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1 **Kaposi's sarcoma-associated herpesvirus glycoprotein K8.1 is critical for infection in a cell-**  
2 **specific manner and functions at the attachment step on keratinocytes**

3

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15

16 Running Head: KSHV K8.1 mediates infection of skin keratinocytes

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**ABSTRACT**

24 Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with Kaposi's sarcoma and  
25 B cell malignancies. K8.1, the major antigenic component of the KSHV virion, has been reported  
26 to play a critical role in the infection of B cells, but otherwise its function remains enigmatic. We  
27 created a K8.1 knockout virus (KSHV $\Delta$ K8.1) in the BAC16 genetic background and analyzed its  
28 infectivity on a range of adherent cells. We observed a strong defect on several epithelial cells,  
29 e.g. the HaCaT keratinocyte cell line, HEK 293T and A549 lung epithelial cells, but no such defect  
30 on other cells, among them e.g. lymphatic and blood endothelial cells. Mechanistically, we  
31 found that reduced infectivity of the K8.1 knockout virus correlated with reduced attachment to  
32 HaCaT cells. The defect in infectivity of KSHV $\Delta$ K8.1 could be rescued by complementation  
33 through expression of K8.1 in KSHV $\Delta$ K8.1 producing cells by means of a lentiviral vector. In a  
34 coculture infection model with iSLK producer cells, KSHV $\Delta$ K8.1 was highly efficient at infecting  
35 the BJAB B cell line but was significantly impaired at infecting the MC116 B cell line, in line with  
36 a previous report. In fusion assays together with the gH/gL glycoprotein complex and gB, the  
37 components of the conserved herpesviral core fusion machinery, we did not observe activation  
38 of membrane fusion by K8.1 or its R8.1 homolog of the rhesus monkey rhadinovirus. In  
39 summary, we found K8.1 to function in a highly cell-specific manner during KSHV entry at the  
40 attachment step, playing an important role in the infection of epithelial cells.

41

## 42 **IMPORTANCE**

43 KSHV is the causative agent of several B cell malignancies and Kaposi's sarcoma. We  
44 analyzed the function of K8.1, the major antigenic component of the KSHV virion, in the  
45 infection of different cells. To do this, we deleted K8.1 from the viral genome. It was found that  
46 K8.1 is critical for the infection of certain epithelial cells, e.g. a skin model cell line, but not for

47 infection of many other cells. K8.1 was found to mediate attachment of the virus to cells where  
48 it plays a role in infection. In contrast, we did not find K8.1 or a related protein from a closely  
49 related monkey virus to activate fusion of the viral and cellular membranes, at least not under  
50 the conditions tested. These findings suggest that K8.1 functions in a highly cell-specific manner  
51 during KSHV entry, playing a crucial role in the attachment of KSHV to e.g. skin epithelial cells.

52

### 53 **INTRODUCTION**

54 KSHV is the etiological agent of Kaposi's sarcoma (KS) and is associated with at least two  
55 B cell malignancies, primary effusion lymphoma and a variant of multicentric Castleman's  
56 disease. An association with KSHV-positive diffuse large B cell lymphoma was also reported (1–  
57 5). A recent report suggests association with osteosarcoma (6), and the virus is associated with  
58 the so-called KSHV inflammatory cytokine syndrome (7). KSHV-associated malignancies  
59 constitute a major disease burden in Sub-Saharan Africa and in immunocompromised  
60 individuals worldwide, in particular in the context of HIV infection (reviewed in (8, 9)). In some  
61 African countries KS is the cancer that is associated with both the highest morbidity and  
62 mortality among all types of cancer (10). A vaccine to prevent either KSHV primary infection or  
63 the development of KSHV-associated diseases would be highly desirable. For both approaches,  
64 understanding the function of the individual glycoproteins at the different stages of KSHV entry  
65 into specific host cells is critical to understand their role during host colonization of individual  
66 tissues.

67 The roles of the individual glycoproteins (GPs) of KSHV during the different steps of the  
68 entry process, attachment, endocytosis, and membrane fusion, have so far only been poorly  
69 defined in the context of different types of host cells. Like other herpesviruses KSHV encodes

70 the glycoproteins gB, gH, and gL that together form the conserved core fusion machinery of the  
71 herpesviruses (11). While gB is the fusion executor, the interaction of KSHV gB with integrins  
72 through an RGD motif may rather contribute to attachment or endocytosis (12–15). The  
73 interaction of gH/gL with receptors from the Eph family can trigger membrane fusion by the  
74 core fusion machinery (16, 17), similar to what is observed with the related human Epstein-Barr  
75 virus or the rhesus monkey rhadinovirus (RRV) (16, 18). This at least holds true within the  
76 experimental limits of these reports as gB always had to be substituted for a gB of a related  
77 virus, either EBV or RRV, due to KSHV gB's extremely low fusogenicity, which makes it  
78 unsuitable for assays measuring cell-cell fusion. Engagement of Eph family receptors also  
79 triggers endocytosis (19–21). The exact function and molecular interactions of a proposed  
80 fusion receptor, xCT, which normally forms a complex with CD98, so far remain unclear, with  
81 different reports claiming either a direct role in fusion or for post-entry gene expression (15,  
82 22).

83         The role of KSHV glycoprotein K8.1, also termed K8.1A or gp35-37 (23), is not well  
84 understood. It binds heparan sulfate (24) and is the major antigenic component of the KSHV  
85 virion (23). A role of K8.1, that is independent from its binding to heparan sulfate, in the  
86 infection of the MC116 B cell line as well as in the infection of primary tonsillar B cells has been  
87 described (25). Otherwise, its function remains enigmatic. For example, it is not known whether  
88 K8.1 plays a role in the fusion process, something that could be suspected due to its potential  
89 positional homology to the gp42-encoding gene of EBV within the KSHV genome. EBV gp42  
90 clearly functions as a trigger of fusion (26). This hypothesis is also supported by homology of a  
91 promoter element directly upstream of KSHV K8.1, EBV gp42, and RF8.1 of the retroperitoneal  
92 fibromatosis herpesvirus of macaques (27), which may hint at a conserved biological role, even

93 if a sequence homology is not readily detectable across the whole protein. Another question is  
94 whether K8.1 only plays a role for infection of some B cell populations or also for infection of  
95 other cell types. We therefore generated a K8.1 deletion mutant and analyzed its relative  
96 infectivity on different primary cells and cell lines. We also investigated K8.1's contribution to  
97 virus attachment and triggering of the fusion machinery.

98

## 99 **RESULTS**

### 100 **Deletion of K8.1 results in decreased infectivity on several cell lines of epithelial origin.**

101 Previously, a role for K8.1 in the infection of B cells was reported by Dollery et al. (25)  
102 but a comprehensive analysis of infection in other cell types was not performed in that study.  
103 We therefore constructed a K8.1 deletion mutant, with a mutation/deletion of the start codon  
104 and signal peptide of K8.1. While probably not necessary for this *in vitro* study, the strategy of  
105 introducing a larger deletion in the signal peptide region was chosen to make sure that no  
106 alternative start codon can give rise to a functional protein and to prevent reversion, also e.g. in  
107 an experimental animal host in future studies. The deletion in the K8.1 locus (Fig. 1A) resulted in  
108 the complete absence of the typical staining pattern with anti-K8.1 antibody observed after  
109 Western blot analysis of crude virus preparations (Fig. 1 B) obtained by centrifugation of cell  
110 culture supernatant from iSLK cells that had been previously transfected to harbor BAC16 wt or  
111  $\Delta$ K8.1 and were induced to enter the lytic cycle. Glycoprotein H (gH) was abundantly detected in  
112 the virus preparation, as was the capsid protein product of ORF65. While ORF65 and gH seemed  
113 slightly more abundant in the  $\Delta$ K8.1 preparation, DNase-resistant genome copy numbers  
114 released into the cell culture supernatant after induction of the lytic cycle were not significantly  
115 different between wt and  $\Delta$ K8.1 virus, even if the  $\Delta$ K8.1 mutant trended lower in this

116 experiment (Fig. 1 C). The “smear-like” staining pattern of K8.1 (Fig. 1 B) almost certainly stems  
117 from its mucin-like degree of O-glycosylation with 18 predicted O-glycosylation sites according  
118 to NetOGlyc 4.0 prediction (28) and is consistent with previous studies (23, 29–31). We  
119 separated recombinantly produced K8.1 ectodomain, which was purified from the cell culture  
120 supernatant of transfected 293T cells, by size exclusion chromatography (Fig. 1 D) and analyzed  
121 the different fractions by gel electrophoresis (Fig. 1 E). This demonstrated that the K8.1  
122 ectodomain exhibits a broad molecular weight distribution range, consistent with highly  
123 heterogenous glycosylation and compatible with the pattern observed in Western blot analysis  
124 of virus preparations (Fig. 1 B).

125 We next compared wt and  $\Delta$ K8.1 virus on a range of different cell lines and primary cells  
126 (Fig. 2 A). While infection of the SLK cell line (a clear renal carcinoma cell line) (32), U2197 (a  
127 histiocytoma cell line) (33), and of primary human foreskin fibroblasts (HFF), human umbilical  
128 vein endothelial cells (HUVEC), and lymphatic endothelial cells (LEC) cells was comparable, the  
129  $\Delta$ K8.1 virus exhibited a significant defect on 293T cells and on A549 cells. On HaCaT cells, the  
130 defect was also very pronounced but due to the overall low susceptibility of HaCaT could only  
131 be shown at higher MOI after spin infection. The discrepancy in susceptibility of HaCaT to  
132 infection by wt and  $\Delta$ K8.1 virus in comparison to SLK cells was also confirmed in separate  
133 experiments with another pair of virus stocks (Fig. 2 B), and it was not dependent on e.g. the  
134 calcium concentration in the culture medium, which reportedly controls differentiation of  
135 HaCaT (34). KS is primarily a cutaneous tumor and skin keratinocytes are infected by KSHV, also  
136 in the context of KS lesions (35–37). HaCaT are a model cell line for keratinized epithelia that  
137 retain the features of primary skin keratinocytes (38).

138 In order to control for possible non-specific off-target effects on viral infectivity that may  
139 result from our genetic manipulation of the K8.1 locus, we performed a reconstitution  
140 experiment. iSLK cells harboring BAC16ΔK8.1 were transduced with either empty lentiviral  
141 vector (control) or a lentiviral vector coding for Tandem-Strep-tagged K8.1. We then compared  
142 infection by KSHV wt or KSHVΔ8.1 produced from cells either transduced with empty lentiviral  
143 vector or with lentiviral vector encoding K8.1. Infectivity of KSHVΔK8.1 on HaCaT, A549 and  
144 293T cells was visibly rescued by reconstitution of K8.1 expression (Fig. 3 A). Calculation of the  
145 50% infectious titer for each virus preparation (Fig. 3 B) from the data in Fig. 3 A demonstrated  
146 that the reconstituted knockout virus did not differ significantly from KSHV wt with regard to  
147 infectivity, while mock-reconstituted virus did. It should be noted that lentiviral transduction  
148 may not lead to uniform expression of the transgene in all cells to the same degree and may be  
149 limited with regard to the achievable expression levels, which likely precludes complete  
150 reconstitution, as K8.1 is expressed to high levels during lytic KSHV replication. K8.1 expression  
151 levels in virus preparations from K8.1-reconstituted KSHVΔK8.1-producing iSLK were similar to  
152 expression levels in the wt virus preparation (Fig. 3 C).

153 **Deletion of K8.1 results in reduced cell-to-cell transmission into the MC116 B cell line**  
154 **but not into the BJAB or Raji B cell lines**

155 Our previous work demonstrated that the BJAB B cell line is infected in a manner that is  
156 highly dependent on the EphA7 receptor and to a lesser degree on the EphA5 receptor, and that  
157 both receptors are engaged by the KSHV gH/gL complex, similar to the interaction with the high-  
158 affinity EphA2 receptor (39). Work by Dollery and colleagues has demonstrated a critical role of  
159 K8.1 for the infection of the MC116 B cell line (25), an EBV-negative Burkitt lymphoma cell line  
160 (40, 41). We compared infection of MC116, BJAB, an EBV-negative Burkitt lymphoma cell line



161 (42), and Raji, an EBV-positive Burkitt lymphoma cell line (43, 44). Neither of the three cell lines  
162 in our hands supported infection with free KSHV wt at levels exceeding 1% infected cells under  
163 conditions that resulted in over 90% infected SLK cells (Fig. 4 A), which disagrees with previous  
164 results for MC116 (45) but may be rooted in e.g. different culture conditions that lead to  
165 differences in susceptibility. It should be noted that this time, we managed to achieve some  
166 very low-level detectable infection of BJAB, unlike in earlier studies by our lab (39) and others  
167 (46), suggesting that these cell lines may indeed vary in their susceptibility to some degree  
168 across passages and subtle changes in culture conditions. Nevertheless, we could not compare  
169 KSHV wt and  $\Delta$ K8.1 as free virus and instead compared the two viruses on these cell lines head  
170 to head in our established cell-to-cell transmission model for KSHV into B cell lines (39) that  
171 roughly follows the established protocol by Myoung et al. (46), who first described the critical  
172 role of cell-to-cell transmission for infection of lymphoblastoid B cell lines. We found that in  
173 agreement with Dollery et al. (25) MC116 infection was highly dependent on K8.1, even if  
174 MC116 in our hands exhibited comparatively low susceptibility (Fig. 4 B middle column pair). On  
175 the other hand, infection of BJAB or Raji was completely independent of K8.1 (Fig. 4 B left and  
176 right column pairs), again demonstrating that the deletion in KSHV $\Delta$ K8.1 does not cause a  
177 general defect in infectivity or induction of the lytic cycle in these cell lines but instead causes a  
178 highly cell-specific defect.

179 **Deletion of K8.1 results in reduced attachment to HaCaT skin epithelial cells.**

180 The defect in infectivity that was observed with KSHV $\Delta$ K8.1 on A549, 293T and  
181 particularly HaCaT cells could conceivably be caused at different stages. As K8.1 is a  
182 glycoprotein, the attachment step, endocytosis of the virus particle or fusion of viral and cellular  
183 membranes come to mind. We analyzed attachment by replicating the spin infection protocol

184 used for the infection assay but immediately after centrifugation at 4° C, the cells were washed  
185 with cold PBS and then harvested for quantification of cell-associated, DNase-resistant viral  
186 nucleic acid as a surrogate marker for attachment. We found that attachment of KSHVΔK8.1 to  
187 HaCaT cells was clearly and significantly reduced by over twofold in comparison to attachment  
188 of KSHV wt (Fig. 5 A). Attachment to A549 and 293T cells was reduced as well, but to a lesser  
189 degree, and the difference did not reach significance for 293T (which may reflect limitations of  
190 the curve-fitting and statistical model, individual values at each concentration were clearly and  
191 uniformly different).

192 **The importance of K8.1 for infection is partially reflected in its binding to cellular**  
193 **membranes.**

194 In order to determine whether K8.1's function is related to its quantitative binding to the  
195 membrane of target cells, which might mediate attachment, or rather an indirect effect of its  
196 presence in the virion, we performed binding assays with a K8.1-Fc fusion protein that consists  
197 of the extracellular domain of K8.1 fused to the Fc part of immunoglobulin G (Fig. 5 B).

198 For comparison, we also used similar molecules, gH-Fc/gL of KSHV that binds to heparan  
199 sulfate and Eph family receptors (19, 47), and as control RRV gH-Fc mutated in the Plxdc1/2  
200 receptor binding site and unable to bind Eph family receptors without gL (RRV gHΔ21-27-Fc)  
201 (18), a molecule that in our hands does not bind to any cell tested so far. Binding of K8.1-Fc and  
202 gH-Fc/gL to cell surfaces should mirror the availability of their respective receptors on these  
203 cells, with the limitation that low-affinity interactions may be missed by such cellular binding  
204 assays. It would be interesting to directly correlate K8.1's contribution to infection with the  
205 expression levels of other known receptors, but the complexity of KSHV's interactions with  
206 different Eph family members (17, 39, 48), integrin combinations (13, 15), lectins (49), and

207 heparan sulfate (24, 47), in addition to reported uncharacterized infection pathways (50),  
208 precludes such a comparison. While we do not include gB, which currently is not available in its  
209 trimeric pre-fusion form, differential binding of gH/gL and K8.1 might explain their relative  
210 importance for infection and may represent a surrogate for comparing expression of their  
211 respective receptors. Binding of K8.1-Fc measured as fold mean fluorescence intensity over  
212 control was clearly most pronounced on 293T cells, followed by HaCaT, two cell lines where the  
213 most pronounced defect of KSHV $\Delta$ K8.1 was observed. For HaCaT, in addition to high K8.1  
214 binding, very low binding of gH-Fc/gL was observed, in line with low expression of EphA2 in this  
215 cell line as reported by the human protein atlas (<https://www.proteinatlas.org/>). For A549, the  
216 third cell line where KSHV $\Delta$ K8.1 showed a clear-cut defect, the picture was less clear. A549  
217 exhibited K8.1 binding that was comparable to that on e.g. SLK or HFF and also interacted avidly  
218 with gH-Fc/gL. Therefore, the relative importance of K8.1 for infection may be partially reflected  
219 by the abundance of known cellular interaction partners like heparan sulfate proteoglycans or  
220 of unknown interaction partners on the cell surface e.g. of HaCaT or 293T cells, but that does  
221 not entirely explain its role, as exemplified by A549 cells, where neither K8.1 binding was  
222 particularly pronounced nor gH/gL binding particularly low.

223 **Presence or absence of K8.1 or its RRV homolog does not appreciably modulate gH/gL-**  
224 **activated membrane fusion by gB.**

225 While our results so far pointed to a function of K8.1 at the attachment step, mediating  
226 binding of the virus to host cell membranes, a function in fusion is also possible. The EBV  
227 glycoprotein gp42 for example, which is located at a similar or even the same position in the  
228 EBV genome compared to K8.1 in the KSHV genome, triggers fusion (26). As KSHV gB is barely  
229 fusogenic in cell-cell fusion assays, analyzing the trigger function of gH/gL on gB-mediated

230 membrane fusion is usually carried out using a gB from a related virus (17, 51). As established in  
231 our group, we tested KSHV gH/gL together with gB of the related rhesus monkey rhadinovirus.  
232 Addition of K8.1 to this combination of glycoproteins did not result in an increase in fusion of  
233 effector cells expressing gH/gL/gB or gH/gB (Fig. 6 A). We tested fusion activity with and without  
234 gL, which results in the ability or inability to use Eph family receptors for fusion (52). This  
235 approach might demonstrate modulation of fusion that might be masked by Eph-mediated  
236 membrane fusion in the presence of the full gH/gL complex. Even so, under none of these  
237 conditions did K8.1 modulate fusion activity. As this only demonstrates a lack of action on  
238 gH/gL-mediated fusion and due to the heterologous gB in the assay, direct activation of gB by  
239 K8.1 might be impaired without any obvious effect in our assay if one postulates that R8.1 may  
240 not interact with KSHV gB as K8.1 does. We therefore tested the positional homolog R8.1 of the  
241 related RRV together with its cognate gH/gL, and gB. Similar to our results with KSHV K8.1, we  
242 did not observe appreciable activation of cell-cell fusion on a range of different target cells by  
243 RRV R8.1 (Fig. 6 B).

244

## 245 **DISCUSSION**

246 Taken together, our results demonstrate that in addition to the known function of K8.1  
247 in the infection of B cells it has a critical function in the infection of epithelial cells, as observed  
248 here for A549, 293T, and the skin model HaCaT cell line, which is not-derived from a tumor but  
249 from a spontaneous immortalization event of cultured skin cells and is regarded as highly  
250 representative of skin epithelium (38, 53). The specificity of the  $\Delta$ K8.1 phenotype for certain  
251 cells is striking as the K8.1 deletion had absolutely no effect on the infectivity of KSHV on  
252 primary fibroblasts, primary endothelial cells and on the SLK and U2197 cell lines. The lack of a

253 phenotype of KSHVΔK8.1 on primary endothelial cells is in agreement with a previous report on  
254 the function of K8.1 (25).

255         While we found a clear correlation of the K8.1 deletion with reduced attachment as  
256 demonstrated for HaCaT cells (Fig. 5 A), we also found reduced infection of MC116 cells in a cell-  
257 to-cell transmission setting, but normal infection of BJAB cells and Raji cells in this setting (Fig. 4  
258 B). This would be compatible with the findings by Dollery et al., who described inhibition of  
259 infection of primary tonsillar B cells by K8.1-targeting antibodies but could not achieve a  
260 complete block, unlike as with MC116 cells (25), suggestive of both K8.1-dependent and K8.1-  
261 independent infection also in primary B cell preparations. These previous findings and the  
262 difference between the BJAB, Raji, and MC116 cell lines argue for the existence of more than  
263 one single mechanism for the infection of B cells, which constitute a diverse population.

264         Our findings in the cell-to-cell transmission setting also suggest a function of K8.1 that  
265 goes beyond the canonical concept of attachment as cell-to-cell transmission is believed to  
266 function through some form of transfer of infectious virus from one cell to the other or even  
267 through a so-called virological synapse, which conceivably might obviate the need for the virus  
268 to attach to the target cell, even if that is so far speculation for KSHV. Nevertheless, this notion  
269 would fit with the report by Dollery et al. that K8.1's function for infection of MC116 is  
270 independent of heparan sulfate binding (25), which is generally believed to play a role for  
271 attachment of viruses to cell membranes. On the other hand, we so far have no indication that  
272 K8.1 or its close homologue, R8.1 of RRV, can trigger the core fusion machinery. Therefore, the  
273 exact mechanism how K8.1 drives infection remains to be further elucidated. There is also the  
274 possibility that while attachment in the original sense is not required during cell-to-cell  
275 transmission, the "hand-over" of virus from one cell to the other still requires strong binding to

276 the target cell. In light of our results and previous reports (24, 25), it is conceivable that K8.1  
277 acts through both its interaction with heparan sulfate proteoglycans and through additional  
278 interactions that may play a comparatively more important role during infection of B cells. It  
279 should also be noted that heparan sulfate proteoglycans contain a highly complex mixture of  
280 sulfated sugars that may differ between cells (reviewed in (54)) and that the preferred heparan  
281 sulfate moieties can be quite specific, as was also observed for gH of KSHV (47).

282 K8.1 may not only mediate attachment to epithelial cells but also aid in endocytosis of  
283 the virus e.g. into MC116 cells, which would be compatible with our findings in adherent cells  
284 and in the cell-to-cell transmission model for B cell lines. In line with this notion, Dollery et al.  
285 did not observe reduced attachment to MC116 cells upon treatment of the virus with antibodies  
286 directed to K8.1 (25). In a similar vein, the observed reduction in infectivity with KSHV $\Delta$ K8.1 for  
287 293T and A549 (Figs 2 and 3) considerably exceeded the reduction in attachment observed  
288 upon deletion of K8.1 (Fig. 5 A). In both scenarios, K8.1 would function more analogous to EBV  
289 gp350, which mediates binding and endocytosis, particularly during B cell infection (55), than to  
290 EBV gp42, which triggers membrane fusion (26).

291 In summary, we not only confirm K8.1's role for the infection of B cells as exemplified by  
292 the MC116 line, but we also demonstrate a critical function during infection of several epithelial  
293 cells, among them skin-derived keratinocytes. Ultimately, animal experiments will be needed to  
294 faithfully elucidate the role of individual glycoproteins for cellular tropism and establishment of  
295 persistent infection. With regard to the development of a glycoprotein-based KSHV vaccine, our  
296 results argue for the inclusion of K8.1 in such an approach as K8.1 clearly functions during  
297 infection of B cells and of skin-derived keratinocytes. Skin keratinocytes have previously been  
298 reported to be susceptible to KSHV and to undergo a certain degree of immortalization through

299 infection (36), and skin is an organ that is prominently affected by KS (8). Epidermal  
300 keratinocytes were shown to express cyclin D mRNA in cutaneous KS lesions (35) and were  
301 shown to be KSHV-infected in such lesions (37), indicating that keratinocytes are at least to  
302 some degree involved in cutaneous KS. The role of cutaneous infection in KSHV transmission is  
303 unclear. Overall, in light of our findings here and previous results (19, 39, 48), targeting the  
304 gH/gL complex to block binding to Eph family receptors together with K8.1 to selectively inhibit  
305 infection of certain B cell populations (25) and impair infection of epithelial cells might be a  
306 good starting point for the design of vaccine antigens.

307

## 308 **MATERIAL AND METHODS**

### 309 **Cells**

310 SLK cells (NIH AIDS Research and Reference Reagent Program), A549 (laboratory of  
311 Stefan Pöhlmann, German Primate Center-Leibniz Institute for Primate Research, Göttingen,  
312 Germany), Human embryonic kidney (HEK) 293T cells (laboratory of Stefan Pöhlmann), HaCaT  
313 human keratinocytes (RRID: CVCL\_0038), human foreskin fibroblasts (HFF) (laboratory of Klaus  
314 Korn, Universitätsklinikum Erlangen, Institute for Clinical and Molecular Virology, Erlangen,  
315 Germany), U2197 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell  
316 Cultures GmbH) were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose,  
317 GlutaMAX, 25mM HEPES (Thermo Fisher Scientific) supplemented with 10% fetal calf serum  
318 (FCS) (Thermo Fisher Scientific), and 50µg/ml gentamicin (PAN Biotech) (D10 medium). For low  
319 calcium conditions, DMEM high glucose without calcium was supplemented as above and  
320 brought to 0.03 mmol calcium from a 2.5 M stock solution. iSLK Bac16 cells (56, 57) (a kind gift  
321 of Jae Jung and Don Ganem) were maintained in DMEM supplemented with 10% FCS, 50µg/ml

322 gentamicin, 2.5µg/ml puromycin (InvivoGen), 250µg/ml G418 (Carl Roth) and 200ug/ml  
323 hygromycin (InvivoGen). Human vascular endothelial cells (HUVEC) were maintained in standard  
324 Endothelial Cell Growth Medium 2 (both PromoCell). Human lymphatic endothelial cells (LEC)  
325 were maintained in Endothelial Cell Growth Medium MV 2 (both PromoCell). BJAB (Leibniz-  
326 Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), Raji  
327 (RRID:CVCL\_0511, a kind gift from Jens Gruber) and MC116 (a kind gift from Frank Neipel) were  
328 cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf  
329 serum (FCS) and 50 g/ml gentamicin (R10 medium).

330

### 331 **Deletion of K8.1 in BAC16**

332 The KSHV K8.1 deletion mutant (KSHVΔK8.1) was generated using a two-step, markerless λ-red-  
333 mediated BAC recombination strategy as described by Tischer et al. (58). KSHVΔK8.1 harbors a  
334 mutation of the K8.1 start codon to CGG followed by a 59-nucleotide deletion resulting in an  
335 additional frameshift. In short, the recombination cassette was generated from the pEPKanS  
336 template by polymerase chain reaction (PCR) with Phusion High Fidelity DNA polymerase  
337 (Thermo Fisher Scientific) using long oligonucleotides (Ultramers; purchased from Integrated  
338 DNA Technologies (IDT)) (KSHV\_K8.1Mut\_F:  
339 TAAAGGGACCGAAGTTAATCCCTTAATCCTCTGGGATTAATAACCCGGGGTGGCGTGCCATGCCAATTGT  
340 CCCACGTATCGAGGATGACGACGATAAGTAGGG, KSHV\_K8.1Mut\_R:  
341 CTCTTGCCAGAATCCCAAATGCGAACGATACGTGGGACAATTGGCATGGCAGCCACCCGGGTTATTAA  
342 TCCAGAGGATCAACCAATTAACCAATTCTGATTAG). The recombination cassette was transformed  
343 into BAC16-carrying *Escherichia coli* strain GS1783, followed by kanamycin selection, and  
344 subsequent second recombination under 1% L(+)-arabinose (Sigma-Aldrich)-induced I-SceI



345 expression. Colonies were verified by PCR of the mutated region followed by sequence analysis  
346 (Macrogen), pulsed-field gel electrophoresis and restriction fragment length polymorphism. For  
347 this purpose, bacmid DNA was isolated by standard alkaline lysis from 5ml liquid cultures.  
348 Subsequently, the integrity of bacmid DNA was analyzed by digestion with restriction  
349 enzyme *Xho*I and separation in 0.8% PFGE agarose (Bio-Rad) gels and 0.5×TBE buffer by pulsed-  
350 field gel electrophoresis at 6 V/cm, 120-degree field angle, switch time linearly ramped from 1s  
351 to 5s over 16 h (CHEF DR III, Bio-Rad). Infectious KSHV recombinants were generated by  
352 transfection of purified bacmid DNA (NucleoBond Xtra Midi (Macherey-Nagel)) into iSLK cells  
353 using GenJet Ver. II (Signagen) according to manufacturer's instructions. After visible GFP  
354 expression, selection was performed using 200 µg/ml hygromycin B (InvivoGen) until only GFP  
355 positive cells remained. Lytic replication of KSHV-BAC16 was induced in D10 medium by addition  
356 of 2.5mM sodium-butyrate and 1µg/ml doxycycline. Supernatant was harvested after the cell  
357 monolayer was destroyed. The BAC sequence was verified by Illumina-based next generation  
358 sequencing.

359

### 360 **Recombinant virus production and Western blot**

361 iSLK BAC16 wt or ΔK8.1 cells were transduced with lentivirus harboring K8.1  
362 overexpressing vector. After selection by blasticidin (InvivoGen), cells were induced by 2.5 mM  
363 sodium-butyrate and 1 µg/ml doxycycline for 3 days. After that, the produced virus was  
364 harvested and subjected to Western blot analysis after concentration by centrifugation through  
365 a 5% Optiprep cushion as described previously (59). Western blot analysis was performed as  
366 described previously (59), ORF65 was detected using a cross-reactive mouse monoclonal  
367 antibody to RRV ORF65 (a kind gift from Scott Wong). K8.1 was detected using monoclonal

368 antibody BS555 (60), a kind gift from Frank Neipel. gH was detected using custom-made  
369 polyclonal rabbit antibodies raised against peptides derived from KSHV gH (Genscript).

370

### 371 **Plasmids**

372 The following plasmids were used in this study: pLenti CMV Blast DEST (706–1) (Ax203, a  
373 gift from Eric Campeau & Paul Kaufman, Addgene plasmid #17451); K8.1-OneStrep (OneStrep  
374 Tag: SAWSHQPFEKGGGSGGGSGGSAWSHQPFEK) in the Ax203 backbone (Ax336); pcDNA6aV5his  
375 encoding KSHV gH (Ax607); pcDNA3.1 encoding codon optimized KHSVgL (Ax252);  
376 pcDNA6aV5his encoding RRV gB (Ax223); VP16-Gal4 (Ax388)(61); The KSHV gH ectodomain  
377 (amino acids 22 through 704) N-terminally fused to the murine IgGkappa signal peptide and C-  
378 terminally fused to FcFcStrep (Strep tag: SAWSHQPFEKGGGSGGGSGGSSWSHQPFEK) (Ax207,  
379 backbone based on the pAB61 vector as described by Birkmann et al. (24))(19); the K8.1  
380 ectodomain (amino acid 1 through 196) fused to FcFcStrep in the same backbone as Ax207  
381 (Ax250); RRV 26-95 N-terminally fused to the murine IgGkappa signal peptide and C-terminally  
382 fused to FcFcStrep, same backbone as Ax207 (Ax176); RRV 26-95 R8.1 ectodomain (amino acid  
383 26 through 237) N-terminally fused to the murine IgGkappa signal peptide and C-terminally  
384 fused to FcFcStrep in the same backbone as Ax207 (Ax243); pLenti CMV Blast DEST encoding a  
385 Gal4-driven TurboGFP-Luciferase fusion reporter gene (Ax526)(51).

386

### 387 **Infection assays and flow cytometry**

388 For infection assay, cells were plated at 25 000 cells/well in 96-well plates. 16h after  
389 plating, the cells were infected with the indicated amounts of virus. For HaCaT cells, spin  
390 infection was used. After adding the virus, HaCaT cells were centrifuged for 2 h at 800 g, 37°C

391 (spin-infection). 48 h post infection, the cells were harvested by brief trypsinization, followed by  
392 adding 5% FCS in PBS to inhibit trypsin activity. Then, the cell suspension was transferred to an  
393 equal volume of PBS supplemented with 4% methanol-free formaldehyde (Carl Roth) for  
394 fixation. A minimum of 5 000 cells was analyzed per well for GFP expression on an ID7000  
395 (Sony). The cell-to-cell transmission assay was based on our previous protocol (39), which in  
396 turn was based on the work by Myoung et al.(46). Briefly, the whole coculture was stained with  
397 anti-CD20 antibody conjugated to Alexa647 and with anti-CD13 antibody conjugated to PE. Only  
398 the GFP-positive cells in the CD20-positive and CD13-negative population were evaluated in  
399 order to separate the iSLK producer cells from B cell target cell lines.

400 50% infectious titers were calculated by non-linear regression using the “[Agonist] vs.  
401 response – FindECanything” model with E constrained to 50 (for calculation of EC50), bottom to  
402 0, and top to 100 (GraphPad Prism 9.4.1). The same model was used for curve fitting.

403

#### 404 **Quantitative realtime-PCR-based viral genome copy number analysis**

405 Concentrated virus samples were treated with DNaseI (0.1 units/ $\mu$ l) to remove non-  
406 encapsidated DNA (37°C, overnight). Subsequently, DNaseI was deactivated, and viral capsids  
407 were disrupted by heating the sample to 95°C for 30 minutes. Realtime-PCR (qPCR) was  
408 performed on a StepOne plus cycler (Thermo Fisher Scientific) in 20 $\mu$ l reactions using the Sensi  
409 FAST Probe Hi-ROX Kit (Bioline) (cycling conditions: 3min in initial denaturation at 95°C, 40  
410 cycles 95°C for 10s and 6°C for 35s). All primer-probe sets were purchased from IDT as complete  
411 PrimeTime qPCR Assays (primer: probe ratio: 4:1). Samples were analyzed in technical  
412 duplicates. A series of six 10-fold dilutions of bacmid DNA was used as the standard for absolute  
413 quantification of viral genome copies based on qPCR of ORF59 of KSHV.

414 **Virus attachment assay**

415 HaCaT and SLK cells were seeded on the 12-well plate at 200 000 cells/well for virus  
416 attachment. After adding indicated amount of virus, cells were centrifuged for 30min at 4122g  
417 (4200rpm) at 4°C. After three washes with ice-cold PBS, genomic DNA was isolated using the  
418 ISOLATE II Genomic DNA Kit (Bioline) according to the manufacturer's instructions. The amount  
419 of viral DNA was determined by qPCR from equal amounts of extracted DNA. For some  
420 experiments, relative values of bound viral genomes to cellular DNA were calculated based on  
421  $\Delta$ Ct values for viral genomic loci (ORF59 for KSHV) and a cellular genomic locus (CCR5), yielding  
422 equivalent results (not shown).

423

424 **Production of recombinant viral proteins**

425 Soluble K8.1-Fc, KSHV gH-Fc/gL, R8.1-Fc, and RRV gH $\Delta$ 21-27-Fc protein were purified by  
426 Strep-Tactin chromatography from 293T cell culture supernatant. 293T cells were transfected by  
427 Polyethylenimine "Max" (PEI) (Polysciences) transfection with the respective expression  
428 plasmids. The protein-containing cell culture supernatant was passed over 0.5ml of a Strep-  
429 Tactin Superflow (IBA Lifescience) matrix in a gravity flow Omniprep column (BioRad). Bound  
430 protein was washed with approximately 50ml phosphate buffered saline pH 7.4 (PBS) and  
431 eluted in 1ml fractions with 3mM desthiobiotin (Sigma-Aldrich) in PBS. Protein-containing  
432 fractions were pooled, and buffer exchange to PBS via VivaSpin columns (Sartorius) was  
433 performed. Protein concentration was determined by absorbance at 280nm. Aliquots were  
434 frozen and stored at -80°C.

435 Soluble K8.1 ectodomain for size exclusion chromatography analysis was produced in  
436 Expi293 (Thermo Fisher). The cells were transfected with a synthetic gene, codon optimized for

437 expression in mammalian cells and encoding glycoprotein K8.1 ectodomain (residues 1-194,  
438 Uniprot O36551), which was cloned into the pCAGGs vector (62). The construct contained  
439 endogenous K8.1 signal peptide to drive its secretion, and a C-terminal Twin-strep tag  
440 (WSHPQFEKGGGSGGGSGGSSAWSHQPFEK) for affinity purification. For transfection, Expi293  
441 cells were diluted to density of  $2 \times 10^6$  cell/ml in FreeStyle Expi293 media (Thermo Fisher) the day  
442 before transfection. One day later, the cells were centrifuged at 200 x g for 10 minutes at room  
443 temperature, and resuspended in pre-warmed FreeStyle media to density of  $3 \times 10^6$  cells/ml. The  
444 resuspended cells were transfected with the expression plasmid using FectoPro transfection  
445 agent (Polyplus) and 1  $\mu$ g of DNA per ml of cell culture, with 1:1 ratio of DNA (mg) per FectoPro  
446 reagent (ml). Five days post-transfection, the supernatant was separated from the cells by  
447 centrifugation at 3,000 x g for 10 minutes at 4°C. Secreted K8.1 was purified from the  
448 supernatant on a Streptactin column (Cytiva) according to manufacturer's protocol. The affinity  
449 purified K8.1 was concentrated to 0.3 ml and loaded onto the Superdex200 10/300 (Cytiva) size  
450 exclusion column, using 10 mM Tris pH8, 50mM NaCl as a running buffer.

451

#### 452 **Binding assays using recombinant viral proteins**

453 Cells were detached by 0.25M EDTA in PBS and transferred to the falcon tube at 200 000  
454 cells/tube. After discarding the supernatant, cells were fixed in 2% methanol-free formaldehyde  
455 (Carl Roth) for 10 min, followed by once washing with PBS. Subsequently, cells were blocked in  
456 5% FCS in PBS for 1 h at 4°C and then incubated with 10  $\mu$ g/m proteins in 5% FCS in PBS for 1 h  
457 at 4°C, followed by two washes with PBS and one wash with 5% FCS in PBS. Then, the cells were  
458 incubated with Alexa Fluor® 647 anti-human Fc Antibody (Thermo Fisher Scientific) at a ratio of  
459 1:200 for 45 min at 4°C in the dark, followed by three washes with PBS. Finally, the cells were

460 stored in 1% methanol-free formaldehyde in PBS until flow cytometry analysis on an ID7000  
461 (Sony).

462

### 463 **Cell-cell fusion assay**

464 SLK, HaCaT, A549, 293T target cells were stably transduced with a lentiviral construct  
465 encoding a Gal4 response element-driven TurboGFP-luciferase reporter (51) and fusion assays  
466 were performed similar to as described previously (18, 51, 61). 293T effector cells were seeded  
467 in 96-well plates at 30 000 cells/well. One day after, 293T effector cells were transfected with a  
468 plasmid encoding the Gal4 DNA binding domain fused to the VP16 transactivator (VP16-Gal4),  
469 and the indicated viral glycoprotein combinations or a pcDNA empty vector control (VP16-Gal4:  
470 31.25 ng/well, K8.1: 25ng/well, KSHV gH: 12.5 ng/well, KSHV gL: 62.5ng/well, RRV gB: 18.75  
471 ng/well, pcDNA only: 118.75 ng/well) using PEI as described before (51). 24 h after transfection,  
472 the medium on 293T effector cells was removed entirely and exchanged to 100µl fresh D10. The  
473 target cells were re-suspended and counted, and 40 000 target cells were added to 293T  
474 effector cells in 100µl fresh D10. Triplicate wells were analyzed for all target-effector  
475 combinations. After 48h, cells were washed once in PBS and lysed in 35 µl 1x Luciferase Cell  
476 culture lysis buffer (E1531, Promega) for 15min at room temperature and centrifuged for 10min  
477 at 4°C. 20 µl of each cell lysate was used to measure luciferase activity using the Beetle-Juice  
478 Luciferase Assay (PJK) according to the manufacturer's instructions on a Biotek Synergy 2 plate  
479 reader.

480

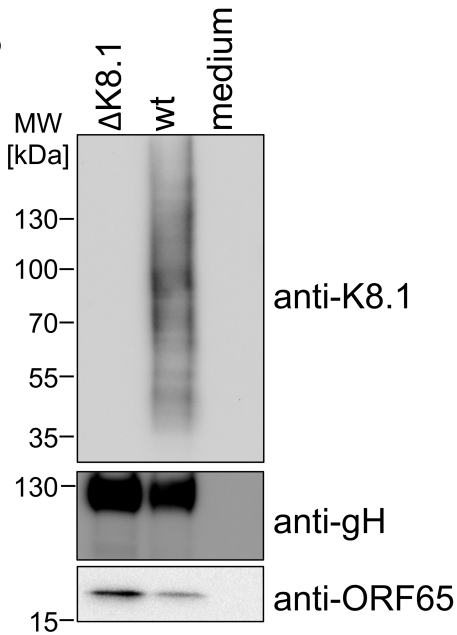
481 **FIGURE LEGENDS**

**A**

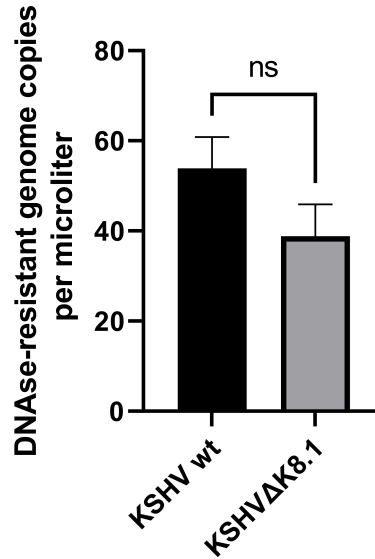
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Nucleotide pos.      75730      75740      75750      75760      75770      75780      75790      75800
(relative to
MK733609.1)
|                |                |                |                |                |                |
K8.1                 M S S T Q I R T E I P V A L L I L C L C L V A C H...
BAC16 KSHV wt        ..ATAACCATGAGTTCACACAGATTTCGCACAGAAATCCCTGTGGCGCTCCTAATCCTATGCCTTTGTCTGGTGGCGTGCCAT..
BAC16 KSHVΔK8.1     ..ATAACCCGG-----GGTGGCGTGCCAT..
  
```

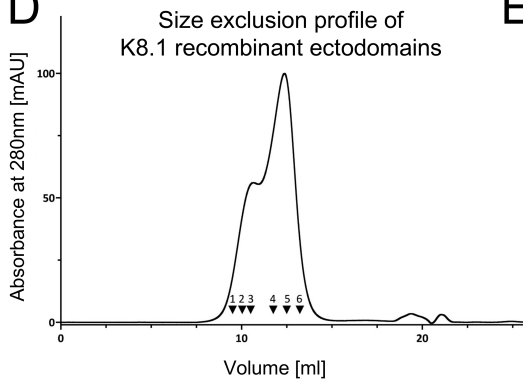
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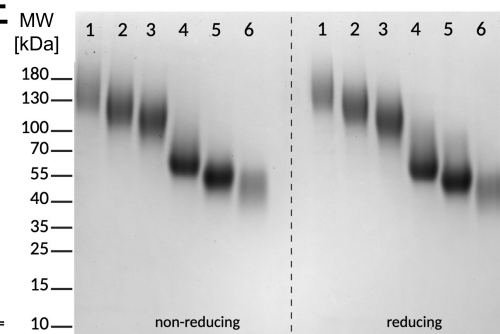
**C**



**D**



**E**



482

483

**Figure 1. Construction of KSHVΔK8.1.** A) Mutation and deletion of the K8.1 start codon

484

and signal peptide in the BAC16 genome. B) Loss of the characteristic high-molecular-weight

485

forms of K8.1 in virus preparations of KSHVΔK8.1 as detected by Western blot. Cell culture

486

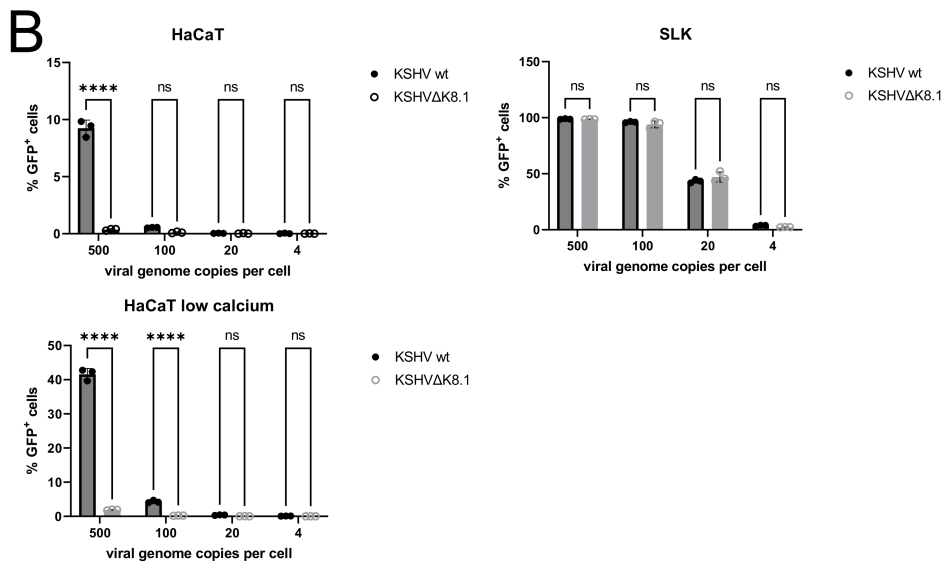
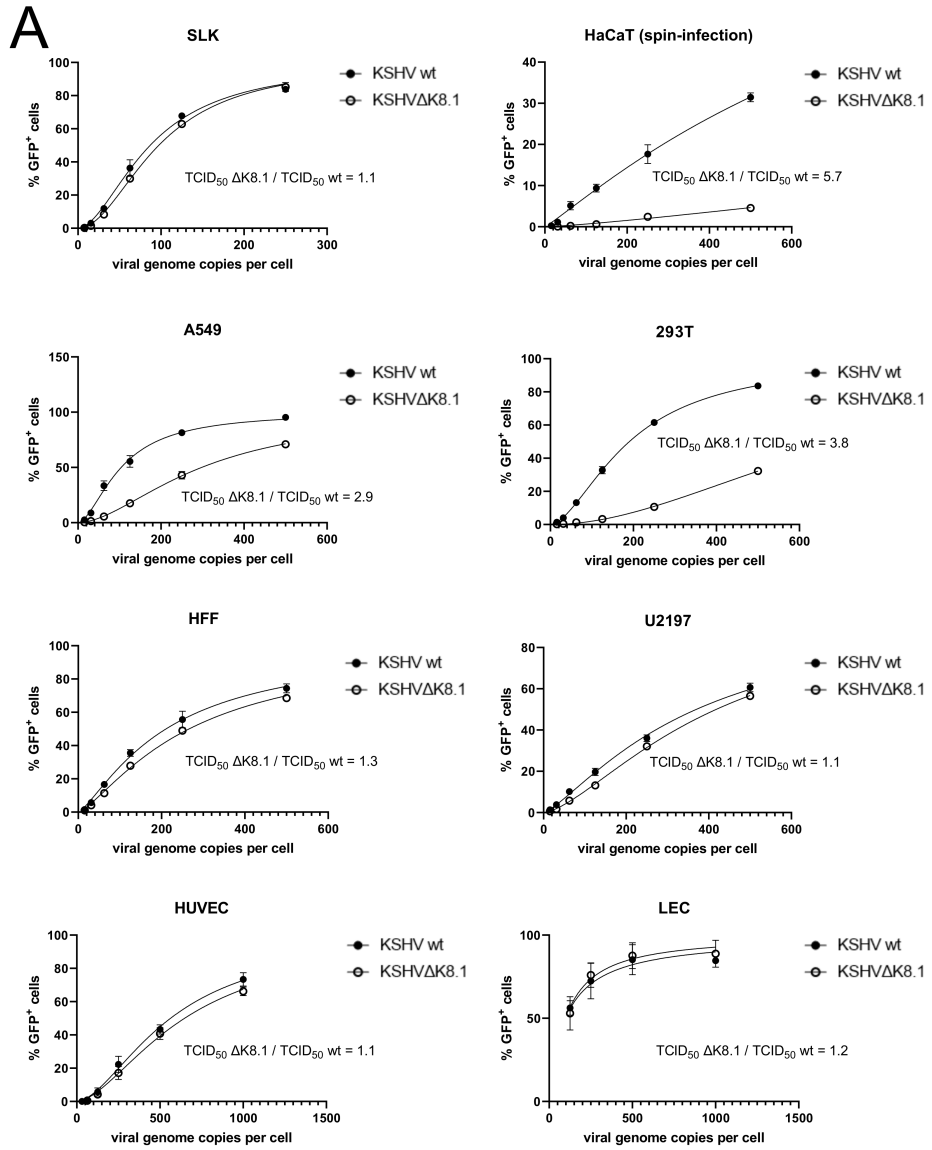
medium serves as a background control. C) Release of encapsidated viral genomes from

487

chemically induced iSLK cells harboring KSHV wt or KSHVΔK8.1 BAC16 (n=3, unpaired t-test). D)

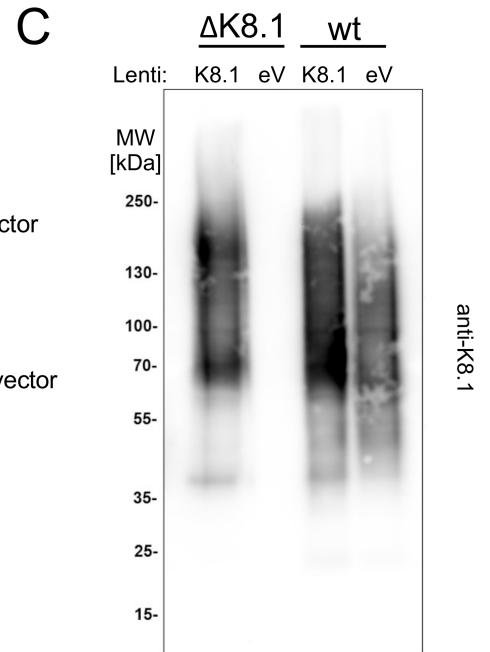
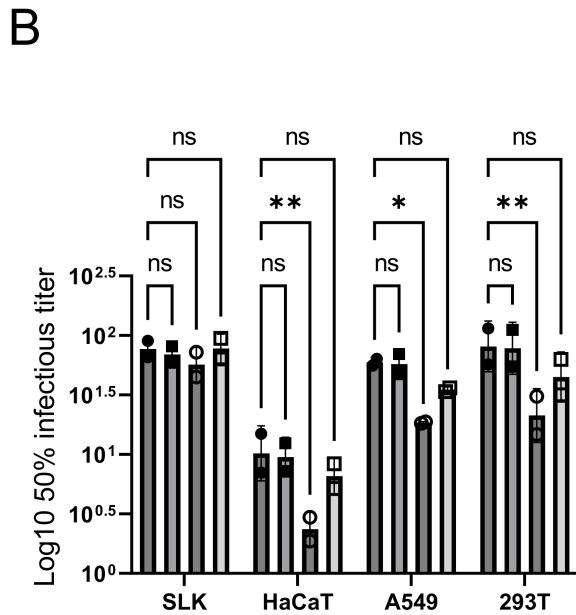
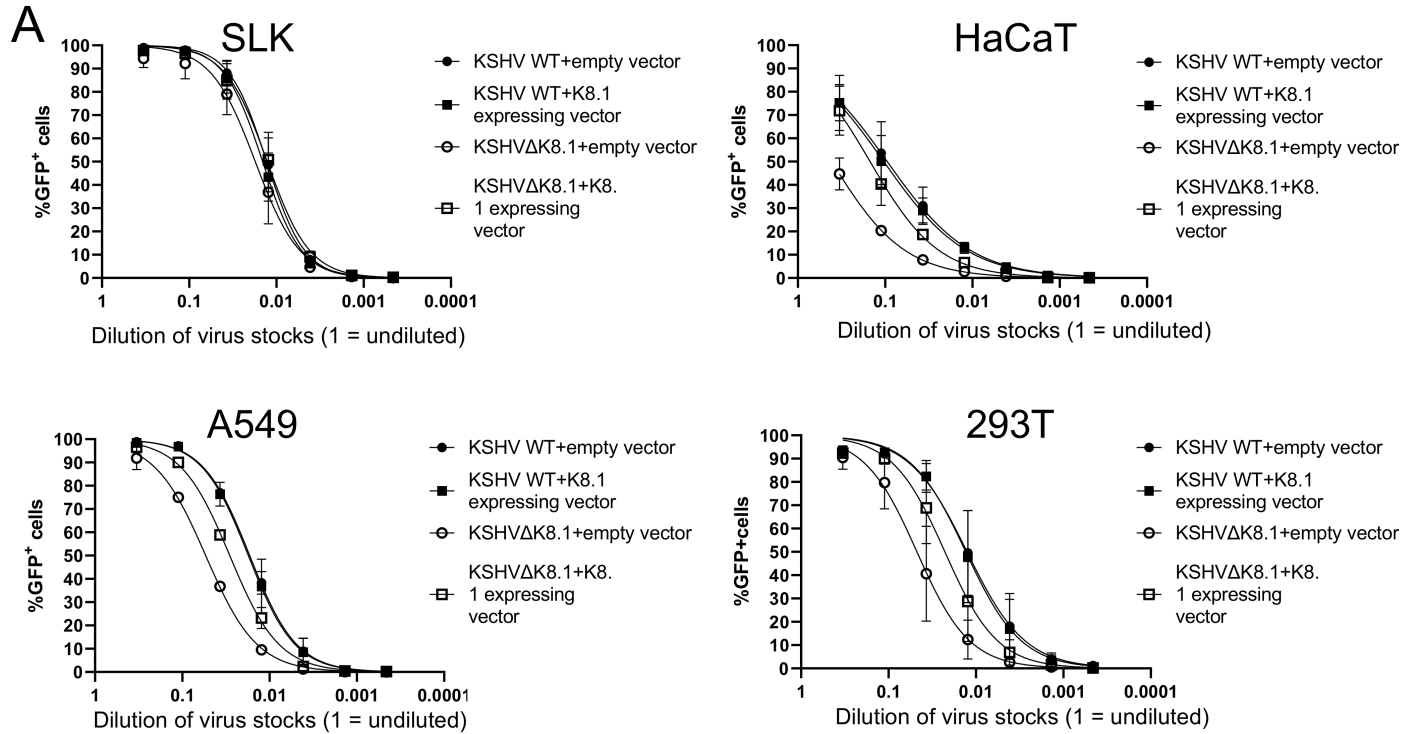
488 Size exclusion chromatography elution profile of purified K8.1 ectodomain, that was  
489 recombinantly produced in Expi293 cells. E) Polyacrylamide gel electrophoresis of fractions (20  
490  $\mu$ l per sample) from (D), stained with Coomassie blue.





492 **Figure 2. Loss of K8.1 results in cell-specific defects in infectivity on different types of**  
493 **adherent cells.** A) Different cell lines and primary cells were infected with BAC16-derived KSHV  
494 wt or KSHV $\Delta$ K8.1 using the same number of virus particles as measured by DNase-resistant  
495 genome copies. Infection was quantified by the percentage of GFP reporter gene-expressing  
496 cells. One representative experiment of two independent experiments with two independent  
497 pairs of virus stocks is shown. B) Repeat experiment comparing infection of SLK and HaCaT  
498 (without spin-infection at normal cell culture conditions and at low calcium) by KSHV wt and  
499 KSHV $\Delta$ K8.1. \*\*\*\* =  $p < 0.0001$ , ns = non-significant. Two-Way ANOVA with Sidak's correction for  
500 multiple comparisons,  $n=3$ .

501



502

503

**Figure 3. Recombinant K8.1 expression in virus producing cells reverts the cell-specific**

504

**defect in infectivity of KSHVΔK8.1.** A) Infection of SLK, HaCaT, A549, and 293T. HaCaT were

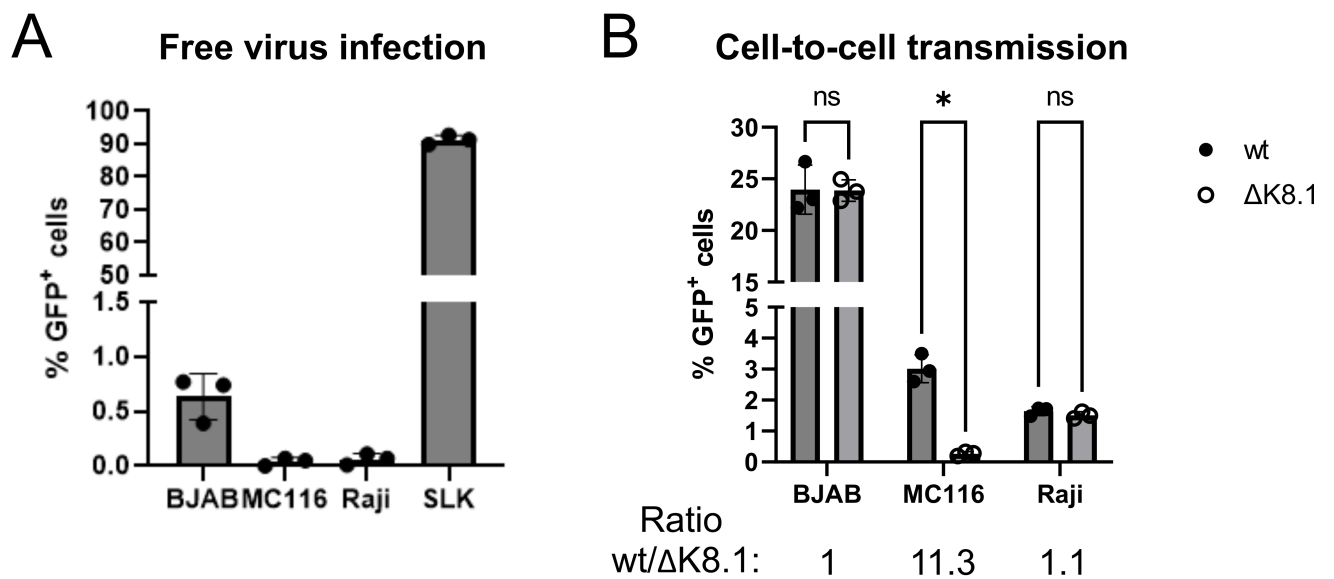
505

infected by spin-infection. Error bars represent the standard deviation of two experiments, each

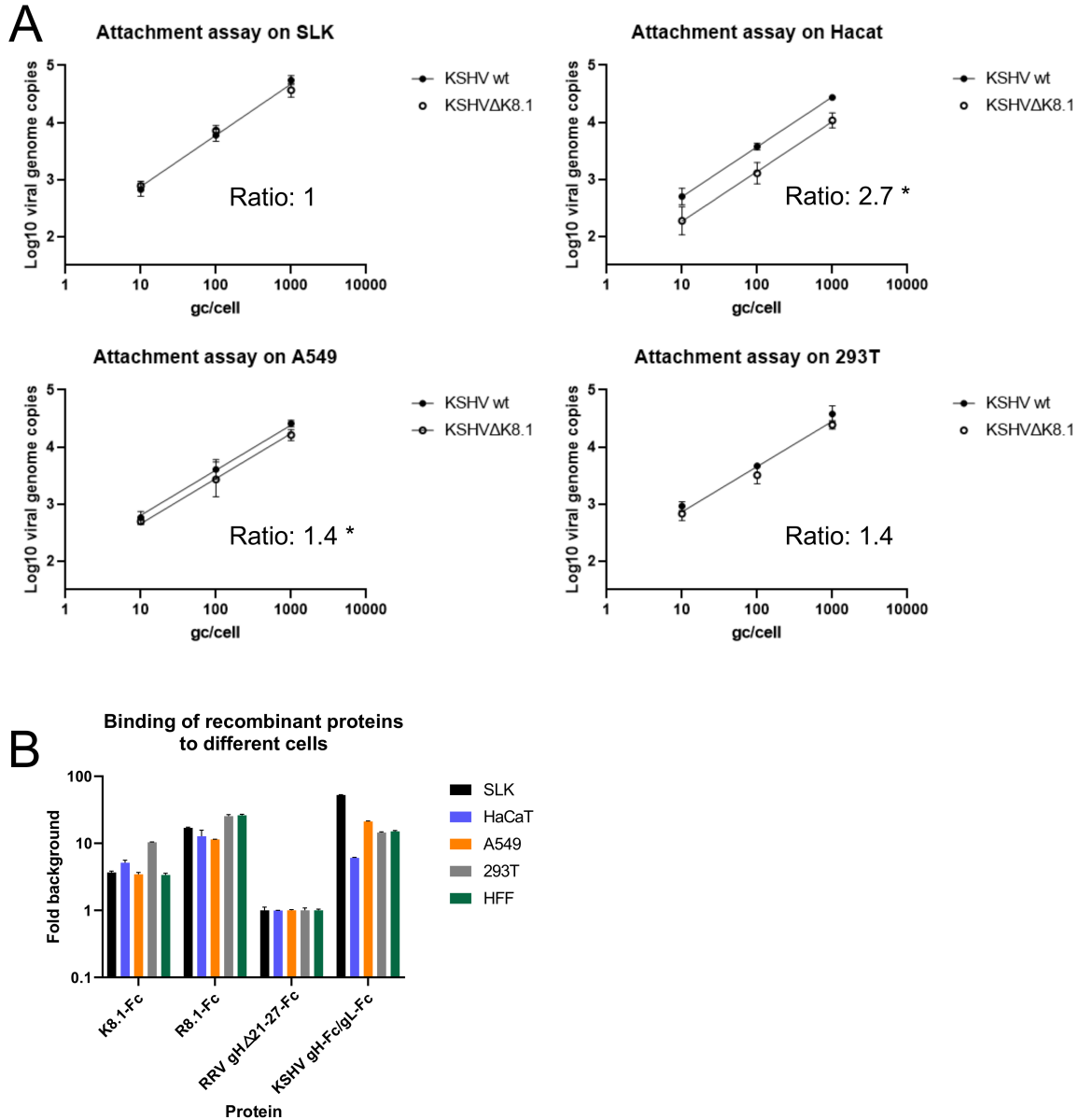
506

performed in triplicates. B) 50% infectious titers were calculated from the data in (A) using curve

507 fitting as described in the methods section for each of the two independent experiments, log-  
 508 transformed, and analyzed by Two-Way ANOVA with Sidak's correction for multiple  
 509 comparisons (n=2, \* = p<0.05; \*\* = p<0.01. C) Western blot analysis of virus preparations from  
 510 chemically induced iSLK producer cells harboring BAC16 KSHV $\Delta$ K8.1 or BAC16 KSHV wt,  
 511 transduced with empty vector (eV) or a lentiviral vector coding for K8.1 tagged with a C-terminal  
 512 tandem StrepTag (K8.1).  
 513



514  
 515 **Figure 4. KSHV $\Delta$ K8.1 exhibits a defect in cell-to-cell transmission into the MC116 B cell**  
 516 **line.** A) Cell-free infection of B cell lines with KSHV is inefficient. Cell lines were infected with the  
 517 same KSHV BAC16 wt virus stock under comparable conditions. B) iSLK cells carrying the BACs  
 518 coding for the respective viruses were chemically induced to enter the lytic cycle and were co-  
 519 cultured with the indicated target cells. Infection was determined after 4 days by flow  
 520 cytometry. Error bars represent the standard deviation; \* p<0.05, Two-Way ANOVA with Sidak's  
 521 correction for multiple comparisons, n=3.



523

524

**Figure 5. KSHVΔK8.1 exhibits reduced attachment on HaCaT cells.** A) SLK, HaCaT, A549

525

or 293T cells were spin-infected at 4° C with the indicated amounts of virus and bound viral DNA

526

was quantified by qPCR. Binding curves for KSHV wt and KSHVΔK8.1 were indistinguishable on

527

SLK but significantly different for HaCaT and A549. (Non-linear fit for semi-log data by least

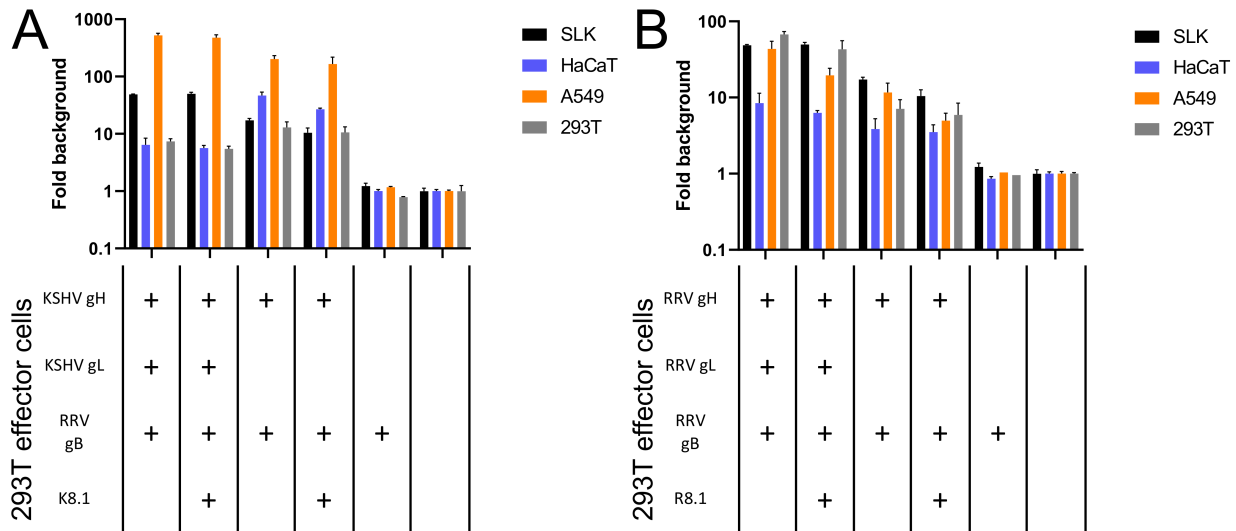
528

squares regression using mean values for each data point, slope shared between curves, ratio

529

calculated from Y-intercept; two curves and \* indicate significant difference with p<0.05; n=6) B)

530 Binding of Fc-fusion proteins K8.1-Fc, gH-Fc/gL, R8.1-Fc or RRV gHΔ21-27-Fc  
 531 (control/background) to the indicated cells was measured by flow cytometry. Error bars  
 532 represent the standard deviation, n=3.  
 533



534  
 535 **Figure 6. Presence or absence of K8.1 or its RRV homolog do not appreciably modulate**  
 536 **gH/gL-activated membrane fusion by gB.** A) KSHV gH/gL together with RRV gB or KSHV gH  
 537 together with RRV gB were expressed in the presence or absence of K8.1 on 293T effector cells,  
 538 and these cells were co-cultured with the indicated target cell populations. Error bars represent  
 539 the standard deviation of triplicate samples. B) RRV gH/gL together with RRV gB or RRV gH  
 540 together with RRV gB were expressed in the presence or absence of R8.1 on 293T effector cells,  
 541 and these cells were co-cultured with the indicated target cell populations. Error bars represent  
 542 the standard deviation of triplicate samples.

543  
 544  
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551

## 552 **DECLARATION OF INTEREST**

553 The authors declare no competing interests. Alexander Hahn is also an employee of GSK, which  
554 did not have any influence on this study.

555

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