Supporting Information

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SI Materials and Methods

Expression Plasmids and Cloning. The flag-tagged Epstein–Barr virus nuclear antigen (FEBNA) 1 expression plasmid was described (1), and the nonflagged Epstein–Barr Virus nuclear antigen (EBNA) 1 expression vector was a gift of John L. Yates (Roswell Park Cancer Institute, Buffalo, NY). The EBNA1 expression vector, with a deletion of amino acids 1–379, designated Δ1–379, was produced by subcloning the coding sequence of EBNA1 amino acids 380–641 into the XhoI and BamHI sites of the pSG5-flag vector (2). The expression plasmid of FEBNA1 with a deletion of the ORF flanking amino acids 100–377, designated Δ100–377, was generated by performing a PCR mutagenesis protocol according to the manufacturer’s protocol (Stratagene). The GFP-EBNA1 expression vector was obtained by introducing the flasking sequence of EBNA2 into the XhoI and BamHI sites of the EGFP-C1 vector (Clontech). The cDNA clone of nucleolin (NCL) was purchased from Thermo Scientific. The full-length cDNA of NCL was amplified by PCR and subcloned into the XhoI and BamHI sites of the pSG5-flag vector or the pDsRed vector (Clontech) to produce flag-tagged NCL (FNCL) or DsRed-NCL expression plasmids. The plasmids that harbor the indicated NCL mutants, including K419A, K610A, and K624/627A, were generated by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The expression vector of yellow fluorescent N-terminal and yellow fluorescent C-terminal tags (YN and YC) are described in Fig. S2. The YN-NCL expression vector was generated by subcloning the full-length cDNA of NCL into the XhoI and BamHI sites of the YN vector, and the YC-EBNA1, YC-Δ100–377, and YC-Δ100–377 expression vectors were generated by subcloning the indicated flagging DNA fragments into the XhoI and BamHI sites of YC, respectively. The oriP-SV40-Luc and SV40-Luc reporter plasmids, and the fragments into the XhoI and BamHI sites of YC, respectively.

Expression Plasmids and Cloning. The flag-tagged Epstein–Barr virus nuclear antigen (FEBNA) 1 expression plasmid was described (1), and the nonflagged Epstein–Barr Virus nuclear antigen (EBNA) 1 expression vector was a gift of John L. Yates (Roswell Park Cancer Institute, Buffalo, NY). The EBNA1 expression vector, with a deletion of amino acids 1–379, designated Δ1–379, was produced by subcloning the coding sequence of EBNA1 amino acids 380–641 into the XhoI and BamHI sites of the pSG5-flag vector (2). The expression plasmid of FEBNA1 with a deletion of the ORF flanking amino acids 100–377, designated Δ100–377, was generated by performing a PCR mutagenesis protocol according to the manufacturer’s protocol (Stratagene). The GFP-EBNA1 expression vector was obtained by introducing the flasking sequence of EBNA2 into the XhoI and BamHI sites of the EGFP-C1 vector (Clontech). The cDNA clone of nucleolin (NCL) was purchased from Thermo Scientific. The full-length cDNA of NCL was amplified by PCR and subcloned into the XhoI and BamHI sites of the pSG5-flag vector or the pDsRed vector (Clontech) to produce flag-tagged NCL (FNCL) or DsRed-NCL expression plasmids. The plasmids that harbor the indicated NCL mutants, including K419A, K610A, and K624/627A, were generated by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The expression vector of yellow fluorescent N-terminal and yellow fluorescent C-terminal tags (YN and YC) are described in Fig. S2. The YN-NCL expression vector was generated by subcloning the full-length cDNA of NCL into the XhoI and BamHI sites of the YN vector, and the YC-EBNA1, YC-Δ100–377, and YC-Δ100–377 expression vectors were generated by subcloning the indicated flagging DNA fragments into the XhoI and BamHI sites of YC, respectively. The oriP-SV40-Luc and SV40-Luc reporter plasmids, and the fragments into the XhoI and BamHI sites of YC, respectively.

Cell Culture and Transfection-Mediated EBNA1-Dependent Transcription Assay. IB4 is an Epstein–Barr Virus (EBV)-transformed lymphoblastoid cell proliferation (LCL) that contains an integrated form of the EBV genome (3, 4). Both LCL#1 and LCL#2 are EBV-immortalized LCLs, which have been described (5). LCLs, BL cell lines, and EBV-immortalized LCLs, which have been described (3, 4). Both LCL#1 and LCL#2 are EBV-immortalized LCLs, which have been described (5). BJAB is an EBV-negative B-cell lymphoma cell line (7). All of the LCLs, BL cell lines, and their derivatives were cultured in RPMI medium 1640 (GIBCO) supplemented with 10% (vol/vol) FCS (Life Technologies). The nuclear colocalized image was visualized by using a confocal microscope (LEICA TCS SP2 AOBS). The co-IP assay was used to identify the physical interaction between EBNA1 with NCL in BJAB-EBNA1 or IB4 cells. The assay was also used to monitor the interaction of ectopically expressed EBNA1 with NCL in BJAB cells that had been cotransfected with the expression vectors of NCL or its mutant derivatives as well as the interaction between NCL and EBNA1 or its mutant derivatives, using the following antibodies: flag epitope (M2; Sigma), EBNA1 (6F9/60; Novas Biologicals), NCL, and NPM1 (5E3; Santa Cruz), respectively. BJAB cells cotransfected with YN-NCL and YC-EBNA1, YC-Δ100–377, or YC-Δ100–377 were monitored for the formation of a fluorescent complex through the association of two ectopically expressed target proteins by confocal microscopy. Cell lysates derived from the indicated cell lines or immunoprecipitated samples were subjected to SDS/PAGE, followed by Western blotting with appropriate antibodies. The antibodies used for internal controls were actin (Santa Cruz) and α-tubulin (Genetext). The proteins were detected and visualized by using an ECL detection kit (Millipore). Whenever necessary, the immunoblotting images were quantified by UN-SCAN-IT.

Large-Scale Immunoprecipitation and Liquid Chromatography-Tandem Mass Spectrometry Analyses. A total of 1 × 10^7 BJAB-EBNA1 or control BJAB cells were subjected to a M2-Sepharose-mediated immunoprecipitation (IP) protocol. The immunoprecipitated samples were resolved on a 4–20% gradient gel by using SDS/PAGE, and FEBNA1-associated cellular proteins were visualized by Coomassie blue staining. The specific bands shown on the gel were excised and subjected to liquid chromatography-tandem mass spectrometry analysis (Protech Taiwan).

Lentivirus-Mediated Expression of NCL and Control shRNAs. Lentiviral plasmids containing NCL or scrambled shRNAs were produced from the National RNAi Core Facility, Academia Sinica Taiwan. The tet-inducible NCL shRNAs, tet-shNCL1 and tet-shNCL2, were purchased from OpenBiosystems. NCL1 targeted the untranslated region, thus allowing overexpression of exogenous NCL from NCL cDNA in a plasmid. The shRNA expressing lentivirus particles were produced by carrying out the protocol suggested by the manufacturer. For NCL depletion, 5 × 10^5 BJAB, IB4, or LCL cells per mL were cultured in six-well plates, and they were transduced with 1 mL of the lentiviral supernatants in the presence of 8 μg/mL polybrene. Each transfectant was replenished with new media and maintained for another 48 h, followed by selection with 5 μg/mL puromycin. For tet-inducible shRNAs, culture medium was further supplemented with 1 μg/mL doxycyclin when performing NCL depletion.

Immunofluorescence Microscopy, Coimmunoprecipitation, Immune Blot, and Bimolecular Fluorescence Complementation Analyses. Immunofluorescence analysis (IFA) was carried out by following the previously described immunostaining protocol (5), using EBNA1 and NCL-specific antibodies, vC-20 and MS-3 (Santa Cruz). In coimmunostainings, Rhodamin-conjugated goat anti-mouse and FITC-conjugated donkey anti-goat (Kirkgaard and Perry Laboratories) were used as fluorochromes, and DNA was counterstained with DRAQ5 (Bio Status). The nuclear colocalized image was visualized by using a confocal microscope (LEICA TCS SP2 AOBS). The co-IP assay was used to identify the physical interaction between EBNA1 with NCL in BJAB-EBNA1 or IB4 cells. The assay was also used to monitor the interaction of ectopically expressed EBNA1 with NCL in BJAB cells that had been cotransfected with the expression vectors of NCL or its mutant derivatives as well as the interaction between NCL and EBNA1 or its mutant derivatives, using the following antibodies: flag epitope (M2; Sigma), EBNA1 (6F9/60; Novas Biologicals), NCL, and NPM1 (5E3; Santa Cruz), respectively. BJAB cells cotransfected with YN-NCL and YC-EBNA1, YC-Δ100–377, or YC-Δ100–377 were monitored for the formation of a fluorescent complex through the association of two ectopically expressed target proteins by confocal microscopy. Cell lysates derived from the indicated cell lines or immunoprecipitated samples were subjected to SDS/PAGE, followed by Western blotting with appropriate antibodies. The antibodies used for internal controls were actin (Santa Cruz) and α-tubulin (Genetext). The proteins were detected and visualized by using an ECL detection kit (Millipore). Whenever necessary, the immunoblotting images were quantified by UN-SCAN-IT.

Chromatin Immunoprecipitation, ATP-agarose–Mediated Pull-Down, and EBV Genome Maintenance Analyses. EBNA1 DNA-binding affinity was assessed by chromatin immunoprecipitation (ChiP) assay, followed by quantitative real-time PCR (qPCR) analysis, as described (1). The primers used to detect of the mini-EBV episome (oriP-SV40-Luc) and full-length EBV genome have been described (1). Briefly, 2 × 10^6 of the indicated cell lines were
subjected to ChIP assay by using antibodies against EBNA1, acetyl-H3 (H3ac) (Millipore), or IgG control (Millipore), following the manufacturer’s protocol. The accumulation of each assayed protein at the promoter of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also gauged by qPCR using a pair of primers that could recognize the known H3ac target site (Millipore). The manufacturer provided the primers used to identify the GAPDH promoter (Millipore). The abundance of each assayed protein at the oriP or GAPDH promoters was determined as a percentage relative to input DNA (% of input). The viral DNA was purified by using a viral DNA isolation kit, following the manufacturer’s protocol (Geneaid Inc. Taiwan). Genome accumulation of the full-length EBV genome and mini-EBV oriP-SV40-Luc was determined by qPCR, using the same primers described above. For ATP-agarose-mediated pull-down assays, the appropriate amount of ATP-conjugated agarose (Sigma) was used to pull down cell lysates from IB4 or BJAB cells that were transfected with the expression vectors for FNCL or its mutant derivatives.

**Statistical Analyses.** The data from transfection-mediated reporter and ChIP assays were represented as the mean ± SEM from three independent experiments. Whenever necessary, statistical comparisons were performed by one-way ANOVA. A P value of less than 0.05 was considered to be statistically significant.

**Measurements of Cell Proliferation.** A total of $5 \times 10^4$ of the indicated cell lines per 200 μL were aliquoted, in triplicate, in 96-well plates. Each type of cell was counted by using the Trypan blue exclusion method every 24 h for five consecutive days.


![Fig. S1. NCL is expressed at similar levels in primary B cells and LCLs, and DNase or RNase treatment destroyed most of cell DNA or RNA. (A) Immune blots for NCL, E1, and actin in primary B cells, LCL#1, LCL#2, LCL#3, and IB4 LCLs. (B) DNase or RNase treatment destroyed most of cell DNA or RNA in the buffer used in the immune precipitation.](image-url)
E1 localizes with NCL. (A) Confocal fluorescence microscopy following eGFP-E1 and DsRed-NCL expression in BJAB cells (Upper) revealed eGFP-E1 and DsRed-NCL to be colocalized in intranuclear bodies. Immunofluorescence used a mouse antibody against E1 and a goat antibody against NCL, followed by FITC conjugated goat anti-mouse antibody (green) and Rodamine conjugated donkey anti-goat antibody (red) in BJAB-FE1 (Middle) or IB4 (Bottom) LCLs. (B) Schematic depiction of the two-component yellow fluorescent protein expression to assess YN-NCL and YC-E1 colocalization in BJAB cells. (C) Immune blots for YN-NCL, YC-E1, YC-E1 Δ1–377, and YC-E1 Δ100–377 are shown. (D) BJAB cells were transfected with expression vectors for YN-NCL and YC-E1 or E1 mutants and assayed by confocal fluorescence microscopy. Nuclei were counterstained with Draq5 (blue).
Fig. S3. NCL increases E1-oriP-dependent transcription. (A) BJAB cells were transfected with FE1, FNCL, or (B) FNCL deletion mutant expression plasmids, an oriP-SV40-Luc reporter, and a CMV-β gal expression plasmid as an internal control. Protein expression and an actin internal control were determined by Western blot. Resulting activities were presented as mean ± SD for data from three experiments. **P < 0.05 vs. control.

Fig. S4. NCL is essential for EBV LCL proliferation. (A) BJAB cell growth was unaffected by NCL knockdown. (B–D) IB4 cells transduced with lentivirus dox regulated shNCL1 or shNCL2 were monitored for growth in the presence or absence of doxycyclin versus shC-transduced IB4 cell controls. Western blots for NCL expression levels are shown. (E and F) LCL#1 and LCL#2 expressing shNCL1 or shNCL2 and monitored for growth versus shC-transduced LCLs.
Fig. S5. ATP-bound NCL associates with E1. (A) ATP or strepavidin-conjugated agarose beads were used to pull down NCL or E1 from IB4 cell lysates. (B) ATP-agarose pull-down assays were used to evaluate NCL or NCL mutant ATP binding. (C) Immunoprecipitation assays evaluated E1 association with NCL in IB4 cells, with or without Antimycin-mediated ATP depletion. Western blots for E1 and NCL in each sample are shown. (D) BJAB cells, transfected with an E1 and oriP-SV40-Luc reporter plasmid, were Antimycin A treated for 2 h, and luciferase activity was monitored. (E) ATP depletion had no effect on SV40-Luc.