Supporting Information

Chakraborty et al. 10.1073/pnas.1119592109

SI Materials and Methods

Generation of EphrinA2 (EphA2) and EphB2 shRNA-Transduced Cells. The lentiviral constructs (Open Biosystems) used are (*i*) TRCN0000006403, (*ii*) TRCN0000006404, (*iii*) TRCN0000006405, (*iv*) TRCN0000006406, and (*v*) TRCN0000006407. For validation of shRNA constructs, HEK293T cells were cotransfected with EphA2 expression plasmid (target) and shRNA lentiviral vector constructs. The construction and production of lentiviral gene transfer vectors were done as previously described (1). Western blot analysis was performed to confirm the level of knockdown. For generating EphB2 shRNA human dermal microvascular endothelial (HMVEC-d) cells, a pool of lentiviral shRNA particles specific for human EphB2 was purchased from Santa Cruz. HMVEC-d cells were transduced with control or EphB2 lentivirus shRNA according to manufacturer's protocol.

Plasmids and Transfection. Briefly, 3 μg of DNA was mixed in 100 μL of 0.25 M $CaCl_2$ and then added to an equal volume of BBS buffered solution (BBS: 50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95). The DNA mixture was added to HEK293T cells grown in six-well plates, in 2 mL complete medium, and incubated overnight at 3% CO_2. The medium was changed and cells were allowed to recover 24 h at 10% CO_2.

Antibodies and Reagents. Mouse anti-integrin α3β1 (mAb 1992), αVβ5 (mAb 2019Z), and αVβ3 (mAb 1976) antibodies were from Chemicon International. Mouse anti-c-Cbl (dilution 1:1,000) and anti-phospho c-Cbl pY731 (dilution-1:500) (phospho-tyrosine) antibodies were from BD Biosciences. Rabbit anti-caveolin-1 antibody (dilution 1:1,000), Alexa 594 conjugate (dilution 1:1,000), DAPI, Alexa 488-conjugated LysoTracker (dilution-1:50), rhodamine-conjugated dextran (0.5 mg/mL), Alexa 594 (dilution 1:1,000) or 488 anti-rabbit (dilution 1:500), and anti-mouse and anti-goat secondary antibodies (1:2,500) were from Molecular Probes and Invitrogen. Goat monoclonal anti-flotillin-1 antibody (dilution 1:100) was from Abcam. Protein G-Sepharose CL-4B was from Amersham Pharmacia Biotech. Anti-goat, anti-rabbit, and anti-mouse antibodies linked to horseradish peroxidase were from KPL. Anti-CD71 mAb hybridoma cell line was from American Type Culture Collection (ATCC). Anti-CD71 mAb secreted in culture medium was purified by Protein A-Sepharose affinity chromatography. Mouse monoclonal anti-Kaposi's sarcoma-associated herpesvirus (KSHV) gpK8.1A (4A4) antibody was generated in our laboratory (2). Rab5, EphA2, and phospho-EphA2 antibodies; rabbit monoclonal N-terminal EphA2 blocking antibody; and phospho-Src, phospho-myosin IIA, myosin IIA, and phospho-PI3-K antibodies (dilution 1:1,000) were obtained from Cell Signaling Technology. Myc antibody (dilution 1:1,000) and tyrosine kinase inhibitor dasatinib were from Santa Cruz Biotechnology. Rabbit N-terminal EphB2 antibody was obtained from Epitomics. Rhotekin RBD-GST beads were from Cytoskeleton. Recombinant soluble EphA2 protein was from R&D Systems. Heparin was from Sigma.

Lipid Raft Extraction. Briefly, HMVEC-d cells were lysed in 0.5 M sodium bicarbonate solution in water (500 mM sodium carbonate, pH 11.0, 2 mM EDTA, 1 mM NaF, 1 mM orthovanadate; Sigma protease mixture inhibitor). Cell lysates transferred into precooled microfuge tubes were homogenized using a Dounce homogenizer (10 strokes) and sonicated for 10 s. A discontinuous density gradient made of five layers of optiprep with different concentrations was prepared as described previously (3). Two milliliters of gradient layer (35% optiprep) was placed at the bottom of the precooled ultracentrifuge tube. Each subsequent optiprep layer was placed over the previous one, using a Pasteur pipette. The tubes were ultracentrifuged at 150,000 × *g* for 4 h, using a Beckman SWI-55 rotor. One-milliter fractions were collected from the top of the centrifuge tube and pooled. Lipid raft-containing fractions were characterized by the presence of caveolin-1 and nonlipid rafts were confirmed by the presence of CD-71 as described before (3).

Measurement of KSHV Entry and Nuclear Delivery by Real-Time DNA PCR. For measuring KSHV entry, cells were washed with HBSS and partially bound uninternalized virus was removed with 0.25% trypsin EDTA for 5 min at 37 °C. For nuclear delivery of KSHV DNA, nuclear fractions were prepared as described previously (4). Internalized KSHV DNA was quantitated by amplification of the ORF73 gene by real-time DNA PCR (5). A paired *t* test was used between control and shRNA- or antibody-treated cells to obtain the *P* values.

Measurement of KSHV Gene Expression by Real-Time Reverse Transcription (RT-PCR). To quantitate viral gene expression, total RNA was subjected to ORF73 expression by real-time RT-PCR, using gene-specific primers and Taqman probes. The relative copy numbers of the transcripts were calculated from the standard curve, using the *Ct* values of different dilutions of in vitro-transcribed transcripts. These values were normalized to GAPDH control reactions.

Immunofluorescence Microscopy. HMVEC-d cells seeded on eight-well chamber slides (Nalge Nunc International) were used. Infected and uninfected cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100, and blocked with Image-iTFX signal enhancer (Invitrogen). The cells were reacted with primary antibodies against the specific proteins, followed by fluorescent dye-conjugated secondary antibodies. For colocalization with dextran, cells were incubated with the fluid-phase marker dextran Texas red (40 kDa, 0.5 mg/mL; Invitrogen) at 37 °C in the presence of KSHV followed by immunostaining with the appropriate antibodies. Cells were imaged with a Nikon fluorescence microscope equipped with a Metamorph digital imaging system. Signal intensity line-scan analysis was performed on the enlarged cells in regions of colocalization, using the Linescan Metamorph digital imaging system.

Colocalization of mean pixel intensities was analyzed for three different fields with a minimum of 40 cells each with the Metamorph pixel intensity calculator. The mean colocalization pixel intensities (for yellow color) measured in arbitrary units (a.u.) that are represented in a graph and a paired *t* test were used to obtain the *P* values between uninfected and KSHV-infected samples.

For confocal analysis, an Olympus 300 microscope was used for imaging, and analysis was performed using Fluoview software (Olympus). All experiments were performed at least three times.

Quantification of Macropinocytic Blebs. Briefly, HMVEC-d cells were infected with KSHV for 5 min and stained with anti-KSHV gpK8.1A monoclonal antibodies and DAPI. DIC images were acquired and the bleb-containing cells were counted visually. At least 10 different microscopic fields were analyzed and represented as a proportion of the total number of DAPI-stained cells.

1. Bottero V, et al. (2009) Kaposi sarcoma-associated herpes virus (KSHV) G protein-coupled receptor (vGPCR) activates the ORF50 lytic switch promoter: A potential positive feedback loop for sustained ORF50 gene expression. *Virology* 392:34–51.
2. Zhu L, Puri V, Chandran B (1999) Characterization of human herpesvirus-8 K8.1A/B glycoproteins by monoclonal antibodies. *Virology* 262:237–249.
3. Chakraborty S, ValiyaVeettil M, Sadagopan S, Paudel N, Chandran B (2011) c-Cbl-mediated selective virus-receptor translocations into lipid rafts regulate productive Kaposi's sarcoma-associated herpesvirus infection in endothelial cells. *J Virol* 85:12410–12430.
4. Naranatt PP, Krishnan HH, Smith MS, Chandran B (2005) Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. *J Virol* 79: 1191–1206.
5. Krishnan HH, et al. (2004) Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. *J Virol* 78:3601–3620.

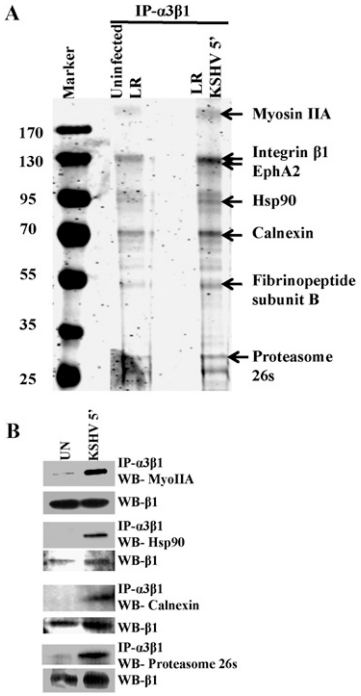

Fig. S1. Mass spectrometry analysis of protein immunoprecipitations with anti-integrin α3β1 antibody and their validation. (*A*) One hundred fifty-microgram lipid raft (LR) fractions from uninfected and KSHV-infected (5 min) HMVEC-d cell lysates were immunoprecipitated with anti-α3β1 antibody and stained with Coomassie brilliant blue, and the bands indicated by arrows were analyzed by mass spectrometry. The identified bands enriched in KSHV-infected LR samples are indicated. (*B*) Similar uninfected and infected cell lysates were immunoprecipitated with anti-α3β1 antibody and Western blotted for the indicated proteins, validating that proteins associated with integrin α3β1 indicated in *A* were enriched in infected LRs.

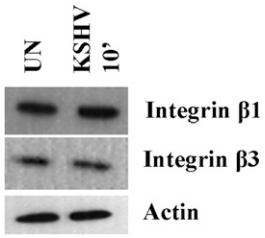

Fig. S2. Determination of integrin β1 and β3 protein levels early during KSHV infection. HMVEC-d cells were uninfected or infected with KSHV (10 DNA copies per cell) for 10 min. Twenty micrograms protein from cell lysates was subjected to Western blot analysis for the indicated proteins. Protein levels of integrin β1 and β3 did not change early during KSHV infection.

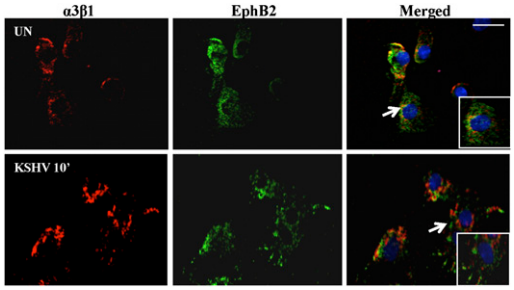

Fig. S3. Status of Ephrin B2 receptor early during KSHV infection. Serum-starved (8 h) HMVEC-d cells were either left uninfected or infected for 10 min with KSHV (10 DNA copies per cell), washed, and processed for immunofluorescence using rabbit anti-EphB2 and mouse anti-α3β1 antibodies for 1 h at 37 °C. Subsequently, cells were stained with either anti-rabbit Alexa fluor 594 or anti-mouse Alexa fluor 488. 2D deconvoluted images showing multiple cells are provided. Arrows represent the cell enlarged and shown in the *Inset*.

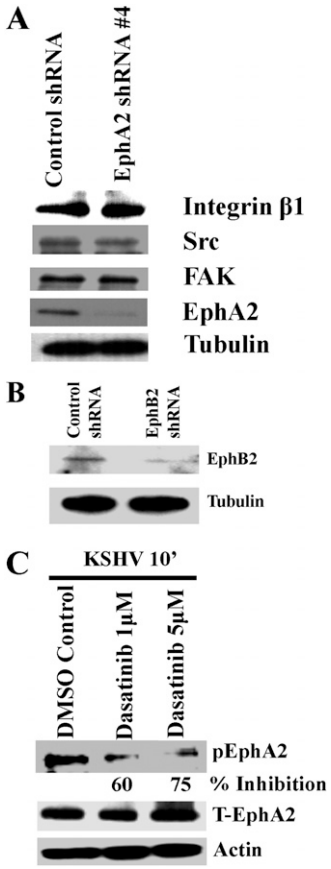

Fig. S4. Validation of EphA2 and EphB2 inhibition in HMVEC-d cells. (*A*) Control shRNA or shEphA2 RNA-4–transduced HMVEC-d cells were analyzed by Western blot for the indicated different cellular signal molecules. (*B*) Western blot showing the knockdown of EphB2 in HMVEC-d cells by EphB2 shRNA. (*C*) Serum-starved HMVEC-d cells were treated with DMSO control or with 1 and 5 μM dasatinib, respectively, for 1 h at 37 °C. After washing, cells were infected with KSHV (10 DNA copies per cell) for 10 min. Twenty micrograms of protein lysates was subjected to Western blot analysis for pEphA2. The blot was stripped and reprobed for total EphA2 and actin.

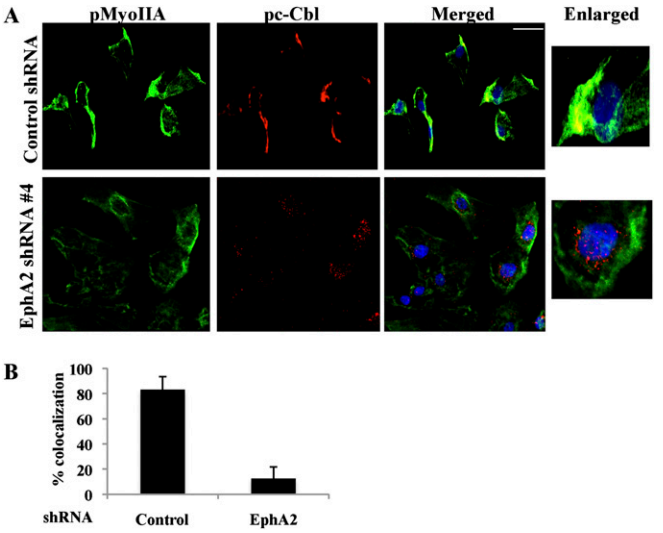

Fig. S5. Effect of EphA2 knockdown on the colocalization of activated myosin and c-Cbl. (*A*) Control shRNA or EphA2 shRNA-4–transduced HMVEC-d cells were infected with KSHV for 5 min and stained with p-myosin IIA and p-c-Cbl antibodies, followed by secondary antibodies conjugated with Alexa 488 and 594, respectively. Representative images are shown. (*B*) Quantification of cells showing myosin and c-Cbl colocalization. At least three different microscopic fields bearing at least 10 cells per field were chosen to analyze the myosin and c-Cbl colocalization efficiency. Error bar represents mean ± SD.

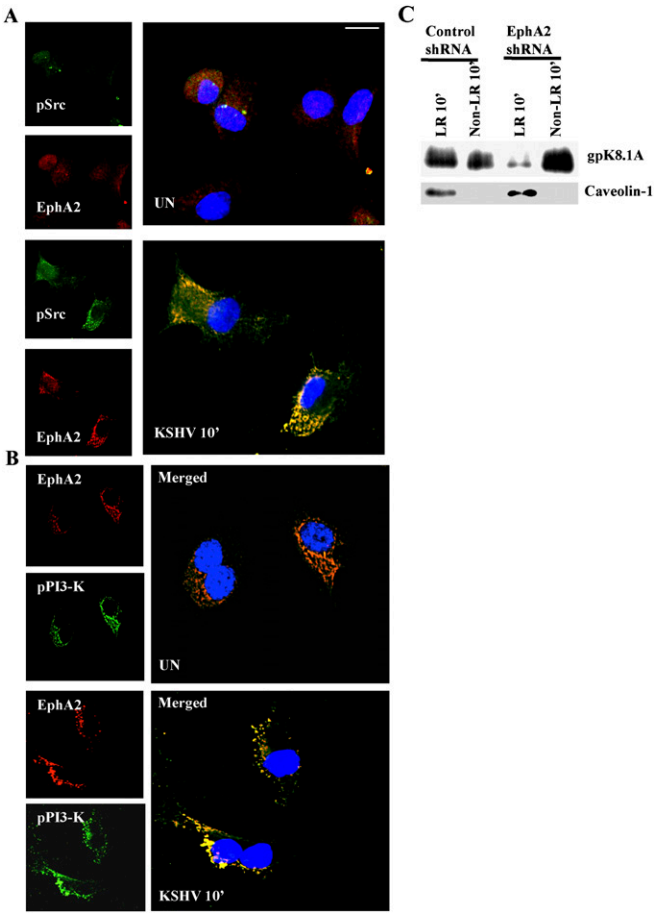

Fig. S6. Colocalization of pSrc, pPI3-K, and pEphA2 early during KSHV infection. (*A* and *B*) Serum-starved HMVEC-d cells were either mock or KSHV infected for 10 min, washed, and reacted with anti-pSrc (*A*) or pPI3-K (*B*) and EphA2 antibodies, followed by secondary antibodies conjugated with Alexa 488 or 594, and observed under an immunofluorescence microscope. (*C*) Control and EphA2 shRNA-transduced HMVEC-d cells were infected with KSHV (10 DNA copies per cell) for 10 min, and LR and non-LR fractions were extracted and tested by Western blot, using anti-KSHV envelope gpK8.1A monoclonal antibodies.

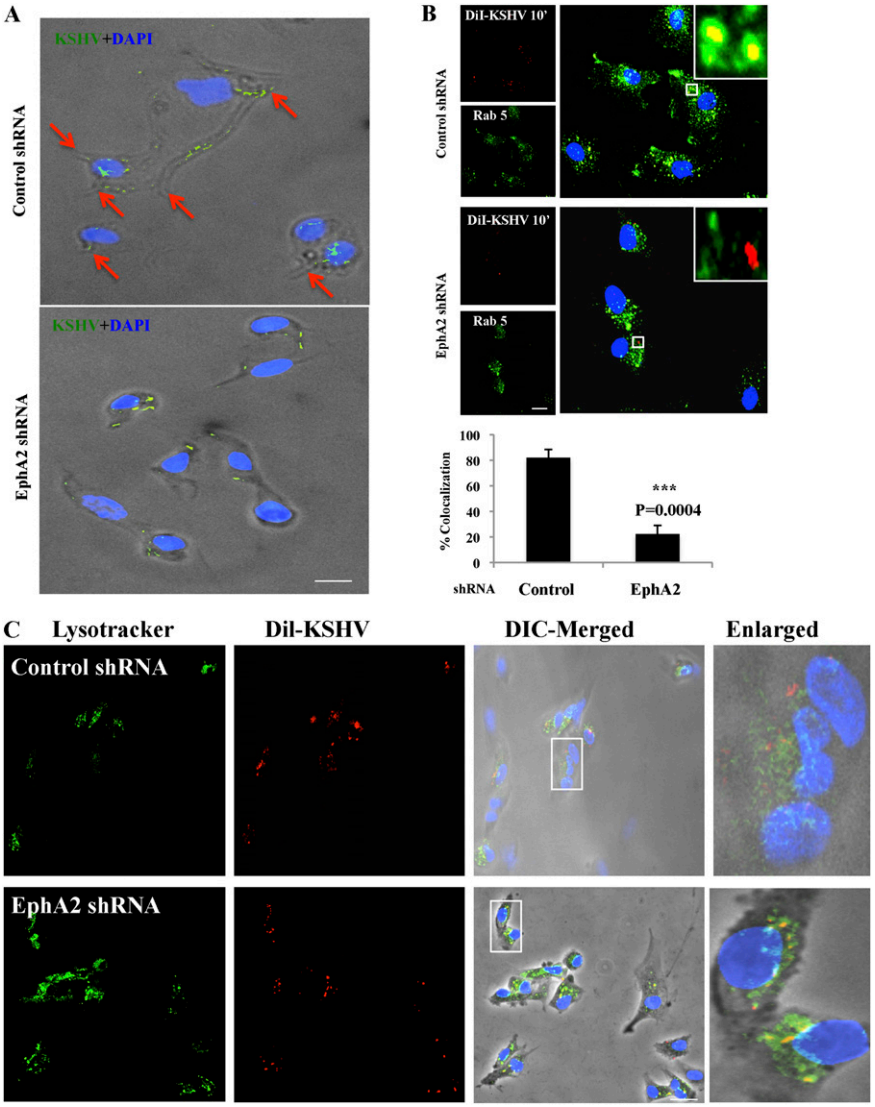

Fig. S7. Effect of EphA2 on macropinocytosis induced by KSHV. (*A*) Control shRNA or EphA2 shRNA-transduced HMVEC-d cells infected with KSHV for 5 min. Cells were stained with anti-KSHV gpK8.1A antibody and DAPI. Representative DIC images are shown and counted visually for macropinocytic blebs. Red arrows indicate the regions of KSHV-induced bleb formation in multiple cells. (*B*) Control shRNA or EphA2 shRNA-4–transduced HMVEC-d cells were infected with DiI-KSHV for 10 min. Cells were washed to remove unbound virus and processed for immunofluorescence using anti-Rab5 antibody. Representative confocal images are shown. Quantitation of KSHV accumulated in Rab5-positive vesicles was performed by counting four different fields having at least 10 cells each and error bar represents mean ± SD. *P* value was obtained by a paired Student's *t* test. (*C*) Control shRNA or EphA2 shRNA-transduced cells were incubated with media containing LysoTracker with DiI-KSHV for 30 min at 37 °C and processed for immunofluorescence. Representative 2D DIC merged images are shown. Boxed areas on the *Left* are enlarged on the *Right*.

Table S1. List of proteins identified by liquid chromatography (LC)-MS/MS analysis of KSHV-infected (5 min p.i.) lipid raft fraction lysate immunoprecipitated with anti-integrin α3β1 antibody

Functional category	Protein identified	PEAK score, %	Approximate mass, Da
Structural	Myosin IIA, nonmuscle	99	226,530
	Actin	98	43,200
	ACTB, Actin	98	40,220
Cellular adhesion	Integrin β1	73	133,240
	Fibrinopeptide B subunit	82	127,500
Tyrosine kinase	Ephrin receptor A2	66	134,500
Cellular chaperones	Heat-shock protein (Hsp90)	99	90,194
	Calnexin	99	67,568
Ubiquitin machinery	Proteasome 26s subunit	55	26,700
	Proteasome	55	115,670

HMVEC-d cells were either uninfected or infected with KSHV for 5 min and LR fractions were isolated as described in *Materials and Methods*. Uninfected and KSHV-infected LR fractions were immunoprecipitated with anti-integrin α3β1 antibodies for 2 h at 4 °C. Prominent bands enriched in KSHV-infected lysates were analyzed by LC-MS/MS. The proteins pulled down with anti-integrin α3β1 antibodies and showing enrichment in LRs during early KSHV infection are classified on the basis of their function.