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Supplemental Data

Regulation of KSHV Lytic Switch Protein Expression by a Virus-Encoded MicroRNA: An Evolutionary Adaptation that Fine-Tunes Lytic Reactivation

Priya Bellare and Don Ganem

Supplemental Experimental Procedures

Cells and Plasmids

HEK293 cells were grown in Dulbecco's Modified Eagle Serum (DMEM from Invitrogen) 10% Fetal Bovine Serum, penicillin, streptomycin, glutamine and β-mercaptoethanol. SLK endothelial cells and HFF cells were also grown in the above medium but which contained puromycin at 10 μg/mL and 0.5 μg/mL respectively. BCBL-1 cells were carried in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine, and ß-mercaptoethanol. For the 3'UTR of RTA, cDNA was made from RNA from BCBL-1 cells and the sequence in the cDNA between the RTA start codon and polyadenylation signal was amplified. This PCR was cloned downstream of *Renilla* luciferase in the Xho1 and Not1 sites of a psiCHECK-2 plasmid about 10 nucleotides upstream of the synthetic polyadenylation site. psiCHECK-2 plasmid also expressed the Firefly luciferase gene that was used as an internal control for transfections and protein amount. Mutations in the 3'UTR were made using QuikChange Site-Directed Mutagenesis kit (Stratagene). For RTA expression in Immunoblot

analyses DNA from BCBL-1 cells was used to PCR amplify the unspliced sequence of RTA (from start of RTA up to the poly adenylation site of the transcript). The PCR was then cloned into the Cla1 and Xba1 sites of pCS2+ vector about 28 nucleotides upstream of the SV40 polyadenylation site.

Luciferase and Immunoblot assays

The mimics of KSHV-encoded microRNAs for these experiments were purchased from Ambion. For the luciferase assay the psiCHECK-2 plasmid and microRNA were cotransfected into HEK293 cells at 0.25 ng/μL and 12.5nM respectively using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Luciferase activity was analyzed 48 hours after transfection and was normalized to a psiCHECK-2 vector lacking the 3'UTR and to a negative control microRNA. Transfections were assayed in duplicate or triplicate and we carried out three independent transfections. For the immunoblot assay, HEK293 cells were cotransfected with the RTA expression plasmid (at about 0.22 ng/μL) and the appropriate microRNA (at about 24nM) using Lipofectamine 2000 (Invitrogen) or a serial transfection of the microRNA using Dharmafect1 (Dharmacon) followed by transfection of the RTA expression plasmid using FuGene (Roche) was done about 18 hours after the microRNA transfection. All transfections were done using the manufacturer's protocol. Cell lysates were prepared 48 hours after the final transfection and then probed with antibody against RTA (Lukac et al., 1998) or β-actin (loading control).

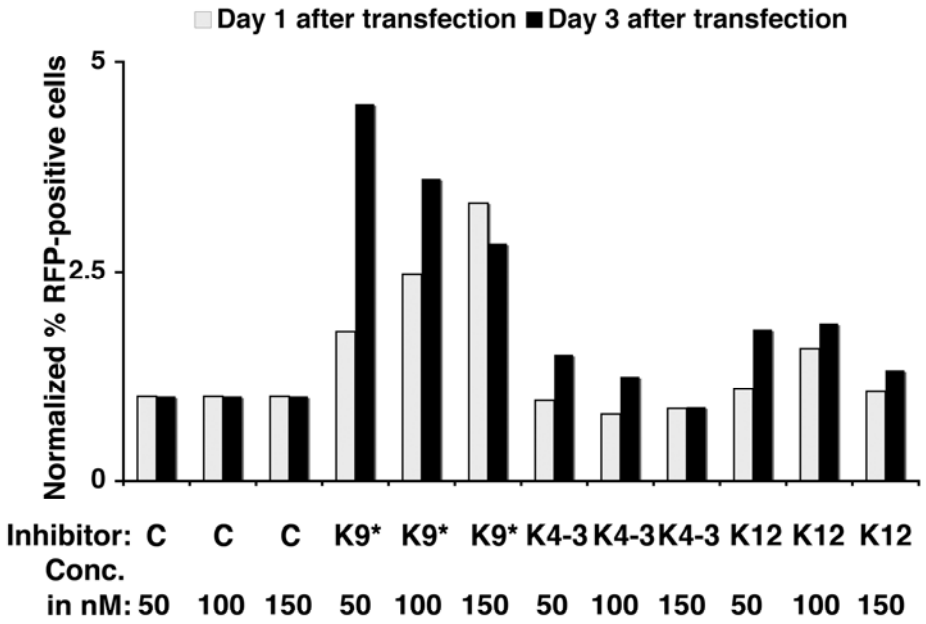

Figure S1. Inhibiting miR-K9* in KSHV-infected SLK endothelial cells stimulates spontaneous lytic reactivation.

SLK endothelial cells latently infected with rKSHV.219 were transfected with different concentrations of inhibitors to the individual KSHV-encoded microRNAs (indicated along the X-axis). Spontaneous lytic reactivation was measured one day or three days after transfection. Lytic reactivation was measured as a % of RFP-positive cells from a population of healthy, GFP-positive cells.

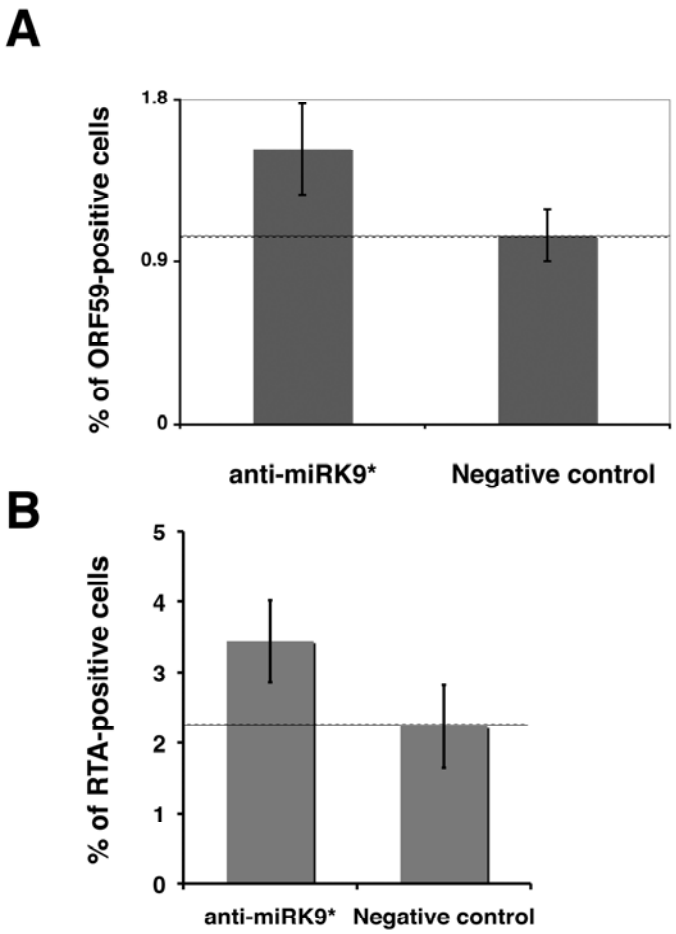

Figure S2. Inhibiting miR-K9* in BCBL-1 cells stimulates spontaneous lytic reactivation. **(A)** An antisense inhibitor to miR-K9* or a negative control inhibitor was transfected twice (about 48 hours apart) into BCBL-1 cells; 48 hrs after the second transfection, the cells were examined by immunofluorescence to visualize ORF59 protein (a delayed-early lytic marker) in the cells. Mean values of % of ORF59 expressing cells from those expressing LANA (latent gene) are derived from two independent transfections.

(B) BCBLs were transfected with negative control inhibitor or inhibitor to miR-K9* microRNA and RTA expression in the cells was analyzed by immunofluorescence 48 to 60 hours after transfection. Mean values of % of RTA expressing cells are derived from three independent transfections.

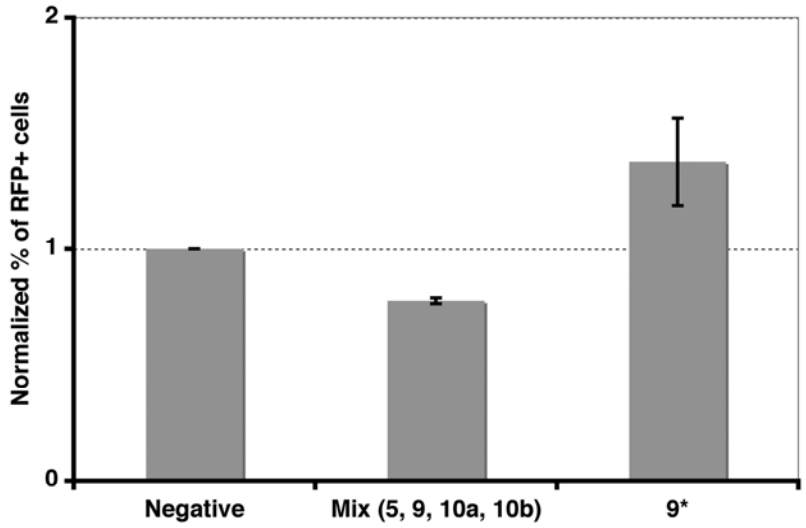

Figure S3. Inhibiting miR-K9* in KSHV-infected HFF fibroblasts that are treated with sodium butyrate increases lytic reactivation.

HFF fibroblast cells infected with rKSHV.219 were transfected with inhibitor to miR-K9* or a mix of inhibitors to miR-K9, miR-K5, miR-K10a and miR-K10b microRNAs (indicated along the X-axis). Two days after transfection cells were treated with 3mM sodium butyrate and lytic reactivation was measured about 24 hours later. Lytic reactivation was measured as a % of RFP-positive cells from a population of healthy, GFP-positive cells. The percentage of RFP-positive cells was derived from two independent transfections.

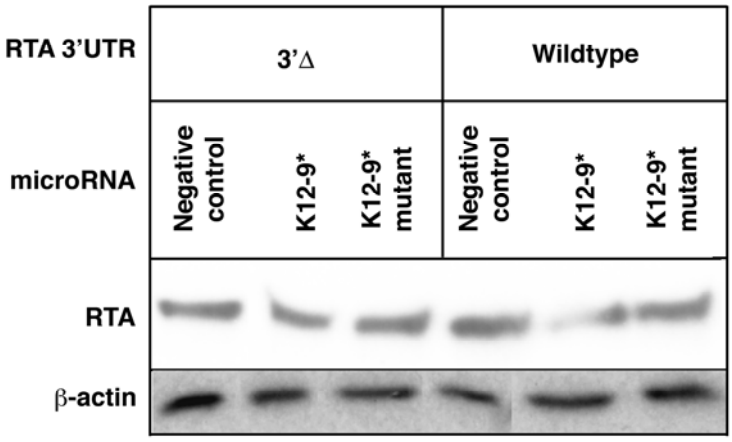

Figure S4. Deleting the predicted target site of miR-K9* from the RTA 3'UTR diminishes miR-K9*-mediated inhibition of RTA accumulation.

HEK293 cells were cotransfected with plasmid encoding full-length (Wildtype) or shortened (3'Δ**)** transcript of RTA with negative control, miR-K9* (K9*) or seed-mutated miR-K9* microRNA. 48 hours later cell lysates were probed for RTA protein and for β-actin as a loading control.

Supplemental References

Lukac, D.M., Renne, R., Kirshner, J.R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.