Supporting Information

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SI Methods

Cell Lines and Reagents. Mutu I (a gift from Alan Rickinson, Birmingham, UK) and Akata (a gift from Kenzo Takada, Sapporo, Japan) are EBV-positive Burkitt lymphoma cell lines. BJAB and DG75 are EBV-negative B-cell lymphoma lines. SNU719 is an EBV-positive gastric carcinoma cell line (1). AGS is a gastric carcinoma cell line obtained from American Type Culture Collection. AGS-Akata (a superinfected EBV-positive gastric carcinoma cell line), HONE-Akata (2) and CNE1-Akata (3) (two different superinfected EBV-positive NPC cell lines) were gifts from Lawrence Young (Birmingham, UK). HeLa is a malignant human epithelial cervical cancer cell line (obtained from American Type Culture Collection). Early and late passage LCLs were obtained by transforming human B cells in vitro with the B95.8 strain of EBV. LCL1 (aka 721) is a late passage line derived more than 30 years ago (kindly provided by Bill Sugden, Madison, WI). LCL2 is an early passage line. IB4 is an EBNA1-independent LCL in which the EBV genome is integrated (a gift from Fred Wang, Boston, MA). Primary peripheral blood B cells were purchased from AllCells.

All cell lines were cultured at 37 °C with 5% CO_2 and 100% humidity in growth media (Invitrogen) supplemented with 10% FBS and 1% penicillin–streptomycin (Sigma-Aldrich), except the primary B cells, which were grown in RPMI medium supplemented with 20% FBS. AGS cells and HeLa cells were cultured in Ham F-12 medium and DMEM medium, respectively. All other cell lines were cultured in RPMI 1640. HONE-Akata and AGS-Akata were maintained under drug selection (G418 sulfate, 0.4 mg/mL).

Geldanamycin, 17-AAG, 17-DMAG (InvivoGen), CHX (Sigma), MG-132 (Calbiochem), and bortezomib (provided by Shigeki Miyamoto, Madison, WI) were dissolved in DMSO. MTX (Immunex) was suspended in isotonic sodium chloride. 3-MA (Sigma) was dissolved in medium just before use.

Plasmids. Plasmid DNA was purified through columns (Qiagen) as described by the manufacturer. The plasmids pcDNA3-EBNA1 and pcDNA3-EBNA1ΔGA have been previously described (4) and express the full-length EBNA1 and a mutant EBNA1 missing most of the Gly-Ala repeat, respectively. The pSG5-EBNA1 vector (a gift from Jeffery Sample, Memphis, TN) (5), pSG5-LMP1 vector (a gift from Lawrence Young, Birmingham, UK) (6), and pSG5-BZLF1 vector (7) are SG5-based vectors (Invitrogen) containing full-length EBNA1, LMP1, and BZLF1 gene sequences, respectively. The plasmid pSG5-EBNA1ΔGA was constructed by ligating the *Mfe*I-to-*Eco*RI fragment, which contains EBNA1ΔGA sequence, of plasmid p1553 (provided by Bill Sugden, Madison, WI) (8) into the *Eco*RI site of the SG5 vector. The *Mfe*I-to-*Eco*RI fragment of p1553 was also ligated into the *Eco*RI site of the retroviral vector, pBABE-puro (Addgene plasmid 1764, generated by Bob Weinberg) to generate pBABE-puro-EBNA1ΔGA.

Reverse Transcription Quantitative PCR. Total RNAs were harvested using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed into cDNA using random primers in a Reverse Transcription II system (Promega) according to the manufacturer's instructions. Expression of viral EBNA1 mRNAs was quantified by quantitative PCR using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primers and probes (9) were as follows: forward primer 5′-TCATCATCATCCGGGTCTCC-3′, reverse primer 5′-CCTACAGGGTGGAAAAATGGC-3′, and probe 5′ (FAM)CGCAGGCCCCCTCCAGGTAGAA(TAMRA)-3′. A predeveloped assay reagent containing premixed primers and a VIC-labeled probe (Applied Biosystems; cat. no. 4310884E) was used to quantify expression of endogenous GAPDH mRNA. PCR reactions were performed in a volume of 25 μL containing 1× Taqman Universal PCR Mastermix (Applied Biosystems), 10 to 25 pmol primers, 5 pmol probe, 1.25 μL of GAPDH predeveloped assay reagent, and 5 μL of cDNA (equivalent to 25 ng of input RNA). Thermal cycling conditions was as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 rounds of amplification (denaturation for 15 s at 95 °C and annealing and extension for 1 min at 60 °C). Template-negative and RT-negative conditions were used as controls. Amplification of EBNA1 cDNAs and the endogenous GAPDH cDNA were continuously monitored by changes in FAM and VIC fluorescent intensities, respectively, with the ABI 7900 software. The corresponding amplification plots were used to determine the threshold cycle value, defined as the number of PCR cycles taken for fluorescent intensity to reach a fixed threshold for each signal. The relative amounts of EBNA1 transcript were normalized to the amount of GAPDH mRNA in the same sample. The normalized value of EBNA1 transcripts in cells treated with vehicle control was assigned as 100.

Cell Transfection. EBV-negative AGS cells, HONE cells, and HeLa cells were transiently transfected using FuGene 6 (Roche) or Lipofectamine 2000 (Invitrogen) with pSG5 (the empty vector), pSG5-EBNA1, pSG5-EBNA1ΔGA, or pSG5-LMP1 according to the manufacturer's protocol. At 4 h after transfection, cells were treated with Hsp90 inhibitors or vehicle control. In some experiments, cells were treated in the absence or presence of MG-132 (50 μM) or 3-MA (10 mM). MG-132 and 3-MA were added into medium 16 and 48 h before cell harvesting, respectively.

Immunoblot Analyses. Total cellular protein was harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses. The primary antibodies used were as follows: LMP1 (cat no. CS1-4; 1:100; Dako), EBNA1 [clone no. IH4EBNA1 (10), 1:50; clone no. 1EB12, 1:1,000, provided by Richard Burgess, Madison, WI], EBNA2 (cat no. NCL-EBV-PE2, 1:100; Vision Biosystems), EBNA3A (cat no. ab16126, 1:200; Abcam), EBNA3C (cat no. ab16128, 1:200; Abcam), EBNA-LP (clone no. JF186, 1:100, gift from Paul Ling, Houston, TX), IKKα (cat no. sc-7606, 1:200; Santa Cruz), Atg5 (cat no. sc-54, 1:200; Santa Cruz), cdc2 (cat no. sc-54, 1:200; Santa Cruz), and anti–β-actin (cat no. A5441, 1:5000, Sigma). Secondary antibodies were horseradish-peroxidase-conjugated secondary anti-mouse IgG (cat no. 31430, 1:10,000; Pierce Biotechnology), anti-rabbit IgG (cat no. 31460, 1:5,000; Pierce Biotechnology), anti-rat IgG (cat no. 31230, 1:5,000; Pierce Biotechnology), or anti-sheep IgG (cat no. AP147P, 1:5,000; Chemicon). Bound antibodies were detected using the ECL system (Pierce Biotechnology). Image quantifications were performed using ImageQuant software.

Immunoprecipitation Assays. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.9, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5% Nonidet P-40) plus EDTA-free protease inhibitor mixture (Roche), phosphatase inhibitor mixture set 1 (Calbiochem), and Benzonase nuclease (Novagen) at concentrations according to the manufacturer's instructions. Cells were incubated on ice for 10 min and microsonicated (three bursts at 20% amplitude for 10 s on ice). Lysate was clarified by centrifugation for 5 min at 24,000 × *g*. Purified mAb was conjugated to

cyanogen bromide-activated Sepharose 4B at a concentration of 2.5 mg mAb per milliliter of swollen gel as described previously (11). Resin composed of a mixture of three anti-EBNA1 mAbs (1EB6, 1EB12, and 3EB13) was applied to SigmaPrep spin column and equilibrated with lysis buffer plus protease and phosphatase inhibitors two times with two column volumes (CVs) of buffer. Cell lysate was added to 50 μL of resin and incubated with shaking at 23 °C for 30 min. Protein-bound resin was collected by centrifugation (82 × *g* for 30 s). The resin was washed with two buffer conditions: two times with two CVs of lysis buffer plus protease and phosphatase inhibitors and two times with two CVs of Tris-HCl, pH 7.9, 0.1 mM EDTA, and 0.25 M NaCl plus protease and phosphatase inhibitors. Proteins were eluted from the immunoaffinity column with one CV of 2× SDS sample buffer, then separated by electrophoresis using 4% to 12% Bis/Tris NuPAGE polyacrylamide gels (Invitrogen). EBNA1 was detected by quantitative Western blot (12) probed with anti-EBNA1 mAb 1EB12 fluorescently labeled with Alexa Fluor 647 (Invitrogen). The Western blot to detect Hsp90 was probed with mAb sc-7947 (Santa Cruz Biotechnology). Prestained molecular weight markers (Multimark; Invitrogen) were included on all gels.

Retroviral Production and LCL Infection. Retrovirus was generated by cotransfecting 293T cells with 3 μg of a plasmid (gift from Richard C. Mulligan, Boston, MA) expressing the HIV Gag-Pol element, 1 μg of a plasmid (gift from Richard C. Mulligan, Boston, MA) encoding the vesicular stomatitis virus G protein, and 10 μg of the pBABE-puro vector control, or a pBABE-puro vector expressing the EBNA1ΔGA protein (pBABE-puro-EBNA1ΔGA). After 48 h, the media was collected and filtered through a filter (0.8 μm pore size) and used to infect LCL1 cells. Two days later, stably infected LCLs pools were selected by incubation with 0.5 μg/mL puromycin (Sigma).

siRNA Experiments. AGS cells were transfected with 80 pmol of siRNA against the human Atg5 message (cat no. sc-41445; Santa Cruz Biotechnology) or a negative control siRNA (cat no. sc-37007, Santa Cruz Biotechnology) using X-tremeGENE (Roche). Two days after delivery of the siRNA, the cells were transfected with 20 pmol Atg5 siRNA (or control siRNA) and 400 ng of pSG5-EBNA1 plasmid using Lipofectamine 2000 (Invitrogen). After 4 h, cells were then treated with Hsp90 inhibitors or vehicle control. Two days later, total protein was harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses.

Cell Cycle Analysis. Cells (2×10^6 cells per condition) were harvested, washed with cold PBS solution, resuspended in 200 μL of PBS solution on ice, and then fixed by drop-wise addition of 2 mL 95% ethanol. After incubation on ice for 30 min, the cells were washed with 2 mL of cold PBS solution, resuspended in 1 mL of PBS solution on ice, followed by addition of 1 μL of RNase A (10 μg/μL; Qiagen). After incubation for 30 min at 37 °C, the cells were spun down, resuspended in 1 mL of propidium iodide, and incubated for 24 h at 4 °C in the dark. The PI-stained nuclei were then analyzed using a Becton Dickinson FACScan unit. The percentage of cells in each phase of the cell cycle was determined using FACS analysis.

Apoptosis Detection. Apoptotic cells were detected using the APO LOGIX kit (cat no. SR100-1; Cell Technology), according to the manufacturer's instructions. Briefly, cells (2×10^5 cells per condition) were harvested and resuspended in 300 μL of medium, followed by addition of 10 μL of the SR-VAD-FMK (30× solution). This reagent is cell-permeable and is used to label apoptotic cells. The samples were incubated for 1 h at 37 °C in the dark. After washing with 2 mL of 1× washing buffer two times, the cells were resuspended in 100 to 150 μL of 1× wash buffer on ice, followed by visualization and counting of fluorescent, apoptotic cells via fluorescence microscope.

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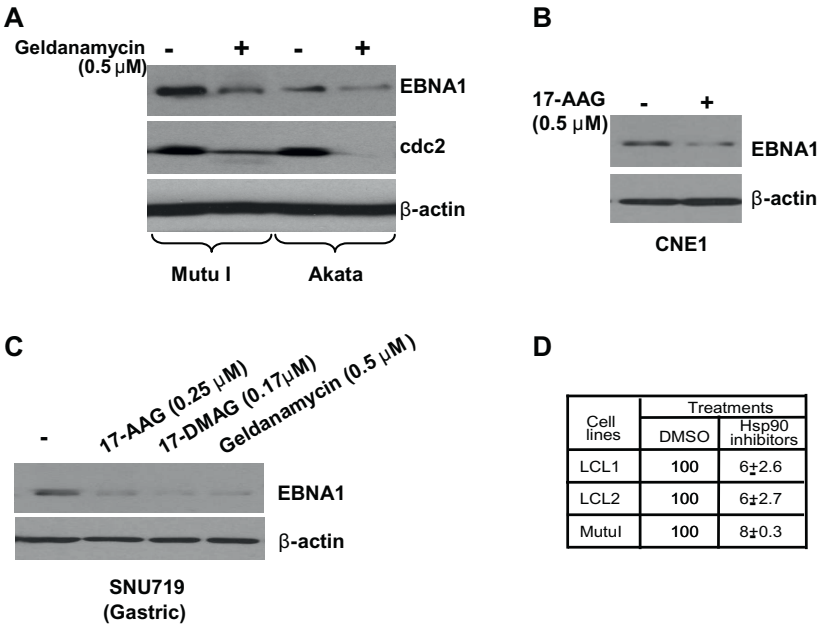

Cell lines	Treatments	
	DMSO	Hsp90 inhibitors
LCL1	100	6±2.6
LCL2	100	6±2.7
Mutul	100	8±0.3

Fig. S1. Hsp90 inhibitors decrease expression of EBNA1. (*A*) Mutu I and Akata Burkitt lymphoma cells were treated with no drug or geldanamycin (0.5 μM) for 96 h. (*B*) EBV-infected NPC cell line, CNE1, was treated with no drug or 17-AAG (0.5 μM) for 48 h. (*C*) SNU719 cells (an EBV-positive gastric carcinoma cell line) were treated with no drug, 17-AAG (0.25 μM), 17-DMAG (0.17 μM), or geldanamycin (0.5 μM) for 48 h. Whole-cell extracts were prepared and immunoblot analysis was performed to analyze the expression of EBNA1, cdc2 (a known cellular substrate of Hsp90), or cellular β-actin as indicated. (*D*) EBNA1 expression was quantitated in cells treated with DMSO versus 17-DMAG (0.17 μM) in three different cell lines in duplicate experiments. EBNA1 expression in DMSO-treated cells is set as 100%.

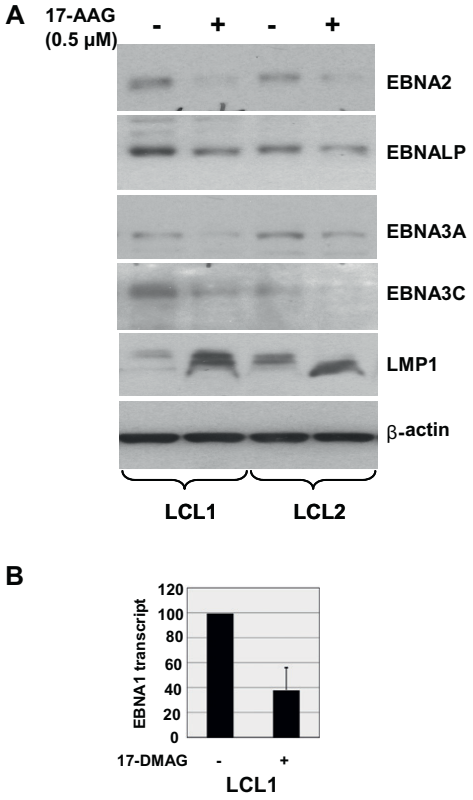

Fig. S2. Hsp90 inhibitors decrease expression of Cp-derived transcripts in B cells with type III latency. (*A*) LCL cells were treated with no drug or 17-AAG (0.5 μM). Immunoblot analysis was performed to analyze expression of EBNA2, -LP, -3A, -3C, LMP1, and β-actin. (*B*) LCL1 cells were treated with 17-DMAG (0.17 μM) for 48 h. Expression of the EBNA1 transcripts was examined by quantitative RT-PCR. The level of EBNA1 transcripts in untreated cells was assigned a value of 100.

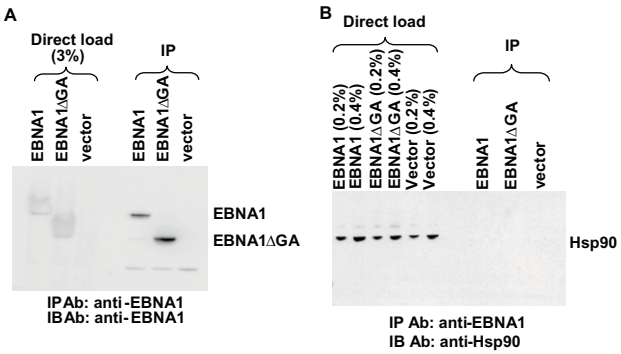

Fig. S3. Hsp90 does not associate with EBNA1. AGS cells were transfected with empty vector (pSG5), pSG5-EBNA1, or pSG5-EBNA1ΔGA. After 48 h, cells were harvested and cell lysate was immunoprecipitated with a pool of anti-EBNA1 Abs. The precipitates were immunoblotted with antibodies against EBNA1 (*A*) or Hsp90 (*B*).

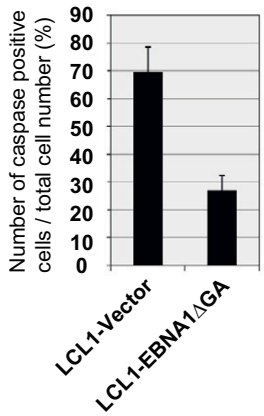

Fig. S4. Expression of an EBNA1 mutant missing the Gly-Ala repeat domain decreases the apoptotic activity in LCL1 cells treated with 17-DMAG. LCL1-vector and LCL1-EBNA1ΔGA cells were treated with either no drug or 17-DMAG (0.03 μM) for 4 d. Numbers of the apoptotic cells were determined using the APO-LOGIX kit via fluorescence microscope. Percentage of apoptotic cell number relative to the total cell number is given.