Supporting Information

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SI Materials and Methods

Cell Culture and Viral Infection. TREx-RTA BCBL-1 cells (1) (provided by Jae Jung, University of California, Los Angeles, CA) were maintained in RPMI medium supplemented with 20% FBS, and Hygromycin B (50 μg/mL). To induce Kaposi's Sarcoma-associated Herpesvirus (KSHV) lytic replication in TREx-RTA BCBL-1, Doxycyclin (2 μg/mL), and TPA (20 ng/mL) were added to the medium (2). Human embryonic kidney cells (HEK) 293T cells were maintained in DMEM supplemented with 10% FBS. Preparations of KSHV viral stocks (3) and de novo KSHV infections and adenoviral RTA expression vector superinfections (4) were performed as previously described (4). Adenoviral vectors expressing RTA or vGPCR were kindly provided by Britt Glaunsinger, University of California, Berkeley, CA. To determine the half life of Drosha protein, 100 μg/mL of cycloheximide was added to culture medium to stop protein biosynthesis in TREx-RTA BCBL-1 cells either during latency, 18 h, or 36 h after induction of lytic infection. The same number of cells was collected for immunoblot analysis (see details for immunoblot experiments below).

Vector Construction and Transfection. The Kaposin B (KapB) expression vectors used in this article were made by cloning the wild-type KapB 3′ UTR (accession: NC_009333.1) or the miRNA-deleted versions of the KapB 3′ UTR (without miR K12-10/12 5p and 3p sequences) into the pCR3.1- HA-KapB vector (a gift from Craig McCormick, Dalhousie University, Halifax, NS, Canada) by overlapping-PCR and confirmed by sequencing. Two different mutations were engineered for each pre-miRNA. Note that "10D-1" indicates that for premiR K12-10, a 22-nt deletion of the sequence corresponding to the guide strand: TAGTGTTGTCCCCCCGAGTGGC (see Fig. S1 for details), whereas "10D-2" indicates a 22-nt deletion of the sequence corresponding to the passenger strand: GAGGCTTGGGGCGATACCACCA; "12D-1" indicates that for premiR K12-12, a 22-nt deletion of the sequence corresponding to the guide strand: ACCAGGCCACCATTCCTCTCCG, whereas "12D-2" indicates a 22-nt deletion of the sequence corresponding to the passenger strand: TGGGGGAGGGTGCCCTGGTTGA. DD-1 is a double-deletion mutant with deleted sequence as shown for 10D-1 plus 12D-1. DD-2 is a double-deletion mutant with deleted sequence as shown for 10D-2 plus 12D-2. The luciferase reporters were made by cloning the KapB 3′ UTR into the pMSCV-Luc2CP vector (5). Drosha (pCK-Drosha-FLAG), and DGCR8 (pFLAG-DGCR8) expression vectors (6) are gifts from V. Narry Kim (Seoul National University, Seoul, Korea). RTA shRNA (pshKRTA) and control shRNA (pshCtrl) expression vectors are gifts from Ren Sun (University of California, Los Angeles, CA) (7). The target sequence for RTA is CGACAACACCCAAACGAAA, and the target sequence for Control is CAACAAGATGAAGAGCACCAA. To express miR K12-10 and miR K12-12, the full-length KapB 3′ UTR was cloned into the pcDNA3.1 vector. 293T cells were transfected with lipofectamine 2000 reagent (Invitrogen). TREx-RTA BCBL-1 cells were transfected by electroporation. 7×10^6 TREx-RTA BCBL-1 cells were collected and resuspended in a 0.4-cm Gene Pulser Cuvettete (Bio-Rad) with 500 μL of serum free RPMI medium. Next, 24 μg of DNA or 700 pmol of siRNA was added to the solution and electroporation was applied at 210V, 950 μF for DNA, and 250V, 950 μF for siRNA. Previously characterized Drosha, DGCR8 or SOX-specific siRNAs were used to knockdown Drosha, DGCR8, and SOX levels (4, 8). The electroporated cells were then transferred to a microtube, centrifuged, and incubated for 20 min at room temperature before being resuspended in 15 mL culture medium. Under these conditions, the transfection efficiency is around 80% as estimated by transfection of an EGFP expression vector (pEGFP-C1; Clontech) and quantifying using flow cytometry.

Luciferase Assay. Cells were transfected with pMSCV-Luc2CP-KapB 3′ UTR reporters. The pcDNA3.1- RLuc vector (5) was also cotransfected as a control for transfection efficiency. Cells were collected 48 h after transfection and analyzed with the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was determined using a Luminoskan Ascent luminometer (Thermo Electronic Cooperation). Results for the reporters are presented with the firefly luciferase activity normalized to *Renilla* luciferase activity.

Immunoblot Analysis. Approximately 5×10^7 Cells were lysed in RIPA buffer (0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 150 mM NaCl, and 10 mM Tris, pH7.2). Then 50 μg of the total lysate was separated in 6% to 12% SDS-polyacrylamide gels, and transferred to PVDF membranes (Millipore). Primary antibodies used in this article are rabbit polyclonal anti-RTA antibody (a gift from Don Ganem, University of California, San Francisco, CA), rabbit anti-KapB antibody (a gift from Craig McCormick, Dalhousie University, Halifax, NS, Canada), rabbit anti-Drosha antibody (Cell Signaling), rabbit anti-DGCR8 antibody (Novas), mouse anti-K8.1 antibody (Advanced Biotech), and mouse anti–β-actin antibody (Abcam). Blots were probed with a 1:1,000 dilution of primary antibody in 5% dehydrated milk in Tris Buffered Saline (TBS) and 1:5,000 dilution for the HRP-conjugated secondary antibodies (Invitrogen). Blots were washed in TBS multiple times, incubated with chemiluminescent substrate (SuperSignal West Pico or West Dura; Thermo Scientific) according to the manufacturer's protocol, and exposed to autoradiography film for visualization of bands.

Northern Blot Analysis. Small RNA Northern blot analysis was performed as previously described (9). Northern blot analysis for Drosha, KapB, and β-actin mRNAs were conducted using total RNA that was separated on a 1.2% agarose formaldehyde gel and then transferred using Whatman TurboBlotter Rapid Downward Transfer Systems. Probes (corresponding to nucleotides position 117,564–117,794 from NC_009333) were generated by PCR using subcloned KapB 3′ UTR plasmid as template and labeled with Amersham Rediprime II DNA labeling system (GE Healthcare). β-Actin, Drosha, and SOX mRNAs were labeled the same way using PCR products (as template) generated from amplification with the following primers (β-actin: TGGATGATGATATCGCCGCGCTC, and ACTTCAGGGTGAGGATGCCTCTC; Drosha: ACGACCAGACTTTGTACCCTTCC, and AAAGTGCCTTGTCCAGGAGGTGC; SOX: AGACTATCTGGTTGACACCCTGG, and CTCACTACCAATAAACTCGCCCAC). Probes for miRs K12-10 and K12-12 were labeled as previously described (10). The intensity of bands were detected by PMI imager (Bio-Rad) and analyzed by Quantity One software (Bio-Rad)

Flow Cytometry and Annexin V Dying Cells Assay. To identify dying cells, TREx-RTA BCBL-1 cells were collected 48 h after transfection. Cells were washed with PBS buffer, and resuspended in 1 mL Annexin V binding buffer (Invitrogen). Six microliters propidium iodide solution 50 μg/mL (Calbiochem) and 5 μL of Alexa Fluor 488 conjugated Annexin V (Invitrogen) were added and incubated for 15 min. Samples were immediately collected and assayed with a FACSCalibur flow cytometer (Becton Dickinson)

using CellQuest Pro Software and further analyzed using FlowJo Software. Dead cells and subcellular debris, as determined from aberrant forward/side scatter profiles, were eliminated from further analysis.

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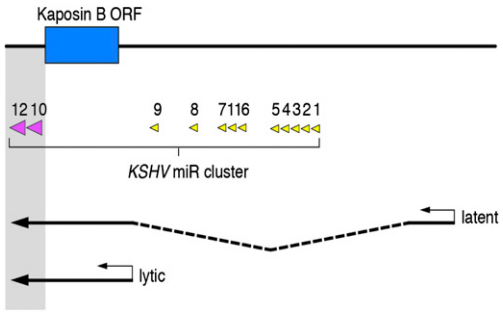

Fig. S1. Transcript map of KapB mRNA and premiRs K12-10 and miR K12-12. There are two promoters that can drive KapB mRNA transcription. One of them is lytic-specific and under the control of KSHV lytic master regulator RTA. We note our results with KapB predict other transcripts from the Kaposin locus, such as KapA or KapC, will contain the premiRs K12-10 and K12-12 and would therefore also likely be subject to the same mode of direct Drosha regulation that we observe for KapB transcripts.

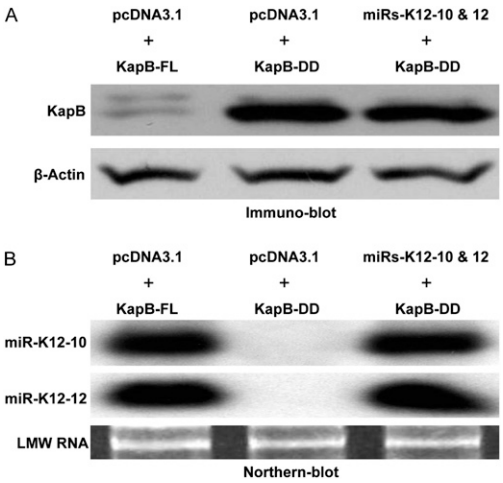

Fig. S2. miRs K12-10 and K12-12 do not mediate *trans* regulation of KapB levels. To control for any possible *trans*-mediated effects of the derivative KapB mRNA miRNAs, KapB-FL and KapB-DD expression vectors were cotransfected into HEK-293T cells with an empty vector control (pcDNA3.1) or with a vector expressing miRs K12-10 and K12-12. Two days after transfection, cells were harvested and immunoblot analysis (*A*) or Northern blot analysis (*B*) was conducted. No difference in KapB expression levels (derived from the KapB-DD construct) is observed in the presence or absence of miRs K12-10 and K12-12. For comparison, KapB protein and miRNAs derived from the intact 3′ UTR (KapB-FL) are shown in the left-most lanes.

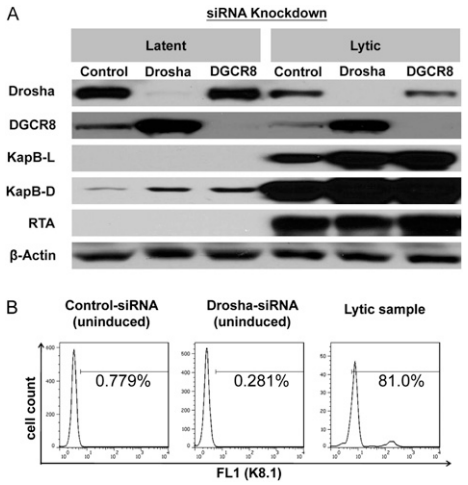

Fig. S3. Knockdown of Drosha does not induce markers of lytic replication. (*A*) TREx-RTA BCBL-1 cells were transfected with siRNAs to knock down Drosha or DGCR8 levels and immunoblot analysis shows that no induction of RTA is observed by this treatment. Chemically mediated lytic induction confirms expected increase in RTA levels. Notably, knockdown of DGCR8, a binding partner of Drosha results in increased KapB protein levels. DGCR8 levels also serve as a positive control for knockdown of Drosha, because DGCR8 mRNA is a direct substrate of Drosha (1). KapB-L indicates a light exposure, and KapB-D indicates a darker exposure of the same KapB immunoblot. (*B*) Flow ctyometric analysis shows no increase in surface levels of KSHV lytic protein K8.1 when Drosha levels are knocked down. Chemical induction of lytic replication confirms expected increase in surface levels of the K8.1 lytic marker.

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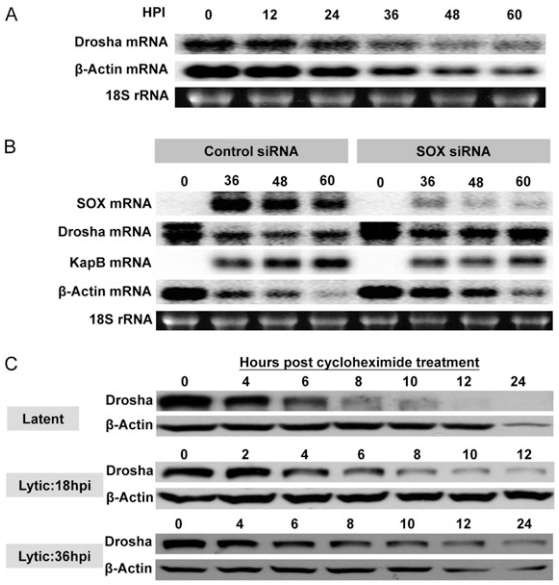

Fig. S4. Decreased mRNA levels and a short protein half-life contribute to lower Drosha levels during lytic infection. (*A*) Northern blot analysis demonstrates that both Drosha RNA and β-actin mRNA levels are lower during KSHV lytic infection. The TREx-RTA BCBL-1 cells were treated with TPA and Doxycyclin, and cells were harvested immediately and every 12 h until 60 h postinduction (HPI). (*B*) KSHV-encoded SOX contributes to decreased Drosha mRNA levels and thus increased KapB mRNA levels. TREx-RTA BCBL-1 cells were transfected with a negative control siRNA or a SOX-specific siRNA, and then lytic replication was induced. The cells were then harvested at different times postinduction and total RNA was purified for Northern blotting analysis. (*C*) Drosha protein has a relatively short half-life. To inhibit translation, cycloheximide was added to the TREx-RTA BCBL-1 cells during latency, 18 or 36 h after lytic induction, and the cells were harvested for immunoblot analysis. The loading for each time point was normalized to total cell number. The calculated approximate half-life is 4 h during either latent or lytic infection. To begin to understand the mechanism of decreased Drosha protein levels observed during lytic infection, we first tested for decreases in Drosha mRNA levels. TREx-RTA BCBL-1 cells were induced to undergo lytic replication and total RNA was harvested at multiple times post-induction. Northern blot analysis shows that Drosha mRNA levels decrease during the course of lytic replication, but ribosomal RNA levels remain constant (*A*). These results are consistent with a smaller pool of Drosha mRNA being available to be translated into protein. KSHV, like many other viruses, can undergo "host shut-off," a process in which viral replication inhibits host protein expression to foster a cellular environment more conducive to virus replication (1). SOX is a viral protein expressed during lytic replication that plays a key role in the host shut-off mediated by KSHV. SOX functions, in part, by inducing mRNA turnover of a majority of mRNA transcripts. To determine if SOX contributes to the decreased Drosha mRNA levels we observe during lytic infection, we used an siRNA approach to knock down SOX levels. As expected, transfection of the siRNA directed against SOX, but not a control siRNA, resulted in decreased levels of the SOX mRNA at multiple times post induction of lytic infection (*B*). Importantly, cells with decreased levels of SOX had reproducibly higher levels of Drosha mRNA and decreased levels of KapB mRNA (*B*). These results suggest that Drosha mRNA is susceptible to SOX-mediated turnover during KSHV lytic infection. Finally, during lytic replication, we consistently observed SOX-mediated decreases in β-actin mRNA levels comparable to Drosha mRNA levels (*A* and *B*), but did not observe robust decreases in β-actin protein levels as we did for Drosha (*A*). Therefore, we hypothesized that differences in the half-life of each respective protein could account for these observations. To test this, we treated TREx-RTA BCBL-1 cells with cycloheximide to block protein biosynthesis and measured the half-life of β-Actin and Drosha proteins (*C*). This analysis confirmed that Drosha has a relatively short half-life of ~4 h both in latent and lytic infections, but β-actin has a longer half-life of >12 h. Thus, these results argue that those proteins with a shorter half-life will generally undergo more dramatic reductions as a result of KSHV-mediated host shut-off. Combined, these finding suggest that SOX-mediated host shutoff accounts for at least part of the decreased Drosha levels observed during KSHV lytic infection.

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