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VIRUS-CELL INTERACTIONS

EphA7 Functions as Receptor on BJAB Cells for Cell-to-Cell Transmission of the Kaposi's Sarcoma-Associated Herpesvirus and for Cell-Free Infection by the Related Rhesus Monkey Rhadinovirus

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ABSTRACT Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma and is associated with two B cell malignancies, primary effusion lymphoma (PEL) and the plasmablastic variant of multicentric Castleman's disease. On several adherent cell types, EphA2 functions as a cellular receptor for the gH/gL glycoprotein complex of KSHV. KSHV gH/gL also has previously been found to interact weakly with other members of the Eph family of receptor tyrosine kinases (Ephs), and other A-type Ephs have been shown to be able to compensate for the absence of EphA2 using overexpression systems. However, whether these interactions are of functional consequence at endogenous protein levels has remained unclear so far. Here, we demonstrate for the first time that endogenously expressed EphA7 in BJAB B cells is critical for the cell-to-cell transmission of KSHV from producer iSLK cells to BJAB target cells. The BJAB lymphoblastoid cell line often serves as a model for B cell infection and expresses only low levels of all Eph family receptors other than EphA7. Endogenous EphA7 could be precipitated from the cellular lysate of BJAB cells using recombinant gH/gL, and knockout of EphA7 significantly reduced transmission of KSHV into BJAB target cells. Knockout of EphA5, the second most expressed A-type Eph in BJAB cells, had a similar, although less pronounced, effect on KSHV infection. Receptor function of EphA7 was conserved for cell-free infection by the related rhesus monkey rhadinovirus (RRV), which is relatively even more dependent on EphA7 for infection of BJAB cells.

IMPORTANCE Infection of B cells is relevant for two KSHV-associated malignancies, the plasmablastic variant of multicentric Castleman's disease and PEL. Therefore, elucidating the process of B cell infection is important for the understanding of KSHV pathogenesis. While the high-affinity receptor for the gH/gL glycoprotein complex, EphA2, has been shown to function as an entry receptor for various types of adherent cells, the gH/gL complex can also interact with other Eph receptor tyrosine kinases with lower avidity. We analyzed the Eph interactions required for infection of BJAB cells, a model for B cell infection by KSHV. We identified EphA7 as the principal Eph receptor for infection of BJAB cells by KSHV and the related rhesus monkey rhadinovirus. While two analyzed PEL cell lines exhibited high EphA2 and low EphA7 expression, a third PEL cell line, BCBL-1, showed high EphA7 and low EphA2 expression, indicating a possible relevance for KSHV pathology.

KEYWORDS B cell, EPH receptors, Kaposi's sarcoma-associated herpesvirus, receptors, virus entry

In addition to Kaposi's sarcoma, Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with a variant of multicentric Castleman's disease (MCD) and with primary effusion lymphoma (PEL) (1). Several publications demonstrate the importance of the

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cellular receptor EphA2 for KSHV entry into various adherent target cells (2–5). While the KSHV gH/gL complex exhibits the highest avidity for EphA2, it can also interact with other members of the Eph family of receptor tyrosine kinases (Ephs), similar to the gH/gL complex of the related rhesus monkey rhadinovirus (RRV) (6). However, it remains unclear to what extent these interactions may be of functional relevance for KSHV infection at endogenous protein levels. Interestingly, although B cells are likely the major KSHV reservoir during lifelong persistence, infection of established B cell lines by cell-free KSHV is extremely inefficient (7–10). While cell-free infection with KSHV, using high levels of input virus and achieving approximately 20% infected cells maximum, was reported for one B cell line (11), coculture of KSHV-producing cells with target cells leads to robust infection of various B cell lines (12). BJAB cells, an Epstein-Barr virus-negative lymphoblastoid B cell line (13), have been used as a model for B cell infection by KSHV (9, 12) and B cell biology in general (14). While BJAB cells, like other established B cell lines, are generally refractory to cell-free KSHV infection (7, 8), KSHV can establish latency in BJAB cells under continuous antibiotic selection after cell-to-cell transmission, free virus infection, or electroporation of viral DNA (12, 15, 16). Our previous work had already demonstrated that cell-to-cell transmission of KSHV into BJAB cells is susceptible to inhibition of the interaction with receptors from the Eph family (6). However, which member of the Eph family of receptor tyrosine kinases is the principal receptor for infection of these cells has remained an open question. Additionally, to further characterize the significance of RRV as an animal model virus for KSHV, we investigated the receptor requirements for BJAB infection by this closely related gamma2 herpesvirus of rhesus macaques (17).

(This article was submitted to an online preprint archive [18].)

RESULTS

Identification of endogenous EphA7 as interaction partner of the gH/gL complexes of KSHV and RRV in BJAB cells. We performed a two-step pulldown from the lysate of BJAB cells using Strep-tagged Fc fusion proteins of soluble versions of the gH proteins (3, 6, 19) of KSHV, RRV 26-95, and RRV 17577 (20) in complex with the respective gL proteins as bait to identify cellular interaction partners (Fig. 1A). In this experiment, we used two gH/gL complexes from two RRV isolates, 26-95 and 17577 (20), as representatives of the two discrete phylogenetic groups of RRV gH and gL sequences described by Shin et al. (21) to identify possible differences in receptor interactions between the isolates. Mass spectrometry analysis identified EphA7 as a prey in all three binding reactions (Fig. 1B). In pulldowns with either of the RRV gH/gL complexes, EphA7 was the only membrane protein identified in excised bands in the 100- to 130-kDa molecular weight range. In the KSHV pulldown, we additionally found several peptides derived from EphA5. A comparison of the mRNA expression profiles of the 14 human Eph family receptors in a data set (22) deposited in the Gene Expression Omnibus database revealed that BJAB cells predominantly express EphA7 (Fig. 1C). We therefore focused our analysis on this member of the Eph receptor family. To confirm our mass spectrometry results, we repeated the pulldown with a similar experimental protocol and tested the precipitate for the presence of EphA7 by Western blot analysis. Using soluble gH/gL complexes of KSHV and RRV 26-95, we pulled down a protein from BJAB lysate, but not from 293T lysate, that reacted with an antibody to EphA7, confirming the mass spectrometry data (Fig. 1D). In contrast, EphA2 was precipitated by KSHV gH/gL from 293T lysate but not from BJAB lysate, which mirrors expression of the two receptors in BJAB or 293T cells, respectively.

EphA7 as a functional receptor for infection of BJAB cells by KSHV and RRV. To test the functional relevance of the gH/gL-EphA7 interaction, we generated EPHA7 knockout (KO) cell pools by transducing BJAB cells with lentiCRISPRv2-based constructs targeting EPHA7. All four tested single guide RNAs (sgRNAs) abrogated EphA7 expression compared to two nontargeting guide RNAs or two guide RNAs targeting EPHA2, as assayed by Western blotting (Fig. 2A). EphA2, the described KSHV receptor for adherent cells, is not expressed to reliably detectable levels in BJAB cells, which is in

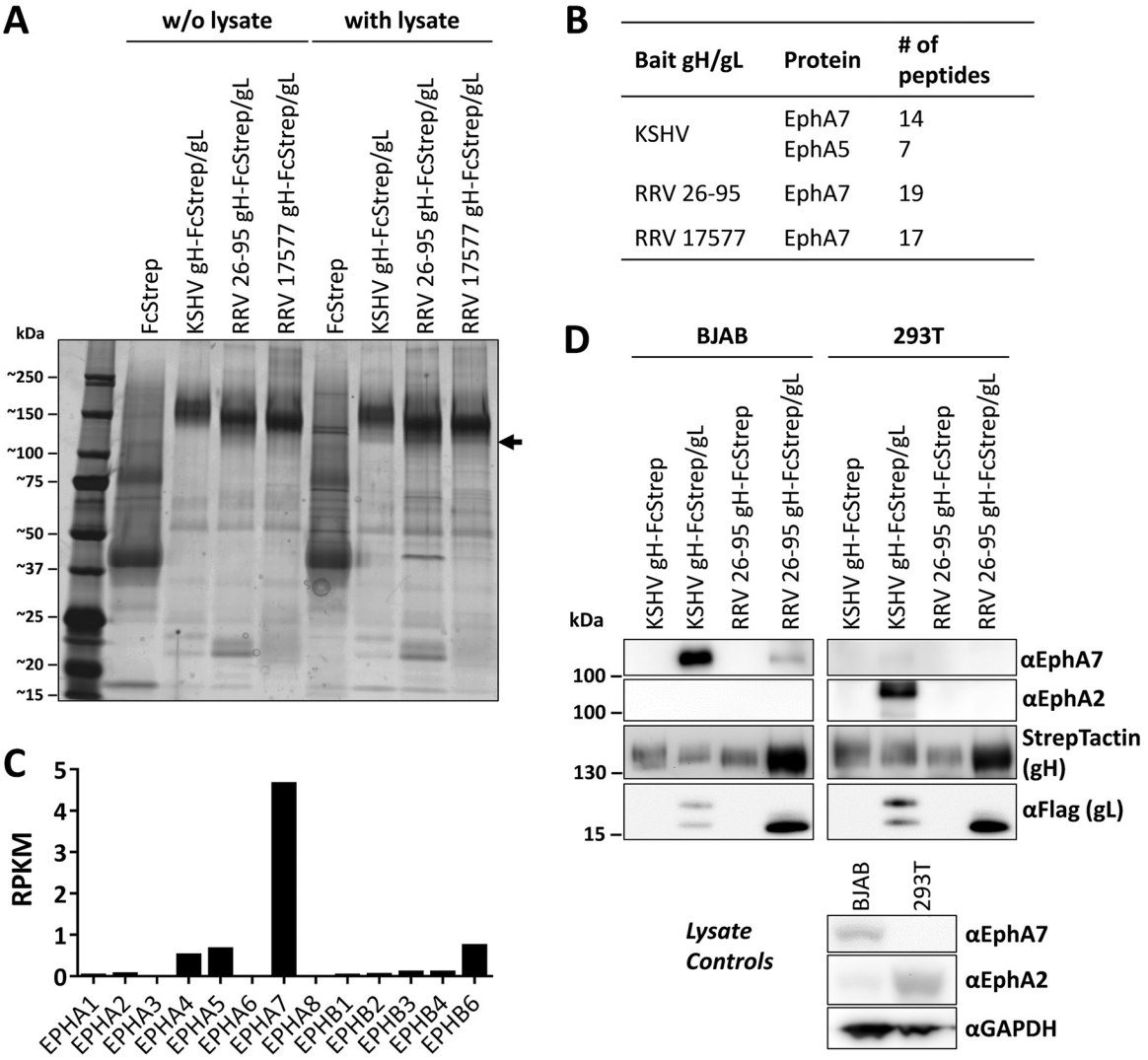

FIG 1 Identification of EphA7 as a gH/gL-interacting protein in BJAB cells. (A) Pulldown from BJAB cells with recombinant soluble gH-FcStrep/gL complexes. The precipitates were analyzed by PAGE and silver stained, and bands at the indicated molecular weights (arrow) were excised and analyzed by mass spectrometry. w/o, without. (B) Proteins and number of peptides per protein identified in the excised bands of each sample. (C) RNA sequencing, in reads per kilobase million (RPKM), of the 14 EPH receptor genes as found in GEO data set series GSE82184 (BJAB control, accession no. GSM2185732). (D) Pulldown from BJAB and 293T lysate using recombinant soluble KSHV or RRV 26-95 gH/gL complexes as bait. Input amounts were normalized to wet cell pellet. Precipitates were analyzed by Western blotting with the indicated antibodies. For KSHV gL, two bands, representing differentially glycosylated forms, were detected. The same lysates used for the pulldown were analyzed by Western blotting as expression controls.

accordance with mRNA expression profiles in databases and the absence of EphA2 in our initial pulldown experiment (Fig. 1). After confirmation of the CRISPR/Cas9 knockout efficiency (Fig. 2A), we used the knockout cell pools to analyze receptor function of EphA7 for infection of BJAB cells (Fig. 2B and C). As BJAB cells are not readily amenable to infection with cell-free KSHV, we resorted to the previously described coculture cell-to-cell transmission system (12). This method allows for efficient infection of BJAB cells by overlaying chemically induced iSLK cells containing recombinant BAC16 KSHV with BJAB cells and resolution of the two populations by flow cytometry after staining for expression of CD13 (as an iSLK cell marker) and CD20 (as a B cell marker) as described by Myoung and Ganem (12). As a control for Eph-independent infection, we included iSLK cells harboring our previously described Eph-detargeted gH-ELAAN mutant (23) in BJAB coculture experiments. To quantify the impact of EPHA7^{KO} on KSHV transmission to BJAB cells, we averaged the percentage of green fluorescent protein-positive (GFP$^+$) cells in the CD13$^-$ CD20$^+$ population individually obtained with all

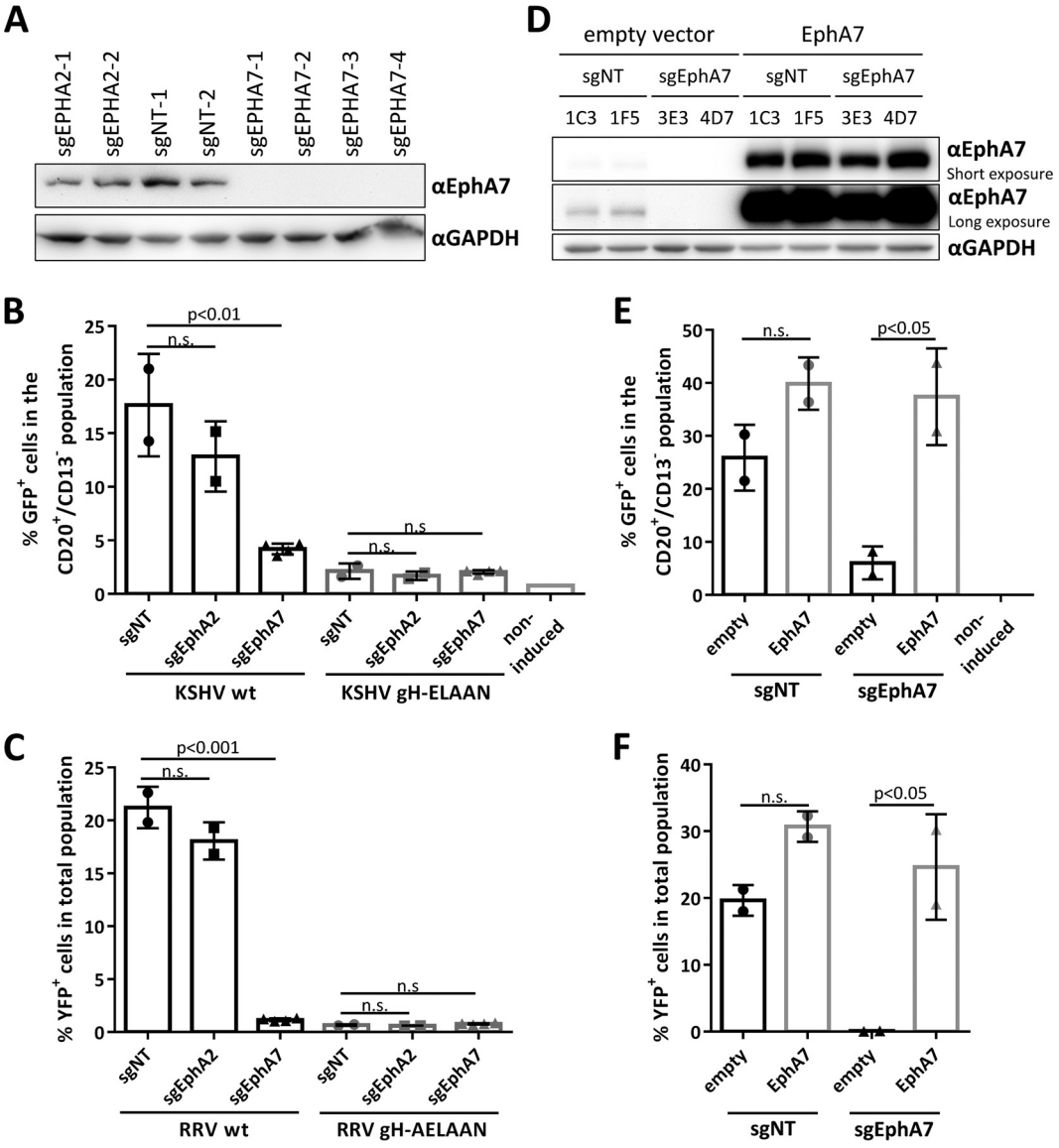

FIG 2 Knockout of EPHA7 reduces infection by KSHV and RRV. (A) Knockout of EPHA7 in BJAB cells. BJAB cells were transduced with the indicated lentiCRISPRv2-based constructs and briefly selected, and cell pools were analyzed by Western blotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) BJAB cells transduced with the lentiCRISPRv2-based constructs targeting the indicated genes were cocultured with iSLK cells harboring either BAC16 KSHV wt or BAC16 KSHV gH-ELAAN. GFP reporter gene expression in the $CD13^{-}$ $C20^{+}$ population, as an indicator of BJAB cell infection, was analyzed by flow cytometry. The means across groups of sgRNAs ($n = 3$ infections per sgRNA) targeting EPHA2 (two sgRNAs), EPHA7 (four sgRNAs), or nontargeting (sgNT; two sgRNAs) are indicated by columns. The standard deviations from the means are indicated by the error bars. The means from the individual triplicate infections for each sgRNA BJAB population within a group are given as symbols within the respective columns. Statistical significance was determined by pairwise comparison to the sgNT control group using one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. n.s., not significant. (C) The same BJAB cell pools as those in panel B were infected with cell-free RRV-YFP wt or RRV-YFP gH-AELAAN. YFP reporter gene expression, as an indicator of infection, was analyzed by flow cytometry. Statistical analysis was performed as described for panel B. (D) Reconstitution of EphA7 expression in monoclonal sgNT or sgEPHA7 cell lines. Monoclonal cells were transduced with a lentivirus carrying an EphA7 expression cassette or an empty vector control and briefly selected, and cell pools were analyzed by Western blotting. Designation of each clone indicates the respective parental sgRNA and clone number. (E) Monoclonal BJAB cell lines stably overexpressing EphA7 were cocultured with iSLK cells harboring BAC16 KSHV wt and analyzed as described for panel B. The means across two clonal cell lines per target gene ($n = 6$ infections per clonal cell line and lentiviral vector in two repeated independent experiments) are indicated by columns. The standard deviations from the means are indicated by the error bars. The means from two independent experiments for each BJAB population within a group (sgRNA, reconstitution) are given as symbols within the respective columns. The indicated individual comparisons were made using two-way ANOVA with Bonferroni correction for multiple comparisons. (F) The same reconstituted, monoclonal cells as those used for panel E were infected with cell-free RRV-YFP wt. YFP reporter gene expression, as an indicator of infection, was analyzed by flow cytometry. Statistical analysis was performed as described for panel E.

different sgRNA constructs targeting one specific gene (four sgRNAs for EPHA7 or two sgRNAs for EPHA2) and compared them to the averaged percentage of infected cells obtained with the two nontargeting control sgRNAs (Fig. 2B). Knockout of EPHA7 resulted in a 76% reduction of infection with wild-type (wt) KSHV, whereas targeting EPHA2, which is not expressed at detectable levels as assayed by Western blotting, resulted in a 27% reduction of wt KSHV infection, which was not significant. Analysis of all BJAB cell pools treated with sgRNAs targeting either EPHA2 or EPHA7 compared to cell pools treated with nontargeting sgRNAs in infection experiments with KSHV gH-ELAAN did not indicate significant changes between any of the groups. RRV 26-95, as opposed to KSHV, readily infects BJAB cells as free virus (6). Therefore, we infected the same set of BJAB knockout cell pools with cell-free wt RRV-yellow fluorescent protein (YFP) and with RRV-YFP gH-AELAAN, an Eph-detargeted RRV mutant (23), analogous to KSHV gH-ELAAN (Fig. 2C). While the results obtained with RRV essentially paralleled those with KSHV, ablating EphA7 expression resulted in an even more pronounced and significant reduction in infection for all EPHA7-targeting constructs compared to the nontargeting controls. The average level of RRV-YFP infection of all BJAB EPHA7^{KO} cell pools was reduced by 95% compared to averaged infection of nontargeting sgRNA controls. Infection levels of RRV-YFP gH-AELAAN equaled RRV wt infection with matched genome copy numbers on BJAB EPHA7^{KO} cells, with no significant differences between cell pools treated with control sgRNAs and sgRNAs targeting EPHA2 or EPHA7. To exclude the contribution of off-target effects to the observed reduction in KSHV and RRV infection, we overexpressed EphA7 by lentiviral gene transduction in monoclonal EphA7^{KO} cells and monoclonal nontargeting control cells (Fig. 2D). EphA7 reconstitution in EphA7^{KO} cells restored KSHV cell-to-cell transmission (Fig. 2E) and cell-free RRV infection (Fig. 2F) to infection levels obtained on nontargeting control cells transduced with an empty vector control when averaged over two single-cell clones per target. On nontargeting controls, EphA7 overexpression had a slightly enhancing, albeit nonsignificant, effect on KSHV and RRV infection compared to that of the empty vector control.

Impact of additional Eph receptors on the infection of BJAB cells by KSHV and RRV. As we observed slight differences in the transmission of KSHV wt and the Eph-detargeted KSHV mutant to EPHA7^{KO} BJAB cells, we wanted to address the question of whether other members of the Eph family of receptor tyrosine kinases play a role in infection of BJAB cells by KSHV. To test this hypothesis, we performed ligand-dependent blocking experiments of KSHV infection, using recombinant ephrinA4, a soluble Fc-fusion protein of a natural Eph ligand, which targets A-type Ephs and blocks infection of adherent cells by cell-free KSHV (3). We treated a reduced set of knockout cell pools with either phosphate-buffered saline (PBS) (control) or ephrinA4 in PBS during the coculture experiment (Fig. 3A). While ephrinA4 treatment significantly inhibited infection of both EPHA2^{KO} BJAB cells and nontargeting control BJAB cells compared to PBS by over 85%, infection of EPHA7^{KO} BJAB cells was reduced by only 20% in the presence of ephrinA4, a level which did not reach significance. It should be noted that knockout of EPHA2 also led to a small (approximately 35%) but significant reduction in infection in this experiment using a reduced set of sgRNA constructs.

As we had detected peptides of EphA5 in the initial pulldown using the KSHV gH/gL complex (Fig. 1A and B), we proceeded to analyze the role of EphA5 in KSHV transmission to BJAB cells. Pulldown experiments with KSHV and RRV 26-95 gH/gL complexes followed by Western blot analysis of precipitates confirmed the binding of KSHV gH/gL but not RRV 26-95 gH/gL to endogenous EphA5 in BJAB lysate. Similar to EphA7, EphA5 was not detected in 293T lysate precipitated with either KSHV gH/gL or RRV gH/gL. EphA2 binding to KSHV gH/gL in 293T lysate was used as a control (Fig. 3B). Stable EPHA5^{KO} cell pools were generated using the lentiviral CRISPR/Cas9 system, and knockout was verified by Western blotting (Fig. 3C). Analogous to experiments with EphA7^{KO} cells, cell pools with confirmed EPHA5 knockout (sgEPHA5-1 and sgEPHA5-2) were tested for cell-to-cell transmission of KSHV in coculture experiments (Fig. 3D) as well as for cell-free infection with RRV (Fig. 3E). Compared to nontargeting controls,

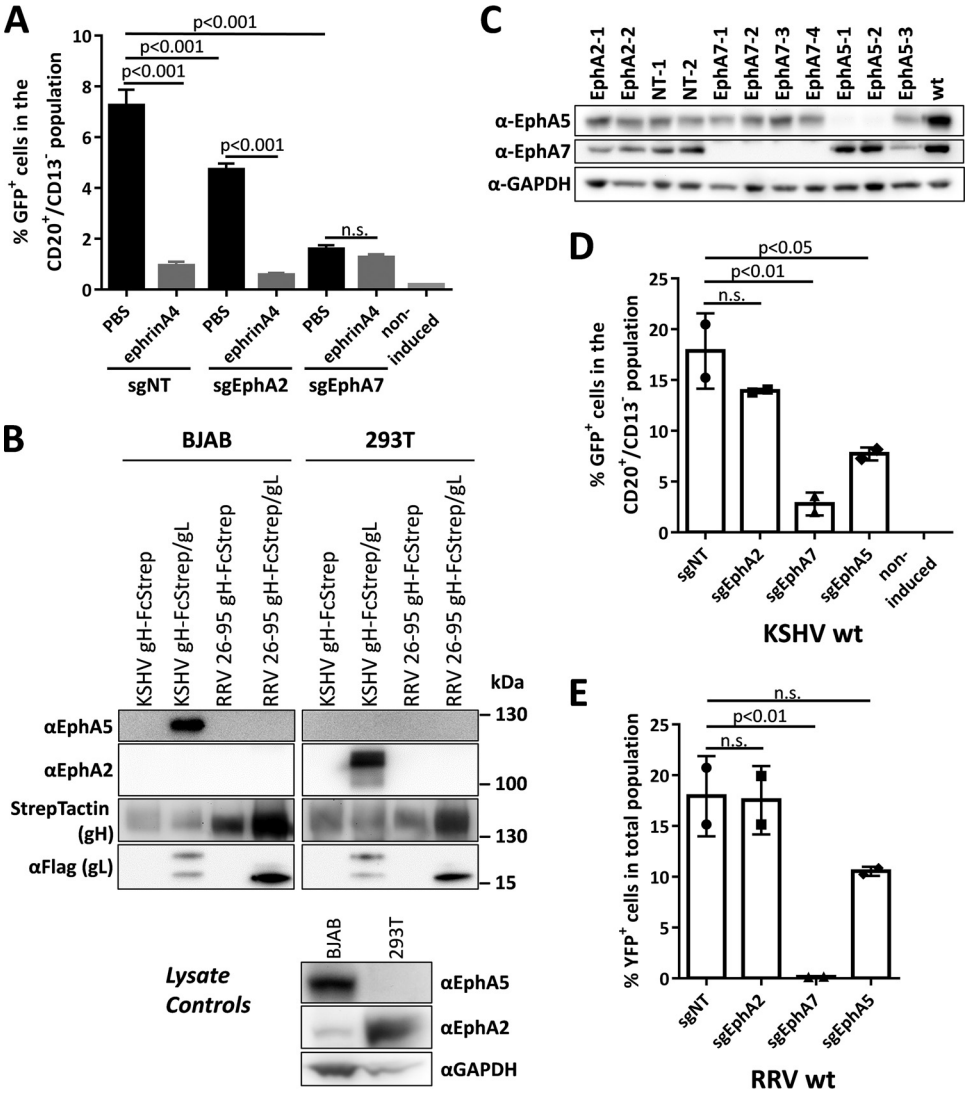

FIG 3 EphA7 is the predominant Eph receptor for infection of BJAB cells by KSHV. (A) BJAB knockout cell pools (sgNT-1, sgEPHA2-1, and sgEPHA7-1) were cocultured with iSLK cells harboring BAC16 KSHV wt. ephrinA4-Fc at a final concentration of 2 μg/ml in PBS, or PBS alone as control, was added to the coculture. The indicated individual comparisons were made using one-way ANOVA with Bonferroni correction for multiple comparisons. (B) Pulldown from BJAB and 293T lysate using recombinant soluble KSHV or RRV 26-95 gH/gL complexes as bait. Input amounts were normalized to wet cell pellet. Precipitates were analyzed by Western blotting with the indicated antibodies. For KSHV gL, two bands, representing differentially glycosylated forms, were detected. (C) Knockout of EPHA7 and EPHA5 in BJAB cells. BJAB cells were transduced with the indicated lentiCRISPRv2-based constructs and briefly selected, and cell pools were analyzed by Western blotting. (D) BJAB cells transduced with the lentiCRISPRv2-based constructs targeting the indicated genes were cocultured with iSLK cells harboring BAC16 KSHV wt. GFP reporter gene expression in the CD13$^-$ CD20$^+$ population, as an indicator of BJAB cell infection, was analyzed by flow cytometry. The means across groups of sgRNAs ($n = 3$ infections per sgRNA) targeting EPHA2 (two sgRNAs), EPHA7 (two sgRNAs, sgEPHA7-3 and sgEPHA7-4), and EPHA5 (two sgRNAs, sgEPHA5-1 and sgEPHA5-2), or nontargeting sgRNAs (sgNT; two sgRNAs), are indicated by columns. The standard deviations from the means are indicated by the error bars. The means from the individual triplicate infections for each sgRNA BJAB population within a group are given as symbols within the respective columns. Statistical significance was determined by pairwise comparison to the sgNT control group using one-way ANOVA with Bonferroni correction for multiple comparisons. (E) The same BJAB cell pools as those use for panel D were infected with cell-free RRV-YFP wt. YFP reporter gene expression, as an indicator of infection, was analyzed by flow cytometry. Statistical analysis was performed as described for panel D.

treatment of BJAB cells with sgRNAs directed against EPHA2, EPHA7, and EPHA5 resulted in reduction of KSHV transmission into BJAB cells of approximately 23%, 84%, and 57%, respectively. In contrast, treatment with sgRNAs directed against EPHA2 had no effect on cell-free RRV infection of BJAB cells, while knockout of EPHA7 and EPHA5 reduced infection with RRV by approximately 99% and 40%, respectively.

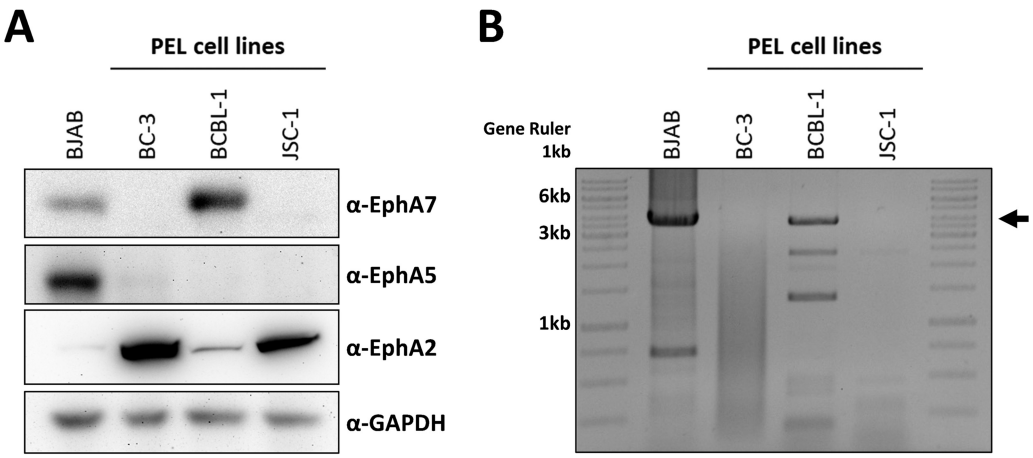

FIG 4 Expression of select Ephs in PEL cells. (A) Lysates prepared from BJAB cells and three PEL cell lines were analyzed by Western blotting with the indicated antibodies. (B) Nonquantitative RT-PCR of EphA7 cDNA using primers in the 5′ and 3′ UTRs common to transcript variant 1 (NM_004440), transcript variant 2 (NM_001288629), predicted transcript variant X1 (XM_005248669), predicted transcript variant X2 (XM_017010365), and predicted transcript variant X4 (XR_001743218.2) with an expected product size of approximately 5.2 kb for predicted transcript variant X4 and 3.9 kb for all other transcript variants (black arrow).

EphA7 expression in PEL cell lines. To address a possible role of EphA7 in KSHV-associated B cell malignancies, we tested three PEL cell lines, BC-3, BCBL-1, and JSC-1, for expression of different Ephs by Western blotting (Fig. 4A). All three PEL cell lines were positive for EphA2, the high-affinity KSHV receptor, and none was positive for EphA5. BCBL-1, which expressed only small amounts of EphA2, were highly positive for EphA7. Sequences of the 3.9-kb fragment obtained after reverse transcription-PCR (RT-PCR) (Fig. 4B, black arrow) were compatible with expression of EphA7 transcript variant 1 (GenBank accession no. NM_004440) and transcript variant 2 (NM_001288629) in BJAB cells and EphA7 transcript variant 1 and the predicted transcript variant X1 (XM_005248669) in BCBL-1 cells, respectively. Sequences obtained from fragments of lower molecular weight did not match deposited EphA7 sequences.

DISCUSSION

The use of EphA7 as a cellular receptor for the infection of BJAB cells by KSHV and RRV is consistent with previous studies that the gH/gL complexes of both viruses can interact with various members of the Eph family of receptor tyrosine kinases. While KSHV gH/gL binds EphA2 with highest affinity, we previously identified trace amounts of peptides of additional Ephs after pulldown with KSHV gH/gL in 293T lysate and demonstrated interaction of these Ephs, as well as EphA7, with KSHV gH/gL in overexpression experiments (6). Similarly, RRV interacts with both A-type and B-type Ephs with various levels of affinity, and the gH/gL complexes of both RRV subtypes precipitated EphA7 from 293T lysate in a previous study (6). While we readily precipitated endogenous EphA7 from BJAB lysate using the gH/gL complexes of either KSHV or RRV 26-95, we were not able to precipitate endogenous EphA7 from 293T lysate under similar conditions in this study. The reason for this lack of detectable EphA7-gH/gL interaction in 293T cells could lie in differences in relative expression levels of different Ephs (Fig. 1), an ability of EphA2 to outcompete for binding to the KSHV gH/gL complex, or the limits of antibody-based detection of EphA7 by Western blotting compared to mass spectrometry-based detection in past studies.

Recent publications suggest that other Ephs, such as EphA4 and EphA5, can substitute for EphA2 as receptors for KSHV in overexpression experiments (2, 24). However, as single knockout was not analyzed in one study (24) or even led to an increase rather than a reduction in KSHV infection in SLK cells both in the presence and absence of EphA2 in the case of EphA4 in a second study (2), a definitive conclusion

regarding the significance of endogenously expressed Eph receptors besides EphA2 for KSHV infection is hard to draw. So far, reduced KSHV infection upon single-gene knockout or knockdown has only been conclusively shown for EPHA2 in several publications (2–5). We now show that knockout of EPHA7 in BJAB cells resulted in a significant reduction in cell-to-cell transmission of KSHV and cell-free infection by RRV. Likewise, endogenously expressed EphA5 interacted with recombinant KSHV gH/gL (Fig. 1A and B and 3B), and knockdown of EphA5 resulted in similar but less pronounced reduction in KSHV transmission. However, we also observed a reduction of RRV infection by approximately 40% without detectable interaction of RRV gH/gL with EphA5 either by mass spectrometry or Western blot analysis. Furthermore, the slight reduction of KSHV transmission after transduction of sgRNAs directed against EPHA2, which reached significance in one set of infections, (Fig. 3A) hints at some residual function of EphA2 at expression levels below the limit of detection achievable in our Western blot analysis.

As the Eph receptor family forms a complex signaling network, in which interaction, (hetero-)oligomerization (25), and modulation between different members have been reported (26), the exact mechanism behind modulation of KSHV and RRV infection may be more complex than the sum of individual contributions by different members of the Eph family. In line with a mechanism that is more complex than a simple additive effect, we did not observe a statistically significant reduction of KSHV infection by treating the iSLK BAC16 KSHV wt/BJAB EPHA7^{KO} coculture with ephrinA4, a natural ligand which interacts with all A-type Ephs and therefore should be able to inhibit the gH/gL interaction with EphA5. Further, while residual KSHV wt infection of EPHA7^{KO} cells was not completely reduced to the levels achieved with the Eph-detargeted KSHV gH-ELAAN mutant on control cells, the difference was marginal (approximately 4% versus approximately 2% infection), and we found RRV infection was essentially abrogated after EphA7 knockout alone.

While EphA7 is reportedly not expressed in mature B cells (27), we found high EphA7 expression in one of three PEL cell lines that we tested. While BC-3 and JSC-1 were strongly positive for EphA2, BCBL-1 showed an expression pattern similar to that seen for BJAB cells with high EphA7 and low EphA2 levels. Thus, KSHV may utilize EphA2 and EphA7 for primary infection of cells that ultimately give rise to PEL. Alternatively, these receptors might be upregulated in infected cells. Of note, many PEL cell isolates harbor oligoclonal KSHV genomes, potentially as a result of superinfection, which was demonstrated to be possible by cell-to-cell transmission (12, 28) and could be promoted by EphA2 or EphA7.

KSHV transmission to BJAB cells and other lymphoblastoid cell lines as well as primary B cells is low even in cell-to-cell systems (12) compared to infection of adherent cells by free KSHV, which has been attributed to a heparan sulfate-dependent attachment defect of KSHV on BJAB (9). Additionally, a role for glycoprotein K8.1A, independent of its heparan sulfate binding activity, has been described for the infection of B cells by KSHV (29). Although infection of BJAB cells by cell-to-cell transmission was not analyzed in these studies, the role of glycoprotein K8.1A in the infection of B cells but not in the infection of other cell types (29, 30) and the described defect at the attachment step (9) hint at possible mechanistic differences specific for B cell infection.

An open question is whether the function of EphA7 in cell-to-cell transmission is somewhat different from the function of, e.g., EphA2 in the infection of epithelial or endothelial cells, and whether the specific nature of cell-to-cell transmission allows for more efficient use of receptors with comparatively lower affinity for gH/gL. This should be addressed in separate studies using loss-of-function methodology. The ability of KSHV to use different Ephs dependent on relative expression levels may parallel the interaction of ephrins with their receptors (31) and receptor usage by RRV (6). Whether there is a direct correlation between affinity and receptor function or whether the mechanism is more complicated will be a subject for future studies. Similarly, the question of whether Eph-associated signaling as described for cell-free KSHV infection

of adherent cells (reviewed in reference 32) also plays a role in KSHV cell-to-cell transmission warrants further analysis.

MATERIALS AND METHODS

Cells and viruses. BJAB cells were obtained from the Leibniz-Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). iSLK cells were a kind gift from Jinjong Myoung (33), 293T cells from Stefan Pöhlmann, the PEL cell line BC-3 (34) from Frank Neipel, PEL cell lines JSC-1 (35) and BCBL-1 (36) from Armin Ensser, and primary rhesus monkey fibroblasts from Rüdiger Behr. BJAB and BCBL-1 cells were propagated in RPMI 1640 medium (Thermo Fisher Scientific) containing L-glutamine supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 50 μg/ml gentamicin (PAN Biotech). JSC-1 and BC-3 cells were maintained in RPMI 1640 containing L-glutamine supplemented with 20% FBS, 50 μg/ml gentamicin, 1 mM sodium pyruvate (PAN Biotech), and 0.05 mM beta-mercaptoethanol (Carl Roth). 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing high glucose, GlutaMAX, 25 mM HEPES (Thermo Fisher Scientific) supplemented with 10% FBS and 50 μg/ml gentamicin. iSLK cells were maintained in DMEM supplemented with 10% FBS, 50 μg/ml gentamicin, 2.5 μg/ml puromycin (InvivoGen), and 250 μg/ml G418 (Carl Roth). RRV-YFP, RRV-YFP gH-AELAAN, and iSLK cells harboring BAC16 KSHV wt or BAC16 KSHV gH-ELAAN were produced as described previously (23).

Coculture and free virus infections. iSLK-BAC16 KSHV wt or iSLK-BAC16 KSHV gH-ELAAN cells were seeded in 48-well plates at 100,000 cells per well. After 5 h, lytic replication was induced with 1 μg/ml doxycycline (Sigma) and 2.5 mM sodium butyrate (Carl Roth) overnight in DMEM containing high glucose, GlutaMAX, 25 mM HEPES, 50 μg/ml gentamicin, and 10% FBS. After 1 day, the induction medium was discarded and the respective BJAB cell pools were added at 60,000 cells per well in 600 μl fresh RPMI medium with 10% FBS and 50 μg/ml gentamicin. After 4 days, cells were harvested for fluorescence-activated cell sorting (FACS) analysis as described below. For blocking experiments, recombinant ephrinA4-Fc protein (R&D Systems) was added at the start of the coculture to a final concentration of 2 μg/ml. For cell-free RRV infection, BJAB cell pools were seeded at 60,000 cells per well in 48-well plates and infected on the same day with RRV preparations normalized to genome copy numbers as described before (23). One day after infection cells were harvested for FACS analysis.

Flow cytometry analysis. After 4 days of coculture, cells were harvested by pipetting, fixed with 2% formaldehyde (Carl Roth) in PBS for 15 min, and washed in PBS. After blocking in 5% FBS in PBS for 30 min, the cells were stained with anti-CD13 (clone WM15; phycoerythrin coupled; BioLegend) and anti-CD20 (clone 2H7; Alexa Fluor 647 coupled; BioLegend) antibodies at a 1:50 dilution in 5% FBS in PBS for 30 to 45 min. After 2 washes in PBS, the cells were resuspended in 2% formaldehyde in PBS. RRV-infected BJAB cell pools were harvested by pipetting, washed in PBS, and fixed in 2% formaldehyde in PBS. The samples were analyzed on an LSRII flow cytometer (BD Biosciences). Flow cytometry data were further analyzed using Flowing software (version 2.5); for details, see Fig. S1 in the supplemental material. KSHV cell-to-cell infection of BJAB cells was assayed by constitutive GFP reporter gene expression in the $CD13^-$ $CD20^+$ population. Cell-free RRV infection was measured by constitutive YFP reporter gene expression. Statistical analysis was carried out using GraphPad Prism, version 6, for Windows (GraphPad Software, La Jolla, California, USA).

Generation of knockout cell pools. Knockout cell pools were generated using the lentiCRISPRv2 system (37) as described previously (38), with the exception that transfection was carried out using polyethylenimine (PEI) MAX (Polysciences) (39). In short, BJAB cells were transduced with lentiviruses harboring the indicated sgRNAs. After 48 h, the selection antibiotic puromycin (Invivogen) was added to a final concentration of 10 μg/ml. After initial selection, the puromycin concentration was reduced to 1 μg/ml. The following sgRNAs were inserted into plentiCRISPRv2 (a kind gift from Feng Zhang [Addgene plasmid number 52961]): 2 nontargeting controls, sgNT-1 (plasmid Ax127; ATCGTTTCCGCTTAACGGCG) and sgNT-2 (Ax128; TTCGCACGATTGCACCTTGG); 4 sgRNAs directed against EPHA7, namely, sgEPHA7-1 (Ax279; GGAGAATGGTTAGTGCCCAT), sgEPHA7-2 (Ax280; GACATGTGTCAGCAGTGCAG), sgEPHA7-3 (Ax281; GGATTTCCTCTCCACCCAAT), and sgEPHA7-4 (Ax282; GATTTCCTCTCCACCCAATG); 2 sgRNAs directed against EPHA2, namely, sgEPHA2-1 (Ax122; CTACAATGTGCGCCGCACCG) and sgEPHA2-2 (Ax123; GGACTTTGCTGCAGCTGGAG); and 3 sgRNAs directed against EPHA5, namely, sgEPHA5-1 (Ax299; GGATTCACGCACTGTCATGG), sgEPHA5-2 (Ax300; GATTGCTTTTCCAAAAAATG), and sgEPHA5-3 (Ax301; GGATTGCTTTTCCAAAAAAT). sgRNA sequences for all sgRNAs directed against EphA7, EphA5, and EPHA2-2 were determined using E-CRISP (40). The lentiCRISPRv2-sgEPHA2-1 plasmid was purchased from GenScript. Nontargeting sgRNA sequences were taken from the GeCKO (version 2) library (37).

EphA7 reconstitution experiments. For the generation of monoclonal BJAB KO cells, cells were seeded at one cell per well in 96-well plates, expanded in RPMI 1640 containing L-glutamine, 10% FBS, 50 μg/ml gentamicin, 1 μg/ml puromycin, and tested for EphA7 knockout by Western blotting. Two monoclonal cell lines per target (nontargeting [sgNT-1] and EphA7 [sgEPHA7-3 and sgEPHA7-4]) were transduced with a lentivirus carrying an EphA7-Strep expression cassette (transcript variant 1 [NM_004440]) (pLenti-CMV-BLAST-EphA7-Strep) or an empty vector control. Lentiviral particles were generated as described previously (38), with the exception that transfection was carried out using PEI. Two days postransduction the selection antibiotic blasticidin (Invivogen) was added to a final concentration of 10 μg/ml. After initial selection the blasticidin concentration was reduced to 1 μg/ml. pLenti-CMV-BLAST-EphA7-Strep was based on pLenti CMV BLAST (Addgene plasmid number 17486; a gift from Eric Campeau and Paul Kaufman), which was previously modified to include the twin-Strep-tag (41) coding sequence following the XbaI recognition site (AGCGCTTGGAGCCATCCACAGTTCGAAAAG GGAGGTGGAAGCGGTGGAGGTAGTGGTGGAAGTGCATGGAGCCATCCTCAGTTTGAAAAGTAACTAGAGGG

CCCA). Mutation of the sgRNA binding sites in the EPHA7 coding sequence (described before [6]) and subcloning into the lentiviral vector were performed using a high-fidelity polymerase (Fusion S7; Biozym) with the following primers: sgRNA mutation_for (ACCGCCGAACGGTTGGGAAGAAATTAGTGGTTTG), sgRNA mutation_reverse (CTTCCCAACCGTTCGGCGGTGAGGAAATCCACTC), EphA7_forward (AAAAAAGCAGGCTC CACCATGGTTTTTCAAACTCGGTACC), EphA7_reverse (TGTGGATGGCTCCAAGCGCTCACTTGAATGCCAGTTC CATG), vector_forward (TCTAGAAGCGCTTGGAGCCATCC), and vector_reverse (CATGGTGGAGCCTGCTTTT TTGTAC). Three-fragment assembly was carried out using the Gibson Assembly master mix (New England Biolabs).

Pulldown experiments and Western blotting. Two-step pulldown with specific elution and reprecipitation using gH-FcStrep/gL complexes (3, 6, 19) for identification by mass spectrometry was performed as described previously (6). One ml of wet BJAB cell pellet was lysed with 5 ml of lysis buffer per sample for 30 min on ice as the starting material for the pulldowns (in total, 4 ml BJAB wet cell pellet was lysed with 20 ml lysis buffer, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM HEPES, pH 7.2). The lysate was cleared by a 1-min spin at 20,000 $\times$ *g* in 2-ml reaction tubes. The supernatant was then incubated for 30 min with agitation with Strep-Tactin Superflow beads (Qiagen). The beads were collected by a brief spin (500 $\times$ *g*), and the supernatant was transferred to a fresh tube. The samples were then centrifuged at 20,000 $\times$ *g* for 30 min. The supernatants were collected and pooled, and 5 ml was incubated overnight at 4°C with agitation with the respective Fc fusion proteins precoupled to Strep-Tactin beads (10 ml of transfected 293T cell supernatant was reacted with approximately 50 μl Strep-Tactin beads overnight before the pulldown experiment) in a 15-ml tube. The beads were then collected by low-speed centrifugation for 5 min, the supernatant was discarded, and the beads were washed with 10 ml 0.75% NP-40 in PBS, which was repeated three times. Bound protein was then eluted for 5 min with 1 ml 2.5 mM desthiobiotin and 0.375% NP-40 in PBS by mixing with the beads, followed by resedimentation of the Strep-Tactin beads by low-speed centrifugation and careful collection of the supernatant. The eluate was incubated for 1 h with approximately 30 μl of protein A agarose beads (GE Lifesciences) at 4°C with agitation. The protein A beads were then collected by centrifugation at 1,500 $\times$ *g* for 1 min and were washed three times with 0.375% NP-40 in PBS. After removal of all washing buffer, 40 μl of SDS sample buffer was added and the samples were heated to 95°C for 3 min. The samples then were analyzed by polyacrylamide gel electrophoresis using 8 to 16% gradient gels (Invitrogen), the gel was silver stained, and excised bands were destained using the SilverQuest staining kit (Life Technologies). Mass spectrometry analysis by liquid chromatography-tandem mass spectrometry of individual gel bands was carried out by the Taplin Mass Spectrometry Core Facility, Harvard Medical School. For pulldown followed by Western blot analysis, cells were lysed with 2 ml of lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 25 mM HEPES, pH 7.3, with addition of protease inhibitor cocktail [Amresco]) per ml of wet cell pellet. The lysate was clarified by centrifugation (21,100 $\times$ *g*, 20 min) and reacted with gH-FcStrep/gL-Flag complexes that were precoupled to Strep-Tactin XT (IBA) beads. After three washes with lysis buffer, the precipitates were analyzed by polyacrylamide gel electrophoresis and Western blotting as described previously (23) using antibodies to EphA7 (clone E-7; sc-393973) and EphA2 (C-20; sc-924), both from Santa Cruz Biotechnology, 1:100 and 1:500, and EphA5 (clone number 86731; MAB541; R&D Systems), 1:500, in NETT gelatin (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100, 0.25% gelatin, pH 7.5) and donkey anti-mouse horseradish peroxidase (HRP)-coupled (Dianova) or goat anti-rabbit HRP-coupled (Life Technologies) secondary antibody in 5% dry milk powder in PBS with 0.05% Tween 20. Membranes were imaged using Immobilon Forte substrate (Merck) on an INTAS ECL ChemoCam system. Similar lysis conditions were used for analysis of gene knockout cell pools by Western blotting.

RNA isolation, cDNA synthesis, and nonquantitative RT-PCR. For RNA isolation, cells were harvested in RNAzol RT (Sigma) and RNA was isolated using the Direct-zol RNA MiniPrep plus kit (Zymo Research) according to the manufacturer's instructions. In short, 0.4 ml of RNase-free water per ml of RNAzol RT, used for homogenization, was added, vortexed for 15 s, and incubated for 10 min at room temperature. After centrifugation (15 min, 12,000 $\times$ *g*, 4°C), the aqueous phase was mixed with an equal volume of 100% ethanol and column purified. In the column, DNase I (30 U) treatment was performed for 15 min at room temperature. RNA was eluted in RNase-free water. One μg RNA was reverse transcribed using the SensiFast cDNA synthesis kit (Bioline) according to the manufacturer's instructions. PCR was performed using Fusion S7 with a primer pair (GGTCTGCAGTCGGAGACTTG and AGCTCTTGGC AACTTGCATT) binding in the 5′ and 3′ untranslated regions (UTRs) of transcript variant 1 (GenBank accession no. NM_004440), transcript variant 2 (NM_001288629), predicted transcript variant X1 (XM_005248669), predicted transcript variant X2 (XM_017010365), and predicted transcript variant X4 (XR_001743218.2). PCR products were analyzed on 1% agarose gels, and bands at the expected sizes were excised and purified using the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel). Sanger sequencing was carried out by Macrogen, Inc.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.00064-19.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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