Supporting Information

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SI Materials and Methods

Plasmids. Latency-associated nuclear antigen 1 (LANA1) constructs containing full-length, N terminus, NCR1, NCR1CR2, and CR2 and deletion mutants were generated by PCR with the BC-1 DNA template (U75698) using the primers described previously (1, 2). The different frames of eGFP were amplified from the pEGFP-C1 vector template (Clontech) by PCR using the following sense primers: 0 frame, eGFP/*Hind*III(S) (CAT AAG CTT GTG AGC AAG GGC GAG GAG CTG); −2 frame, eGFP-2/*EcoR*V(S) (CCG ATA TCC GTG AGC AAG GGC GAG GAG CTG) or eGFP-2/*Hind*III(S) (CCA AGC TTC GTG AGC AAG GGC GAG GAG CTG); −1 frame, eGFP-1/*EcoR*V(S) (GAT ATC TGA GCA GGG CGA GGA GCT GTT) or eGFP-1/*Hind*III(S) (CCA AGC TTT GAG CAA GGG CGA GGA GCT GTT); and antisense primers, eGFP/*Hind*III(AS) (CCA AGC TTC TTG TAC AGC TCG TCC ATG CC) or eGFP/*Xho*I(AS) (CTT CTC GAG CTT GTA CAG CTC GTC CAT GC). PCR products were then inserted into the *EcoR*V/*Hind*III or *Hind*III/*Xho*I sites of various LANA1. To construct DsRed in different frames, PCR products for DsRed were amplified from pDs-Red-Monomer-Hyg-N1 (Clontech) using the following primers: DsRed/*Xho*I(S) (CCC TCG AGG ACA ACA CCG AGG ACG TCA TC), DsRed-1/*Xho*I(S) (CCC TCG AGG GAC AAC ACC GAG GAC GTC ATC) (for −2 reporter), and DsRed/*Apa*I(AS) (AAT GGG CCC CTG GGA GCC GGA GTG GCG GGC). PCR products were digested with *Xho*I/*Apa*I and inserted into the CR2–eGFP(0) construct. For the forced −2 frame control CR2 (1288–2304 aa) expression plasmid [pCMV-tag2A.CR2$^{(-2)}$] (Fig. 3*A*), the CR2 insert from pCMV-tag2B.LANA1 CR2 (2) was ligated into the pCMV-tag2A vector to change its frame.

To examine recoding efficiency, LANA1 CR2 fragments were amplified by PCR using paired primers CR2.WT(S) (GGA TCC GGC GAT GGA AAC AAA ACG TTG AGC ATC) and -(AS) (GAG CTC TCC TGC TCC TGC TCC TCC TGC TG) for WT and primers CR2.IFC(S) (GGA TCC GCG ATG GAA ACA AAA CGT TGA GCA TC) and -(AS) (GAG CTC CCT GCT CCT GCT CCT CCT GCT G) for the in-frame control. PCR products were cloned into the p2-Luc and p2-Luci vectors (3). These parental cloning vectors and control constructs (p2-Luc, p2-Luc-HIV, -MMTV, -AZ1, p2-Luci, p2-Luci-HIV, -MMTV, and -AZ1) were previously described (3). The full-length pEpstein–Barr nuclear antigen 1 (EBNA1)-GFP (B95.8 strain) plasmid was kindly provided by Neil Blake, University of Liverpool, Liverpool, United Kingdom. For EBNA1 NGAr–eGFP constructs, the following primers were used: EBNA1.N(S) (TTC GAA TTC ATG TCT GAC GAG GGG CCA GGT ACA) and EBNA.NGAr(AS) (AAG CTT GAT CGG TGG AGA CCC GGA TG). All enzyme digestion sites used in this study for the plasmid constructions were underlined in the primer sequences. A dual N-terminal GST and C-terminal maltose binding protein (MBP)/His-tagged EBNA.N was cloned into a modified pSPORT vector by PCR using the following primers: N.BHI(S) (CGG GAT CCC CAT GTC TGA CGA GGG GCC AGG T) and N.RI(AS) (CGG AAT TCG CAG CCA ATG CAA CTT GGA CGT TT). The PCR fragment was inserted into the *Bam*HI and *Eco*RI sites located between the GST (0 frame) and MBP/His genes (−1 frame) to investigate the −2 frameshifting of EBNA1 (4). For M2, M3, and M2M3 mutants of the EBNA1 N terminus, overlapping PCR was performed using the following primer pairs: M2.F (GGG GGT GAT AAC CAC GGA CGA GGA CGG), M2.R (CCG TCC TCG TCC GTG GTT ATC ACC CCC T), M3.F (CAA GAC ATA GAG ACG GTG TCC GGA GAC C), and M3.R (GGT CTC CGG ACA CCG TCT CTA TGT CTT G). All enzyme digestion sites used in this study for the plasmid constructions were underlined in the primer sequences.

In Vitro Transcription/Translation. pcDNA.LANA1 C-terminal truncations were generated by digesting and linearizing a pcDNA full-length LANA1 construct at the following restriction sites: *Hinc*II (320 aa), *Acl*I (434 aa), *Bsmb*I (928 aa), *Nru*I (980 aa), and *Xho*I (1162 aa). RNA concentrations were determined by UV spectroscopic measurement at 260 nm, and molar equivalents of each RNA sample were calculated (based on nucleotide length and concentration) for use in uncoupled in vitro translation with Rabbit Reticulocyte Lysate System (Promega) and [^{35}S]-methionine (Amersham). Reaction products were resolved on SDS/PAGE gel, dried, exposed overnight to screens, and read using PhosphorImager SI (Molecular Dynamics).

Generation and Purification of Antibodies. Rabbit polyclonal antibodies for LANA1$_{ARF}$ (RαLANA1$_{ARF}$, CM826) and EBNA1$_{ARF}$ (RαEBNA1$_{ARF}$, CM827) were generated commercially (Covance) by inoculating rabbits with peptides corresponding to the predicted −2 frame of LANA1 CR2 (CKK-ACP-SSRMSSSSRMSS) and glycine–alanine residue (GAr) (CKK-ACP-QEQEEGQEGQE-QEG). For a mouse monoclonal IgM antibody (MαLANA1$_{ARF}$), the standard method of immunizing mice was used with the keyhole limpet hemocyanin-derivative peptide (Epitope Recognition Immunoreagent Core facility, University of Alabama). Antibodies were further purified with either a SulfoLink immobilization kit (Pierce) or Nab protein A/G Spin kit (Pierce) according to the manufacturers' protocol. Briefly, 0.1 mg of peptide was dissolved in coupling buffer and loaded to the column. The sample was incubated with resin at room temperature (RT) for 1 h. Resin was washed with binding/wash buffer 4 times and eluted with 0.2 M glycine·HCl (pH 2.5), and then 1/10th volume of 1 M Tris·HCl (pH 9.0) buffer was added for the neutralization.

Immunofluorescence and Microscopy. For conventional immunofluorescence microscopy following 24–48 h of transfection, cells were rinsed and fixed with 4% (wt/vol) paraformaldehyde for 20 min, permeablized with PBS with 0.1% Triton X-100, and blocked with 10% (vol/vol) normal goat serum. For confocal images, cells were fixed and permeabilized with ice-cold methanol for 10 min at −20 °C. After two PBS washes, cells were treated with 2 N HCl at RT for 20 min followed by a PBS wash and neutralization with 0.1 M boric acid, pH 8.5, for 10 min. Following an additional PBS wash, cells were blocked with 10% (vol/vol) FBS in PBS. Fixed cells were incubated with antibodies, αNucleolin (kindly provided by Nancy Maizels, University of Washington, Seattle), αSC-35 (1:1,000; Sigma-Aldrich), αPML (promyelocytic leukemia) (Santa Cruz) (1:100 dilution), α*N*-term LANA1 (1:2,000; Novus, 4C11), MαLANA1$_{ARF}$ (1:200), and RαLANA1$_{ARF}$ (1:8,000) at RT for 2 h or at 4 °C overnight. Secondary antibodies (Alexa Fluor 568 and Alexa Fluor 488; Life Technologies) (1:1,000) were applied for 1 h at RT. Antisera was diluted in blocking buffer with 10% (vol/vol) normal goat serum (or FBS) in PBS either with or without 1% Tween 20 (PBS-T), and intervening washes of slides were carried out twice with PBS-T for 30 min. Cells were counterstained with DAPI and examined under a fluorescent microscope (AX70, Olympus). For confocal microscopy, the nuclei were stained with DRAQ5 (Molecular Probes) for 15 min at RT, rinsed in PBS, and mounted with Gelvatol mounting medium for fluorescence. Confocal images

were acquired using a Leica TCS SP confocal microscope (Center for Biologic Imaging, University of Pittsburgh).

Luciferase Assay for Recoding Efficiency and GFP Fluorescence Analysis. Transfected HEK293 cells were harvested at 48 h after transfection. Renilla and Firefly luminescence activities were analyzed using the dual luciferase reporter assay (Promega) according to the manufacturer's protocol, on a microplate reader, Synergy 2 (Bio-tek). Frameshift efficiencies were calculated using the method described previously (3). All assays were performed in triplicate in each of three separate transfections. For eGFP fluorescence analysis, 293 cells were transfected with LANA1 constructs fused to eGFP using Lipofectamine 2000 (Invitrogen).

For the fluorimetry, cells (1.7×10^6) were harvested at 48 h after transfection and lysed in buffer (50 mM Tris·HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 3 mM EDTA, 1% Triton X-100, 1 mM NaF, and 1 mM Na Orthovanadate) supplemented with protease inhibitors (Roche). The eGFP signal from cell lysates (40 μg) in 100 μL of PBS was analyzed by a fluorescence reader, either Synergy 2 (Biotek) or Safire (Tecan). The background signal (autofluorescence) was determined on the same plate and was subtracted.

Detection of LANA1$_{ARF}$ by Immunoprecipitation (IP)–Immunoblot (IB). HEK293 cells transiently transfected with LANA1 constructs and KSHV-negative (BJAB) and -positive primary effusion lymphoma (PEL) cell lines (BC-1, BCBL-1, JSC-1) were harvested and lysed with IP lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM NaF, 1 mM NaVO3 with protease inhibitors). To disrupt the binding of proteins to DNA, NaCl was added (final concentration, 250 mM) to the lysate and mixed gently. The lysate was incubated for 20 min on ice and diluted with an equal volume of ice-cold water. After brief sonication, the lysate was centrifuged at 17,000×*g* for 20 min at 4 °C. The supernatant was further cleared with IgG isotype antibody control (3 h at 4 °C) and 50% slurry of A/G agarose beads (Santa Cruz) for an additional 1 h at 4 °C. Following centrifugation at 4,700×*g* for 1 min, the precleared lysate was incubated with primary antibody (α*N*-term, Novus, 4C11), αCR2 (CM810), αCR2–3 (ABI, LN53), and RαLANA1$_{ARF}$ overnight with rotation. The next day, 50% slurry of A/G agarose beads were added for another 2 h and washed four times with IP lysis buffer. Beads were resuspended in 2× SDS loading buffer, and proteins were separated by SDS/PAGE. IB analysis was performed with MαLANA1$_{ARF}$ antibody using secondary mouse anti-IgM (Santa Cruz).

EBNA1 N Terminus Protein Expression and Purification. EBNA1 N terminus expression plasmid (pSport-GST-EBNA1.*N*-MH) was expressed in 293F cells (Invitrogen) using Freestyle Max reagent (Invitrogen) as described by the manufacturer. Cells were lysed in PBS + 0.5% Triton X-100 with protease inhibitors by sonication 6 d after transfection. The fusion protein was purified by affinity chromatography using Glutathione Sepharose 4B (GE Healthcare) and Ni-NTA Agarose (Novagen).

1. Kwun HJ, et al. (2011) The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV) latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation. *Virology* 412(2):357–365.
2. Kwun HJ, et al. (2007) Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mimics Epstein-Barr virus EBNA1 immune evasion through central repeat domain effects on protein processing. *J Virol* 81(15):8225–8235.
3. Grentzmann G, Ingram JA, Kelly PJ, Gesteland RF, Atkins JF (1998) A dual-luciferase reporter system for studying recoding signals. *RNA* 4(4):479–486.
4. Baranov PV, et al. (2005) Programmed ribosomal frameshifting in decoding the SARS-CoV genome. *Virology* 332:498–510.

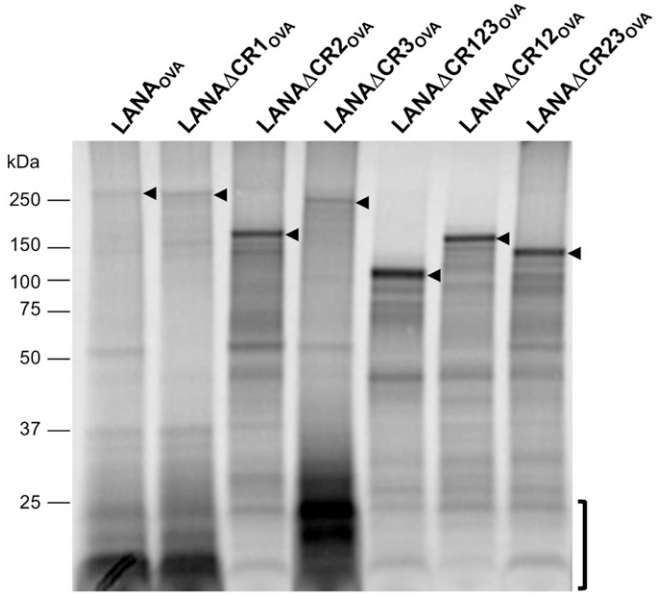

Fig. S1. LANA1 generates ARF proteins. RNAs of full-length and central repeat (CR)-deleted LANA1 constructs were used for in vitro translation analysis (1). RNAs containing the CR2 subdomain showed incorporation of [^{35}S]-methionine into low molecular-weight products below 37 kDa (bracket). Arrowheads show canonical frame of LANA1 expression. Unexpected translation products occur in the presence of the CR2 domain (LANAova, LANAΔCR1ova, LANAΔCR3ova) but not with the CR2 deletion (LANAΔCR2ova, LANAΔCR123ova, LANAΔCR12ova, LANAΔCR23ova).

1. Kwun HJ, et al. (2011) The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV) latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation. *Virology* 412(2):357–365.

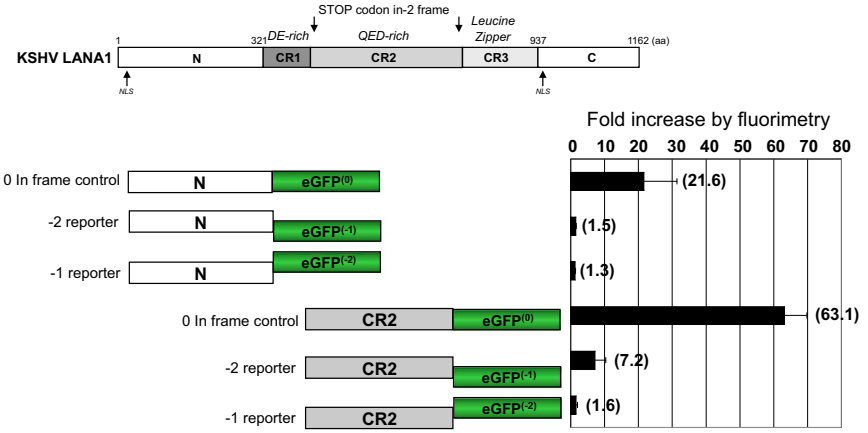

Fig. S2. ARF occurs in LANA1 CR2. The N or CR2 domain of LANA1 was fused to three frames of eGFP (0, −1, −2). All constructs were transfected into HEK293 cells, and GFP fluorescence was analyzed 48 h after transfection. The N terminus of LANA1 does not show the eGFP signal with either −1 or −2 reporters by fluorimetry.

BC1

MSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSRSSRMSRSSRMSRSSRMS**SSRMSSSSRMSSSSRMSSSSRMSSSSRMSSSSRMNR**SSRRSRSSRRSRSRS*

BCBL1

MSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSR**SSRMSSSSRMSSSSRMSSSSRMSS**SRRSRSSRRSRSRS*

JSC1

MSSSRMSSSRMSSSRMSSSRMSSSRMSSSRMSRSSRMS**SSRMSSSSRMNR**SSRRSRSSRRSRSSRRSRSSRRSRSRS*

Fig. S3. Variation of the LANA1$_{ARF}$ SR-rich sequence in the CR2 domain of KSHV strains from PEL cells. The peptide sequence of the LANA1$_{ARF}$ antibody epitope differs depending on the length of central repeats in PEL cell lines. The BC-1 cell line has four complete overlapping LANA1$_{ARF}$ antibody epitopes (SSRMSSSSRMSS, red, underlined), the BCBL-1 has three (red, underlined), and no complete epitope is present in JSC-1 (green, underlined).

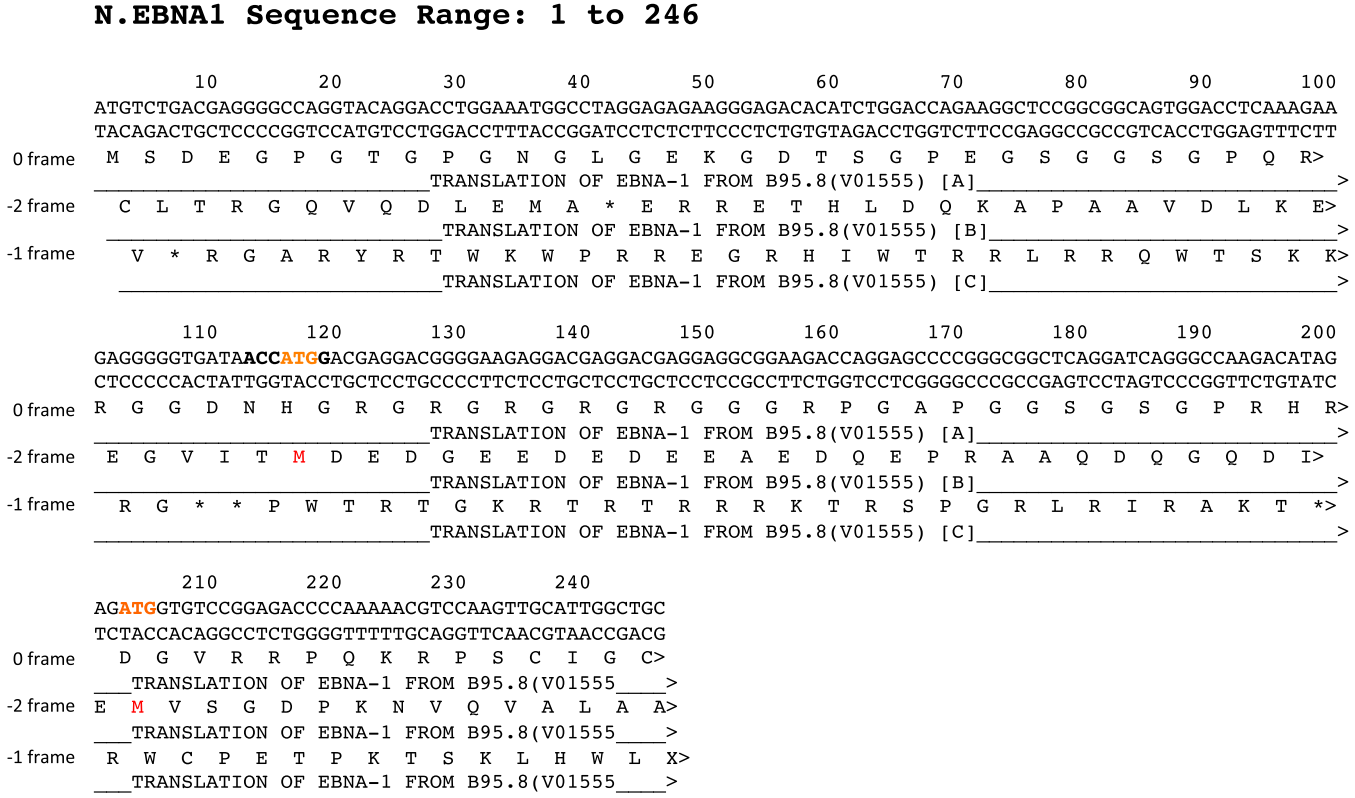

Fig. S4. Three frames of the N terminus (nt 1–246) EBNA1 sequence. ATG sequences for two methionines at −2 frame nt 116–118 and nt 203–205 in the N terminus of EBNA1 that serve as start sites for the −2 frame are shown in orange (ATG) and red (Met). A −2 frame methionine (nt116–119) in the EBNA1 N domain has a favorable Kozak context (ACCATGG, bold) (1) as an alternative translation start site.

1. Ossevoort M, et al. (2007) The nested open reading frame in the Epstein-Barr virus nuclear antigen-1 mRNA encodes a protein capable of inhibiting antigen presentation in cis. *Mol Immunol* 44(14):3588–3596.

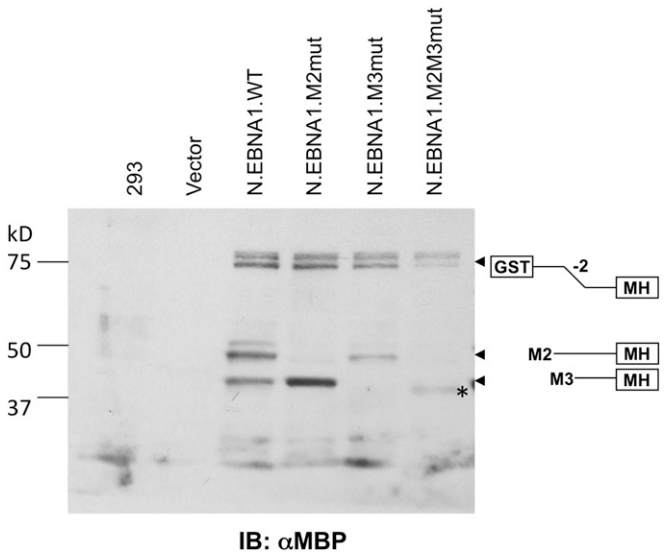

Fig. S5. Both alternative translation initiation and ribosomal frameshifting occurs in the N terminus of EBNA1. The N terminus (nt 1–246) EBNA1 sequence was fused in between the 0 frame of GST and the −1 frame of MBP/His tag to test −2 frameshifting. The GST$^{(0)}$-EBNA1.*N*-MH$^{(-1)}$construct (N.EBNA1.WT) was expressed in HEK293 cells, and three isoforms (~75, ~45, and ~41 kDa) produced by −2 frameshifting in the EBNA1 N-terminal sequence were detected by MBP antibody. A single mutation of the −2 frame methionine to threonine [nt116–119 (M2) or nt 203–205 (M3)] in the EBNA1 N domain parallels with the loss of the bands at 45 and ~41 kDa, respectively, suggesting that two methionines (M2 and M3) of the −2 frame serve as alternative translation start sites. Double-mutant N.EBNA1.M2M3mut is no longer able to make alternative translation initiation products (45 and 41 kDa), whereas the low-migrating form at ~75 kDa is still present. An asterisk denotes the MBP (40 kDa) band alone initiated by double mutations.

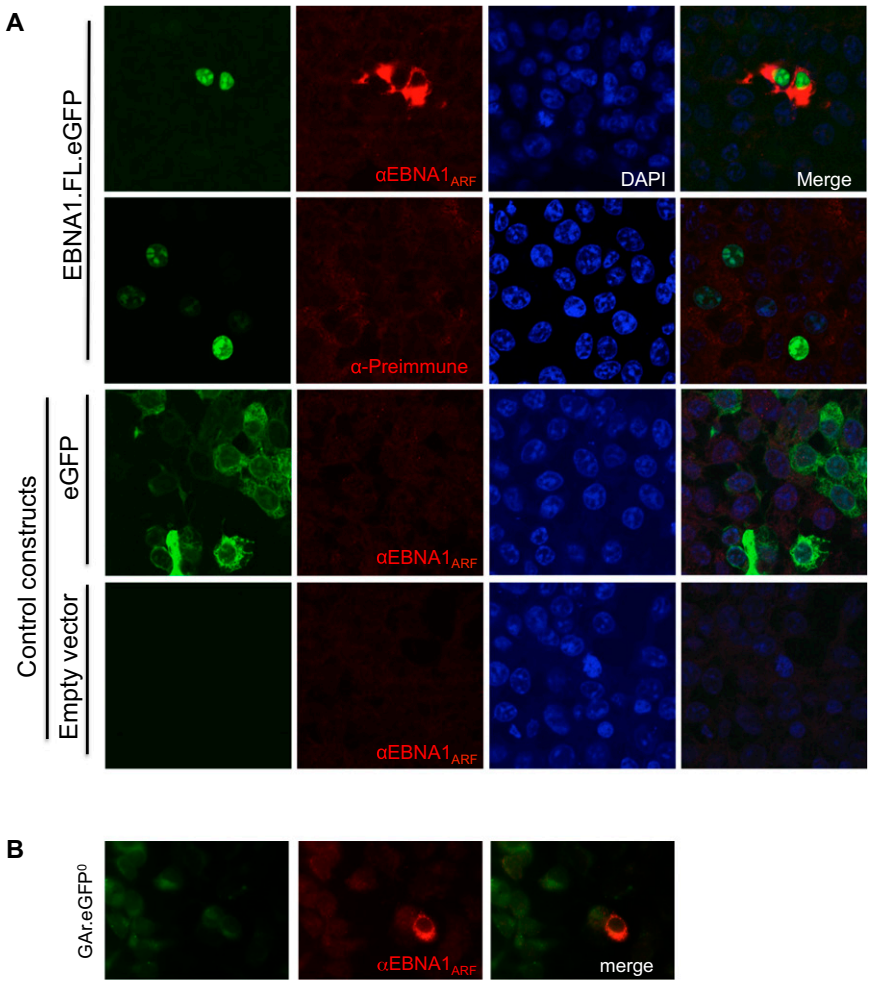

Fig. S6. Specificity of $EBNA1_{ARF}$ antibody. (*A*) Full-length EBNA1-eGFP, eGFP, and empty vector were expressed in HEK293 cells. The canonical 0 frame EBNA1 was detected in the nucleus (green) and eGFP in the cytoplasm (column 1). The $EBNA1_{ARF}$ antibody detected $EBNA1_{ARF}$ in the cytoplasm (red, column 2) and did not cross-react with the controls (eGFP, empty vector). Rabbit preimmune serum (red, *Second row*) was used as a control and shows the specificity of the $EBNA1_{ARF}$ antibody. (*B*) The $EBNA1_{ARF}$ antibody detects the ARF protein from GAr sequences in the cytoplasm.

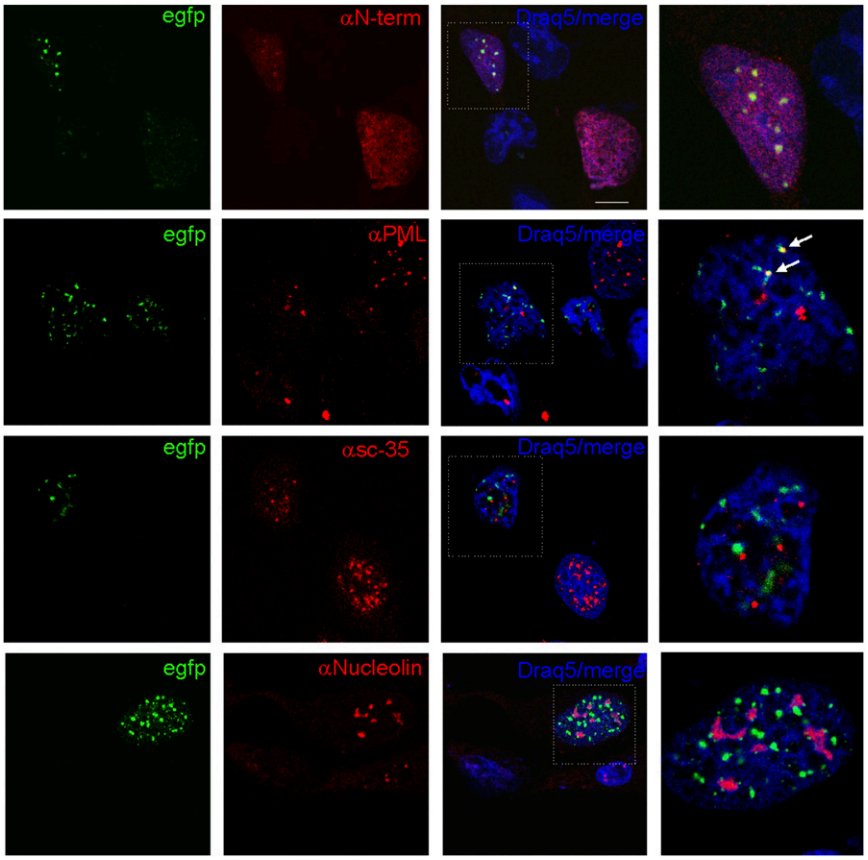

Fig. S7. Subnuclear localization of LANA1$_{ARF}$. Confocal fluorescent images of LANA1$_{ARF}$ fluorescence in the −2 reporter (NCR1CR2-eGFP$_{-1}$) (green) (Fig. 2 *A* and *C*) showed a speckled nuclear staining pattern distinct from 0 frame canonical LANA1 protein (red, α*N*-term, second panel in *Top* row). LANA1$_{ARF}$ (green) subnuclear localization was investigated using different nuclear body markers such as PML, the splicing factor SC-35, and nucleolin. [Scale bar (*Top*, third panel), 5 μm.]

Table S1. Repeat sequences of episome maintenance proteins from different gammaherpesviruses

Genus/species	Common name	Repeat sequences			Accession no.
		Frame	Sequence	Length, aa	
Lymphocryptoviruses					
Epstein–Barr virus	EBV/HHV-4	0	GA	236	NC_007605
		−2	EGQ	238	
		−1	SRG	237	
Macacine herpesvirus 4	rhHHV-4	0	GSR	154	NC_006146.1
		−2	EVA(Q)	77	
		−1	SR	60	
Cercopithecine herpesvirus 12	CeHV-12	0	GSR	213	HPU23857
		−2	EVA(Q)	106	
		−1	RS(K/Q)	85	
Rhadinoviruses					
Human herpesvirus 8	KSHV/HHV-8	0	DE/QED/EQ	617	U52064.1
		−2	SR(M/H)	326	
		−1	GAT, GA(V/R)	157, 173	
Saimiriine herpesvirus 2	HVS	0	RE/GE/EA	272	NC_001350.1
		−2	EK/KL(R)	282	
		−1	RK	144	
Ateline herpesvirus 3	AtHV-3	0	DEG	171	AAC95598
		−2	GET(K)	201	
		−1	GR	172	
*Macacine herpesvirus 5**	CeHV-17	0	EP	514	DQ792459.1
		−2	SL(K/Q)	497	
		−1	AR(G)	<18	
Macaviruses					
Alcelaphine herpesvirus 1	AlHV-1	0	PEG/ED/GE	965	AF005370.1
		−2	KR, KE	61, 134	
		−1	R	>102	
Ovine herpesvirus 2	OvHV-2	0	GEP(V)	331	AY839756
		−2	KE(D/L), KR(E)	<59, 93	
		−1	SR, GR	<83, 122	

Content of the amino acid repeat sequences of different frames are indicated as specific amino acids (aa). A, alanine; E, glutamic acid; G, glycine; H, histidine; K, aspartic acid; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine. To evade immune recognition, episome maintenance proteins have evolved different strategies including inhibiting antigen presentation, proteasomal degradation, pre-mRNA processing, reducing self-protein synthesis, and retarding mRNA translation (1).

*Only viruses of the RV1 lineage of *Macacine herpesvirus 5* contain an internal amino acid repeat.

1. Blake N (2010) Immune evasion by gammaherpesvirus genome maintenance proteins. *J Gen Virol* 91(Pt 4):829–846.