 protein (**Fig 1F**). Similar effect was observed in rNef protein treated Akata-Bx1 (**Fig 1G**) and TREx BCBL1-Rta (**Fig 1H**) cells. In parallel, we also confirmed the earlier

finding that treatment of rNef protein suppresses KSHV lytic reactivation in Dox-treated TREx BCBL1-Rta cells without any cytotoxicity (**Fig S1A,B**).

PLK1 is required for maintenance of KSHV latency.

KSHV infection is more common among people living with HIV (PLWH) than in the general population in the world[35,36,37]. To investigate the role of PLK1 in regulating KSHV infection, we depleted the expression of endogenous PLK1 in TREx-BCBL1 Rta cells using its siRNA (siPLK1), while non-target siRNA (siNT) was used as a control. PLK1 depletion led to more reduction of cell viability, especially in KSHV-reactivated cells induced by doxycycline treatment (**Fig 2A**), while siPLK1 knockdown efficiency was confirmed by immunoblotting (**Fig 2B**). Consistently, PLK1 depletion further enhanced KSHV lytic reactivation through measurement of K8.1 lytic protein by immunoblotting, but it only occurred in doxycycline-treated cells (**Fig 2B**). It seemed that PLK1 loss is not sufficient to induce KSHV reactivation but rather further enhance it induced by other stimulus. We further tested the effect of PLK1 depletion on KSHV reactivation in iSLK.r219 cells, which showed the similar effect on KSHV lytic reactivation (**Fig 2C,D**), but there was no difference in terms of cell viability irrespective of reactivation (**Fig S2A**). Alternatively, we also confirmed the effect of PLK1 by gain-of-function analysis. pLEX-PLK1 (V5-tagged) was transiently transfected in iSLK.r219 cells, causing the compromised KSHV lytic reactivation comparing to those transfected with the control vector (pLEX-FLAG expressing FLAG peptide) (**Fig 2E,G**). Overexpression of PLK1 was confirmed by both protein immunoblotting (**Fig 2F**) and mRNA qPCR (**Fig S2B**) assays.

PLK1 inhibition promotes KSHV lytic reactivation.

Our recent work suggests that PLK1 inhibition reactivates latent HIV[14]. Based on the observation that PLK1 depletion facilitates KSHV lytic reactivation, we further evaluated such

effect of PLK1 inhibitors. We used a PLK1-specific inhibitor, SBE 13 HCl (SBE), which targets PLK1 inactive kinase conformation[38]. SBE treatment further enhanced Dox-induced KSHV lytic reactivation in TREx BCBL1-Rta cells in a dose dependent manner (**Fig 3A,B**), but SBE alone is not sufficient to do so (data not shown). SBE didn't cause any severe cytotoxicity (**Figure S3A**). We further confirmed SBE's effect on KSHV lytic reactivation in BCBL1 cells treated with TPA + Sodium butyrate (TPA/SB) or mock treated (**Fig 3C**). In BCBL cells, SBE alone caused some weak effect on KSHV reactivation but significantly enhanced TPA/SB's reactivation potency. Lastly, we carried out the similar test of SBE in iSLK.r219 cells. Consistently, SBE also further enhanced the Dox-induced KSHV lytic reactivation in these cells (**Fig 3D,E**). Recently, PLK1-BET dual inhibitors have been developed to target both PLK1 and BRD4, two major protein targets in anticancer therapies[39,40,41]. We tested one of such PLK1-BET dual inhibitors, UMB158, which possesses the novel scaffold (UMB series)[28], previously studied by our group. Indeed, UMB158 treatment also promotes the Dox-induced KSHV reactivation in iSLK.r219 cells at the lower dose compared to SBE (**Fig 3F**). We also tested two other PLK1-BET dual inhibitors, Bi-6727 and Bi-2536, which also benefits the Dox-induced KSHV lytic reactivation in TREx BCBL1-Rta cells (**Fig S4A-D**).

PLK1 inhibition promotes the cell death of KSHV-reactivated cells.

It has been well reported that PLK1 supports cell survival[42,43,14]. In this work, we wondered whether PLK1 inhibition would synergize with viral cytopathic effect and further promote cell death of KSHV-reactivated cells. Indeed, we confirmed that SBE treatment causes more cell death of KSHV-reactivated TREx BCBL1-Rta cells induced by doxycycline compared to mock treatment without KSHV reactivation (**Fig 4A**), measured by LIVE/DEAD assay. Alternatively, we also determined the viability of cells treated with PLK1 inhibitors by measuring cellular ATP

level. Un-infected BJAB cells were included for such analysis. SBE treatment causes more reduction of cell viability of KSHV-reactivated TREx BCBL1-Rta cells induced by doxycycline compared to mock treated TREx BCBL1-Rta and BJAB cells (**Fig 4B**). Similar effect was also observed in TPA/SB-treated BCBL1 cells **(Fig 4C)**. UMB158 demonstrated the comparable effect as SBE in KSHV-reactivated TREx BCBL1-Rta cells (**Fig 4D**). To pinpoint the types of cell death, we further carried out the YO-PRO/PI staining assays for detection of apoptosis/necrosis. SBE or UMB158 treatment led to more cell apoptosis (FITC-YO PRO signal) of KSHV-reactivated TREx BCBL1-Rta cells compared to DMSO treatment (**Fig 4E, F**).

PLK1 promotes KSHV latency through regulation of Myc and histone methylation.

The proto-oncogene myc encodes the c-Myc, a transcription factor, which regulates cellular growth, proliferation, differentiation, and apoptosis[44,12]. It is known that PLK1 regulates c-Myc activities[13], while c-Myc suppresses KSHV lytic reactivation[45]. To evaluate the role of c-Myc in mediating the effect of PLK1 inhibition on promoting KSHV lytic reactivation and cell death of KSHV-reactivated cells, we measured the endogenous c-Myc protein level in SBE-treated, KSHV-infected cell lines (TREx BCBL1-Rta, iSLK.BAC16, and iSLK.r219 cells). SBE treatment led to the reduction of c-Myc protein level in these cells in a dose-dependent manner (**Fig 5A**). Specifically, SBE has more drastic effect in TREx BCBL1-Rta cells. It is also known that c-Myc protein modulates histone lysine methyltransferases[46]. It has been reported that two specific histone marks, H3K27Me3 and H3K9Me3, are involved in KSHV latency[47,48]. We further measured the protein level of H3K27Me3 and H3K9Me3 marks in SBE-treated, above KSHV-infected cell lines with KSHV reactivation induced by doxycycline. SBE caused the consistent reduction of the H3K27Me3 mark in all these cells (**Fig 5B**) but not the H3K9Me3 mark (**Fig 5C**). Furthermore, we measured the level of H3K27Me3 mark specifically associating with certain loci

of KSHV genome by using ChIP-PCR assays. We confirmed that SBE indeed results in the reduction of H3K27Me3 mark associating with the promoter regions of KSHV lytic genes (ORF50, ORF59) as well as the OriLyt site (**Fig 5D**), which also correlates with the reduction of PLK1 association at these sites due to SBE treatment (**Fig 5E**). In parallel, we failed to identify any significant change of H3K9Me3 mark at these sites (**Fig S5A**). SBE treatment also modulates the expression of certain cell apoptosis and cell-cycle genes, explaining its effect on cell survival of KSHV-reactivated cells (**Fig 5F, G**).

PLK1 inhibitors synergize with STAT3 inhibitors to induce KSHV lytic reactivation.

It has been reported that STAT3 inhibition is sufficient to induce KSHV lytic reactivation[49]. We postulated that the combination of PLK1 inhibitors and STAT3 inhibitors would formulate an effective regimen to reactivate latent KSHV. We tested two STAT-3 inhibitors, cryptotanshinone and static, along with the PLK1 inhibitor, SBE. We treated BCBL1 cells with either cryptotanshinone (5, 10uM) or stattic (0.25, 0.5uM) along with lower dose of SBE (2uM). Both of STAT3 inhibitors have similar effect on lytic reactivation of latent KSHV and their combination with PLK1 inhibitor further enhanced the reactivation potency (**Fig 6A-C**). These findings suggest that STAT3 inhibitors can be paired with PLK1 inhibitors to maximize the efficiency of KSHV lytic reactivation.

PLK1 inhibition also promotes the reactivation of latent EBV.

As KSHV is closely related to EBV[50], we also determined whether PLK1 plays a similar role in regulating EBV infection. We depleted the endogenous PLK1 protein in Akata/Bx cells by transient transfection of its shRNA (shPLK1) or non-target shRNA (shNT), whose knockdown efficiency was confirmed by immunoblotting (**Fig 7A**). Such PLK1 knockdown also led to the increased expression of EBV b-ZIP protein in (ZEBRA) in Akata/Bx cells treated with human IgG

to induce EBV reactivation. Next, we identified that SBE treatment dramatically increases the expression of a series of EBV lytic genes (**Fig 7B, C**). UMB158 showed the similar effect (**Fig 7D**). Furthermore, treatment of UMB158 or SBE indeed induced more cell death (less cell viability) of EBV-reactivated Akata/Bx cells (**Fig 7E, F**).

Discussion

There was clear evidence of poor overall survival of primary effusion lymphoma (PEL). Recently, one study showed that only 10.2 months of survival for HIV-infected PEL patients receiving multi-drug anticancer treatment[51]. It has been reported that there is a strong correlation between KSHV and HIV co-infection with cancer progression[52]. Although the antiretroviral therapy is effective to control HIV infection[53], the complications associated with KSHV co-infection remains a significant problem.

This study were based on our previous work showing that PLK1 is a unique host kinase that is elevated in HIV infections and supports the survival of HIV-infected cells[14]. We demonstrated that PLK1 expression in B cells is induced in the context of HIV infection (Fig 1). HIV-encoded Nef protein can trigger PLK1 induction in B cells while suppressing KSHV reactivation. PLK1 pharmacological inhibition can promote reactivation of latent KSHV/EBV and the cell death of KSHV/EBV-reactivated tumor cells (Fig 4, 7), supported by the results that knockdown of PLK1 using its siRNAs and shRNAs led to the similar effect (Fig 2, 7). KSHV is marked by a broad cell tropism, including epithelial and endothelial cells, fibroblasts, monocytes, and B cells[54,7]. Beyond B cells, the similar effect of PLK1 inhibition is observed in other cell types (Fig 2).

It is known that Myc is deregulated in ~30% of human cancers, including B and T cell lymphomas[44,55] and also is required for the maintenance of KSHV latency[45]. Recently, it has been reported that PLK1 inhibition significantly reduces the downstream c-Myc phosphorylation and

impairs BRD4 binding to the c-Myc gene inhibiting c-Myc transcription[56]. Our study demonstrated that PLK1 inhibition induced KSHV reactivation involves c-Myc (Fig 5), which further affects the level of H3K27me3 suppressive mark that associates with the repressed transcription of KSHV lytic genes[57]. In this study we successfully delineated that the connection of PLK1 inhibition with c-Myc dysregulation and the subsequent change of histone methylation marks.

We showed that cell apoptosis is likely the predominant type of cell death caused by KSHV reactivation due to PLK1 inhibition (Fig 4, 8). We also checked the effect of PLK1 inhibition on expression of several cell survival genes regulated by c-Myc, including BAX and Bcl2, further supporting the cellular effect of PLK1 inhibition. As c-Myc also contributes to the release of cell cycle brakes[58], we postulated that PLK1 inhibition causes the cytotoxicity of KSHV-reactivated cell through affecting the expression of G2/M checkpoint genes (Fig 5).

As we observed, PLK1 inhibition alone is not sufficient to induce KSHV reactivation. KSHV infection induces STAT3 phosphorylation[59]. It has been shown that inhibition of STAT3 causes KSHV reactivation[60]. We identified that PLK1 inhibition can synergize with STAT3 inhibition to efficiently reactivate latent KSHV (Fig 6), which formulates an attractive regimen for reactivating latent KSHV/EBV and eliminating KSHV/EBV-reactivated tumor cells through the viral oncolytic approach, worthy of further investigation *in vivo*.

Acknowledge

We would like to thank Dr. Prashant Desai, Dr. Renfeng Li, for HEK293.r219 cells and Akata/Bx cells. We would also thank Dr. Jae Jung for TREx BCBL1-Rta cells, and Dr. SJ Gao for iSLK.r219 and iSLK.BAC16 cells. This work was supported by grants to N.S. [R03DE029716] and J.Z. [R01DE025447, R01AI150448] from the National Institute of Health.

Figures

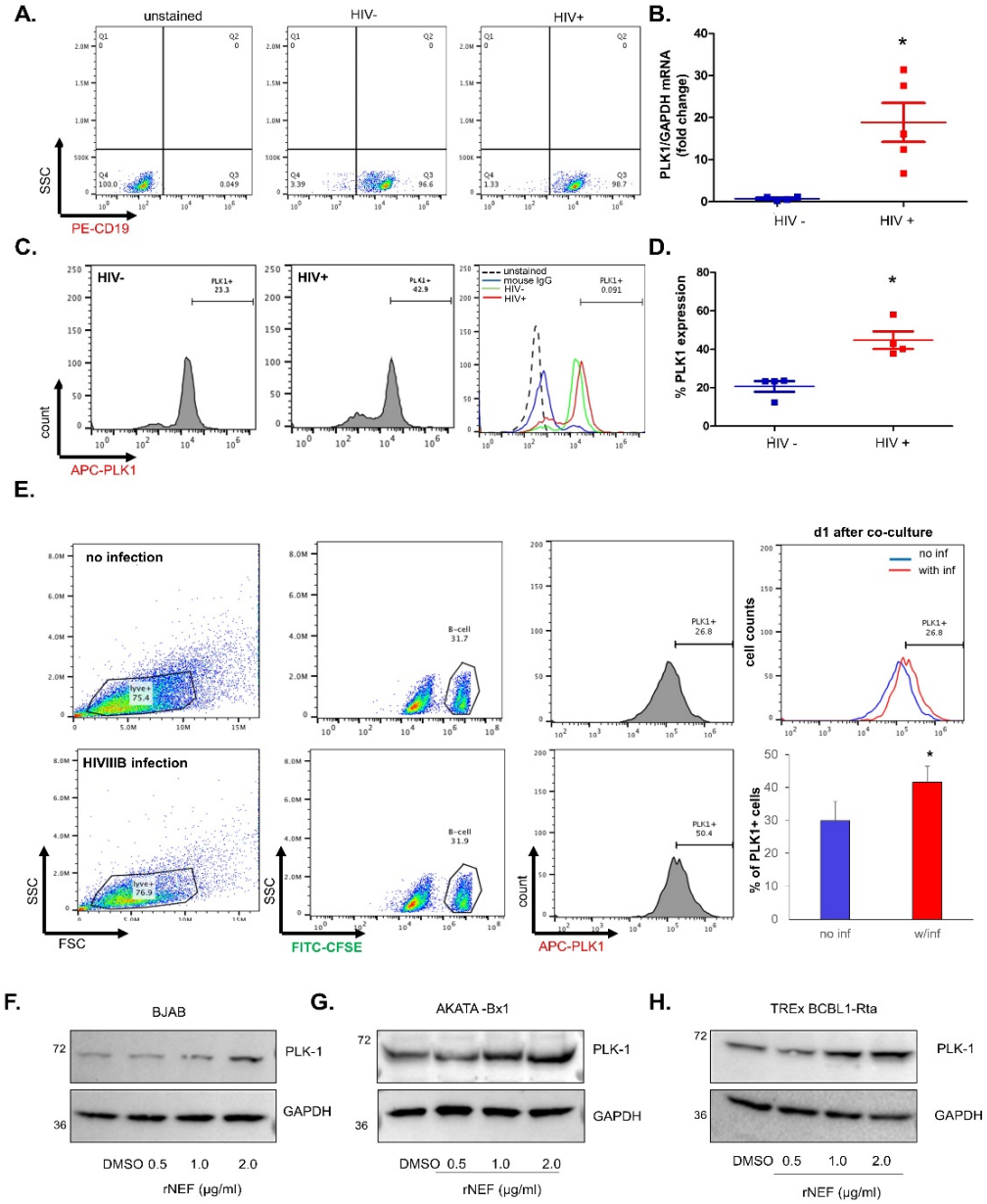

Figure 1. PLK1 expression is induced in B cells in the context of HIV infection. (A) B cells were isolated from healthy (HIV-) and HIV infected (HIV+) patient's PBMCs. Conjugated PE-CD19 antibodies were used for B cell enrichment, confirmed by flow cytometry analysis. (B) PLK1 mRNA level from isolated B cells was measured by using qPCR analysis (5 donors, *p<0.01). (C) PLK1 protein from isolated B cells was measured by immunostaining and flow cytometry analysis. (D) PLK1 protein level was quantified (4 donors, *p<0.01). (E) HIV-1 IIIB

infected Jurkat cells were co-cultured with BJAB cells pre-stained with CFSE. PLK1 protein from CFSE+ BJAB cells was measured by immunostaining and flow cytometry analysis (n=6, *$p<0.05$). (F, G, H) PLK1 protein in BJAB, Akata/Bx , TREx BCBL1-Rta cells incubated with recombinant Nef (rNef) protein was measured by immunoblotting.

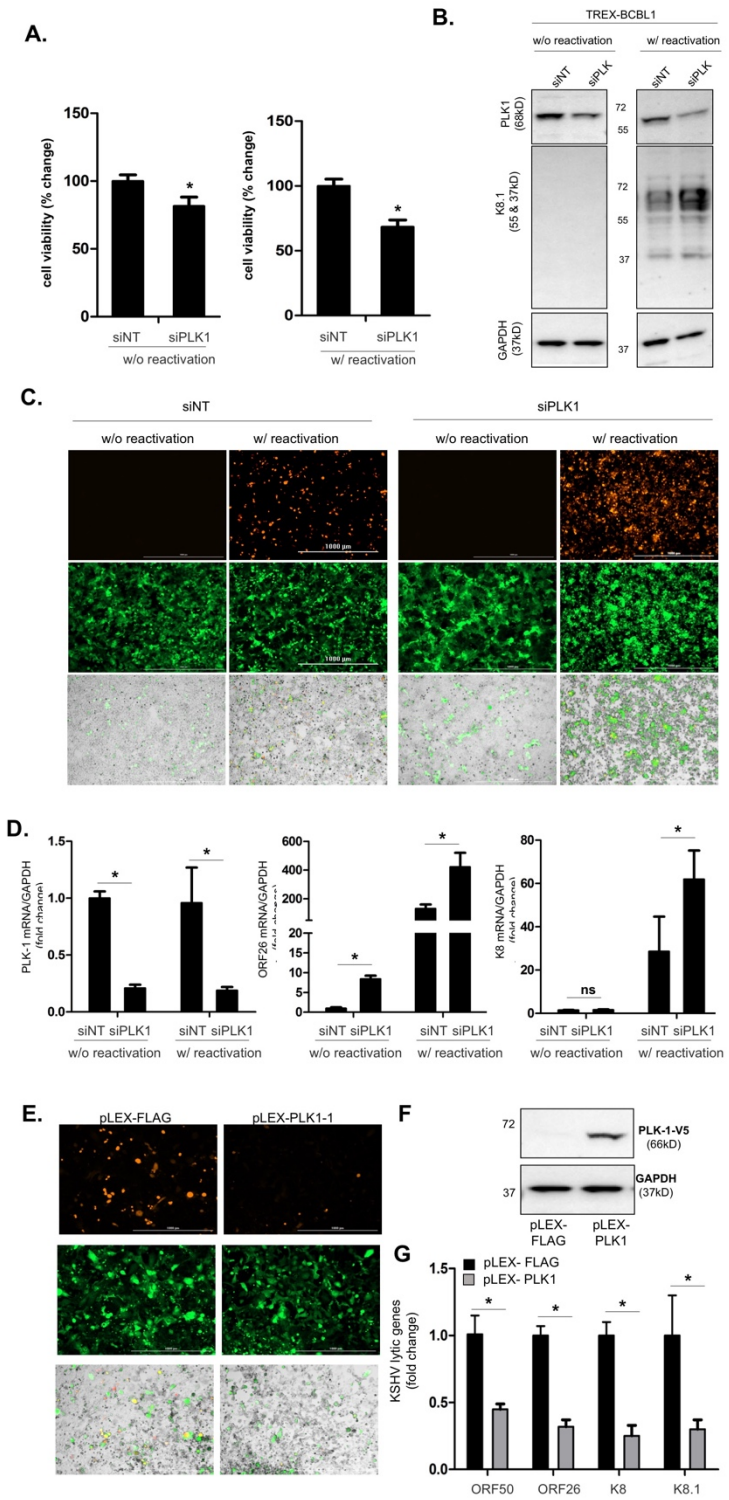

Figure 2. PLK1 is required for maintenance of KSHV latency. (A) TREx BCBL1-Rta cells were transiently transfected with the indicated siRNAs (siNT or siPLK1) and treated with

doxycycline to induce KSHV reactivation. Cell viability was analyzed at both reactivated (w/) and non-reactivated (w/o) conditions. (B) PLK1 knockdown and its impact on expression of KSHV lytic protein K8.1 was confirmed by immunoblotting. (C) iSLK.r219 cells were transiently transfected with indicated siRNAs and treated with doxycycline to induce FKSHV reactivation. RFP and GFP-expressing cells were illustrated. (D) The above iSLK.r219 cells (C) were subjected to qPCR analysis to measure the expression of KSHV lytic genes. (E) iSLK.r219 cells were transiently transfected with pLEX-FLAG or pLEX-PLK1 vector, and treated with doxycycline to induce KSHV reactivation. RFP and GFP-expressing cells were illustrated. (F) PLK1 overexpression was confirmed by immunoblotting. (G) The above iSLK.r219 cells (F) were subjected to qPCR analysis to measure the expression of KSHV lytic genes ($n=6$, $*p<0.05$).

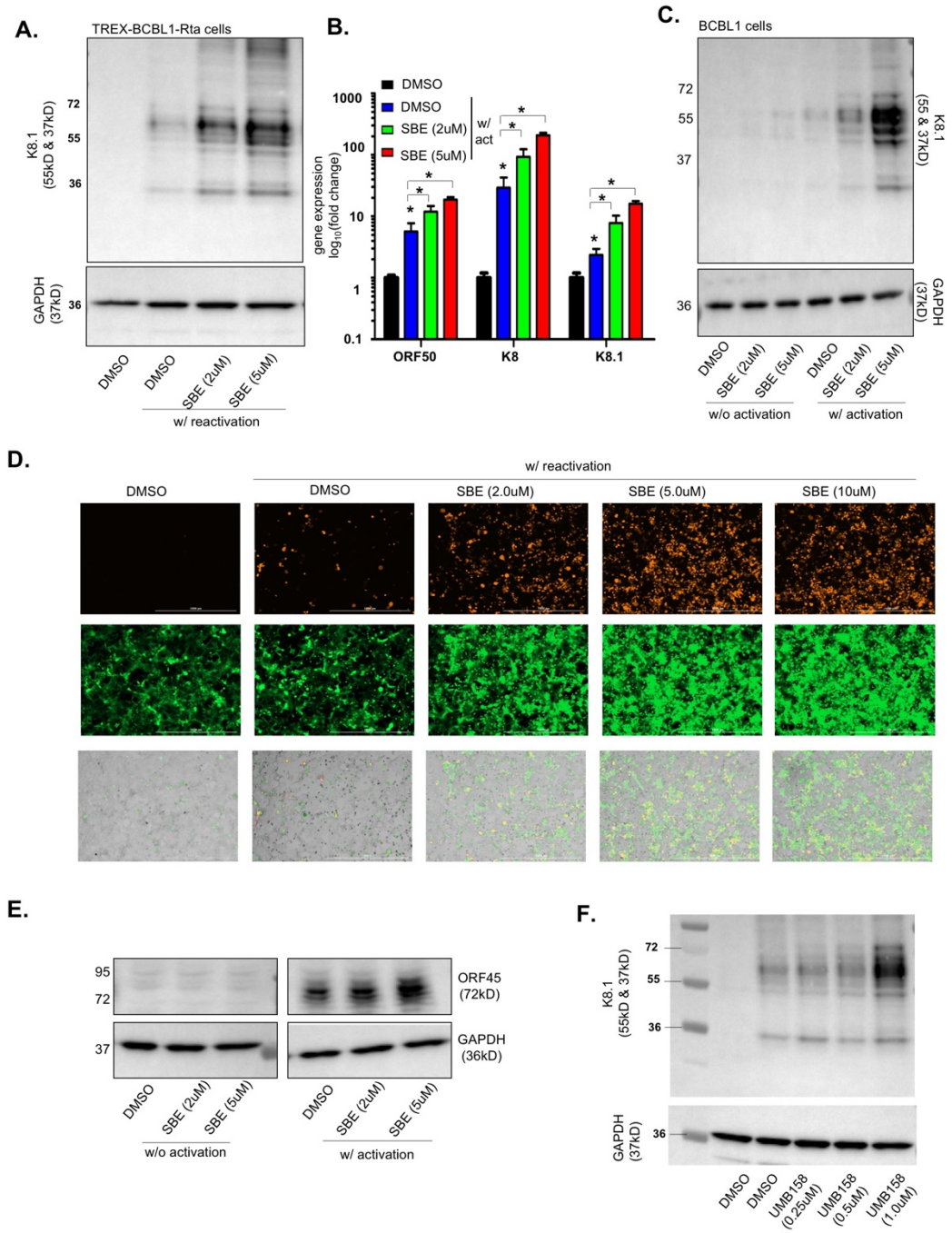

Figure 3. PLK1 inhibition promotes KSHV lytic reactivation. (A) TREx BCBL1-Rta cells were treated with PLK1-specific inhibitor, SBE 13 HCl (SBE), and doxycycline to induce KSHV reactivation. Expression of KSHV lytic gene K8.1 was analyzed by immunoblotting. (B) Effect of SBE on expression of KSHV lytic genes (ORF50, K8, K8.1) were measured by qPCR analysis.

(C) BCBL1 cells were treated with SBE, and TPA (20ng/ml) + Sodium butyrate (1mM) to induce KSHV reactivation. Expression of KSHV lytic gene K8.1 was analyzed by immunoblotting. (D) iSLK.r219 cells were treated with SBE and doxycycline to induced KSHV reactivation. RFP and GFP-expressing cells were illustrated. (E) Expression of KSHV lytic gene K8.1 in iSLK.r219 cells (D) was analyzed by immunoblotting. (F) Expression of KSHV lytic gene K8.1 in iSLK.r219 cells treated with UMB158 and doxycycline was analyzed by immunoblotting.

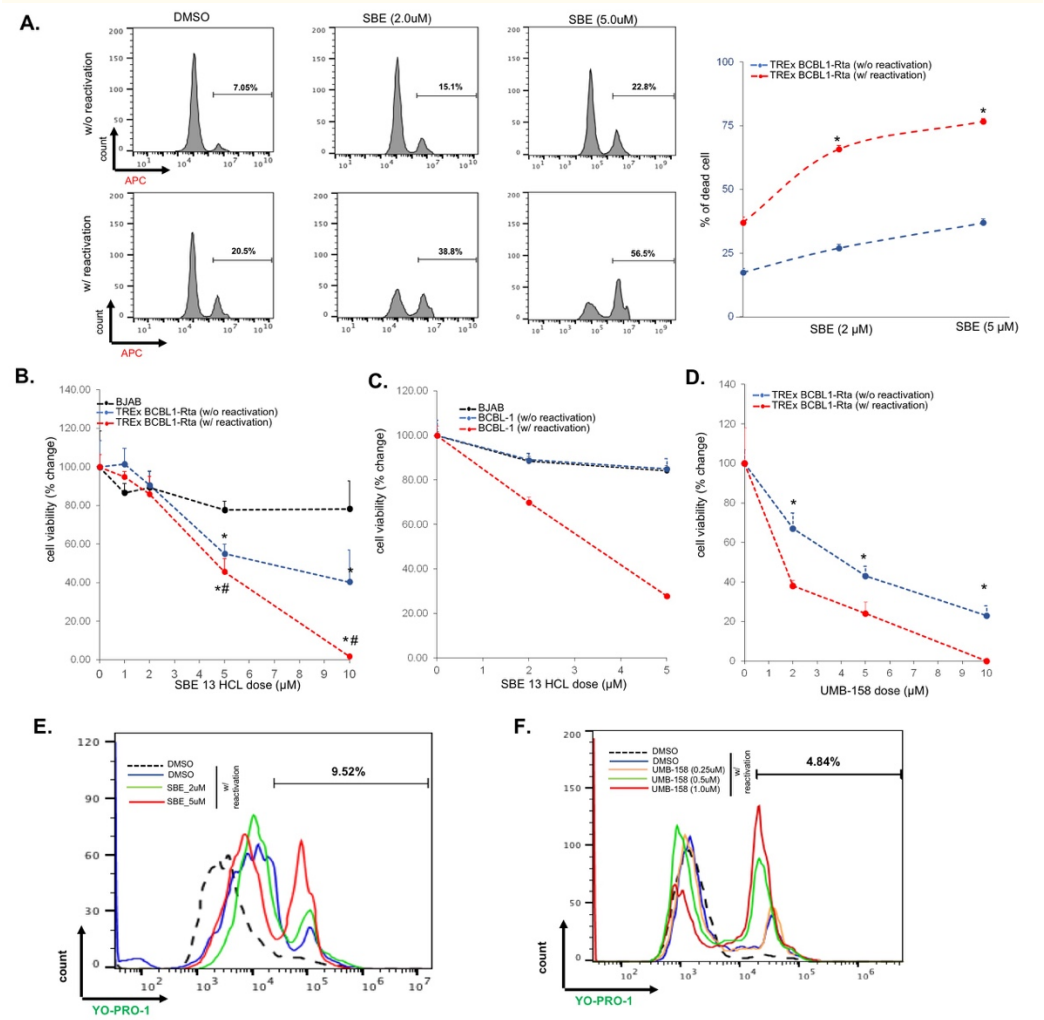

Figure 4. PLK1 inhibition promotes death of KSHV-reactivated cells. (A) Cell death of TREx BCBL1-Rta cells treated with SBE in the presence or absence of doxycycline were analyzed by using LIVE/DEAD™ dead cell staining (n=6, *p<0.01). (B) ATP-based assays were used to measure the cell viability of SBE-treated BJAB cells (black) and TREx BCBL1-Rta cells without (blue) or with (red) doxycycline induction to reactivate latent KSHV. (C) The similar ATP-based assays (B) was performed for SBE-treated BCBL1 cells without (blue) or with (red) TPA/SB induction to reactivate latent KSHV. (D) ATP-based assays were used to measure the cell viability of UMB158-treated TREx BCBL1-Rta cells without (blue) or with (red) doxycycline induction.

(E,F) Cell apoptosis of TREx BCBL1-Rta cells treated with SBE (E) or UMB158 (F) in the presence or absence of doxycycline was analyzed by YO-PRO/PI staining assays.

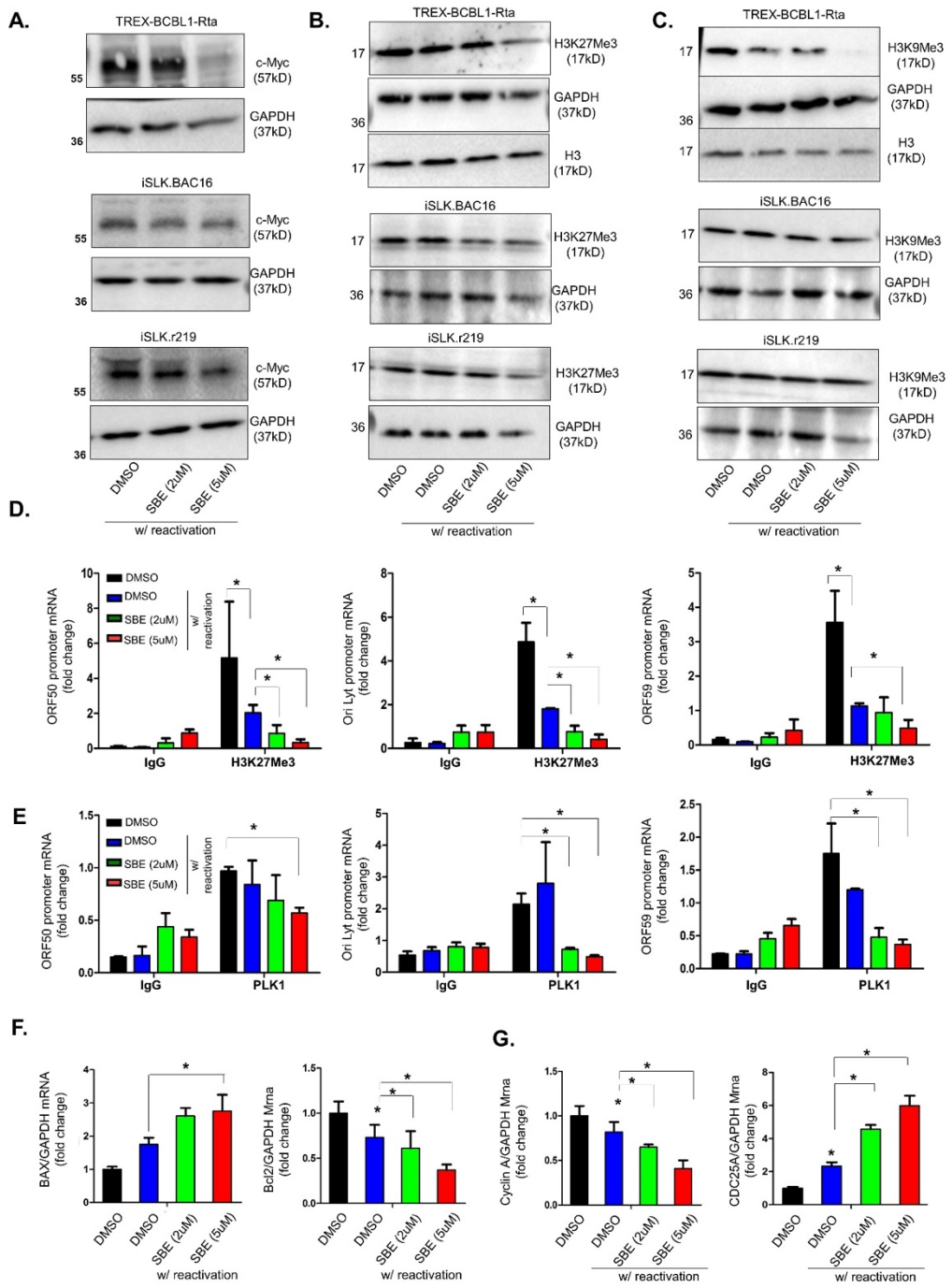

Figure 5. PLK1 inhibition destabilizes c-Myc and reduces H3K27me3 suppressive mark. (A) Expression of c-Myc in TREx BCBL1-Rta, iSLK.BAC16, and iSLK.r219 cells treated with SBE in the presence or absence of doxycycline was measured by immunoblotting. (B, C) Level of H3K27me3 (B) and H3K9me3 (C) suppressive marks in TREx BCBL1-Rta, iSLK.BAC16, and

iSLK.r219 cells treated with SBE in the presence or absence of doxycycline were measured by immune-blotting. (D, E) Level of H3K27me3 (D) and PLK1 (E) at promoter region of KSHV lytic genes (ORF50, ORF59) and OriLyt loci in TREx BCBL1-Rta cells treated with SBE in the presence or absence of doxycycline was analyzed by ChIP-PCR analysis (n=6, *$p<0.01$). (F, G) Expression of apoptosis-related genes, BAX and Bcl2 (F), and cell-cycle checkpoint genes, Cyclin A and CDC25A (G), in TREx BCBL1-Rta cells treated with SBE in the presence or absence of doxycycline was analyzed by qPCR analysis (n=6, *$p<0.01$).

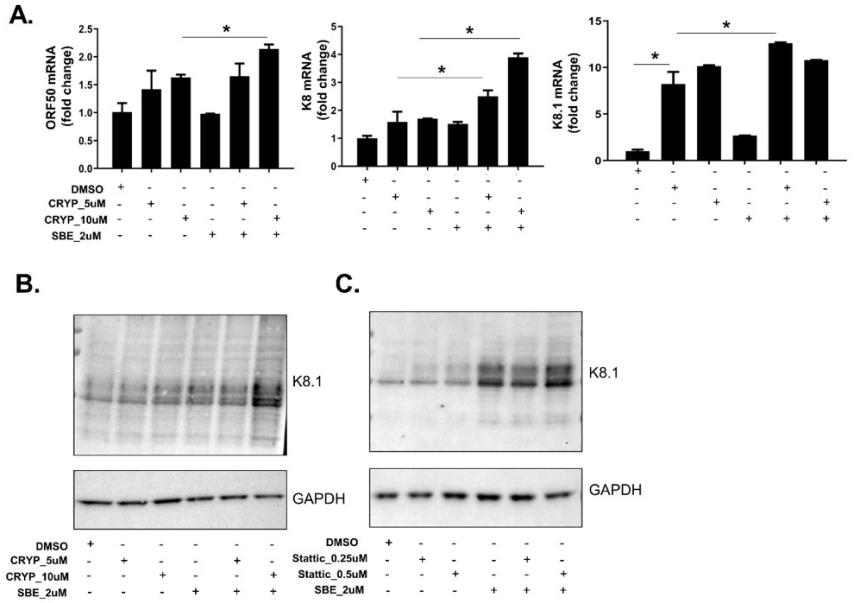

Figure 6. PLK1 inhibition synergizes STAT3 inhibition to induce KSHV reactivation. (A) BCBL1 cells were treated with either cryptotanshinone (CRYP) alone or in combination with SBE, followed by the qPCR analysis to measure expression of KSHV lytic genes (ORF50, K8, K8.1). (B) Expression of KSHV lytic gene K8.1 in BCBL cells (A) was measured by immunoblotting. (C) BCBL1 cells were treated with either Stattic alone or in combination with SBE, followed by the immunoblotting to measure expression of KSHV lytic gene K8.1.

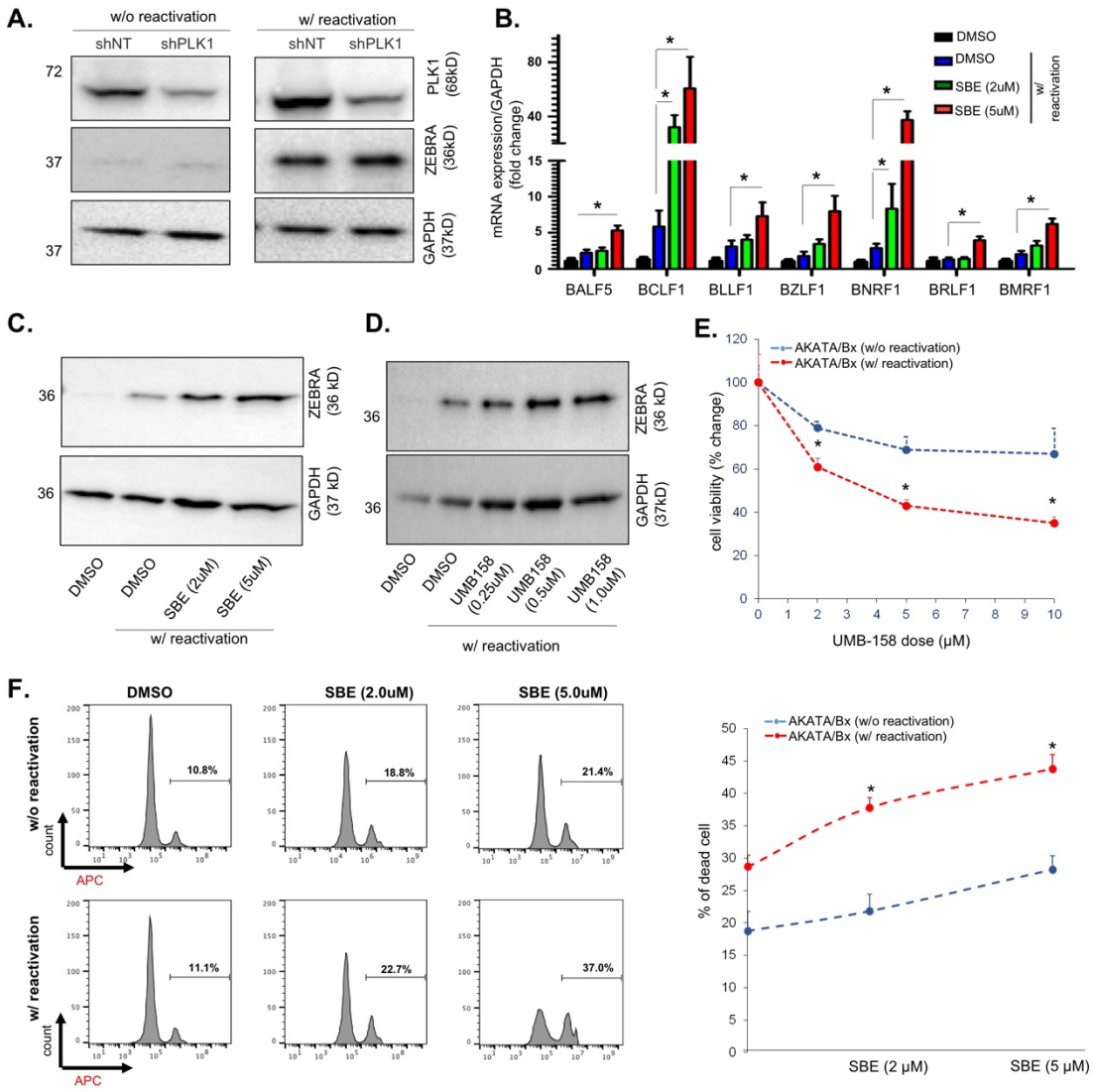

Figure 7. PLK1 depletion or inhibition also promotes EBV reactivation. (A) Akata/Bx cells were transiently transfected with the indicated shRNAs (shNT or shPLK1), which was induced by doxycycline. These cells were further treated with human IgG (hIgG) to reactivate latent EBV. PLK1 knockdown and its impact on expression of EBV lytic gene ZEBRA was analyzed by immunoblotting. (B) Expression of EBV lytic genes in AKATA/Bx cells (A) was analyzed by qPCR analysis (n=6, *p<0.01). (C, D) Expression of EBV lytic gene ZEBRA in Akata/Bx cells treated with SBE (C) or UMB158 (D) in the presence or absence of hIgG was analyzed by immunoblotting. (E) ATP-based cell viability assay was performed for Akata/Bx

cells treated with UMB158 in the presence or absence of hIgG (n=6, *p<0.01). (F) Cell death of Akata/Bx cells treated with SBE in the presence or absence of hIgG was measured by the LIVE/DEAD™ dead cell staining (n=6, *p<0.01).

Supplementary Figures

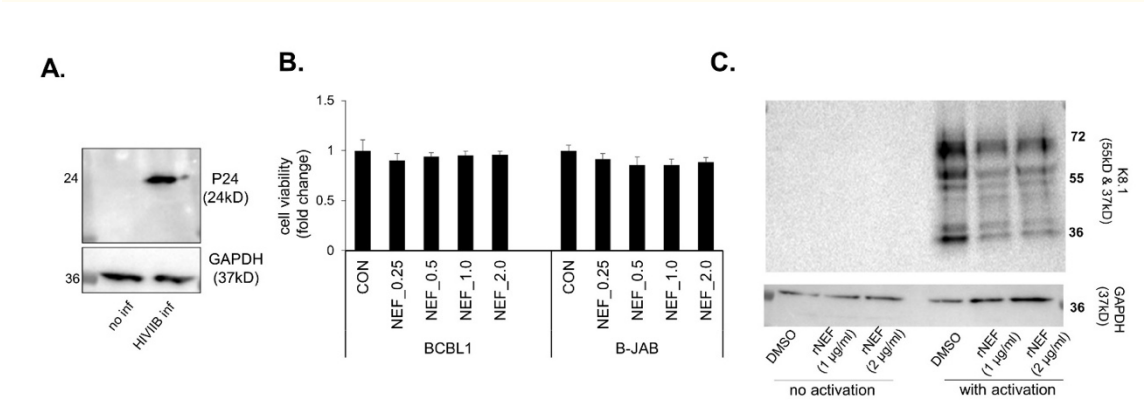

Figure S1. (A) Immunoblotting showed the expression of HIV P24 protein in Jurkat cells infected with HIV-1 IIIB. (B) Cell viability was measured by ATP-based assay for BCBL1 and BJAB cells incubated with rNef protein. (B) Immunoblotting showed the expression of KSHV lytic gene K8.1 in rNef-treated TREx-BCBL1 Rta cells with or without doxycycline.

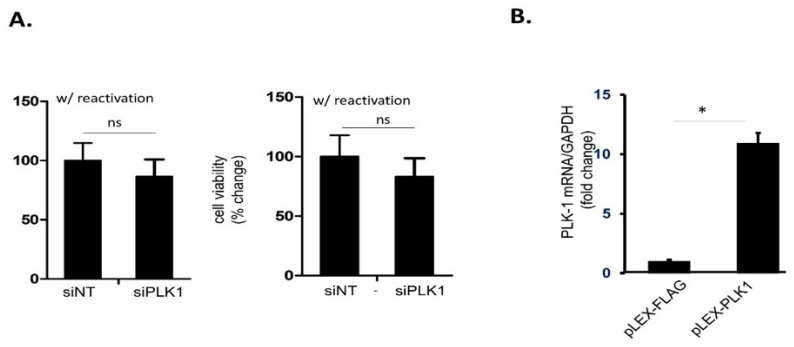

Figure S2. (A) Cell viability was measured by ATP-based assay after transient transfection of either siNT or siPLK1 w/ or w/o reactivation in iSLK.r219 cells (n=4, *p<0.01). (B) q-PCR analysis confirmed the PLK1 expression in iSLK.r219 cells transiently transfected with pLEX-FLAG or pLEX-PLK1 vector (n=6, *p<0.001).

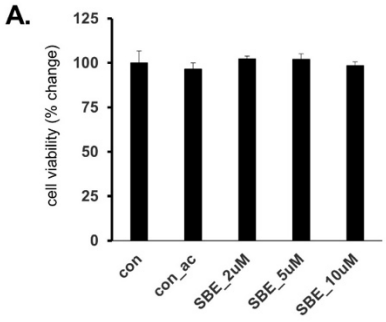

Figure S3. (A) Cell viability was measured by ATP-based assay for SBE-treated iSLK.r219 cells. (n=6, *p<0.01).

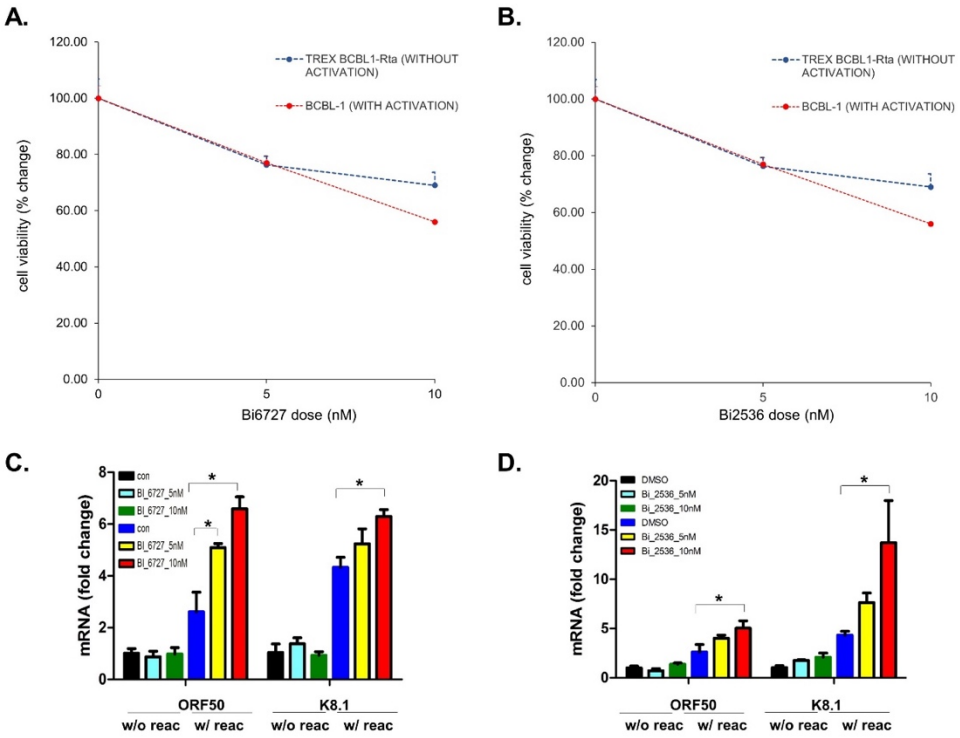

Figure S4. (A, B) Cell viability was measured by ATP-based assays for TREx BCBL1-Rta cells treated with Bi-6727 (A) and Bi-2536 (B) in the presence or absence of doxycycline (n=6, *p<0.05). (C, D) Expression of KSHV lytic genes (ORF50, K8.1) in TREx BCBL1-Rta cells treated with Bi-6727 (C) and Bi-2536 (D) in the presence or absence of doxycycline were analyzed by qPCR (n=5, *p<0.01).

A.

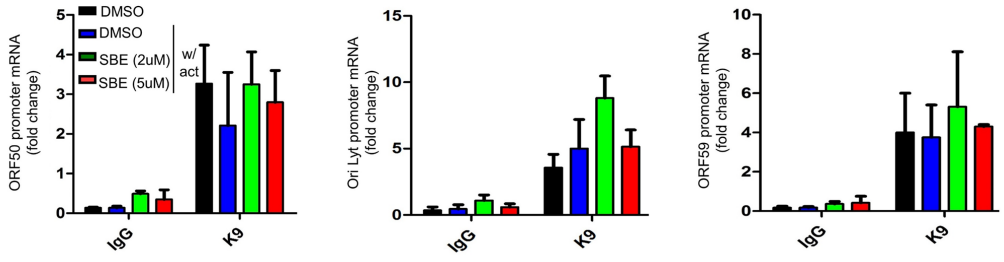

Figure S5. ChIP-PCR analysis of H3K9me3 level at the promoter region of KSHV lytic genes (ORF50, ORF59) and the OriLyt loci in TREx BCBL1-Rta cells treated with SBE in the presence or absence of doxycycline (n=6, *p<0.01).

Table S1: Primers used in this study.

Gene	Forward (5' to 3')	Reverse (5' to 3')
EBV_BZLF1	AATGCCGGGCCAAGTTTAAGCAAC	TTGGGCACATCTGCTTCAACAGGA
EBV_BRLF1	TGGCTTGGAAGACTTTCTGAGGCT	AATCTCCACACTCCCGGCTGTAAA
EBV_BMRF1	ATACGGTCAGTCCATCTCCT	CACTTTCTTGGGGTGCTT
EBV_BALF5	GCGGCCCCGGAGTTGTTA	CGTGGCCGTGGATCATTATTTC
EBV_BNRF1	GCAAACATACAGGAGGAAAG	CAGCAGGTTCTCAGCAATC
EBV_BLLF1	GCCTTGGAGAATATAACCTTG	CATTACTGTCTCGGGTCTTGG
EBV_BcLF1	GTGGATCAGGCCGTTATTGA	CCTCAAACCCGTGGATCATA
EBV_EBNA1	TGAGTCGTCTCCCCTTTGGA	CCTTAGCGGGCCAGGTTGTG
KSHV_ORF50	CCTTCGGCCCGGGGTCT	CGGTGGCAGTTGCGTATACTCT
KSHV_K8	CCTGGACGCTCTCTCACACA	GGATCTGCGAGTTGGAAGCT
KSHV_ORF26	AGCCGAAAGGATTCCACCAT	TCCGTGTTGTCTACGTCCAG
KSHV_LANA	TTACCTCCACCGGCACTCTT	GGATGGGATGGAGGGATTG

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