Supporting Information

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SI Text

SI Methods. ***Cell lines and culture conditions.*** HEK 293 cells (CRL-1573) obtained from American Type Culture Collection (ATCC) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Invitrogen) with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37 °C with 5% CO_2 and split every 2 or 3 d. The body cavity lymphoma cell line BCBL-1 carrying episomal KSHV genome was established and maintained in RPMI1640 (Invitrogen) with 10% fetal bovine serum, 2 mM L-glutamine, 55 uM 2-mercaptoethanol (Invitrogen, 21985023), and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37 °C with 5% CO_2, and split at a density of 2×10^5 cells/mL before cells reached 1×10^6 cells/mL (1). To induce productive replication of KSHV genome, BCBL-1 cells were treated with 500 uM sodium-valproate (Sigma, P4543).

Plasmid constructions. 3′UTR luciferase reporter constructs. ORF56 and ORF57 3′UTR sequences have been experimentally characterized (2, 3). Other uncharacterized 3′UTRs were cloned according to NCBI's annotations. For ORF6 3′UTR, we used the first canonical AATAAA following the annotated ORF6 coding sequence as the polyadenylation sequence. Candidate 3′ UTRs were inserted immediately downstream of Renilla luciferase (Rluc) reporter of psiCHECK2 dual luciferase reporter plasmid (Promega). We PCR amplified 3′UTRs with the following primer pairs: UTR6-F/R (psiCHECK-6.3U), UTR9-F/R (psiCHECK-9.3U), UTR40/41-F/R (psiCHECK-40/41.3U), UTR44-F/R (psiCHECK-44.3U), UTR56-F/R [psiCHECK-56.3Ug (genomic sequence, with an intron) and psiCHECK-56.3U (without an intron)], UTR57-F/UTR56-R (psiCHECK-57.3U), and UTR59-F/R (psiCHECK-59.3U).

C-ORF luciferase reporter constructs. We extended Rluc ORF to cover miR-K5 target site, originally situated in ORF56 3′ UTR. To do so, we abolished three stop codons in the region between the Rluc stop codon and miR-K5 site of psiCHECK-56.3U by changing only three nucleotides using primers CORF1-F and CORF2-F.

N-ORF luciferase reporter constructs. To generate N-ORF constructs expressing MYC-ORF57-Rluc fusion proteins, we first replaced the starting methionine (ATG) of Rluc ORF of psiCHECK2 with Leucine (TTG) using a primer pair of Rluc (A1T)-F/R. This replacement prevents initiation of translation from Rluc ORF. The modified Rluc ORF was named Rluc (A1T). Next, a fragment with a MYC tag followed by nucleotides 1-103 of ORF57 cDNA was PCR amplified using primers NORF-F and NORF-R and in-frame inserted into NheI site immediately upstream of the Rluc(A1T).

Genomic DNA expression constructs. Our experiments suggest that miR-K5 and miR-K6-3p affect CMV (Cytomegalovirus) and EF-1α promoter activity. To use SV40 promoter for expressing ORF57 and ORF56 protein, we replaced Rluc of psiCHECK2 with ORF57 and ORF56 genomic locus consisting of sequences from the starting methionine of ORF to the polyadenylation sequence, AATAAA. For MYC-57 construct, ORF57 genomic locus was PCR amplified with a MYC tag at its N terminus using a primer pair of MYC57-F/R, digested with XbaI and NotI, and cloned into NheI and NotI sites of psiCHECK2. 3FLAG-56 constructs were assembled as described below. First, ORF56 coding sequence, PCR amplified from genomic DNA using primers 56-F and 56-R, was cloned into NotI and EcoRI sites of p3xFLAG-CMV10 (Sigma). Next, a 3FLAG-ORF56 fragment flanking with AvrII and XhoI sites, amplified using primers 3FLAG56-F and 3FLAG56-R, was cloned into NheI and XhoI sites of psiCHECK2. This resulting construct was cut by XhoI and PmeI and then ligated with ORF56 3′UTR genomic sequence, released from aforementioned psiCHECK2-56.3Ug using XhoI and PmeI.

Mutagenesis. Potential miRNA target site mutants, 56-MUT1 (tK5), 56-MUT2, 56-MUT3 (tK6-3p), and 56-MUT4, were introduced by the following primer pairs: 56-MUT1-F/R, 56-MUT2-F/R, 56-MUT3-F/R, and 56-MUT4-F/R, respectively.

Western blotting. Proteins were resolved in 10% Tris-HCl SDS-PAGE gels (BioRad) and electotransferred onto PVDF membranes (Immobilon-P, Millipore). The membranes were blocked with 10% nonfat dry milk (BioRad) in TBST20 (20 mM Tris-HCl, pH7.5, 137 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. Primary mouse anti-FLAG M2 (diluted 1:1,000; Sigma, F1804), mouse anti-c-MYC (9E10) (diluted 1:500; Santa Cruz Biotechnology, SC-40), or goat antifirefly luciferase (diluted 1:1,000; Promega, G745A) was applied in TBST20 with 2% nonfat dry milk over night at 4 °C. Blots were washed extensively in TBST20 and incubated with goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (diluted 1:3,000; BioRad, 170-6,516) or donkey anti-goat IgG HRP conjugate (diluted 1:5,000; Santa Cruz Biotechnology, SC-2020) in TBST20 for 1 h at room temperature. After extensive wash in TBST20, blots were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare) followed by exposure to Amersham Hyperfilm ECL (GE Healthcare).

RNA isolation and RT-PCR. Total RNA from BCBL-1 and HEK293 mock transfected or transfected with 3FLAG-56 or MYC-57 (1.0 ug plasmid and 5 uL Lipofectamine 2000 used in a 6-well format for 24 h transfection) was extracted using RNeasy mini kit with on-column DNase I digestion (Qiagen). A second DNase treatment by TURBO DNA-free kit (Applied Biosystems) was carried out to remove trace amounts of contaminated genomic DNA. Recovered RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). An equal amount of RNA (200 ng) was reverse transcribed in the presence (+RT) or absence (−RT) of MultiScribe reverse transcriptase using High Capacity cDNA RT Kit (Applied Biosystems). One-tenth of the reverse transcription reaction (+RT or −RT) was used as a template for Platinum Taq PCR (Invitrogen) using primer 56 (RT56-F and RT56/57-R) and primer 57 (RT57-F and RT56/57-R). One-tenth of PCR products were separated in 1.5% agarose gels.

Mature KSHV miRNA sequences and oligonucleotides. Mature miRNA sequences. The seed of miRNA (nucleotides 2-8) is underlined.

miR-K1: A<u>UUACAGG</u>AAACUGGGUGUAAGC
miR-K2: A<u>ACUGUAG</u>UCCGGGUCGAUCUG
miR-K3-5p: U<u>CACAUUC</u>UGAGGACGGCAGCGA
miR-K3-3p: U<u>CGCGGUC</u>ACAGAAUGUGACA
miR-K4-5p: A<u>GCUAAAC</u>CGCAGUACUCUAGG

miR-K4-3p: UAGAAUACUGAGGCCUAGCUGA
miR-K5: UAGGAUGCCUGGAACUUGCCGG
miR-K6-5p: CCAGCAGCACCUAAUCCAUCGG
miR-K6-3p: UGAUGGUUUUCGGGCUGUUGAG
miR-K7: UGAUCCCAUGUUGCUGGCGCU
miR-K8: CUAGGCGCGACUGAGAGAGCAC
miR-K9-5p: ACCCAGCUGCGUAAACCCCGCU
miR-K9-3p: CUGGGUAUACGCAGCUGCGUAA
miR-K10a.1: UAGUGUUGUCCCCCCGAGUGGC
miR-K10a.2: UUAGUGUUGUCCCCCCGAGUGGC
miR-K10b.1: UGGUGUUGUCCCCCCGAGUGGC
miR-K10b.2: UUGGUGUUGUCCCCCCGAGUGGC
miR-K11: UUAAUGCUUAGCCUGUGUCCGA
miR-K12-5p: AACCAGGCCACCAUUCCUCUCCG
miR-K12-3p: UGGGGGAGGGUGCCCUGGUUGA
miR-K5.m34: UAUUAUGCCUGGAACUUGCCGG

Primers used for PCR cloning candidate 3′UTRs into psiCHECK2 dual luciferase reporter. Restriction sites are underlined.

UTR6-F: CTACTCGAGTTGTGTACCCGTAACGATGGC
UTR6-R: CATAGCGGCCGCAGTGACAGCTTTAACTCCAAGG
UTR9-F: TACGCAGGTCGACTGACTCAGACGCGGAAACAGC
UTR9-R: GACAGTTTAAACTGAAAGCAATAAAGACAAATGTGTG
UTR40/41-F: CTACTCGAGAAAAAGGGAAACAATGGGGGG
UTR40/41-R: CATAGCGGCCGCGTGATACCATCTTTACCAAGCACC
UTR44-F: CTACTCGAGCCCGTACCCCTCTCTTAGGACACTGATGTGTTTGGGGCGGCCGCTATG
UTR44-R: CATAGCGGCCGCCCCAAACACATCAGTGTCCTAAGAGAGGGGTACGGGCTCGAGTAG
UTR56-F: CTACTCGAGTCCCACTATATAACCTGGCTGCC
UTR56-R: GACAGTTTAAACAGAGAAAGGCTACGTGAGTTACAT
UTR57-F: GTCTCGAGGGATTGCCAAACCCCATGGCA
UTR59-F: CTACTCGAGCACCATGTGCCGCCTGGACAG
UTR59-R: GACAGTTTAAACACCGACAGGAGACATGAGATACAT

Primers used for PCR cloning N-ORF into psiCHECK2 dual luciferase reporter. Restriction sites are underlined.

NORF-F: GTGCTAGCCACCATGGAGCAGAAGCTGATT
NORF-R: TCACTAGTGCGTCCGTCTCGTCGTCCCT

Primers used for PCR cloning genomic expression constructs, MYC-57 and 3FLAG-56. Restriction sites are underlined.

MYC57-F: GCTCTAGACCACCATGGAGCAGAAGCTGATTTC
MYC57-R: CATAGCGGCCGCAGAGAAAGGCTACGTGAGTTACAT
56-F: CATAGCGGCCGCGTTGGAGACGACATACCGCCGC
56-R: TGGAATTCTTAACTGGCCAGTCCCACTGGT
3FLAG56-F: GTCCTAGGCCACCATGGACTACAAAGACCATGAC
3FLAG56-R: CTACTCGAGTTAACTGGCCAGTCCCACTGGT

Primers used for generating C-ORF by multi site-directed mutagenesis and for introducing mutations into Rluc by site-directed mutagenesis. Mutated nucleotides are shown in lowcase letters.

CORF1-F: CCAGGTTCCCAAAAgAGCCCGCGGCATAC
CORF2-F: GCGCGTGCTGAAGAACGAGCAGgAATTCaAGGCGATCGCTCGAGTCCCAC
Rluc(A1T)-F: CTATAGGCTAGCCACCtTGGCTTCCAAGGTGTAC
Rluc(A1T)-R: GTACACCTTGGAAGCCAaGGTGGCTAGCCTATAG

Primers used for introducing mutations into potential miR-K5 and miR-K6-3p binding sites of ORF57 coding sequence and ORF56 3′UTR by site-directed mutagenesis. Mutated nucleotides are shown in lowcase letters.

56-MUT1-F: TAGACATGGACATTATGAAGGGaATatTAGAGGGTAAGTCCTCGTCTAC
56-MUT1-R: GTAGACGAGGACTTACCCTCTAatATtCCCTTCATAATGTCCATGTCTA
56-MUT2-F: CGGCAGACGAGCGCATaaGgGGTACCCAGTCGG
56-MUT2-R: CCGACTGGGTACCcCttATGCGCTCGTCTGCCG
56-MUT3-F: CGCCCGGCGGACCtcaCCCCCACCCCTCA
56-MUT3-R: TGAGGGGTGGGGGtgaGGTCCGCCGGGCG
56-MUT4-F: CCTCCCCCTGAAAAGCtcaCTCGGCGCAGACCAC
56-MUT4-R: GTGGTCTGCGCCGAGtgaGCTTTTCAGGGGGAGG

Primers used for RT-PCR analyses in Fig. S3.

RT56-F: AGACACTTCCTCTGCATCAACC
RT57-F: GACATTATGAAGGGCATCCTAGAG
RT56/57-R: GGACCTGGGTCGAGACAGTG

1. Renne R, et al. (1996) Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat Med* 2:342–346.
2. Kirshner JR, Lukac DM, Chang J, Ganem D (2000) Kaposi's sarcoma-associated herpesvirus open reading frame 57 encodes a posttranscriptional regulator with multiple distinct activities. *J Virol* 74:3586–3597.
3. Majerciak V, Yamanegi K, Zheng ZM (2006) Gene structure and expression of Kaposi's sarcoma-associated herpesvirus ORF56, ORF57, ORF58, and ORF59. *J Virol* 80:11968–11981.

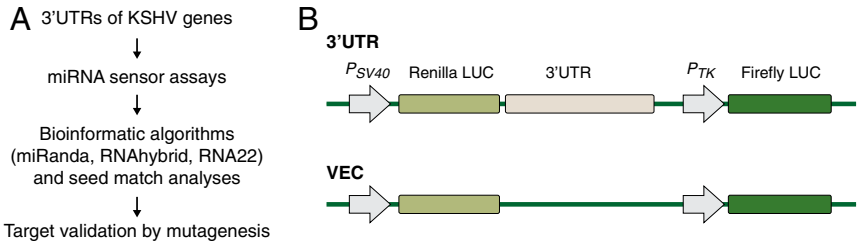

Fig. S1. Dual luciferase screens for viral targets of KSHV miRNAs. (*A*) Strategy for identification of KSHV miRNA target sequences in viral 3′UTRs. We systematically screened for the miRNA-induced repression of luciferase reporters containing 3′UTRs of the viral genes listed in Table S1. The identified 3′UTRs were then scanned against the corresponding miRNAs to search potential miRNA target sequences using a combination of bioinformatic algorithms and seed match analyses. The predicted miRNA target sites were further validated by mutations of miRNAs and the target sites in 3′UTRs. (*B*) Dual luciferase reporter constructs for miRNA sensor assays. Each viral 3′UTR was cloned downstream of a Renilla luciferase (LUC) reporter, driven by a SV40 promoter. In the same vector, Firefly LUC expression, driven by a TK promoter, serves as an internal control to normalize for transfection efficiency and sample preparation.

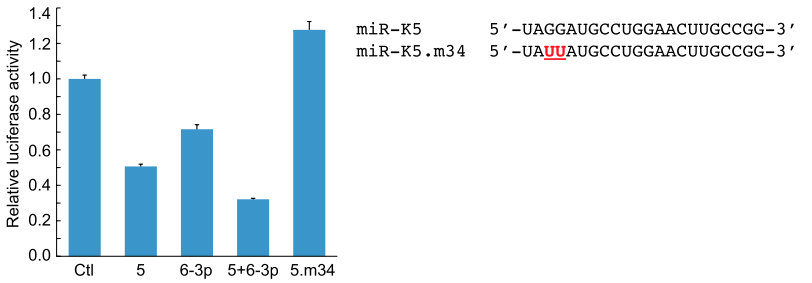

Fig. S2. KSHV miR-K5 and miR-K6-3p repress the expression of ORF56 3′UTR reporter in an additive manner. On the right, miR-K5 and miR-K5.m34 (bearing two substitutions in the seed region, underlined in red) sequences are shown.

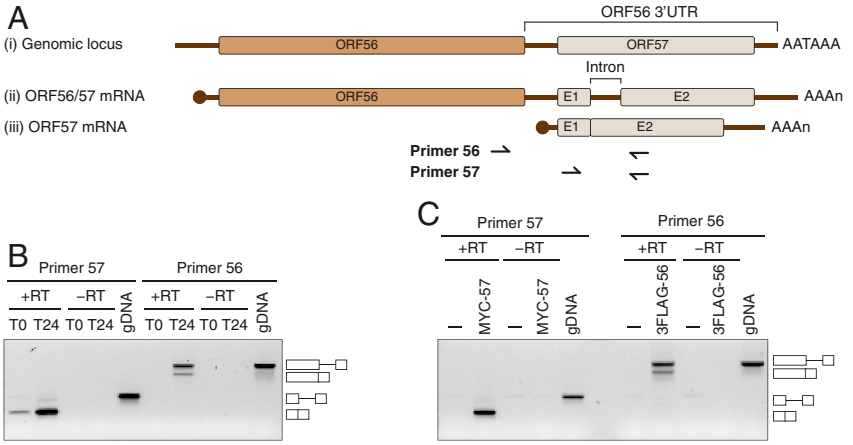

Fig. S3. Differential splicing of the bicistronic ORF56/57 transcript and monocistronic ORF57 transcript. (*A*) ORF56/57 genomic locus and mRNA transcripts derived from this locus, an inefficiently spliced ORF56/57 transcript and an efficiently spliced ORF57 transcript. Primer sets 56 and 57 for RT-PCR analyses are illustrated as arrows in the bottom of the diagram. (*B*) (*C*) RT-PCR analyses of ORF56/ORF57 and ORF57 transcripts in KSHV-infected BCBL-1 cells and genomic DNA-transfected HEK293 cells. (*B*) BCBL-1 cells harboring episomal KSHV genome were uninduced (T0) or induced to lytic replication for 24 h (T24) by treatment of sodium valproate. (*C*) HEK293 cells were either mock transfected (–) or transfected with 3FLAG-56 (Fig. 3*A*) and MYC-57 (Fig. 4*A*) for 24 h. RT reactions, carried out in the presence (+RT) or absence (–RT) of reverse transcriptase, were used as a template for PCR amplification using Primer 56 or 57. 3FLAG-56 and MYC-57 plasmid were used as a genomic DNA template (gDNA) for PCR amplification. In (*B*), a small amount of spliced RT-PCR product of ORF57 observed in uninduced (T0) BCBL-1 cells was probably due to spontaneously lytic replication of KSHV, which is observed in 1–5% of cells in this in vitro culture condition.

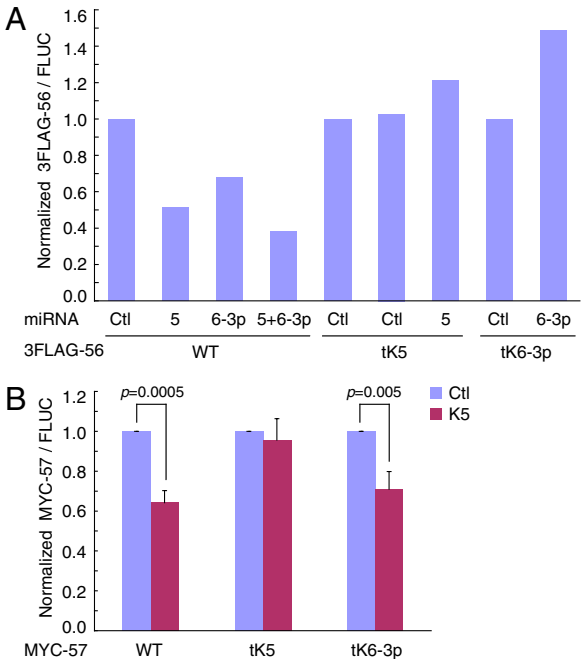

Fig. S4. (*A*) Quantitation of relative 3FLAG-56 band intensities in experiment of Fig. 3*B*. (*B*) Quantitation of relative MYC-57 band intensities in experiment of Fig. 4*B*. In (*B*), mean values and error bars (standard deviation, SD) were derived from three independent measurements, one of which was shown in Fig. 4*B*. Statistical significance is indicated (*p* value, two-tailed *t* test). Relative band intensities were calculated as described below. Band intensities of Western blot films were analyzed using ImageJ program. The band intensity of 3FLAG-56 (*A*) or MYC-57 (*B*) was first normalized to that of Firefly luciferase (FLUC) transfection control. The value obtained from KSHV miRNA (K5 and K6-3p) transfection was then normalized to the value obtained from control (Ctl) miRNA transfection for each plasmid construct (WT, tK5, and tK6-3p), which was set at 1.

Table S1. Seed match analyses of KSHV delayed-early genes governing lytic replication of KSHV genome

		Seed matches				
Gene	Function	8mer	7mer-m8	7mer-A1	6mer	Total
ORF6	single-strand DNA binding	-	K10a.1, K12-3p	K10a.2	K10a.2	4
ORF9	DNA polymerase	-	K1, K8, K10a.2, K10b.1	K5, K10a.1	K1, K5(x2), K6-5p, K10b.1, K10b.2(x2), K12-5p	14
ORF40/41	primase associated	-	-	-	-	0
ORF44	helicase	-	-	-	-	0
ORF56	primase	K5	K3-5p, K12-3p	-	K4-3p, K6-3p(x2), K8, K9-3p, K10a.1, K12-3p(x5)	14
ORF59	polymerase processivity	-	K6-5p	K6-5p	K6-5p(x3)	5
ORF57	Posttranscriptional regulator	-	-	-	-	0

Numbers of seed-matched sequences of KSHV miRNAs in viral 3′UTRs are shown (e.g., ORF59 3′UTR contains a total of five seed matches for miR-K6-5p: one 7mer-m8, one 7mer-A1, and three 6mers). Seed match 6mer represents perfect Watson-Crick (W-C) basepairing to miRNA bases 2-7 (positions in the miRNA are numbered 5′-3′.); 7mer-A1 represents 6mer and an adenosine opposite miRNA base 1; 7mer-m8 represents perfect W-C basepairing to miRNA bases 2-8; 8mer represents perfect W-C basepairing to miRNA bases 2-8 and an adenosine opposite miRNA base1