Supporting Information

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SI Materials and Methods

Cell Lines. BJAB cells were maintained in RPMI medium containing 10% bovine growth serum (BGS; HyClone) and 15 μg/mL gentamicin. Kaposi's sarcoma-associated herpesvirus (KSHV)–infected body cavity-based B-cell lymphoma-1 (BCBL-1) cells were maintained in RPMI medium containing 20% BGS and 15 μg/mL gentamicin. 293T cells were maintained in DMEM containing 10% Tet-proof BGS (Clontech) and 15 μg/mL gentamicin. Flp-In 293 T-Rex (also referred to as 293/FRT; Invitrogen) cells were maintained in DMEM containing 10% Tet-proof BGS and 15 μg/mL gentamicin.

Plasmids. To construct the vectors expressing WT latency-associated nuclear antigen (LANA) or LANA mutants, the NruI site of plasmid cDNA5-ZZ-FLAG (pcDNA5-ZZ-FLAG) (1) was destroyed, and the resultant plasmid was designated pcDNA5-ZZ-FLAG-mNru. The coding sequences for LANAΔ33–929 and LANAΔ33–888 (2) were PCR-amplified, inserted into pZZ-FLAG-mNru, and termed pZZ-Δ33–929-FLAG and pZZ-Δ33–888-FLAG, respectively. pSG5-T7LANAΔ264–929, pSG5-T7LANAΔ332–929, and pSG5-T7LANA (3) were digested with AscI and NruI, and the smaller fragments were inserted into pZZ-Δ33–929-FLAG between the AscI and NruI sites to generate pZZ-Δ264–929-FLAG, pZZ-Δ332–929-FLAG, and pZZ-full-length (FL)-LANA-FLAG. These constructs include the initial LANA codons beginning with ATG. Human replication factor C (RFC) 1, RFC2, RFC3, RFC4, and RFC5 were PCR-amplified from insect cell expression vectors (4) and inserted into pcDNA4-Flag (1) to generate pcDNA4-RFC1, pcDNA4-RFC2, pcDNA4-RFC3, pcDNA4-RFC4, and pcDNA4-RFC5, respectively.

For expression of FL-LANA for purification, pcDNAEF monomeric Azami-Green (mAG), a derivative of pcDNA3.1hygro (Invitrogen) carrying the HindIII/XbaI fragment of CSII-EF-MCS version 4-3 (5) between its NruI and XbaI sites, was used after the HindIII end was first blunted with T4 DNA polymerase. The BamHI fragment of pZZ-FL-LANA-FLAG carrying the C-terminal three-quarters of the LANA coding sequence was inserted into the BamHI site of pcDNAEFmAG in the appropriate direction, resulting in pcDNAEFmAGLANAC1. The C-terminal region of LANA between NruI and XhoI was substituted with the NruI/XhoI fragment from pZZ-Δ33–888-FLAG to generate pcDNA-mAG-LANACNX104, which can express an N-terminal truncated version of LANA tagged with mAG and His6 at the N-terminal end and a FLAG peptide sequence at the C-terminal end. The sequence from ATG to the internal BamHI of LANA was PCR-amplified with primers LANABamHIRV (GGATCCCTCAGACGGGGATG) and LANANFW (GGATCCATGGCGCCCCCGGGAATG) from pZZ-FL-LANA-FLAG DNA and inserted into the BamHI site of pcDNA-mAG-LANACNX104 in the appropriate direction. The resultant plasmid, termed pcDNA-mAG-LANA-FL, can express full-length LANA with mAG and His6 at the N-terminal end and FLAG at the C-terminal end. pUC19GAP1 was generated by annealing the oligonucleotides (GATCCCTCAGCGTCGACTTTTTTTTTTTTTTTTTTTTTTTTCCTCAGCA and AGCTTGCTGAG-GAAAAAAAAAAAAAAAAAAAAAAAAAAGTCGACGCTGAGG) and insertion into BamHI- and HindIII-digested pUC19. pTAGAP terminal repeat (TR) 1–3 was constructed by insertion of the HindIII/EcoRI fragment of pUC19GAP1 between HindIII and EcoRI of pCR2.1-TOPO (Invitrogen) and then inserting the Not1 fragment from p8TR (6), containing one copy of the KSHV TR, into the NotI site.

shRNA sequences were designed using "siRNA Target Finder," which was provided by Ambion (with guanine and cytosine percent between 45% and 65%), and cloned into the BglII and HindIII sites of pSuperior.puro vector (Oligoengine). Inducible shRNA cell lines were established as described earlier (1). The shRNA sequence for human RFC1 knockdown is GATCCCCTACCAAGTGGAAAGAAACT TTCAAGAGAAGTTTCTTTCCACTTGGTATTTTTA, with the targeted sequence underlined, and the additional generic sequence from Oligoengine. The control, scrambled shRNA sequence for human RFC1 is GATCCCCCGTCAATACAATAATGAGAGGTTCAAGAGACCTCTCATTATTGTATTGACGTTTTTA, with the control, scrambled sequence generated by Oligoengine underlined. The resultant vectors with this sequence were termed pSuperior-RFC1 and pSuperior-RFC1-scrambled, which were then digested with SpeI to remove the puromycin resistance cassette and subsequently digested with NotI and XhoI, and the ∼1.5-kb fragment was purified and ligated into NotI- and XhoI-digested pCDNA6-TR (Invitrogen), generating pCDNA6-TR-RFC1 or pCDNA6-TR-RFC1-scrambled. pCDNA6-TR-RFC1 is capable of achieving inducible RFC1 knockdown using a single vector. In the absence of tetracycline, the Tet repressor forms a homodimer that binds with high affinity to the $TetO_2$ sequence in the H1 promoter of the pSUPERIOR vector. Binding of the Tet repressor homodimer to the $TetO_2$ sequence represses transcription of the RNA hairpin precursor of the siRNA duplex targeted to silence the gene of interest. After addition of tetracycline, the tetracycline binds with high affinity to the Tet repressor homodimer at a 1:1 stoichiometry, resulting in a conformational change in the repressor, rendering it unable to bind to the Tet operator.

Plasmids containing a single WT TR copy or a single TR copy that contains 20-bp deletions of the two adjacent LANA binding sites (LBSs) were previously described (7).

Generation of Stable Cell Lines. Stable 293 cell lines were obtained according to the protocol provided by the manufacturer (Invitrogen). Briefly, pOG44 (Invitrogen) was cotransfected into the Flp-In 293 T-REx cell (Invitrogen) with pZZ-FLAG-mNru, pZZ-Δ33–929-FLAG and pZZ-Δ33–888-FLAG, pZZ-Δ264–929-FLAG, pZZ-Δ264–929-FLAG, or pZZ-FL-LANA-FLAG. Transfections were performed in a six-well dish at 75% cell confluence using 1.5 μg of DNA in a ratio of pOG44 to LANA vectors of 1 μg to 0.5 μg. Selection was performed with DMEM supplemented with 10% TET-proof BGS, 0.5 μg/mL blasticidin (selection for the Tet-repressor expression vector, pCDNA6-TR, integrated in the Flp-In 293 T-REx cells), 50 μg/mL hygromycin (selection for pCDNA5 vector-encoding target sequence in the flip-in site), and 15 μg/mL gentamicin. Single colonies were picked and screened for the inducible expression of ZZ-FLAG–tagged proteins upon doxycycline (DOX) treatment. Clones that exhibited the best inducible gene expression were used for large-scale cultures and subsequent protein purification. Drug selection was removed for large-scale cultures.

To establish BJAB stable cell lines with inducible RFC1 knockdown, BJAB cells or BJAB cells stably expressing T7LANA were transfected with 5 μg of pTripz-RFC1 (catalog no. RHS4696-100897382, clone ID V3THS_390989; Thermo Fisher Scientific) by nucleofection, using Amaxa nucleofector II (solution V, program C-009), into 10 million cells. Twenty-four hours posttransfection, cells were placed under selection using 1 μg/mL puromycin, for

which resistance is encoded by the vector. Clonal cell lines were then generated and analyzed for inducible RFC1 knockdown.

To establish BCBL-1 stable cell lines with inducible RFC1 knockdown, 10 million cells were transfected with 5 μg of pTripz-RFC1 (Thermo Fisher Scientific) using Amaxa nucleofector II (solution V, program T-001). Twenty-four hours post-transfection, cells were placed under selection with 0.75 μg/mL puromycin after seeding cells into 96-well plates at a concentration of 10 cells per milliliter to obtain ∼1.5 cells per well in 150 μL per well. Clonal stable cell lines were selected and analyzed for inducible knockdown of RFC1 by Western blot analysis.

Tandem Affinity Purification and MS. Tandem affinity purification (TAP) of LANA complexes from human cells was performed. Stable cell lines capable of expressing ZZ-LANA-FLAG upon DOX induction were used. Doses of DOX were used to induce LANA expression close to physiological levels, similar to the naturally KSHV-infected primary effusion lymphoma cell line BCBL-1. Briefly, 8×10^8 cells were collected and thoroughly washed with PBS. To isolate nuclei, cells were resuspended in hypotonic buffer [10 mM Tris·HCl, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4, protease mixture (pH 7.9)], and swollen cells were treated by douncing. After centrifugation at 4,000 × *g* for 20 min, the pellet was harvested and resuspended in hypertonic buffer [20 mM Tris·HCl, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM NaF, 1 mM Na_3VO_4, 0.5% Nonidet P-40 (American Bioanalytical), 0.2 mM EDTA, 1 mM DTT, protease mixture (pH 7.9)]. After vigorous vortexing, the nuclear extract was harvested after centrifugation at 15,000 × *g* for 20 min at 4 °C. Next, 0.4 mL of packed IgG beads (GE Healthcare) (and ethidium bromide at 10 μg/mL in Fig. 1*D*) was added to the supernatant, followed by gentle rotation overnight at 4 °C for 12 h. The protein-bound IgG beads were washed three times using a buffer composed of the aforementioned hypotonic and hypertonic buffers at a ratio of 2:1. Bound proteins were eluted after incubation with tobacco etch virus (TEV) protease (Invitrogen) at 4 °C for 8 h. The TEV protease cleaves between the ZZ-tag and LANA or between the LANA mutants, leaving the ZZ-tag on the beads. Next, proteins were bound to anti-FLAG antibody–conjugated beads (Sigma). Beads were then incubated with 4 U/mL DNaseI (Sigma) at 4 °C for ∼12 h to eliminate potential protein associations caused by any "bridging" DNA bound to LANA. After incubation, beads were pelleted and washed twice for 5 min each time using washing buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA]. Anti-FLAG bead-bound proteins were then eluted using 2 × 70 μL of elution buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2 μg/μL 3× FLAG peptide], resolved by SDS/PAGE in a 4–12% gradient gel, and visualized by silver staining. Another gel run in parallel was stained using colloidal blue dye, and bands were cut out and subjected to MS analysis. The titrated expression and TAP of the ZZ-FLAG LANA mutants were achieved following the same procedure in parallel. The TAP and MS were performed twice for LANA and each mutant.

Immunoprecipitation. Cells were collected, and nuclear extract lysates were used for immunoprecipitation after preparation as described for the TAP procedure. Nuclear extracts were incubated with relevant antibody using 2 μg of anti-LANA antibody affinity-purified against MBP-LANA888-1162aa for 10 h at 4 °C in the presence of 4 U/mL DNaseI and then incubated with protein A+G Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. The beads were next washed with buffer composed of the TAP hypotonic and hypertonic buffers at a ratio of 2:1 three times for 5 min each time and subjected to SDS/PAGE. Immunoblotting was performed using standard procedures. RFC1 was detected using RFC1 antibody (sc-20993; Santa Cruz Biotechnology).

Fluorescence Microscopy. A total of 2×10^5 cells were collected in a microfuge at 450 × *g* for 5 min. After aspiration of the supernatant, 1 mL of hypotonic buffer (1% Na citrate, 1 mM $MgCl_2$, 1 mM $CaCl_2$) was added. After incubation for 5 min at room temperature (RT), cells were spread onto slides using a cytospin (Thermoshandon) and fixed in 4% paraformaldehyde in PBS for 10 min at RT. After three washes with PBS, cells were permeabilized using 0.5% Triton X-100 in PBS for 5 min at 4 °C. To detect LANA, primary monoclonal antibody IA-2-12 (a gift from Mary Ballestas, University of Alabama School of Medicine, Birmingham, AL) at a ratio of 1:2,000 was incubated with cells for 2 h at RT. Cells were then incubated with secondary anti-mouse Alexa488 or Alexa568 antibody (Abcam). Cells were counterstained with DAPI (Invitrogen), and coverslips were applied with Aqua-Poly/Mount (Polysciences). Quantification of LANA dots was performed in three separate experiments for each cell line; 100 cells were assessed for the presence of dots in each experiment, and the SD was determined. Images were captured using confocal microscopy at a magnification of 630× using a Zeiss AxioPlan 2 microscope.

DNA Replication Assay. DNA replication assays were performed as described previously (8), with minor modifications. For the replication assay in 293T cells (Fig. 2*A*), cells in 15-cm dishes at 50% confluence were first transfected with GenEscort (Wisegen) using polyethylenimine (PEI) solution (20 μg of PEI/10 μg of DNA) with amounts of vector DNA expressing LANA or LANA mutants that resulted in similar LANA expression levels (8 μg of empty vector, 4 μg of pSG5-T7LANAΔ264–929, 4 μg of pSG5-T7LANAΔ332–929, 8 μg of pSG5-T7LANAΔ262–320, 8 μg of pSG5-T7LANAΔ288–320, and 10 μg of pSG5-T7LANA per 15-cm dish). Twenty-four hours later, p8TR-gB (8) (4 μg of DNA per 15-cm dish) was transfected into each dish. For transfection, the frozen 1 mg/mL PEI stock solution in PBS was thawed in a 37 °C water bath for 5 min to avoid any precipitation. DNA was added to 500 μL of DMEM (without serum), and PEI was separately added to 500 μL of DMEM (without serum). After 10 min at RT, the two solutions were mixed, incubated for 15–20 min at RT, and gently mixed by tapping, and the solution was added to a 15-cm dish containing cells. Twenty-four hours after p8TR-gB was transfected, the cells were trypsinized. One-third of the cells were collected for normalization of transfection efficiency, and the rest of the cells were further cultured in 15-cm dishes for an additional 48 h. Cells were subsequently collected and analyzed for DNA replication. The results represent the average of four experiments.

For the DNA replication assay performed in BJAB cells, cells carrying RFC1 shRNA were induced with 1 μg/mL DOX for 3 d. Ten million uninduced control cells or RFC1 knockdown cells were transfected by nucleofection with 3 μg of p8TR-gB using Amaxa nucleofactor program O-17 and with solution V using 2 μg of DNA. After transfection, cells were seeded into 25-cm^2 flasks in 5 mL of medium. Twenty-four hours after transfection, half of the cells were collected and used to normalize transfection efficiency and the other half of the cells were further cultured. Seventy-two hours posttransfection, low-molecular weight DNA was harvested from cells by the Hirt method (9). Hirt DNAs (9) were assayed for replication using real-time PCR as previously described. The results represent the average of four experiments.

Episome Maintenance Assays. BJAB or BJAB/LANA cells were induced for RFC1 knockdown with 1 μg/mL DOX for 3 d. Ten million BJAB or BJAB/LANA cells with or without RFC1 knockdown were then transfected by nucleofection with 2 μg of p8TR using Amaxa nucleofactor program O-17 and solution V. Twenty-four hours posttransfection, cells were seeded into microtiter plates at 1,000, 100, or 10 cells per well in medium containing G418 at 0.6 mg/mL or G418 at 0.6 mg/mL and

0.5 μg/mL DOX. Macroscopic colony formation was assessed after 16 d. G418-resistant cell lines were also expanded. Gardella gel analysis (10) was performed on G418-resistant cells using cell lines expanded from plates initially seeded at 100 cells per well. Cells were loaded into loading gel wells made of agarose containing DNase-free protease (Sigma) and SDS, which results in in situ lysis of cells, followed by electrophoresis in Tris·borate·EDTA buffer. DNA was transferred to a nylon membrane, and KSHV DNA was detected using a ^{32}P-labeled TR probe.

The BCBL-1 stable cell lines clone 2, clone 3, and clone 4 were induced with DOX (500 ng/mL) for 3 d. The knockdown of RFC1 was confirmed by Western blot analysis. On day 0, 2 million uninduced or DOX-induced cells were seeded in 30 mL (6.6×10^4 cells per milliliter) of RPMI 1640 medium containing 10% Tet-proof FBS (Clontech) in a T75 flask with or without DOX (day 0) and continuously expanded. Cells were sampled at the indicated time points and analyzed by real-time PCR or Gardella gel assay (10) using the standard protocol described above. Each PCR was performed in triplicate, and the data shown are representative of three experiments. For the microtiter outgrowth assay, clone 3 and clone 4 were induced with 1 μg/mL DOX for 3 d and then were seeded into 96-well microtiter plates at 1,000, 100, or 10 cells per well in medium containing 0.5 μg/mL DOX. Colony formation was monitored both macroscopically and microscopically, and was recorded 12 d after seeding. Wells containing at least 20 cells were scored as positive.

GFP Decay Assay. BJAB cells infected with recombinant KSHV containing a GFP expression cassette (11) were cultured in RPMI medium containing 10% BGS, 15 μg/mL gentamicin, and 10 μg/mL puromycin. To generate BJAB cells infected with KSHV with RFC-inducible knockdown or with an inducible scrambled RFC1 negative control sequence, 2 μg of pCDNA6-TR-RFC1 or 2 μg of pCDNA6-TR-RFC1-scrambled was cotransfected with 4 μg of pHygro-BABE into 10 million cells by nucleofection using Amaxa nucleofactor program O-17 and solution V. Ten minutes after transfection, cells were seeded into T75 culture flasks and puromycin selection continued. At 24 h posttransfection, hygromycin was added into the culture at a concentration of 100 μg/mL and puromycin selection continued. After 7 d of selection, the cells were treated with 1 μg/mL DOX and tested for RFC1 knockdown efficiency by Western blot analysis. Cells were then kept in continuous culture under hygromycin selection. BJAB/KSHV cells expressing RFC shRNA or scrambled shRNA and Tet repressor were treated with DOX for 3 d either to induce RFC1 knockdown or as a negative control (with the scrambled sequence); puromycin and hygromycin were then removed from the culture medium to permit episome loss, which was monitored by detection of GFP expression using flow cytometry. Each time point was assessed in triplicate, and the result shown in Fig. 4*A* is representative of two experiments.

Preparation of Purified FL-LANA. To generate FL-LANA, pcDNA-mAG-LANA-FL expressing double-tagged LANA was transiently transfected into 3×10^7 293T cells in three 150-mm plates in DMEM containing 10% FCS at about 50% confluence as described by Uno et al. (5). Cells were collected 48 h after transfection, washed once with TD buffer [25 mM Tris·HCl (pH 7.4), 0.136 M NaCl, 6.7 mM KCl, 0.7 mM Na_2HPO_4], and suspended in 2.4 mL of buffer B [50 mM Tris·HCl (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF, 20 μg/mL leupeptin]. Cells were lysed by addition of Nonidet P-40 and NaCl to a final concentration of 0.5% and 0.5 M, respectively, at 4 °C for 30 min. The cell lysate, obtained by centrifugation at 130,000 × *g* for 30 min, was diluted by the addition of 1.5× buffer H lacking NaCl and then loaded on Q-Sepharose (5 mL; GE Healthcare) at 0.2 M NaCl in buffer H [25 mM Hepes (pH 8.0), 1 mM EDTA, 0.01% Nonidet P-40, 0.1 mM PMSF, 20 μg/mL leupeptin]. The column was subsequently washed with 0.5 M NaCl, and mAG-LANA was eluted behind the bulk protein peak. The mAG-LANA–enriched fractions were pooled and loaded onto Ni-NTA-Sepharose HP (1 mL, GE Healthcare). The mAG-LANA, eluted at 20 mM imidazole, was further loaded onto an anti-Flag–agarose column (1 mL; Sigma) and eluted with 100 μg/mL FLAG peptide (Sigma). The eluted mAG-LANA was concentrated with ResourceQ (0.15 mL; GE Healthcare) using a 1.5-mL gradient of NaCl from 0.2 to 0.8 M. A total of 160 μg/mL purified mAG-LANA at its peak fraction was obtained. Purified protein was analyzed by SDS/PAGE and Coomassie blue staining.

In Vitro Binding of LANA to RFC. To prepare RFC-prebound beads, 2 μL of anti-GFP beads was incubated with 7 μL of High 5 insect cell lysate that was either uninfected or infected with baculoviruses for expression of GFP-RFC1 and RFC2–RFC5 as described (4). Baculovirus for expression of GFP-RFC1 was constructed by insertion of the PCR-amplified GFP coding sequence from pEGFP1 (Invitrogen) at the N-terminal end of human RFC1. The beads were then washed and suspended with buffer H containing 0.1 M NaCl. Anti-GFP beads (anti-GFP monoclonal antibody agarose; MBL) prebound with or without GFP-RFC1 were mixed with 100 ng of purified proliferating cell nuclear antigen (PCNA) (12) or 320 ng of mAG-LANA in 30 μL of buffer H with 0.1 M NaCl and incubated at 4 °C for 1 h. The beads were collected by centrifugation, washed three times with buffer H containing 0.1 M NaCl, and suspended in 20 μL of 1× SDS loading buffer (13). Five microliters of each sample was used for electrophoresis, and proteins were visualized by silver staining or immunoblotting.

For the binding assay in the presence of nuclease, the washing and incubation buffer was changed to mHBSG [10 mM Hepes (pH 7.5), 0.01% Tween 20, 10 mM $MgCl_2$, 0.2 mM EDTA, 150 mM NaCl, 10% glycerol]. The RFC prebound beads were incubated with 320 ng of mAG-LANA or 250 ng of control His-tagged human minichromosome maintenance complex component 2 (MCM2) with or without 1.25 units of turbo nuclease (Accelagen). Bound proteins were visualized by silver staining.

PCNA Loading Assay. LANA was incubated with DNA in a binding reaction performed in 20 μL of DNA binding reaction mixture [50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 10% glycerol] containing 1 μg/mL polydI/dC (GE Healthcare). DNA was biotin-labeled after digestion with Cfr10I (pUC19GAP1) or BglII (pTAGAPTR1-3), and the ends were filled using biotinylated dCTP with Klenow polymerase. To determine the optimal concentration of LANA for the in vitro PCNA loading assay, magnetic-SA beads (15 μg, Dynabeads M-280 Streptavidin; Invitrogen) bound with equimolar amounts of pUC19GAP1 (~60 ng) or pTAGAPTR1-3 (~72 ng) or without DNA were incubated at RT for 30 min after mixing with 130 ng, 260 ng, or 390 ng of LANA. The DNA bead-bound fraction was recovered in 16 μL of 1× SDS loading buffer, and 8-μL aliquots were resolved by electrophoresis in a 10% SDS polyacrylamide gel, followed by immunoblotting with anti-FLAG antibody (M2; Sigma).

Magnetic-SA beads (15 μg) bound with pTAGAPTR1-3 (~72 ng) were prebound with or without 260 ng of LANA in 20 μL of DNA binding reaction mixture with 1 μg/mL polydI/dC (per reaction) at RT for 30 min. The LANA prebound or unbound beads were suspended in loading reaction mixture [10 mM Hepes (pH 7.5), 0.01% Tween 20, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.15 M NaCl, 50 mM creatine phosphate, 25 μg/mL creatine phosphokinase, 0.4 mM DTT, 2 mM ATP] containing 480 ng of PCNA and 0, 3, 6, 9, 12, or 15 ng of RFC, and were incubated at 32 °C for 30 min after mixing with RFC. The DNA bead-bound fraction was recovered in 16 μL of 1× SDS loading buffer, and

8-μL aliquots were resolved by electrophoresis in a 10% SDS polyacrylamide gel followed by immunoblotting with anti-FLAG and anti-PCNA antibody, respectively.

ChIP Assays. Formaldehyde cross-linking and ChIP assays of cells were performed as described (14, 15), with some modifications. Twenty million BJAB or BCBL-1 cells in a T175 flask at exponential growth stage were cross-linked by the addition of formaldehyde to a final concentration of 1% directly into the medium and were gently shaken at a speed of 80 rpm for 5 min at RT. Cross-linking was stopped with the addition of glycine at a final concentration of 125 mM, and cells were shaken at RT for another 10 min. Cells were collected by centrifugation (2,000 × *g* for 4 min) and washed three times with ice-cold 1× PBS. Cell pellets were resuspended in cell lysis buffer [5 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (KOH) (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40] containing protease mixture (Roche). After incubation for 10 min on ice, samples were dounced several times on ice with a type B homogenizer. Nuclei were collected by centrifugation (2000 × *g* for 15 min). Nuclei were resuspended in nuclear lysis buffer [50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS] containing protease mixture (Roche) and incubated on ice for 10 min. Chromatin was then sonicated to an average length of ∼600 bp (which was later confirmed on a 1.5% agarose gel), keeping samples on ice. Debris was cleared by centrifugation at 13,000 × *g* for 10 min at 4 °C. Supernatant was transferred to a new tube and diluted fivefold in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.1), 167 mM NaCl plus protease inhibitors]. To reduce nonspecific background, samples were precleared with 80 μL of a salmon sperm DNA/protein A agarose slurry for 30 min at 4 °C with agitation. Beads were collected by brief centrifugation at 1,000 × *g* for 5 min at 4 °C, and supernatant fraction was saved. Twenty percent of the total supernatant was used for total input control, and the rest of the supernatant was divided into two fractions: one for incubation with 5 μg of species-matched IgG control and the second for incubation with 5 μg of specific antibody [affinity-purified anti-LANA antibody, anti-RFC1 rabbit polyclonal antibody (Bethyl), or anti-PCNA rabbit polyclonal antibody PC10 (Cell Signaling)]. Incubations were performed overnight at 4 °C with rotation. Immune complexes were collected using 60 μL of salmon sperm DNA/protein A agarose slurry for 1 h at 4 °C with rotation. Beads were then washed at RT consecutively for 3–5 min on a rotating platform with 1 mL of each of the following solutions: (*i*) once in low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 150 mM NaCl], (*ii*) once in high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 500 mM NaCl], (*iii*) once in LiCl wash buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8.0)], and (*iv*) twice in 1× Tris EDTA buffer. Complexes were eluted by adding 250 μL of elution buffer [1% SDS, 0.1 M $NaHCO_3$] to the pelleted beads. After centrifugation at 13,000 × *g* for 3 min at RT, supernatants were transferred to new tubes. Complexes were then eluted a second time, and the two elutions were combined. The formaldehyde cross-linking was then reversed with the addition of 1 μL of 10 mg/mL RNase; 5 M NaCl was added to adjust the final concentration to 0.3 M NaCl and incubated at 65 °C for 4–5 h. Two and one-half volumes of 100% ethanol was then added, and DNA was precipitated overnight at −20 °C. DNA was collected by centrifugation, and the pellet was resuspended in 100 μL of water. Next, 2 μL of 0.5 M EDTA, 4 μL of 1 M Tris (pH 6.5), and 1 μL of 20 mg/mL proteinase K were added and incubated for 1–2 h at 45 °C. DNA was purified using QiaQuick spin columns (Qiagen) and eluted in 50 μL of 10 mM Tris (pH 8.0). Two microliters of DNA was used in quantitative PCR reactions (forward primer 5′-GGGGGACCCCGGGCAGCGAG-3′ and reverse primer 5′-232GGCTCCCCCAAACAGGCTCA-3′ flanking KSHV TR nucleotides 677–766). PCR was performed after melting at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For quantification, 5%, 1%, or 0.2% of input DNA was used to generate a standard curve. For measuring the LANA-directed TR loading of RFC and PCNA by ChIP assay, 10 million BJAB, BJAB/LANA, or BJAB/LANAΔ262–320 cells were transfected with 5 μg of p8TR using the conditions described above. Thirty-six hours posttransfection, ChIP was performed using RFC1 and PCNA antibodies. For assessing the role of the LBS in LANA-directed TR loading of RFC and PCNA, 10 million BJAB or BJAB/LANA cells were transfected with 5 μg of plasmid containing a copy of TR DNA or a copy of TR DNA deleted for the LBS using the conditions above. Twenty-four hours posttransfection, ChIP was performed using affinity-purified anti-LANA, RFC1, or PCNA antibody.

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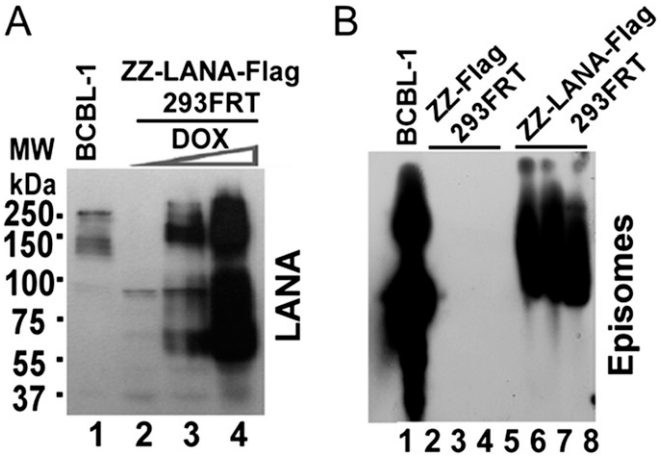

Fig. S1. ZZ-LANA-FLAG maintains episomes. (*A*) LANA Western blot of 293FRT/ZZ-LANA-FLAG cells before or after induction with increasing amounts of DOX. A similar number of BCBL-1 primary effusion lymphoma cells are shown in lane 1. (*B*) Episome maintenance was assessed in 293FRT/ZZ-LANA-FLAG or 293FRT/ZZ-FLAG cells. Cells were transfected with p8TR plasmid containing a puromycin resistance cassette and subjected to puromycin selection. After 20 d, a Gardella gel assay (10) was performed on puromycin-resistant cells. BCBL-1 cells are shown in lane 1. Lanes 2–8 contain cells from independently derived, puromycin-resistant ZZ-FLAG 293FRT or ZZ-LANA-FLAG 293FRT cell lines.

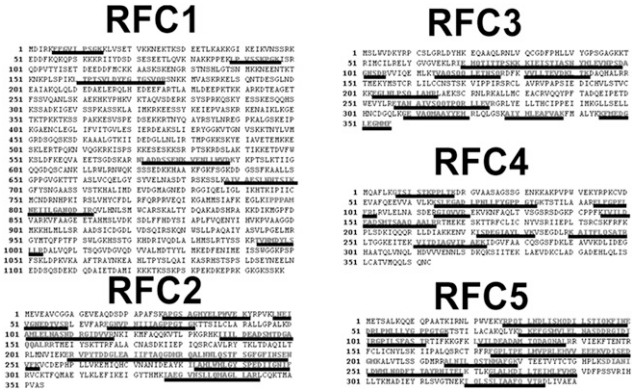

Fig. S2. ZZ-LANA-FLAG interacts with RFC. RFC peptides identified by MS following LANA TAP are indicated (underlined).

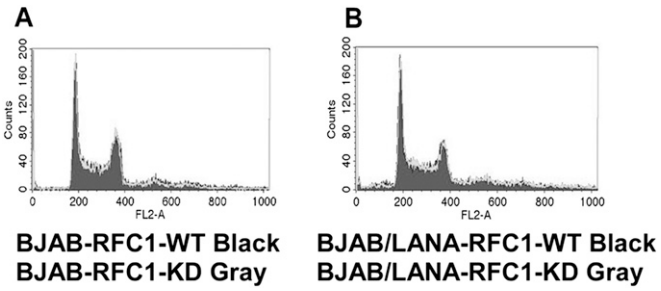

Fig. S3. RFC1 knockdown did not alter the cell cycle of BJAB or BJAB/LANA cells. Cell cycle analyses of BJAB cells (*A*) or BJAB/LANA cells (*B*) with or without induction of RFC1 knockdown are shown.

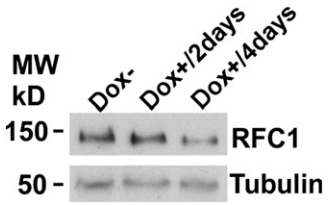

Fig. S4. Western blot for RFC1 or tubulin in BJAB/KSHV cells with or without incubation with DOX.

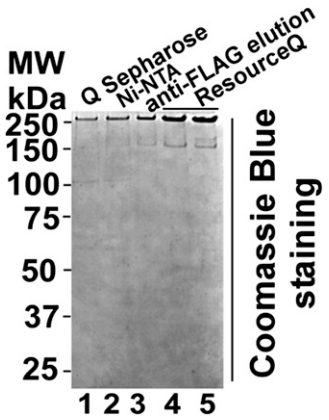

Fig. S5. Purification of LANA. Purification of recombinant FL-LANA from 293T cells. Successive steps in LANA purification are shown: lane 1, Q-Sepharose; lane 2, nickel-nitrilotriacetic acid (Ni-NTA) following imidazole; lane 3, anti-FLAG elution; and lanes 4 and 5, concentration using ResourceQ and NaCl gradient elution.

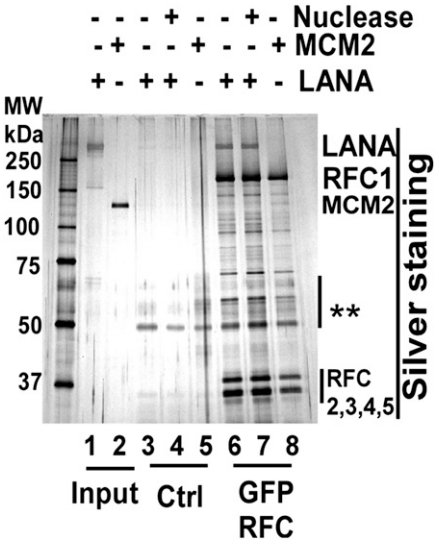

Fig. S6. Nuclease treatment does not disrupt LANA interaction with RFC. Assessment of RFC binding to LANA or to negative control (Ctrl) minichromosome maintenance complex component 2 (MCM2) after incubation with anti-GFP beads with or without bound GFP-RFC. Incubations were performed in the presence or absence of nuclease. A total of 30 ng or 320 ng of LANA was used for input or incubations with beads, respectively. A total of 20 ng or 250 ng of MCM2 was used for input or incubations with beads, respectively. The double asterisk indicates heavy chain (lanes 3–8) and degraded GFP-RFC1 (lanes 6–8).

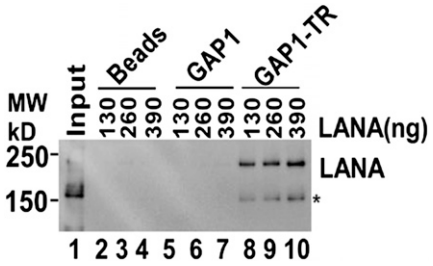

Fig. S7. Purified LANA specifically binds GAP1-TR DNA. Magnetic beads or beads bound with pUC19GAP1 or pTAGAPTR1-3 were incubated with LANA, and binding was assessed by immunoblotting with anti-FLAG antibody. The asterisk indicates degradation product.

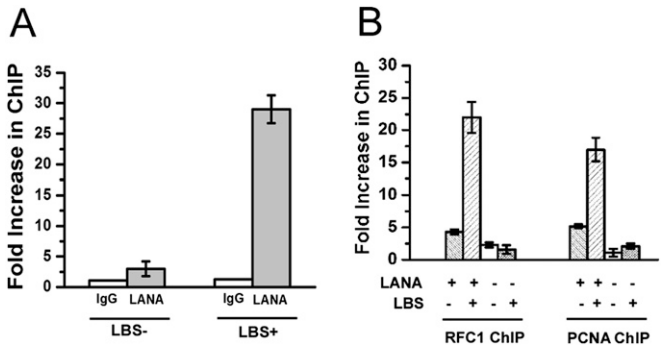

Fig. S8. Enrichment of LANA, RFC, and PCNA at TR DNA is dependent on the presence of the LBS. (*A*) ChIP assay for LANA binding to TR DNA after transfection of TR DNA or TR DNA deleted for the LBS into BJAB/LANA cells. (*B*) ChIP assay for RFC1 or PCNA binding to TR DNA after transfection of TR DNA or TR DNA deleted for the LBS into BJAB (LANA-negative) or BJAB/LANA cells. Averages of three experiments, with SDs, are shown.