Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs

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Supplementary information

Methods

Microarray analysis

Arrays scans were extracted using Agilent Feature Extraction Software and normalized using intensity dependent (Lowess) normalization. Data was then imported into Genespring GX and normalized following the default Agilent normalization chip. Each spot was divided by the control channel value (Universal Reference RNA, Stratagene, Cy3). To normalize each array, each measurement was divided by the 50.0th percentile for all measurements marked present of that sample. Present and absent calls were made by the default Agilent chip setting in Genespring GX using Feature Extraction data. To calculate the expression level relative to the negative control miRNA, the normalized ratio of test miRNA to control miRNA was generated. A ratio of 1.0 represents the same expression between the test and control miRNA. To identify downregulated genes in the presence of exogenous miRNAs, array data from transient miRNA transfections in BJAB cells was subjected to a t-test with a p-value cutoff of 0.05 (using Genespring GX t-test). A second list was generated containing genes with a 1.5-fold reduction in at least one of the biological replicate arrays. The t-test

and fold change lists were combined and separated into 5 clusters using k-means clustering in Genespring GX. The k-means cluster containing all of the genes meeting the t-test was selected (also contained a subset of fold change filtered genes). This list was the starting point for the rank sum analysis. Array data from the stable BJAB cell lines expressing miRNAs, HUVECs latently infected with KSHV, and BCBL-1 cells were ranked (based on expression ratio) if the normalized expression data was less or greater than 1.0 in the appropriate condition. Genes meeting all four expression criteria are shown in Fig. 1a. Expression data from BJAB and BCBL-1 experiments can be accessed using the GEO accession number GSE12967. HUVEC expression data can be accessed at http://puma.princeton.edu/.

Cloning

The BCLAF1 3'UTR was cloned using primers BCLAF1-R1 and BCLAF1-F1T. The BCLAF 3'UTR was cloned into a custom pMIR-Report (Ambion) vector containing a Gateway cassette using Gateway cloning (Invitrogen). The miR-K5 seed mutations in the BCLAF 3'UTR was generated with the QuikChange mutatgenesis system (Stratagene). See oligo list for sequences.

Viral replication

BCBL-1 cells were electroporated with miRNA inhibitors (Ambion) and stimulated for lytic reactivation using valproate. Cell supernatant was filtered, a spike-in DNA ("Alien" DNA) was added and total DNA was purified. Virion and spike-in DNA was measured using Syber Greener (Invitrogen). Uninduced SLK cells

infected with (rKSHV.214) were gated for GFP+ and RFP+ cells after transfection with siRNAs against BCLAF1 or miRNA inhibitors.

Oligonucleotides

PCR primers for BCLAF1 3'UTR:
BCLAF1-F1T: CACCTTACAACAGAG CAGAACTTGCACC
BCLAF-R1: GGGAAAGGCATTAAAGCCTGC

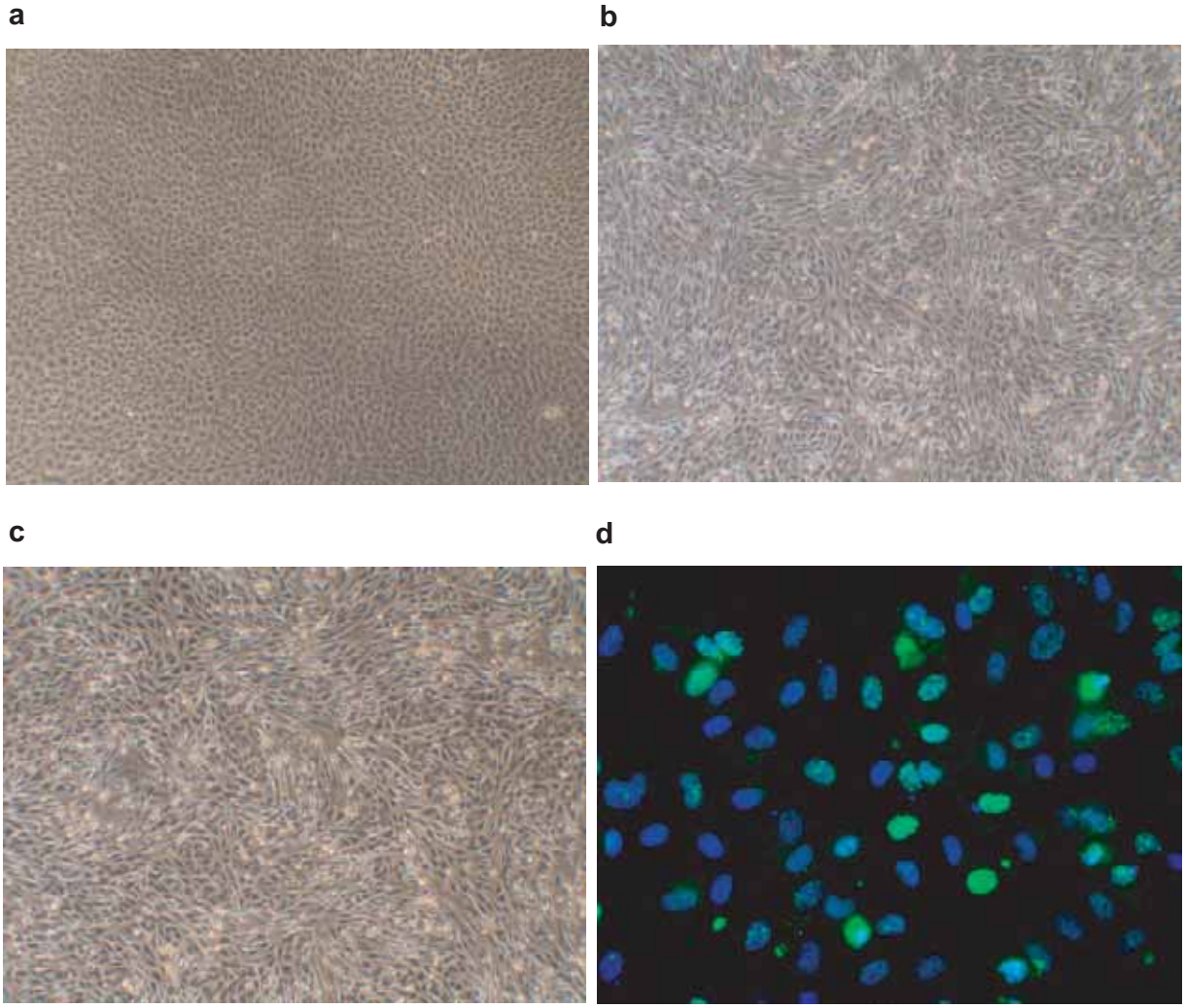
Oligos used for BCLAF1 3'UTR mutagenesis:
bclaf1-qkt5:
GCCATGACTCTTTAGGTGTGTATGTGTACCTTTCAGTACGACAGGAATTTTT
ATACTAAAAGCAAAATGTTT
bclaf1-m52t:
GCCATGACTCTTTAGGTGTGTATGTGTACCTTTCAGCATAATAGGAATTTTTA
TACTAAAAGCAAAATGTTT

Oligo used for duplicating miR-K5 target in BCLAF1 3'UTR:
btfm5x2sh-t:
CTAGTGTGTATGTGTACCTTTCAGCATCCTAGGAATTTTGTGTATGTGTACCT
TTCAGCATCCTAGGAATTTA

Primers for Syber green detection of KSHV DNA and spike-in control:
PANCHIPT: GGTGACCCAACATAGTGATTCGG
PANCHIPB: AAACCAGAAGCGGCAAGAAGGC
alien-f: AGGACCCGATCAACAACATC
alien-r: ATCGCGTTCTTGTTCAGCTT

Oligos for taqman detection of miRNAs:
mirrev: GTGCAGGGTCCGAGGT
mk5 for: GCCCTAGGATGCCTGGAACT
mk5 RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGGCA
mk5 taqman: TGGATACGACCCGGCA
mk9 RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTACGC
mk9 for: GCCCCTGGGTATACGCAGCT
mk9 taqman: TGGATACGACTTACGC

Supplementary Figure 1



Supplementary Figure 1 | Analysis of KSHV infection of HUVEC cells. Brightfield images of cells used in western blot in Figure 2a show mock infected (a) and two independent latent KSHV infections (b and c) show spindling phenotype in infected cells. d, Immunofluorescence images show DAPI in blue and LANA in green in HUVEC cells infected with KSHV. Quantitation reveals approximately 75% of cells are positive for LANA staining.

Supplementary Figure 2

a

b

miRNA	BJAB trans	BJAB stable	BCBL
5	+	+	+
9	+	+	-
10a	-	NA	-
10b	+	NA	+

c

miRNA	8mer	7mer-m8	7mer-A1	6mer
5	1	0	1	1
9	0	0	0	1
10a	0	0	0	2
10b	0	0	0	0

d

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BCLAF:      5' AUUUGAGAAAAAUAUUACCCAG 3'
                 |   |           |||||||
miR-K9:     3' AAUGCGUCGACGCAUAUGGGUC 5'

BCLAF:      5' UGCUCUUUCAUCUUAAACACUU 3'
                   | ||          ||||||
miR-K10a:   3' CGGUGAGCCCCCCUGUUGUGAU 5'

BCLAF:      5' AAACUAAGCUAGUUUAACACUU 3'
                        |   |   ||||||
miR-K10a:   3' CGGUGAGCCCCCCUGUUGUGAU 5'
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e

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BCLAF:     5' ATCTTTTGGGGTTGGCCAGCTTCC 3'
                 | :|:||||  || ||:| :|
miR-K10b:  3' CGGUGAGCCCC--CCUGUUGUGGU 5'


BCLAF:     5' CCCAUUGGUGGGGAU-GUACCU 3'
               |||:| | |||||: ::|||
miR-K10b:  3' CGGUGAGCCCCCCUGUUGUGGU 5'
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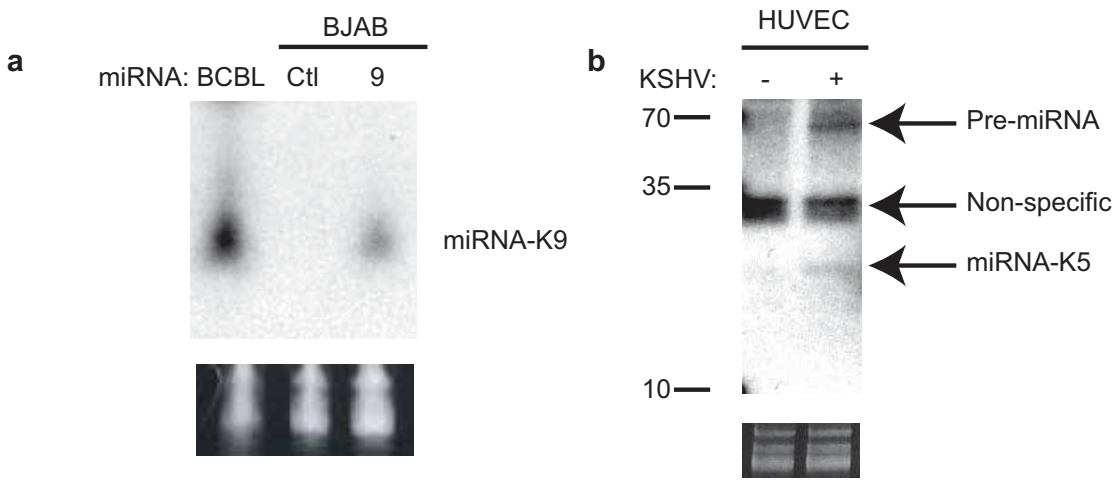
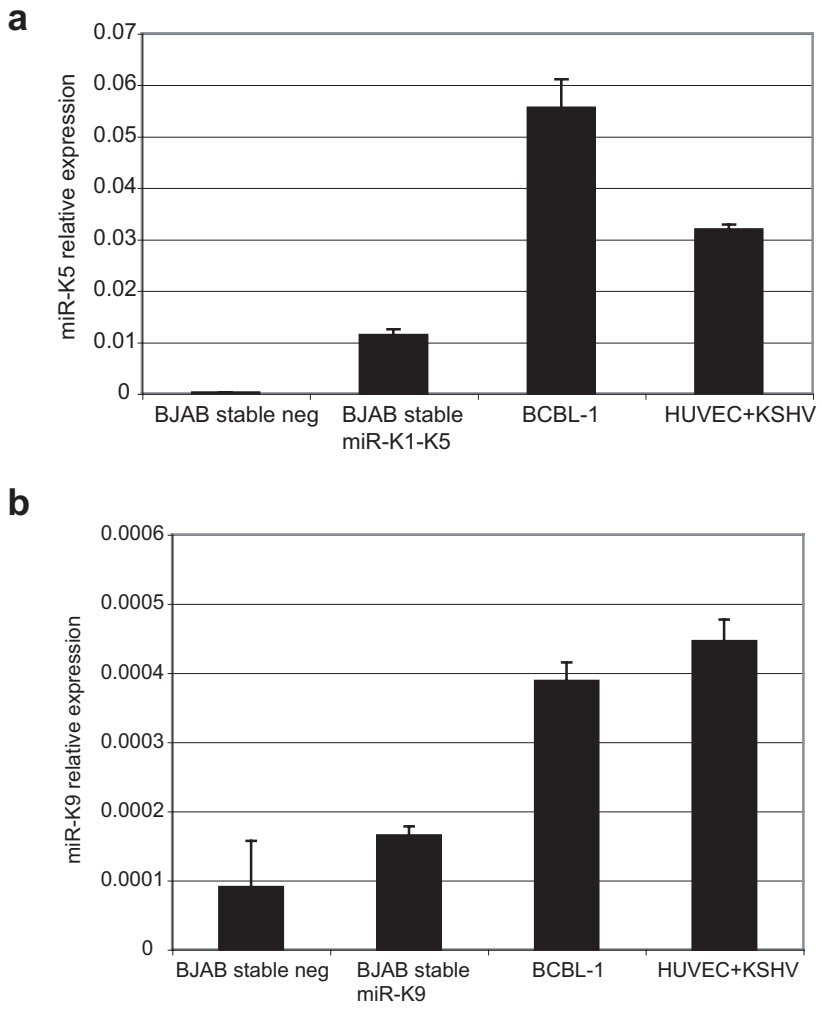
Supplementary Figure 2 | Expression and seed matching data for BCLAF1 and KSHV miRNAs. a, Heatmap showing expression data for BCLAF1 in the presence of transient (BJAB trans) expression of miRNA, stable expression (BJAB stable) of one or more miRNAs, or inhibition of individual miRNA in latently infected BCBL-1 cells (BCBL). Expression values are log base 2. b, Table of expression of BCLAF1 in conditions as in (a). "+" refers to decreased expression in presence of miRNA (BJAB columns) and "+" refers to inhibited expression in presence of miRNA inhibitor (BCBL column). c, Number of seed matched sequences in the 3' UTR of BCLAF1 for the shown miRNAs. "8mer" denotes complementary sequences to miRNA bases 2-8 and an adenosine complementary to miRNA base 1. "7mer-m8" denotes complementary sequences to miRNA bases 2-8. "7mer-A1" denotes complementary sequences to miRNA bases 2-7 and an adenosine complementary to miRNA base 1. "6mer" denotes complementary sequences to miRNA bases 2-7. d, Sequence alignments showing the seed matches described in c. e, miRanda predictions for miR-K10b and BCLAF1.

Supplementary Figure 3



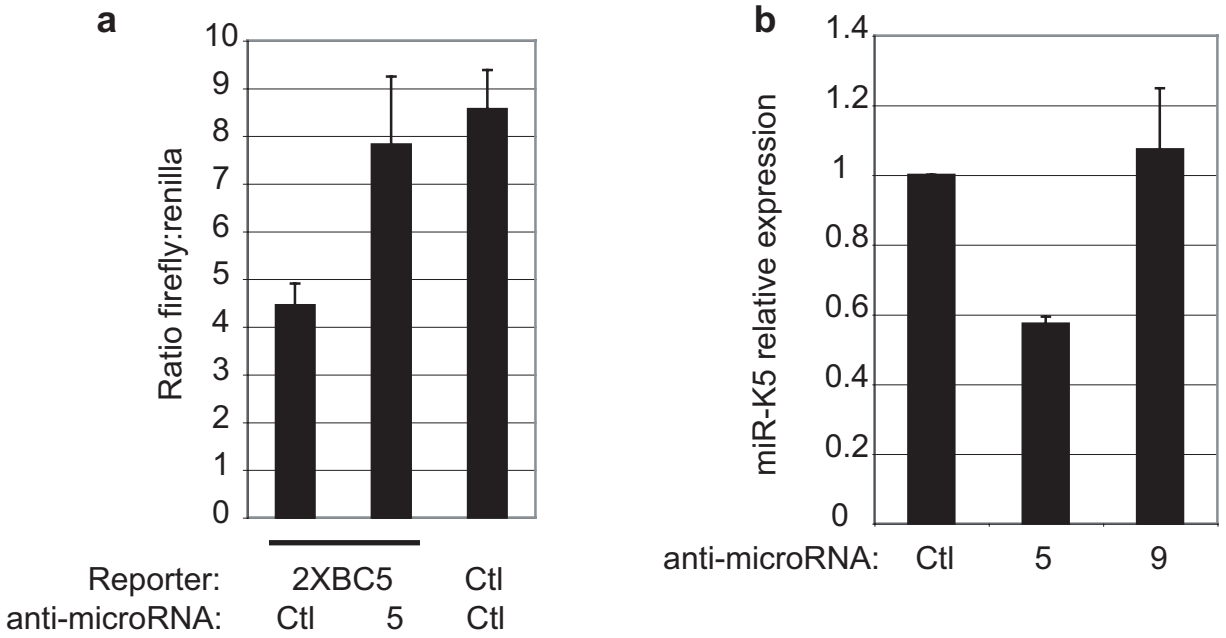
Supplementary Figure 3 | Northern analysis of miRNA expression. a, Northern blot showing expression of infected BCBL-1 cells, BJAB stable lines transduced with empty vector (Ctl) or a region containing miR-K9. b, Northern blot showing expression of miRNA-K5 in HUVEC cells after de novo infection.

Supplementary Figure 4



Supplementary Figure 4 | Taqman quantitiation of miRNA expression. Relative miRNA expression was normalized to internal control small RNA (RNU48) and plots reflect expression using 2^-ΔCt method. Expression for miR-K5 (a) and miR-K9 (b) are shown for cells described in Supplementary Figure 4. Values reflect average (n=3) with errors bars showing 1 std. dev.

Supplementary Figure 5



Supplementary Figure 5 | Analysis of inhibition by miRNA inhibitors. a, The firefly luciferase reporter containing two copies of the miR-K5 target site was transfected into SLK endothelial cells infected with rKSHV.214. In the presence of miR-K5, cells were co-transfected with a negative control miRNA inhibitor (Ctl) or the inhibitor to miR-K5 (5). The control (Ctl) reporter lacks the miR-K5 target site. b, Relative miRNA expression was normalized to internal control small RNA (RNU48) using taqman reactions. The RNA used in the reactions was from BCBL-1 cells electroporated with individual miRNA inhibitors (100 nM final concentration). Values reflect average (n=3) with errors bars showing 1 std. dev.