



HAL
open science

Human herpesvirus 8 molecular mimicry of ephrin ligands facilitates cell entry and triggers EphA2 signaling

Taylor P. Light, Delphine Brun, Pablo Guardado-Calvo, Riccardo Pederzoli, Ahmed Haouz, Frank Neipel, Felix Rey, Kalina Hristova, Marija Backovic

► **To cite this version:**

Taylor P. Light, Delphine Brun, Pablo Guardado-Calvo, Riccardo Pederzoli, Ahmed Haouz, et al.. Human herpesvirus 8 molecular mimicry of ephrin ligands facilitates cell entry and triggers EphA2 signaling. 2021. pasteur-03388352v1

HAL Id: pasteur-03388352

<https://pasteur.hal.science/pasteur-03388352v1>

Preprint submitted on 14 Jun 2021 (v1), last revised 20 Oct 2021 (v2)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

Human herpesvirus 8 molecular mimicry of ephrin ligands facilitates cell entry and triggers EphA2 signaling

Taylor P. Light¹, Delphine Brun², Pablo Guardado-Calvo², Riccardo Pederzoli², Ahmed Haouz³, Frank Neipel⁴, Felix Rey², Kalina Hristova^{1*}, Marija Backovic^{2*}

¹ Department of Materials Science and Engineering and Institute for NanoBioTechnology, John Hopkins University, Baltimore, Maryland, USA

² Unité de Virologie Structurale, Institut Pasteur, Département de Virologie, 28 rue du Dr Roux, 75015 Paris, France; CNRS, UMR3569, Paris, France.

³ Crystallography Platform C2RT, Institut Pasteur, 25 rue du Dr. Roux, Paris 75015, Paris, France; CNRS UMR 3528

⁴ Virologisches Institut, Universitaetsklinikum Erlangen, Schlossgarten 4, D-91054 Erlangen, Germany.

*Corresponding authors

23 ABSTRACT

24 Human herpesvirus 8 (HHV-8) is an oncogenic virus that enters cells by fusion of the viral and
25 endosomal cellular membranes in a process mediated by viral surface glycoproteins. One of the cellular
26 receptors hijacked by HHV-8 to gain access to cells is the EphA2 tyrosine kinase receptor, and the
27 mechanistic basis of EphA2-mediated viral entry remains unclear. Using X-ray structure analysis,
28 targeted mutagenesis and binding studies, we here show that the HHV-8 envelope glycoprotein
29 complex gH/gL binds with sub-nanomolar affinity to EphA2 via molecular mimicry of the receptor's
30 cellular ligands, ephrins, revealing a pivotal role for the conserved gH residue E52 and the amino-
31 terminal peptide of gL. Using FSI-FRET and cell contraction assays, we further demonstrate that the
32 gH/gL complex also functionally mimics ephrin ligand by inducing EphA2 receptor association via its
33 dimerization interface, thus triggering receptor signaling for cytoskeleton remodeling. These results
34 now provide novel insight into the entry mechanism of HHV-8, opening avenues for the search of
35 therapeutic agents that could interfere with HHV-8 related diseases.

36 INTRODUCTION

37

38 Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated virus, is a member
39 of *Rhadinovirus* genus that belongs to the *Gammaherpesvirinae* subfamily of *Herpesviridae* ¹. HHV-8 is
40 an oncogenic virus and etiological agent of Kaposi's sarcoma (KS), malignancy of endothelial cells
41 named after the Hungarian dermatologist who first described the disease in 1872 ². Because KS has
42 different clinical manifestations, two main forms are distinguished - the classic KS that is a relatively
43 indolent and rare tumor, appearing as skin lesions mostly in elderly men, and the epidemic or HIV-
44 associated KS, an aggressive form that spreads extensively through skin, lymph nodes, intestines and
45 lungs ³. The KS affects up to 30% of untreated HIV-positive individuals ^{4,5}, and is nowadays one of the
46 most frequent malignancies in men and children in subequatorial African countries ⁶.

47 Behind the ability of HHV-8 to spread to diverse tissues lies its wide tropism demonstrated *in*
48 *vivo* for epithelial and endothelial cells, fibroblasts, B- and T-lymphocytes, monocytes, macrophages
49 and dendritic cells (reviewed in ⁷). As other herpesviruses, HHV-8 attaches to cells via its envelope
50 glycoproteins that engage in numerous low affinity interactions with ubiquitous cellular factors such
51 as heparan sulfate proteoglycans ⁸. Following these initial contacts, the viral envelope glycoprotein(s)
52 bind to specific cell receptor(s) in an interaction that provides trigger for membrane fusion. The
53 envelope glycoproteins B (gB) and the non-covalent heterodimer made of glycoproteins H and L
54 (gH/gL) constitute the conserved core fusion machinery of all herpesviruses, whose function is to
55 induce merger of the viral and cellular membranes. The release of herpesvirus capsids occurs at the
56 level of plasma membrane or as in the case of HHV-8, after endocytosis, by fusion of the viral and
57 endosomal membranes ⁹. In all herpesviruses gB is the fusogen protein, while gH/gL plays a role in
58 regulation of gB activity ¹⁰. What is particular to HHV-8 is the simultaneous employment of several viral
59 glycoproteins - HHV-8-specific K8.1A glycoprotein, as well as the core fusion machinery components –
60 that engage diverse cellular receptors (gB binds to integrins and DC-SIGN, gH/gL to EphA receptors)
61 increasing the HHV-8 target repertoire and providing the virus with a set of tools for well-orchestrated
62 entry (reviewed in ¹¹).

63 The X-ray structures of gH/gL from alpha-, beta- and gammaherpesviruses ¹²⁻¹⁵, revealed a
64 tightly bound protein complex, in which the gH ectodomain, composed of four domains forms a tight
65 complex with gL via its N-terminal, membrane-distal domain. Despite low gH and gL sequence
66 conservation across the herpesvirus family, the structures are remarkably similar, indicating
67 conservation of a function. The current fusion model posits that upon binding of the virus to the
68 cellular receptor (via gH/gL, gB, or a virus-specific glycoprotein), gH/gL in a still unknown way relays
69 the signal and switches gB fusion activity on, setting membrane fusion in motion ¹⁶. As mentioned
70 above, HHV-8 gH/gL also functions as a receptor binding protein that interacts with cellular Eph

71 receptors that belong to a superfamily of transmembrane tyrosine kinases, named Eph for their
72 expression in erythropoietin-producing human hepatocellular carcinoma cell line ¹⁷.

73 The physiological ligands of Eph receptors are membrane-tethered proteins called ephrins
74 (acronym for Eph family receptor interacting proteins). Ephrin-A ligands are attached to the membrane
75 by glycosylphosphatidylinositol anchors and bind to Eph receptors classified as type-A Eph receptors,
76 while ephrin-B ligands possess a transmembrane and an intracellular PDZ domain and interact with
77 receptors designated as Eph type-B receptors ^{18,19}. There are 5 ephrin-A ligands and 9 Eph type-A
78 receptors in humans. HHV-8 binds with the highest affinity to EphA2, and less to the related EphA4
79 and EphA7 receptors ²⁰⁻²². In addition, the EphA2 receptor serves as a receptor for two other
80 gammaherpesviruses - human herpesvirus 4 (HHV-4 also known as Epstein-Barr virus (EBV) and rhesus
81 monkey rhadinovirus ^{23,24}. The interactions are in all cases established via gH/gL. Unrelated pathogens
82 such as Hepatitis C virus, some bacteria and yeast also depend on EphA2 receptor for entry ²⁵⁻²⁸.

83 Eph receptor-ephrin ligand interactions mediate short-distance cell-cell communications and
84 lead to cytoskeleton rearrangements and rapid changes in cell mobility and / or morphology ²⁹. Some
85 of the typical outcomes of ephrin-A1 ligand activation of EphA2 receptor are cell retraction ³⁰⁻³³ and
86 endocytosis of receptor-ligand complexes ³⁴. These processes are especially active and important
87 during development, and in adulthood many of the same circuits get repurposed for functions in bone
88 homeostasis, angiogenesis, synaptic plasticity (reviewed in ²⁹). Since motility and angiogenesis
89 contribute to tumorigenesis and other pathologies, Eph receptors and ephrin ligands are in the
90 spotlight as targets for therapeutic intervention ^{35,36}.

91 EphA2 was identified as HHV-8 entry receptor by Hahn *et al.* who showed that deletion of the
92 EphA2 gene abolished infection of endothelial cells, and that binding of gH/gL to EphA2 on cells led to
93 increased EphA2 phosphorylation and endocytosis facilitating viral entry. The presence of the
94 intracellular kinase domain was shown to be important for HHV-8 entry in epithelial 293 cells ²⁰. In
95 this respect, HHV-8 gH/gL does not play the role of a classical herpesvirus receptor binding protein,
96 directly activating gB upon binding to a cellular receptor to induce fusion of the viral and plasma
97 membranes, such as gD in alphaherpesviruses. The merger of the HHV-8 and cellular membranes
98 occurs at low pH within an endosome, and is spatially and temporally separated from the gH/gL
99 interactions with EphA2 that occur at the cell surface. HHV-8 gH/gL instead activates EphA2 receptors
100 that initiate signaling pathways leading to rapid internalization of the virus and cytoskeletal
101 rearrangements that create a cellular environment conducive for the virus and capsid intracellular
102 transport ³⁷.

103 All Eph receptors contain an elongated ectodomain made of – as beads on a string - a ligand-
104 binding domain (LBD), a cysteine-rich domain (CRD) and two fibronectin (FN) domains, followed by a
105 transmembrane anchor, a short juxtamembrane region containing several conserved tyrosine
106 residues, an intracellular Tyr kinase domain, a sterile alpha motif (SAM) that has a propensity to

107 oligomerize, and a PDZ domain involved in protein-protein interactions³⁸. The LBD consists of a rigid,
108 conserved β -sandwich, decorated with variable loops that dictate ligand specificity^{39,40}. In a similar
109 fashion, the protruding flexible loops of ephrin ligands are arranged around a compact, eight-stranded
110 central β -barrel⁴¹. We employ the accepted nomenclature for the secondary structure elements for
111 Eph receptors⁴² and ephrin ligands throughout the text⁴¹. In both cases single letters designate β -
112 strands and helices, and double letters are used to label loops that connect the secondary structure
113 elements (Fig. S1). The ephrin ligand interactions with the Eph receptors are driven by an 18-residue
114 long and mostly hydrophobic loop that connects strands G and H – the GH loop – of the ligand, which
115 inserts into a complementary hydrophobic cavity presented at the surface of the receptor LBD⁴². The
116 ephrin ligand GH loop carries a conserved E119 that establishes polar interactions, critical for high
117 affinity binding, with a strictly conserved R103 on the loop of the Eph type-A receptors. This R103
118 happens to be in the loop connecting strands G and H in EphA receptors and is also designated as GH
119 loop⁴³. To avoid confusion, we use superscripts to indicate the molecule of the residue or feature
120 described (R103^{EphA2}, E119^{ephrin}, GH^{EphA2}, GH^{ephrin} etc).

121 At the molecular level, as in the case of other receptor tyrosine kinases, ephrin ligand binding
122 induces oligomerization of Eph receptors, promoting trans-phosphorylation and signal transduction
123 into the cell¹⁹. Structural and functional studies revealed that ephrin ligand binding to Eph receptors
124 results first in formation of so-called tetramers made of two ‘Eph-ephrin’ complexes in which each Eph
125 receptor interacts with 2 ephrin molecules – its cognate ligand with high affinity, and via low affinity
126 interactions with ephrin from the other complex¹⁸ (Fig. S2). In such tetrameric arrangement that is
127 brought about by ligands, two Eph receptors are arranged into a dimer (also known as homodimer),
128 stabilized by contacts at the EphA2 LBD dimerization interface (DIN). Eph-ephrin tetramers can
129 polymerize into clusters via the interface in the downstream CRD referred to as the clustering interface
130 (CIN)⁴⁴⁻⁴⁶. Since the clustering involves an EphA2 region outside of the LBD, this interface is known as
131 ligand independent. Indeed EphA2 receptor was shown to exist on cells in the absence of ligand in a
132 monomer-dimer equilibrium⁴⁷, with the unliganded dimers stabilized via the CIN (Fig. S2).

133 The cellular response to EphA2 receptor activation is ligand- and cell-type dependent and
134 modulated by factors such as the spatial distribution of the receptor in the membrane⁴⁸, residues in
135 the intracellular domain that are phosphorylated, size and type of the EphA2 receptor oligomers, to
136 just name some²⁹. EphA2 receptor dimers were shown to be already active⁴⁹, and there is evidence
137 that oligomers made of 6-8 EphA2 receptor molecules activate, while larger aggregates may dampen
138 the signaling in some cell types⁵⁰. Different ligands (monomeric, dimeric ephrin-A1, and agonist or
139 antagonist peptides) were shown to stabilize distinct dimeric or oligomeric EphA2 receptor assemblies
140 (Fig. S2), further indicating that the signaling properties may be defined by the nature of the EphA2
141 dimers and oligomers^{47,51}.

142 The structures of several Eph receptor – ephrin ligand complexes have been determined
143 ^{39,40,42,44,45}, but how viral antigens such as HHV-8 gH/gL interact with EphA2 was completely unknown
144 until recently. The 3.2Å X-ray structure of a HHV8 gH/gL-EphA2 complex was published while we were
145 preparing this manuscript ⁵². Our goal has been to explore the events that emulate the early stages of
146 HHV-8 entry. We sought to obtain the structural details on the gH/gL-EphA2 complex, and to determine
147 if and how HHV-8 gH/gL affects assembly of EphA2 receptors in the membranes of living cells, taking
148 advantage of the FSI-FRET system that allows following lateral interactions of membrane proteins *in*
149 *vivo* ⁵³. We report here a 2.7Å resolution X-ray structure of the HHV-8 gH/gL ectodomain bound to the
150 LBD of EphA2 together with results of structure-guided mutagenesis and cell-based studies that
151 highlight E52^{gH} as a key residue for high-affinity binding to the receptor. Based on our analyses and the
152 similarities we observed between the binding modes of gH/gL and the ephrin ligand to EphA2, we
153 provide evidence that this structural similarity extends into functional mimicry - soluble gH/gL induces
154 cell contraction and stabilizes the same type of EphA2 receptor dimers on cells, as was shown for the
155 ephrin-A1 ligand ⁴⁷. This is the first time that a non-ephrin protein in its monomeric form has been
156 shown to activate Eph receptors, underscoring that signaling via EphA2 dimers, and not large
157 oligomers, may be more common than thought. The results presented here now lay a path for further
158 exploration of downstream events and the investigation of whether HHV-8 activation of Eph receptors
159 may play a role beyond ensuring a productive infection, for example contributing to virus
160 oncogenicity/oncogenic transformation of the cell.

161 RESULTS

162

163 Structure determination of the EphA2 LBD - gH/gL tertiary complex

164 Recombinant HHV-8 gH/gL ectodomains and EphA2 LBD were expressed in insect cells and the
165 proteins were purified as described in detail in Material and Methods (MM). To maximize the tertiary
166 complex (gH/gL-bound to EphA2 LBD) formation, the gH/gL was mixed with an excess of LBD which
167 was then removed by size exclusion chromatography. Multiangle light scattering measurements⁵⁴
168 demonstrated a 1:1:1 tertiary complex stoichiometry for gL/gH bound to EphA2 LBD, as well as to the
169 EphA2 ectodomain (Fig. S3).

170 The tertiary complex crystallized in an orthorhombic space group ($C 2 2 2_1$) and the crystals,
171 which contained one molecule per asymmetric unit, diffracted to 2.7Å. The initial phases were
172 calculated by molecular replacement using the EphA2 LBD (PDB: 3HEI) and an HHV-8 gH/gL theoretical
173 model derived from the EBV gH/gL X-ray structure (PDB: 3PHF) using the Phyre2 program for protein
174 modelling and structure prediction⁵⁵. The partial molecular replacement solution was extended by
175 iterative cycles of auto- and manual building as explained in MM. The final map displayed clear electron
176 density for residues 27-200 of EphA2, residues 21-128 for gL, and residues 35-696 gH with the
177 exception of several short regions of poor density that precluded unambiguous placement of the
178 polypeptide chain (Fig. 1A). The N-terminus of gL contained two additional residues (Arg and Ser)
179 carried over from the expression vector. Around 40 gL residues at its C-terminus were not resolved
180 and are likely to be disordered as predicted by IUPred2A server^{56,57}. The atomic model was refined to
181 a $R_{\text{work}}/R_{\text{free}}$ of 0.22/24 (Table S1).

182

183 The gH/gL-EphA2 LBD complex structure

184 The tertiary complex forms an extended structure 15 nm long and around 4.6 nm across its
185 widest part, in the gH region (Fig. 1B). EphA2 LBD adopts a jelly-roll fold as originally described³⁹ – its
186 N- and C-termini point in the same direction and away from the gH/gL binding site, consistent with the
187 expected location of the remaining EphA2 domains. Two antiparallel 5-stranded β -sheets pack into a
188 compact β -sandwich, with loops of different lengths connecting the strands. The HI^{EphA2} loop is well
189 ordered and forms the DIN, while the JK^{EphA2} loop, which carries a short J' helix, is not resolved in our
190 tertiary complex structure likely due to its already reported structural plasticity⁴⁰ and / or
191 displacement by gL (Fig.1B). Apart from the JK^{EphA2} loop, the EphA2 LBD does not change conformation
192 upon binding to gH/gL.

193 The gH/gL complex has an architecture already described for other herpesvirus orthologs – the γ -
194 herpesvirus EBV gH/gL¹⁵, β -herpesvirus human CMV¹³, and α -herpesviruses HSV-2, PrV and VZV^{14,58,59}.

195 The N-terminal domain I (DI) of gH is separated by a linker (hinge) helix from the rest of the ectodomain
196 (domains II, III and the membrane-proximal domain IV) (Fig. 1B). The HHV-8 gH/gL resembles the most
197 its EBV counterpart, consistent with the highest sequence conservation between the two, followed by
198 the β -herpesvirus CMV gH/gL complex and less so the α -herpesvirus complexes (Fig. S4). The rmsd
199 values and Z-scores calculated from the superimposition of individual gH domains and gL are given in
200 Fig. S4 (superimposing the entire gH/gL ectodomains is not informative because of the different
201 orientations of the domains with respect to each other). Clear electron density was observed at 4 N-
202 linked glycosylation sites (N46^{gH}, N267^{gH}, N688^{gH}, and N118^{gL}) allowing placement of 1 or 2 N-
203 acetylglucosamine residues.

204

205 Binding interface between gH/gL and EphA2

206 The EphA2 LBD and gH/gL form an intricate interface structure made of a seven-stranded
207 mixed β -sheet containing strands contributed by all 3 proteins. The N-terminal segment of gH co-folds
208 with gL forming a mixed five-stranded β -sheet composed of two gH and three gL β -strands. The third
209 gL β -strand further engages in contacts with the D β -strand of EphA2 (Fig. 2A).

210 gL binds to the EphA2 LBD via its N-terminal segment (residues 21-30) and residues from its
211 β 2 and β 3 strands (Fig. 2A) (the full list of contact residues is given in Table S2 and is represented in
212 Fig. S5). The gL N-terminal segment is restrained by C26 and C27 that form disulfide bonds with C74
213 and C54, respectively. Immediately upstream this anchoring point there is an elongated, hydrophobic
214 'tail' (residues 21-25) that inserts into a hydrophobic channel formed by the EphA2 strands D and E
215 and gL strands β 2 and β 3 (the 'roof'). The buried surface area for the gL residues 19-32 is around 480
216 \AA^2 . Of the 14 hydrogen bonds formed between gL and EphA2, 7 are contributed by the gL N-terminal
217 segment, 6 by strand β 3 and 1 by the C-terminal η 4 Asn128.

218 Below the gL 'tail', the single gH residue that makes contacts with EphA2 – E52^{gH} – forms a salt
219 bridge with R103^{EphA2}, clamping the bottom of the tunnel (the 'base'). E52^{gH} is also involved in polar
220 interactions with residues V22^{gL}, H47^{gL} and F48^{gL}, thus being a center point (a hub) interlaying gL and
221 EphA2 (Table S2).

222

223 Biolayer Interferometry (BLI) analyses of EphA2 and gH/gL interactions in solution

224 To investigate the role of the gH/gL and EphA2 residues implicated in the interactions observed
225 in the crystal structure, we tested a series of mutants that were conceived to induce large
226 perturbations into gL and EphA2 by introducing N-glycosylation sites. We resorted to such drastic
227 changes because most EphA2 point mutations already tested in immunoprecipitation assays had only
228 moderate effects on gH/gL binding⁶⁰. The point mutation R103A^{EphA2} has been reported to abolish the

229 binding to gH/gL⁵² and served as a positive control. Since E52^{gH} is the only gH residue contacting EphA2,
230 we also introduced point mutations E52A^{gH} and E52R^{gH} to specifically target this site.

231 The variants with the following N-glycosylation sites were generated: N57^{EphA2}, N190^{EphA2},
232 N30^{gH} or N68^{gH}, and point mutant variants R103A^{EphA2}, E52A^{gH} bound to gL (E52A^{gH}/gL) and E52R^{gH}/gL
233 (Fig. 2B). The recombinant proteins were expressed in mammalian cells. The introduced sites N30^{gH}
234 and N68^{gH} were glycosylated as clearly observed by gL shift to a higher molecular weight on SDS-PAGE
235 gels, while the change in the migration was harder to detect for EphA2 ectodomains possibly because
236 its larger size and small difference introduced by an additional glycosylation. The N190^{EphA2} mutation
237 was already reported to perturb the interactions with ephrin ligands⁴⁴. The constructs were
238 engineered so that gL contained a strep tag for complex purification, as before, and gH contained a
239 histidine tag at C-terminus for immobilization onto BLI sensors via the end distal to the EphA2 binding
240 site (Fig. S6). Further details on protein production and BLI parameters are given in MM.

241 We determined the dissociation constant (Kd) <1nM by doing a series of BLI measurements
242 for wild type (WT) gH/gL binding to the EphA2 ectodomain (res. 27 - 534) or its LBD (res. 27-202) (Fig.
243 2C). The low Kd observed for the WT proteins was dominated by a slow k_{off} rate. We obtained a Kd in
244 the subnanomolar range when the measurements were done at pH 5.5 (Fig. S7A), or when the system
245 was inverted i.e. EphA2 LBD or ectodomains were immobilized via a histidine-tag to the sensor, and
246 gH/gL was in solution (Fig. S7B).

247 Each of the 3 mutations introduced in EphA2 significantly reduced the binding as anticipated
248 (Fig. 2D). The Q30N^{gH} mutation in the gL N-terminal segment also diminished binding, consistent with
249 the presence of a carbohydrate at this position blocking the interactions with the strand D^{EphA2} and
250 DE^{EphA2} loop. Introduction of the N-linked carbohydrate at residue N68^{gH} in its β 2- β 3 turn did not affect
251 binding as expected, because of its location proximal to the binding site but in an exposed loop (Fig.
252 2B). The E52R^{gH}/gL and E52A^{gH}/gL variants resulted in weaker or absence of interactions with EphA2
253 ectodomains, respectively (Fig. 2D). These results demonstrated that the binding interface between
254 EphA2 and gH/gL seen in the crystal is in agreement with the one mapped by measurements in
255 solution.

256

257 The gH/gL molecular mimicry of ephrin-A ligands

258 The EphA2 binding site for ephrin-A1 ligand and gH/gL largely overlap, with the former
259 including a more extensive surface area and a larger number of contacts established by EphA2 β -
260 strands D and E, CD and DE loops, as well as the LM loop (Fig. 3, Fig. S1 and S5). Comparative analyses
261 of the gH/gL-EphA2 and ephrin-A1-EphA2 complexes further demonstrate that the structural elements
262 employed by gH and gL resemble the ephrin-A1 ligand mode of binding to EphA2 receptor. The GH^{ephrin-}
263 A1 loop, which is the principal interaction region with the EphA2 receptor, occupies the same space as

264 the gL ‘tail’, while the salt bridge established between the conserved R103^{EphA2} and E119^{ephrin-A1} is
265 replaced at the same location by a salt bridge between R103^{EphA2} and E52^{gH} (Fig. 3). The conserved
266 E52^{gH} and E119^{ephrin-A1} occupy equivalent position in respect to R103^{EphA2}, but the chain segments
267 carrying the conserved E52^{gH} and E119^{ephrin-A1} run in opposite directions so that the following residues,
268 F53^{gH} and F120^{ephrin-A1}, do not superpose. Both F53^{gH} and F120^{ephrin-A1} are engaged in π - π stacking
269 interactions with F108^{ephrin-A1} and H53^{gL}, respectively; in addition, the F108^{EphA2} establishes π - π
270 interactions with Y21^{gL} or F111^{ephrin-A1}, indicating a common mechanism for stabilization of the loop
271 that presents the critically important glutamic acid residue for interactions with EphA2.

272

273 HHV-8 gH/gL induces constitutive EphA2 dimerization on the cell surface

274 Since binding of ephrin ligands to Eph receptors induces formation of higher-order receptor
275 oligomers, we sought to determine if gH/gL alters EphA2 interactions at the cell surface in a similar
276 fashion. The method we applied was developed to probe the stability and association (stoichiometry)
277 of protein complexes in cell membranes, and is referred to as FSI-FRET (Fully Quantified Spectral
278 Imaging (FSI) Förster Resonance Energy Transfer (FRET))⁵³. The measurements are carried out on the
279 membranes of live cells containing the proteins of interest tagged with donor or acceptor fluorescent
280 probes at the intracellular end. The lateral interactions of EphA2 molecules in the absence and
281 presence of various ligands were already investigated using this approach⁴⁷.

282 We performed FSI-FRET measurements in HEK293T cells co-transfected with EphA2-
283 mTurquoise (donor probe) and EphA2-eYFP (acceptor probe). Recombinant HHV-8 gH/gL was added
284 at the final concentration of 200 nM, significantly exceeding the apparent subnanomolar K_d value (Fig.
285 2C), thus ensuring that all the EphA2 molecules were occupied by gH/gL. The measured FRET
286 efficiencies (corrected for “proximity FRET” as discussed in MM (equation 1), and the concentration of
287 donor-tagged and acceptor-tagged EphA2 molecules) were used to construct dimerization curves by
288 fitting with a monomer-dimer equilibrium model⁵³ (the raw FRET data is shown in Fig. S8). Dimer
289 formation is characterized by two parameters: the two-dimensional dissociation constant, K_{diss} , and
290 the structural parameter “Intrinsic FRET”, \tilde{E} . The K_{diss} is a measure of the dimerization propensity of
291 EphA2 at the plasma membrane. The Intrinsic FRET is the FRET efficiency in an EphA2 dimer with a
292 donor and an acceptor, which depends on the positioning of the fluorescent proteins (attached to the
293 C-terminus of the intracellular domain) of the EphA2 dimer. The Intrinsic FRET is strictly a structural
294 parameter and therefore does not have any implications on the dimerization propensity of the full
295 length EphA2 receptor [64, 65].

296 The dimerization curve calculated from the FRET data for EphA2 WT in the presence of gH/gL
297 is shown in Fig. 4A and is compared to the data for EphA2 WT in the absence of ligand. The best-fit K_{diss}
298 and the best-fit Intrinsic FRET, determined from the FRET data, are presented in Table 1. As previously

299 reported⁵¹, EphA2 WT in the absence of ligand exists in monomer-dimer equilibrium with a K_{diss} is 301
300 ± 67 receptors/ μm^2 . We found EphA2 in the presence of gH/gL to be 100% dimeric (“constitutive
301 dimer”) over the EphA2 concentration range observed in the experiments, precluding reliable
302 measurement and calculation of the dissociation constant. In control experiments, we added soluble
303 EphA2 LBD and also precomplexed gH/gL with EphA2 LBD (gH/gL-LBD) (Fig. 4B, C). As anticipated,
304 soluble LBD had no effect on EphA2 dimerization and the effect of gH/gL was also abolished when
305 precomplexed with EphA2 LBD, as the dimerization curves and the best-fit K_{diss} value were
306 indistinguishable from those determined in the absence of gH/gL (Table 1).

307 Along with K_{diss} , which measures the strength of the EphA2 association, these experiments give
308 information about conformational changes that affect the relative disposition of the fluorescent
309 proteins attached to the C-termini of EphA2, inside the cell. This information is contained in the
310 structural parameter “Intrinsic FRET”. A lower Intrinsic FRET value is observed upon gH/gL binding,
311 reflecting that the distance (d) between the fluorescent proteins is greater when gH/gL is bound to
312 EphA2. Since the fluorescent proteins are attached to the C-termini of EphA2 via flexible linkers, this
313 is a demonstration of a structural change in the EphA2 dimer induced by gH/gL binding, which is
314 transmitted across the membrane to the intracellular domains, involving an apparent increase in the
315 separation between the C-termini of EphA2. This implies that the conformation of the intracellular
316 domains in the EphA2 dimer are altered in response to gH/gL binding. The presence of soluble EphA2
317 LBD and precomplexed gH/gL-LBD had no effect on the Intrinsic FRET values (Table 1).

318 Since FRET has limited utility in discerning the oligomer size, we used Fluorescence Intensity
319 Fluctuation (FIF) to directly assess the oligomer size of EphA2 in the presence of gH/gL. FIF calculates
320 molecular brightness of eYFP-tagged receptors in regions of the cell membrane. The molecular
321 brightness, defined as the ratio of the variance of the fluorescence intensity within a membrane region
322 to the mean fluorescence intensity in this region, is known to scale with the oligomer size⁶¹. The
323 cumulative (over all measured EphA2 concentrations) distributions of molecular brightness for EphA2
324 and EphA2 with gH/gL obtained from small sections of the plasma membrane in hundreds of cells are
325 compared in Fig. 4D. Consistent with the fact that EphA2 exists in a monomer/dimer equilibrium in the
326 absence of ligand⁵¹, the EphA2 brightness distribution is between the distributions of LAT (Linker for
327 Activation of T-cells, a monomer control)⁶² and E-cadherin (a dimer control)⁶³. The FIF data for these
328 controls have been published previously⁶⁴ and are shown here for comparison (Fig. 4D). We found
329 that gH/gL shifts the maximum of the histogram to higher molecular brightness relative to EphA2
330 (untreated), such that it virtually overlaps with the dimeric E-cadherin distribution. Therefore, the FIF
331 measurements indicate that EphA2 is a constitutive dimer in the presence of gH/gL, consistent with
332 the FRET data. We see no indication for the formation of higher order oligomers, which would have
333 resulted in a brightness distribution shifted to higher values than the ones measured for E-cadherin.

334 Taken together, the FRET and FIF data demonstrate that gH/gL significantly stabilizes EphA2 dimers
335 but does not induce EphA2 oligomerization.

336

337 Residues E52^{gH} and R103^{EphA2} are critical for EphA2 dimerization on cells

338 To test the importance of residue E52^{gH} for binding of gH/gL to EphA2 in native membranes,
339 FSI-FRET experiments were also performed with the E52R^{gH}/gL recombinant protein, which exhibited
340 significantly reduced binding to the soluble EphA2 ectodomains in BLI experiments (Fig. 2D). The
341 dimerization curve for EphA2 WT in the presence of E52R^{gH}/gL is shown in Figure 5A and the fit
342 parameters in Table 1. Constitutive EphA2 receptor dimerization was not observed as we did with
343 gH/gL. Rather, EphA2 interactions were reduced to levels similar to the case of no ligand, indicating
344 that the presence of E52R^{gH}/gL did not result in EphA2 dimer stabilization. This is consistent with the
345 finding that the binding of this E52R^{gH}/gL variant to EphA2 was disrupted, and/or with the idea that
346 bound E52R^{gH}/gL did not enhance dimer stability. However, the measured Intrinsic FRET was slightly
347 increased, as compared to no treatment, suggesting a decrease in the separation between the
348 attached fluorescent proteins, and thus between the C-termini of EphA2. This effect could be due to
349 structural perturbations in the EphA2 dimer in response to possible E52R^{gH}/gL binding at the high
350 E52R^{gH}/gL (200nM) concentrations used, which could have propagated to the intracellular domain of
351 EphA2.

352 In addition, we sought to test the importance of the residue R103^{EphA2} for binding to gH/gL
353 using the FSI-FRET method. The dimerization curve when the cells were transfected with EphA2
354 harboring the R103E mutation in the gH/gL binding site, in the presence of saturating gH/gL
355 concentrations, is shown in Fig. 5B, and the fit parameters are shown in Table 1. The dimerization
356 propensity for R103E^{EphA2} variant in the presence of gH/gL is the same as for EphA2 in the absence of
357 ligand (Table 1), indicating that either gH/gL binding to the R103E^{EphA2} mutant is disrupted, as also seen
358 in the BLI experiments, and/or that binding did not lead to dimer stabilization. These data further
359 corroborate our findings that R103^{EphA2} plays an essential role in gH/gL binding. Here again, we
360 observed an increase in the Intrinsic FRET, which indicates that the fluorescent proteins are in closer
361 proximity, as compared to EphA2 WT in the absence of ligand (Table 1). Similar to the behavior of the
362 E52R^{gH}/gL variant, this effect could be a consequence of R103A^{EphA2} binding to gH/gL, at the high gH/gL
363 concentrations used, that would be transmitted to the EphA2 intracellular domains.

364

365 HHV-8 gH/gL induced EphA2 dimers on cell surface are stabilized via the 'dimerization'
366 interface

367 We showed that when bound to gH/gL, EphA2 is a constitutive dimer. To determine if the
368 EphA2 dimers form via one of the already described interaction surfaces – the dimerization (DIN) or

369 clustering interface (CIN), as reported previously⁴⁷ (Fig. S2), we transfected HEK293T cells with the
370 EphA2 variants with perturbed dimerization (G131Y) or clustering (L223R/L254R/V255R) interfaces
371 and treated them with soluble gH/gL ectodomains. The binding of these variants to gH/gL in solution,
372 as measured by BLI, was not affected by the mutations, all of which reside outside of the gH/gL binding
373 site (Fig. S2). The dimerization curves and FRET efficiencies for these mutants in the presence of gH/gL
374 are shown in Fig. 5 and Fig. S8, respectively, with the fit parameters listed in Table 1. We observed a
375 significant decrease in the dimerization due to the G131Y^{EphA2} mutation (Table 1; Fig. 5C), and a small
376 effect due to the L223R/L254R/V255R^{EphA2} mutations (Table 1; Fig. 5D). Thus, G131^{EphA2} plays an
377 important role in the stabilization of EphA2 dimers bound to gH/gL, implying that the EphA2 dimers
378 are formed via DIN. This also supports our finding that gH/gL induces EphA2 dimers and not higher
379 order oligomers, as these oligomers are known to engage both the DIN and the CIN (Fig. S2).

380

381 HHV-8 gH/gL binding to EphA2 expressed on cells induces cell contraction

382 The interactions between Eph receptors and ephrin ligands is known to stimulate cell
383 contraction, a signaling response that plays a role in developmental processes including axon guidance
384 and tissue patterning⁶⁵. To test if the recombinant gH/gL proteins induce similar effects, we performed
385 the assays in HEK293T cells, which express very low amounts of EphA2 (generally below the Western
386 blot detection limit⁴⁷). Therefore, we generated a HEK293T cell line that stably expressed EphA2, and
387 measured cell contraction induced by gH/gL and the E52R^{gH}/gL variant that bound weakly to EphA2
388 (Fig. 2D). The EphA2 LBD, alone or precomplexed with gH/gL (gH/gL-LBD) was used as negative control,
389 and dimeric ephrin-A1-Fc as a positive control (described in the MM section). Untransfected HEK293T
390 cells were treated with gH/gL in control experiments. The cells were fixed with paraformaldehyde
391 (PFA), permeabilized, stained for actin and imaged (representative images are displayed in Fig. 6A).
392 Histograms showing the mean cell area measured for each condition show that EphA2-HEK293T cells
393 stimulated with gH/gL were significantly smaller in size compared to every other condition except for
394 cells stimulated with dimeric ephrin-A1-Fc (Fig. 6B). This demonstrates that gH/gL triggered
395 downstream signaling through EphA2, reminiscent to the ephrin-A1 induced signaling. Notably,
396 untransfected HEK293T cells stimulated with gH/gL were also smaller than cells that were not
397 stimulated, but not to the same extent as cells that stably express low levels of EphA2. When
398 stimulated with E52R^{gH}/gL, EphA2-HEK293T cells were slightly smaller than untreated cells, but not to
399 the same extent as gH/gL-stimulated cells. The mean cell area determined for EphA2-HEK293T cells in
400 response to E52R^{gH}/gL was not statistically different from the area of untransfected HEK293T cells
401 stimulated with gH/gL. Little to no differences in cell area are observed in EphA2-HEK293T cells upon
402 incubation with EphA2 LBD or with the preformed gH/gL-LBD complex.

403 To corroborate our findings in the fixed cells and exclude possible artifacts induced by PFA
404 fixation, we performed a cell contraction assay without fixation using live HEK293T cells transiently

405 transfected with full-length EphA2-eYFP. Soluble recombinant ectodomains of gH/gL, EphA2 LBD, or
406 the gH/gL-LBD complex were added to the media. As in the fixed cell contraction assay, significant live
407 cell area reduction was observed only when free gH/gL was added (Fig. 6C, D). These studies confirm
408 that gH/gL mimics ephrin-A1 binding and signaling through EphA2.

409 Discussion

410 The structure of the HHV-8 gH/gL complex bound to EphA2 LBD revealed two major gL
411 elements important for binding - the N-terminal segment (res 22-30) and strands β 2 and β 3, the latter
412 forming a mixed β -sheet with the EphA2 LBD (Fig. 2A). The gL residues form extensive van der Waals
413 contacts and 14 hydrogen bonds with the LBD in total (Table S2A). Sequence alignment of gL from the
414 gammaherpesvirus family shows preference for hydrophobic residues (Ala, Ile, Val) in the N-terminal
415 segment (Fig. S10) consistent with the constraints imposed by packing of these side chains within the
416 'channel' formed by the hydrophobic residues from the EphA2 and gL β strands ('roof') (Fig. 2). In a
417 cell-cell fusion assay, Su *et al.* reported that gH/gL from other gammaherpesvirus genera, the bovine
418 Alcelaphine gammaherpesvirus 1 (AIHV-1) from the *Macavirus* genus, and Equid gammaherpesvirus 2
419 (EHV-2) from the *Percavirus* genus, bind to human EphA2 to trigger fusion, and suggested a potential
420 for the spillover of animal herpesviruses to humans⁵². They also demonstrated that this was not the
421 case for the gH/gL from murid herpesvirus 4 (MHV68), which with HHV-8 belongs to the *Rhadinovirus*
422 genus. We performed comparative sequence analyses and found that the N-terminal segment of
423 MHV68 gL contains a number of charged residues (NH₃⁺-KILPKHCC...), which could preclude it from
424 fitting into the human EphA2 binding 'channel'. The same is true for gL from another rodent
425 herpesvirus, cricetid gammaherpesvirus 2, whose N-terminus (NH₃⁺-IIGSFLARPCC) also contains a
426 charged residue (Fig. S10), and we predict that this gH/gL complex would have weak binding affinity
427 for human EphA2 as well. We therefore propose that the amino acid composition of the N-terminal gL
428 segment can serve as a predictor for the potential for binding to human EphA2, and that the presence
429 of charged or polar amino acids would weaken or abrogate the binding.

430 The C-terminal gL segment (residues 129-167) was not resolved in the structure, likely because
431 the residues reside within a flexible region that seems to point away from the complex and is not
432 involved in contacts neither with gH nor EphA2. This finding is consistent with the co-
433 immunoprecipitation experiments done with HHV-8 gH co-expressed with the gL Δ 135-164 variant,
434 which could still form a complex with gH and EphA2⁶⁶. This segment is absent from the EBV gL (Fig.
435 S10) indicating a possibly virus-specific function.

436 Based on a series of BLI measurements we determined the K_d for WT HHV-8 gH/gL and EphA2
437 ectodomains to be in subnanomolar range. Decreasing the pH to 5.5, the endosomal pH at which the
438 viral and endosomal membranes fuse to release the HHV-8 capsids into the cytoplasm⁶⁷, did not affect
439 the K_d (Fig S7A), suggesting that gH/gL dissociation from EphA2 is not required for fusion. This would
440 imply that gH/gL bound to EphA2 could still activate gB, or that there is a fraction of unliganded gH/gL
441 that could interact with gB. The subnanomolar K_d value was obtained when the entire EphA2
442 ectodomain instead of the LBD alone was used, demonstrating that the EphA2 binding site for gH/gL

443 resides in the LBD and that gH/gL does not interact with the downstream EphA2 domains. Higher
444 dissociation constants for HHV-8 gH/gL and EphA2 of 3 or 9nM (depending on the orientation of the
445 molecules) and 16nM were reported by two other groups, respectively ^{52,60}. The discrepancies might
446 be due to the overestimation of the gH/gL concentration, which was purified via a tag on gH, resulting
447 in the protein preparation that contained gH/gL and free gH ⁶⁰. Su *et al.* measured the affinities by SPR
448 with the gH/gL immobilized to the chips by chemical coupling, procedure that modifies the protein
449 surface and thus likely the binding site in a fraction of gH/gL ⁵². We designed the experiment to be able
450 to separate the gH/gL complex from free gH by purifying the complex via the affinity tag on gL. The
451 gH/gL was then immobilized to the sensors via the C-terminal affinity tag on gH, which is distal to the
452 EphA2 binding site (Fig. S6). We therefore think that our data represent so far the most accurate
453 quantification of the strength of gH/gL EphA2 interactions. The subnanomolar Kd for the WT proteins
454 were obtained also in an inverted system i.e. when the EphA2 ectodomain or LBD were immobilized
455 via affinity tag on its C-terminus and gH/gL was added as analyte (Fig S7B).

456 The residues of gH implicated in interactions with Eph receptors had been identified previously
457 in a mutagenesis study ⁶⁶. In particular, E52^{gH} and F53^{gH} in HHV-8, and the equivalent residues - E54^{gH}
458 and F55^{gH} in rhesus RRV gH - had been found essential for EphA2 binding *in vitro*²⁴. These residues are
459 well conserved in gH of gamma-herpesviruses ²⁴. The recombinant HHV-8 and RRV viruses carrying the
460 mutations in this gH region were still able to attach to cells and infect them, although with one order
461 of magnitude reduction in efficiency, by being re-targeted to other cellular attachment factors and
462 receptors. We noticed that the gH β -turn (SIELEFNGT) including E52^{gH} and F53^{gH} (underlined), carries
463 resemblance to the motif located within a GH^{ephrin-A1} loop that is the main interaction structural
464 element inserting into the EphA2 cavity (Fig. 3). A conserved glutamic acid residue in the same GH^{ephrin-}
465 ^{A1} loop (E119^{ephrin-A1}) forms salt bridge with the conserved R103 in the GH^{EphA2} loop (Fig. 3B, Fig. S1),
466 which is the most important residue for ephrin binding, as its mutation to glutamic residue entirely
467 abolished the interaction ⁴³. In our structure the R103^{EphA2} forms 2 hydrogen bonds and 1 salt bridge
468 with the E52^{gH}, which is substantially less than the gL contribution (14 HB and extensive buried surface
469 area) (Table S2). In EBV gH/gL the equivalent residue E30^{gH} is located away from the EphA2 binding
470 interface due to a different organization of the N-terminal part of gH compared to the HHV-8 gH,
471 possibly contributing to the reported weaker affinity with a Kd in the μ M range ⁵² (the EBV gH and
472 EphA2 in fact do not form any contacts). To determine to what extent the HHV-8 E52^{gH} influences
473 interactions with EphA2, we performed biophysical (gH/gL and EphA2 ectodomains in solution) and
474 cellular assays (EphA2 full-length membrane bound and gH/gL ectodomains in solution). We produced
475 recombinant gH/gL variants carrying the mutations E52R^{gH} or E52A^{gH} and showed that both had
476 reduced binding to EphA2 ectodomains in solution (Fig. 2D). When the cells expressing full-length
477 EphA2 were treated with the recombinant HHV-8 E52R^{gH}/gL, the changes reported for ephrin ligands
478 ⁶⁸ and induced by the WT gH/gL, such as increased EphA2 dimerization and cell contraction (Fig. 4A,

479 Fig. 6), were not observed. Our data therefore demonstrate that the E52^{BH} is an important molecular
480 determinant for EphA2 binding, further highlighting the gH/gL mimicry with ephrin ligands. While the
481 single structural element of ephrins, the GH loop, engages in polar as well as hydrophobic interactions
482 with EphA2, in HHV-8 gH/gL the same contributions are made by E52^{BH} and the N-terminal segment of
483 gL.

484 EphA2 receptors are present on the cell surface in a dynamic monomer-dimer equilibrium,
485 which shifts towards dimers and dimer-made oligomers as a function of ephrin ligand concentration
486 ^{18,69}. EphA2 signaling oligomers can be stabilized via one of the two opposite interfaces - the DIN and
487 the CIN. In the absence of ligand, the EphA2 dimers are stabilized via CIN ⁴⁷. The initial formation of a
488 tetramer – a pair of EphA2 receptor-ligand complexes - is thought to be driven by the ephrins that
489 behave as bivalent ligands interacting with their EphA2 partner molecule through the high-affinity site,
490 and through a secondary site with the other EphA2 molecule from the same tetramer, holding together
491 the whole assembly (Fig. S2). In this arrangement the EphA2 dimers were reported to be stabilized via
492 the GH^{Epha2} loops (DIN), while the larger clusters that form as the ligand concentration increases are
493 formed via contacts between adjacent CRD interfaces (CIN) (Fig. S2) ^{44,45}. In other words, the
494 association via DIN results in EphA2 dimers, while CIN interactions can result in EphA2 dimers or larger
495 oligomers. (Fig. S2). Our data indicate that once the gH/gL binds, a structural transition is induced and
496 EphA2 dimers are formed via DIN. To explore why EphA2 dimers and not larger oligomers were
497 observed in our experiments, we constructed a model in which unliganded EphA2 ectodomains (PDB
498 2X10) ²⁹ were superimposed onto the EphA2 LBD bound to the gH/gL, preserving the packing of the
499 LBD-gH/gL we observed in the crystal (Fig. S9). This theoretical model reflects the putative
500 arrangement EphA2 receptors expressed on cells would adopt upon binding to gH/gL, and indicates
501 that the CINs would be too far apart to mediate EphA2 clustering, corroborating our ‘gH/gL induced
502 EphA2 dimer’ finding.

503 The EphA2 dimers we observe in the crystal structure and in FSI-FRET studies, formed via the
504 DIN, would presume ligand-driven stabilization resembling the formation of the Eph-ephrin tetramers
505 described above. Analyses of the contacts in our structure show however the gH/gL molecules do not
506 make contacts with the second EphA2 from the DIN-stabilized tetramer, but rather with an EphA2 from
507 the neighboring EphA2 tetramer (Fig. S9). This poses question of how the gH/gL-induced EphA2 dimers
508 are stabilized, and why CIN-stabilized EphA2 dimers bound to gH/gL are not observed on the cell-
509 surface instead. We analyzed the DINs in unliganded EphA2 receptor and in complex with ephrin
510 ligands and HHV-8 gH/gL. When EphA2 is bound to an ephrin ligand, the DIN is stabilized by increased
511 buried surface area and more hydrogen bonds compared to unliganded EphA2 (Table S3). The values
512 obtained for gH/gL induced EphA2 dimerization (16 HBs and 693 Å² interface) are comparable to the
513 stabilization of the same interface when bivalent ephrin ligands are bound, indicating that binding of
514 gH/gL to EphA2 LBD stabilizes the DIN sufficiently even though gH/gL does not seem to behave as a

515 bivalent ligand bridging the two EphA2-gH/gL together. Our model would indicate, although this
516 remains speculative, that gH/gL would preferentially bind to the monomeric EphA2 receptors on cells,
517 shifting the equilibrium between the CIN-stabilized, unliganded EphA2 dimers to monomeric EphA2,
518 which once bound to gH/gL would be stabilized via the DIN.

519 Ephrin ligands are expressed on cells as membrane-bound monomers, and Eph receptor
520 clustering and activation at high level are *in vitro* typically induced by addition of soluble dimeric or
521 pre-clustered ephrin ectodomains that mimic their ephrin high concentration when expressed at the
522 cell surface. Ephrin-A1 ligand was also found as a soluble molecule, being released from cancerous
523 cells by cleavage by cellular proteases. Such soluble, monomeric ephrin (m-ephrin) was demonstrated
524 to be a functional ligand, able to activate the EphA2 receptor by inducing tyrosine phosphorylation ⁷⁰,
525 internalization of EphA2 and cell retraction, overall decreasing the cellular oncogenic potential ⁷¹.
526 These beneficial cellular responses are characterized as the outcome of the so-called canonical Eph
527 receptor activation ⁷². The structural and functional mimicry of HHV-8 gH/gL and ephrin-A1 would
528 suggest that the HHV-8 interactions with EphA2 trigger canonical signaling pathways, consistent with
529 the observed increased endocytosis and overall EphA2 phosphorylation upon virus binding ²⁰, as well
530 as gH/gL induced cell contraction that we report in Fig. 6. Chen *et al.* reported a different outcome
531 when ephrin-A1 ligands were presented to EphA2-expressing cells in a polarized manner; the Src
532 mediated signaling was activated instead, promoting cell motility and malignancy via phosphorylation
533 of serine and not tyrosine residues (the so-called non-canonical EphA2 activation) ⁴⁸. In that light it is
534 interesting that HHV-8 binding to fibroblasts was also reported to result in Src recruitment by androgen
535 receptor, a steroid-activated transcription factor that interacts with the intracellular domain of EphA2.
536 Src activation in this case led to phosphorylation of the S897^{EphA2} in the intracellular EphA2 domain i.e.
537 activation of the non-canonical pathway, which was a prerequisite for HHV-8 infection ⁷³. These
538 conflicting observations raise the question of what type of signaling HHV-8 gH/gL activates to enter
539 the cells. The canonical and non-canonical pathways were thought to be mutually exclusive, but
540 Barquilla *et al.* recently reported that the two can co-exists and that the EphA2 canonical signaling can
541 be rewired in prostate cancer cells by androgenic receptors leading to the phosphorylation of the same
542 S897^{EphA2} residue ³⁰ implicated in HHV-8 entry ⁷³. The authors proposed that this EphA2 reprogramming
543 could have implications for the disease progression. It will be important to discern if a similar interplay
544 of the two seemingly antagonistic EphA2 pathways exist in cells infected with HHV-8. It is also possible
545 that there is a temporal regulation and that different EphA2 signaling pathways are activated during
546 the primary infection (canonical, which would stimulate virus internalization via endocytosis) and
547 reactivation (non-canonical, which would increase cellular oncogenic potential).

548 In this report we present the structure of the HHV-8 gH/gL bound to EphA2, and show that the
549 gH/gL induces dimerization of EphA2 expressed by cells as well as morphological changes at cellular
550 level, resembling the action mode of ephrin ligands. It is possible that the membrane anchored gH/gL

551 at the virion surface could induce EphA2 oligomerization into even larger aggregates, in particular if
552 membrane regions with higher local gH/gL concentration exist in virions, emulating the conditions of
553 high ephrin ligand concentration. What is clear however is that at the mechanistic level HHV-8 gH/gL
554 and ephrin ligands induce formation of the same EphA2 dimers, and that these dimers are already
555 functional leading to cytoskeletal rearrangements and cell contraction. How the structural changes
556 that gH/gL binding initiates are transmitted to the EphA2 intracellular domain, and which types of
557 signaling cascades are elicited and when during the virus life cycle are the questions that need to be
558 addressed next.

559

560 Material and methods

561

562 Protein expression and purification for crystallization

563 **HHV8 gH/gL:** The segments coding the ectodomain of HHV8 gH (residues 26 to 704) and the entire gL
564 (residues 21 – 167) were each cloned from the already described plasmids²⁰ into the pT350 vector⁷⁴
565 for expression in Schneider S2 *Drosophila* cells (S2 cells). The Cys58 on gL was predicted to be unpaired
566 based on the sequence alignment with EBV gL and was mutated to Ser to prevent potential formation
567 of disulphide-linked dimers. The pT350 vector contains inducible metallothionein promoter activated
568 by divalent cations, the exogenous *Drosophila* Bip signal peptide (MKLCILLAVVAFVGLSLG *RS*),
569 underlined, that drives protein secretion appended to the N-terminus of the protein upstream of the
570 5' cloning site. The *RS* are vector residues that code for the *Bgl*III cloning site. A double strep tag (DST)
571 for affinity purification at the C-terminus was added downstream of the 3' cloning site. The sequence
572 of the DST in our pT350 plasmid is FE**DDDDK***AGWSHPQFEK***GGGSGGGSGGG***WSHPQFEK*, where
573 DDDDK is the enterokinase cleavage site and the sequences in bold correspond to the two strep tags
574 separated by a GS linker (italics). The residues FE come from the vector *Bst*BI cloning site.

575 HHV-8 gH can be secreted without being bound to gL⁷⁵, and the complex purification via a gH
576 tag resulted in a mixture of the gH/gL complex and free gH, which were difficult to separate on size
577 exclusion chromatography (SEC) due to the small size difference (gH has a molecular weight of 75 kDa,
578 and gL 20 kDa). This is why it was crucial for gH/gL purification to add the tag for affinity purification
579 only on gL (Fig. 1A). Thus, the gL was cloned to contain the DST at the C-terminus, and gH has a stop
580 codon before the DST. The resulting expression plasmids were named gH^{nt}pT350 and gLC58SstpT350,
581 respectively, where 'nt' and 'st' stand for no tag and strep tag, respectively. Such choice was made to
582 allow affinity purification of the gH/gL complex via gL.

583 It is worth noting that due to the design of the pT350 vector for cloning via restriction digestion
584 the expressed proteins end up having two extra residues *RS* (*Bgl*III site) at the N-terminus. The
585 gLC58SstpT350 and gH^{nt}pT350 both contain the *RS* before the first authentic residues of the mature gL
586 (Y21) and gH (L26).

587 The gH^{nt}pT350 and gLC58SstpT350 plasmids were co-transfected in the S2 cells along with the
588 plasmid encoding puromycin resistance, and the stably transfected cell lines were established by
589 puromycin selection during 3-4 weeks following the previously established protocol⁷⁶. The expression
590 was induced by addition of 0.5mM CuSO₄, and the protein was purified from the supernatant 7-10
591 days post induction. After purification on Streptactin column (IBA Biosciences) and Superdex S200
592 16/60 GL column (GE life sciences), around 1 milligram of the gH/gL heterodimer was obtained per

593 liter of cell culture (the same amount of the aggregated protein was present). SEC running buffer was
594 10 mM Tris, 50mM NaCl pH 8.0.

595
596 **EphA2 LBD:** The EphA2 gene segment that encodes for residues 23-202 was amplified from the vector
597 ²⁰ and cloned into the pT350 vector for expression in S2 cells (EphA2LBDstpT350 construct). The
598 expressed protein contained the DST at the C-terminus. Affinity and SEC purifications were done as
599 described for gH/gL above. Around 10 milligrams of pure EphA2 LBD were obtained from 1 liter of cell
600 culture.

601
602 **Preparation and purification of the trimeric complex EphA2 LBD-HHV8 gH/gL for crystallization:** HHV-
603 8 gH has 14 predicted N-glycosylation sites, gL one and EphA2 LBD none. To increase the probability
604 that the complex would crystallize, the gH/gL complex was enzymatically deglycosylated with
605 recombinant Endoglycosydase D (endo- β -N-acetylglucosaminidase from *Streptococcus pneumoniae*
606 i.e. EndoD ⁷⁷), in 100mM sodium-citrate buffer pH 5.0, 150mM NaCl for 18h at 25°C. The ratio of
607 protein to EndoD was 40 to 1 (w:w). To remove the EndoD and exchange the reaction buffer, the
608 deglycosylated complex was purified on Superdex S200 column in 10 mM Tris, 50mM NaCl pH 8.0, and
609 then mixed with the purified EphA2 LBD in 1:1.3 molar ratio (gH/gL : EphA2 LBD). The complex was
610 incubated at 4°C for 1h to over-night, and then re-purified on Superdex S200 to separate the excess
611 EphA2 LBD. The running buffer in all SEC purifications was 10mM Tris pH 8, 50mM NaCl. The trimeric
612 complex was stable on SEC and presence of gH/gL and the EphA2 LBD in the complex peak was verified
613 by SDS-PAGE gel analysis.

614
615 Crystallization

616 The tertiary complex in 10 mM Tris pH 8, 50 mM NaCl was concentrated to 5.1 mg/ml in Vivaspin
617 concentrators with the 10 kDa cut-off. Crystallization screening was performed at the Institut Pasteur
618 Core facility for crystallization ⁷⁸ by vapor diffusion, in sitting drops of 0.4 μ l containing equal volumes
619 of the protein and reservoir solution. The drops were dispensed in 96-well Greiner plates by a
620 Mosquito robot (TTPLabtech, Melbourn, UK) and images were recorder by a Rock-Imager 1000
621 (Formulatrix, Bedford, MA, USA). Initially, crystals were found in numerous conditions, but none
622 diffracted better than 5Å. To improve these crystals, Hampton additive screen HT (HR2-138) was set
623 up next based on one of the initial hits, and crystals grown in 0.1M Na-malonate pH 5, 14.2% PEG 3350
624 in the presence of 14 mM adenosine-5'-triphosphate disodium salt hydrate diffracted to 2.7 Å. These
625 crystals were transferred into the crystallization solution supplemented by 20% ethylene glycol as a
626 cryo-protectant and flash-frozen in liquid nitrogen.

627

628 Data collection and structure determination

629 Data collected at the Proxima 1 beamline at the French national synchrotron facility (SOLEIL, St Aubin,
630 France) were indexed, integrated, scaled and merged using XDS⁷⁹ and AIMLESS⁸⁰. Molecular
631 replacement was done with Phaser within Phenix^{81 82} using as search models the EphA2 LBD structure
632 (Protein Data Bank [PDB] accession number 3HEI) and the HHV8 gH/gL model that was generated in
633 Phyre2⁵⁵ based on the sequence similarity with the EBV gH/gL (PDB code 3PHF). The partial solution
634 containing EphA2 LBD and parts of gH was obtained, and was extended by iterative rounds of model
635 building (Autobuild⁸³ in Phenix and manual building in Coot⁸⁴) and refinement using Buster⁸⁵ and
636 Phenix⁸². The final model converged to R_{work}/R_{free} of 0.22/0.24. The final map displayed clear electron
637 density for residues 27-200 of EphA2 (with a break in the J helix region (residues 148-162) and GH loop
638 (G111)), residues 21-128 for gL, and residues 35-696 gH with the exception of several short regions of
639 poor density that precluded unambiguous placement of the polypeptide chain (Fig. 1A).

640 The crystallization conditions, crystal parameters, data statistics, and refinement parameters
641 are shown in Table S1. Superpositions of structures and all structural figures were generated with
642 PyMOL (version 1.3r1)⁸⁶. The atomic coordinates and structure factors for trimeric complex EphA2
643 LBD-HHV8 gH/gL have been deposited in the RCSB Protein Data Bank with the PDB code 7B7N.

644

645 Expression of HHV8 gH/gL and EphA2 variants in mammalian cells

646 To avoid lengthy selection of the stably transfected S2 cells expressing recombinant proteins (4-5
647 weeks), a panel of gH/gL and EphA2 variants to be tested in biophysical assays was ordered as synthetic
648 genes (GenScript) cloned in pcDNA3.1 (+) vector for transient expression in mammalian Expi293 cells
649 (5-7 days). The reason mammalian cells were not used for production of the proteins used for
650 crystallizations is that gH/gL complex is heavily glycosylated and the complex sugars added by
651 mammalian cells are typically detrimental for protein crystallization and cannot be enzymatically
652 removed unlike the simple Endo-D and Endo-H sensitive carbohydrates added by insect cells.

653 The mammalian cell expression constructs for gH and gL (residues 21 – 167, and 26-704 respectively)
654 contained at the N-termini an exogenous murine Ig κ -chain leader sequence that targets protein to
655 secretory pathway (Coloma et al., 1992) (METDTLLLWVLLLWVPGSTG). Different affinity tags were
656 added to their C-termini - for gH construct (gH^{his}pcDNA3.1) an enterokinase cleavage site (underlined)
657 flanked by two GS linkers (italics) was followed by an octa-his tag (bold) (*GS* DDDDK *SGS* **HHHHHHHH**),
658 and for gL a DST (gLstpcDNA3.1; the DST sequence is the same as in the gLC58SstpT350 construct
659 described above). The EphA2 variants contained the endogenous signal peptide at the N-terminus and
660 ended at residue 534 followed by a DST tag.

661

662 Biophysical analyses

663 **SEC-MALS measurements:** The complexes were formed by incubating gH^{his}/gLst and EphA2st, both
664 produced in mammalian cells as described above, in 1:1.3 molar ratio for 30 minutes at 4°C (~100 µg
665 of gH/gL were mixed with 80 µg of EphA2 ectodomains or 30 µg of the EphA2 LBD in PBS in total
666 volume of 200 µl). Assembled complexes were injected into Superdex 200 10/300 GL column (GE life
667 sciences) using 500 µl loop, and run in PBS at a flow rate of 0.4 ml/min. The samples passed through a
668 Wyatt DAWN Heleos II EOS 18-angle laser photometer coupled to a Wyatt Optilab TrEX differential
669 refractive index detector. Data were analyzed with Astra 6 software (Wyatt Technology Corp).

670

671 **Biolayer interferometry measurements:** Biolayer interferometry (BLI) was applied to determine the
672 effect of gH/gL and EphA2 mutations on the complex dissociation constant (K_d). The measurements
673 were carried out on an Octet RED384 instrument (ForteBio). Affinity and SEC purified gH^{his}/gLst
674 produced in mammalian Expi293 cells was immobilized on Ni²⁺-NTA sensors (ForteBio) in PBS. The
675 loaded and equilibrated biosensors were dipped into analyte solutions containing 250 nM to 1 nM
676 EphA2 variants in PBS containing 0.2 mg/ml BSA (the assay buffer).

677 Association and dissociation were monitored for 250 and 500 seconds respectively. Sensor reference
678 measurement was recorded from a sensor not loaded with gH/gL and dipped in PBS. Sample reference
679 was recorded from a sensor loaded with gH/gL that was dipped in the assay buffer. Specific signals
680 were calculated by double-referencing, that is, subtracting non-specific signals obtained for the sensor
681 and sample references from the signals recorded for the gH/gL-loaded sensors dipped in EphA2 analyte
682 solutions. Association and dissociation profiles, as well as steady-state signal versus concentration
683 curves, were fitted assuming a 1:1 binding model.

684 Cell culture used in FRET and contraction experiments

685 Human embryonic kidney (HEK) 293T cells were purchased from American Type Culture Collection
686 (Manassas, VA; CRL-3216). The cells were cultured at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle
687 Medium (DMEM; Thermo Scientific; 31600-034) that contained 3.5 g/L D-glucose, 1.5 g/L sodium
688 bicarbonate, and 10% fetal bovine serum (FBS; Sigma-Aldrich; F4135). The cells were passed up to 25
689 times and then discarded.

690

691 Fixed cell contraction assay

692 HEK293T cells transiently transfected with WT EphA2-eYFP were selected with 1.6 mg/ml G-418
693 solution (Roche; 4727878001) for 12 days to generate a stable cell line. The concentration of G-418
694 was determined using a kill curve. HEK293T cells or EphA2 HEK293T stable cells were seeded (1 x 10⁴
695 cells/well) into 8-well tissue culture chambered coverglass slides (Thermo Scientific; 12565338) and
696 cultured for 36 hours. The cells were then washed twice with serum-free, phenol red-free media and

697 were serum starved for 12 hours overnight. The cells were washed twice with PBS, followed by
698 treatment for 10 min at 37°C with PBS or 200 nM gH/gL, 200 nM gH E52R/gL, 200 nM LBD, 200 nM
699 gH/gL-LBD, or 0.5 µg/ml ephrin-A1-Fc in PBS. The cells were then fixed in 4% paraformaldehyde in PBS
700 for 15 min at 37°C, permeabilized in 0.1% Triton X-100 in PBS for 15 min 37°C, and incubated with
701 blocking solution (5% FBS, 1% BSA in PBS) for 30 min at room temperature. Then, the cells were stained
702 for actin using rhodamine-conjugated phalloidin (Thermo Fisher Scientific; R415) for 90 min at room
703 temperature. The lyophilized phalloidin-rhodamine powder was reconstituted in DMSO to make a 40x
704 stock solution and then diluted with blocking buffer to make a 1x solution which was added to the
705 fixed cells. The cells were washed twice with PBS in between each step. Finally, starvation media was
706 added to each well prior to imaging. Actin-stained fixed cells were imaged with a Leica TCS SP8 confocal
707 microscope equipped with a HyD hybrid detector and a 63x objective. The measurements were
708 performed with a 552 nm excitation diode laser at 0.5% power using the dsRed setting which measures
709 the fluorescence between wavelengths of 562 and 700 nm. The scanning speed was at 200 Hz, the
710 pixel depth at 12-bits, the zoom factor at 1, and the image size at 1024x1024 pixels. Cell area was
711 determined using the ImageJ software (NIH, Bethesda, MD) by drawing a polygon around the
712 membrane of the cells. Statistical significance was determined by one-way ANOVA followed by a
713 Tukey's test using the GraphPad Prism software.

714 Live cell contraction assay

715 HEK293T cells were seeded, transfected with EphA2 WT-eYFP, and serum-starved overnight in the
716 same manner as described in the FRET section. Ten minutes prior to imaging, the media was replaced
717 with starvation media or starvation media containing 200 nM gH/gL, 200 nM LBD, or 200 nM gH/gL-
718 LBD. Cells were imaged with a Zeiss Axio Observer Inverted two-photon microscope using a 63x
719 objective at a wavelength of 960 nm to excite the eYFP fluorophore. Cell area and statistical
720 significance was determined in the same manner used for the fixed cell contraction assay.

721

722 FSI-FRET measurements and analysis

723 For FRET experiments, the cells were seeded in 35 mm glass bottom collagen-coated petri dishes
724 (MatTek Corporation, MA) at a density of 2×10^5 cells/dish and cultured for 24 hours. The cells were
725 co-transfected with EphA2-mTurquoise (mTURQ, the donor) and EphA2-enhanced yellow fluorescent
726 protein (eYFP, the acceptor) in pcDNA, as described ^{87,88}, using 0.5-2 µg of total DNA and the
727 Lipofectamine 3000 reagent (Invitrogen, CA). In control experiments, cells were transfected with either
728 EphA2-mTURQ or EphA2-eYFP and used for calibration as described ⁵³. Twelve hours following
729 transfection, the cells were washed twice with serum-free, phenol red-free media and serum-starved
730 in the same media for 12 hours overnight. Immediately before imaging, the starvation media was

731 replaced with hypo-osmotic media (10% starvation media, 90% diH₂O, 25 mM HEPES) to ‘unwrinkle’
732 the highly ruffled cell membrane under reversible conditions as described⁸⁹. The soluble proteins were
733 premixed with the hypo-osmotic media before adding to the cells. Cells were incubated for 10 minutes
734 and then imaged under these conditions for approximately 1 hour.

735 Spectral images of HEK293T cells under reversible osmotic conditions were obtained following
736 published protocols⁵³ with a spectrally resolved two-photon microscope (Zeiss Inverted Axio Observer)
737 equipped with line-scanning capabilities (OptiMis True Line Spectral Imaging system, Aurora Spectral
738 Technologies, WI)⁹⁰. Fluorophores were excited by a mode-locked laser (MaiTai™, Spectra-Physics,
739 Santa Clara, CA) that generates femtosecond pulses between wavelengths 690 nm to 1040 nm. Two
740 images were collected for each cell: one at 840 nm to excite the donor and a second one at 960 nm to
741 primarily excite the acceptor. Solutions of purified soluble fluorescent proteins (mTURQ and eYFP) at
742 known concentrations were produced following a published protocol⁹¹ and imaged at each of these
743 excitation wavelengths. A linear fit generated from the pixel-level intensities of the solution standards
744 was used to calibrate the effective three-dimensional protein concentration which can be converted
745 into two-dimensional membrane protein concentrations in the cell membrane as described⁵³. The
746 calibration curve along with the cell images were used to calculate the FRET efficiency and the
747 concentration of donors and acceptors present in the cell membrane⁵³. Regions of the cell membrane
748 not in contact with neighboring cells were selected and analyzed to avoid interactions with proteins
749 on adjacent cells.

750 The measured FRET efficiencies (E_{app}) were corrected for ‘proximity FRET’ (E_{prox}) as described previously
751⁹². The proximity FRET accounts for donor-tagged molecules and acceptor-tagged molecules coming
752 into close enough proximity to observe a FRET signal (within 100 Å) but not interacting directly. The
753 corrected FRET due to specific interactions between the labeled proteins is given by:

$$754 \quad FRET = \frac{E_{prox} - E_{app}}{2E_{prox} - E_{prox}E_{app} - 1} \quad (1)$$

755 The corrected FRET depends on the fraction of membrane protein dimers, f_D , and on the acceptor
756 fraction, x_A , according to:

$$757 \quad FRET = f_D x_A \tilde{E} \quad (2)$$

758 The ‘Intrinsic FRET’ (\tilde{E}) is a structural parameter that depends on the distance between and orientation
759 of the two fluorophores in the dimer but is not dependent on the dimerization propensity⁹²⁻⁹⁴.

760 When the dimeric fraction is 100% ($f_D = 1$), the corrected FRET does not depend on the concentration
761 of the labeled proteins and thus equation (2) can be simplified further:

$$762 \quad FRET = x_A \tilde{E}. \quad (3)$$

763 The dependence of the Intrinsic FRET on the distance between the fluorescent proteins in the dimer,
764 d , is given by ⁹³:

$$765 \quad \tilde{E} = \frac{1}{1 + \left(\frac{d}{R_0}\right)^6} \quad (4)$$

766 where R_0 is the Forster radius for the mTurquoise-eYFP FRET pair, 54.5 Å. Since the fluorescent proteins
767 are attached to the C-terminus of the membrane proteins via long flexible linkers, we assume free
768 rotation of the fluorescent proteins.

769 By rearranging equation (2), the dimeric fraction, f_D , can be determined from the corrected FRET
770 efficiencies and concentrations according to:

$$771 \quad f_D = \frac{FRET}{x_A \tilde{E}} \quad (5)$$

772 In the case of dimers, the following equation is used to determine the two unknowns K_{diss} and \tilde{E} as
773 described previously ⁵³:

$$774 \quad \frac{FRET}{x_A} = \frac{1}{[R_{total}]} \left([R_{total}] - \frac{K_{diss}}{4} \left(\sqrt{1 + 8[R_{total}]/K_{diss}} - 1 \right) \tilde{E} \right) \quad (6)$$

775

776 Fluorescence intensity fluctuations (FIF) spectroscopy measurements and analysis

777 HEK293T cells were seeded as described in the FRET section at a density of 4×10^5 cells/dish. The cells
778 were transiently transfected 24 hours later with 1 µg of EphA2-eYFP using Lipofectamine 3000 and
779 then washed and serum starved twelve hours later. The starvation media was replaced with a 75%
780 hypo-osmotic media (25% starvation media, 75% diH₂O, 25 mM HEPES) containing 200 nM gH/gL prior
781 to imaging. A TCS SP8 confocal microscope (Leica) using the photon counting capabilities of the HyD
782 hybrid detector was used to collect images of the cell basolateral membranes. The measurements
783 were performed with a 488 nm excitation diode laser and the emission spectra of YFP were collected
784 from 520-580 nm. The scanning speed was at 20Hz, the pixel depth at 12-bits, the zoom factor at 2,
785 and the image size at 1024x1024.

786 The cell images were analyzed using the FIF software described in ⁶¹. The software performed
787 segmentation of the basolateral membrane into 15x15 pixel regions of interest. Each cell is outlined
788 by researcher prior to segmentation. The segmented data was analyzed using the brightness and
789 concentration calculator in the FIF software ⁶¹. The molecular brightness, ϵ , was calculated according
790 to:

$$791 \quad \epsilon = \frac{\sigma^2 - \sigma_D^2}{\langle I \rangle} \quad (7)$$

792 where σ^2 is the variance of fluorescence across segments, σ_D^2 is the variance of the noise of the
793 detector, and $\langle I \rangle$ is the average fluorescence intensity. For a photon-counting detector as used
794 here, the brightness is ^{64,95}:

795
$$\varepsilon = \frac{\sigma^2}{\langle I \rangle} - 1 \quad (8)$$

796 The brightness values, calculated for thousands of regions of interest, were potted as histograms.

797 REFERENCES

- 798 1 Pellett, P. E. & Roizman, B. in *Fields Virology* Vol. 2 (eds D.M. Knip & P.M. Howley) Ch. 59,
799 1802-1823 (Lippincott Williams & Wilkins, 2013).
- 800 2 Kaposi, M. Idiopathisches multiples Pigmentsarkom der Haut. *Archive für Dermatologie und*
801 *Syphillis* **4**, 265-173 (1872).
- 802 3 Board, P. A. T. E. Kaposi Sarcoma Treatment (PDQ(R)): Health Professional Version. (National
803 Cancer Institute, Bethesda (MD), 2018).
- 804 4 Centers for Disease, C. Update on acquired immune deficiency syndrome (AIDS)--United
805 States. *MMWR Morb Mortal Wkly Rep* **31**, 507-508, 513-504 (1982).
- 806 5 Beral, V., Peterman, T. A., Berkelman, R. L. & Jaffe, H. W. Kaposi's sarcoma among persons with
807 AIDS: a sexually transmitted infection? *Lancet* **335**, 123-128, doi:10.1016/0140-
808 6736(90)90001-I (1990).
- 809 6 Mesri, E. A., Cesarman, E. & Boshoff, C. Kaposi's sarcoma and its associated herpesvirus. *Nat*
810 *Rev Cancer* **10**, 707-719, doi:10.1038/nrc2888 (2010).
- 811 7 Mohl, B. S., Chen, J. & Longnecker, R. Gammaherpesvirus entry and fusion: A tale how two
812 human pathogenic viruses enter their host cells. *Adv Virus Res* **104**, 313-343,
813 doi:10.1016/bs.aivir.2019.05.006 (2019).
- 814 8 Veettil, M. V., Bandyopadhyay, C., Dutta, D. & Chandran, B. Interaction of KSHV with host cell
815 surface receptors and cell entry. *Viruses* **6**, 4024-4046, doi:10.3390/v6104024 (2014).
- 816 9 Dollery, S. J. Towards Understanding KSHV Fusion and Entry. *Viruses-Basel* **11**, doi:ARTN 1073
817 10.3390/v11111073 (2019).
- 818 10 Connolly, S. A., Jardetzky, T. S. & Longnecker, R. The structural basis of herpesvirus entry. *Nat*
819 *Rev Microbiol*, doi:10.1038/s41579-020-00448-w (2020).
- 820 11 Dollery, S. J. Towards Understanding KSHV Fusion and Entry. *Viruses* **11**,
821 doi:10.3390/v11111073 (2019).
- 822 12 Xing, Y. *et al.* A site of varicella-zoster virus vulnerability identified by structural studies of
823 neutralizing antibodies bound to the glycoprotein complex gHgL. *Proc Natl Acad Sci U S A* **112**,
824 6056-6061, doi:10.1073/pnas.1501176112 (2015).
- 825 13 Ciferri, C. *et al.* Structural and biochemical studies of HCMV gH/gL/gO and Pentamer reveal
826 mutually exclusive cell entry complexes. *Proc Natl Acad Sci U S A* **112**, 1767-1772,
827 doi:10.1073/pnas.1424818112 (2015).
- 828 14 Chowdary, T. K. *et al.* Crystal structure of the conserved herpesvirus fusion regulator complex
829 gH-gL. *Nat Struct Mol Biol* **17**, 882-888, doi:10.1038/nsmb.1837
830 nsmb.1837 [pii] (2010).
- 831 15 Matsuura, H., Kirschner, A. N., Longnecker, R. & Jardetzky, T. S. Crystal structure of the Epstein-
832 Barr virus (EBV) glycoprotein H/glycoprotein L (gH/gL) complex. *Proc Natl Acad Sci U S A* **107**,
833 22641-22646, doi:10.1073/pnas.1011806108
834 1011806108 [pii] (2010).
- 835 16 Sathiyamoorthy, K., Chen, J., Longnecker, R. & Jardetzky, T. S. The COMPLEXity in herpesvirus
836 entry. *Curr Opin Virol* **24**, 97-104, doi:10.1016/j.coviro.2017.04.006 (2017).
- 837 17 Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph
838 Nomenclature Committee. *Cell* **90**, 403-404, doi:10.1016/s0092-8674(00)80500-0 (1997).
- 839 18 Himanen, J. P. Ectodomain structures of Eph receptors. *Semin Cell Dev Biol* **23**, 35-42,
840 doi:10.1016/j.semcdb.2011.10.025 (2012).
- 841 19 Himanen, J. P., Saha, N. & Nikolov, D. B. Cell-cell signaling via Eph receptors and ephrins. *Curr*
842 *Opin Cell Biol* **19**, 534-542, doi:10.1016/j.ceb.2007.08.004 (2007).
- 843 20 Hahn, A. S. *et al.* The ephrin receptor tyrosine kinase A2 is a cellular receptor for Kaposi's
844 sarcoma-associated herpesvirus. *Nat Med* **18**, 961-966, doi:10.1038/nm.2805
845 nm.2805 [pii] (2012).
- 846 21 Chen, J., Zhang, X., Schaller, S., Jardetzky, T. S. & Longnecker, R. Ephrin Receptor A4 is a New
847 Kaposi's Sarcoma-Associated Herpesvirus Virus Entry Receptor. *MBio* **10**,
848 doi:10.1128/mBio.02892-18 (2019).

- 849 22 Grosskopf, A. K. *et al.* EphA7 Functions as Receptor on BJAB Cells for Cell-to-Cell Transmission
850 of the Kaposi's Sarcoma-Associated Herpesvirus and for Cell-Free Infection by the Related
851 Rhesus Monkey Rhadinovirus. *J Virol* **93**, doi:10.1128/JVI.00064-19 (2019).
- 852 23 Chen, J. *et al.* Ephrin receptor A2 is a functional entry receptor for Epstein-Barr virus. *Nat*
853 *Microbiol* **3**, 172-180, doi:10.1038/s41564-017-0081-7 (2018).
- 854 24 Hahn, A. S. & Desrosiers, R. C. Rhesus monkey rhadinovirus uses eph family receptors for entry
855 into B cells and endothelial cells but not fibroblasts. *PLoS Pathog* **9**, e1003360,
856 doi:10.1371/journal.ppat.1003360 (2013).
- 857 25 Lupberger, J. *et al.* EGFR and EphA2 are host factors for hepatitis C virus entry and possible
858 targets for antiviral therapy. *Nat Med* **17**, 589-595, doi:10.1038/nm.2341
859 nm.2341 [pii] (2011).
- 860 26 Subbarayal, P. *et al.* EphrinA2 receptor (EphA2) is an invasion and intracellular signaling
861 receptor for *Chlamydia trachomatis*. *PLoS Pathog* **11**, e1004846,
862 doi:10.1371/journal.ppat.1004846 (2015).
- 863 27 Aaron, P. A., Jamklang, M., Uhrig, J. P. & Gelli, A. The blood-brain barrier internalises
864 *Cryptococcus neoformans* via the EphA2-tyrosine kinase receptor. *Cell Microbiol* **20**,
865 doi:10.1111/cmi.12811 (2018).
- 866 28 Kaushansky, A. *et al.* Malaria parasites target the hepatocyte receptor EphA2 for successful
867 host infection. *Science* **350**, 1089-1092, doi:10.1126/science.aad3318 (2015).
- 868 29 Kania, A. & Klein, R. Mechanisms of ephrin-Eph signalling in development, physiology and
869 disease. *Nat Rev Mol Cell Biol* **17**, 240-256, doi:10.1038/nrm.2015.16 (2016).
- 870 30 Barquilla, A. *et al.* Protein kinase A can block EphA2 receptor-mediated cell repulsion by
871 increasing EphA2 S897 phosphorylation. *Mol Biol Cell* **27**, 2757-2770, doi:10.1091/mbc.E16-
872 01-0048 (2016).
- 873 31 Miao, H., Burnett, E., Kinch, M., Simon, E. & Wang, B. Activation of EphA2 kinase suppresses
874 integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol* **2**, 62-69,
875 doi:10.1038/35000008 (2000).
- 876 32 Huang, X., Wu, D., Jin, H., Stupack, D. & Wang, J. Y. Induction of cell retraction by the combined
877 actions of Abl-CrkII and Rho-ROCK1 signaling. *J Cell Biol* **183**, 711-723,
878 doi:10.1083/jcb.200801192 (2008).
- 879 33 Astin, J. W. *et al.* Competition amongst Eph receptors regulates contact inhibition of
880 locomotion and invasiveness in prostate cancer cells. *Nat Cell Biol* **12**, 1194-1204,
881 doi:10.1038/ncb2122 (2010).
- 882 34 Pitulescu, M. E. & Adams, R. H. Eph/ephrin molecules--a hub for signaling and endocytosis.
883 *Genes Dev* **24**, 2480-2492, doi:10.1101/gad.1973910 (2010).
- 884 35 Saha, N., Robev, D., Mason, E. O., Himanen, J. P. & Nikolov, D. B. Therapeutic potential of
885 targeting the Eph/ephrin signaling complex. *Int J Biochem Cell Biol* **105**, 123-133,
886 doi:10.1016/j.biocel.2018.10.006 (2018).
- 887 36 Barquilla, A. & Pasquale, E. B. Eph receptors and ephrins: therapeutic opportunities. *Annu Rev*
888 *Pharmacol Toxicol* **55**, 465-487, doi:10.1146/annurev-pharmtox-011112-140226 (2015).
- 889 37 Kumar, B. & Chandran, B. KSHV Entry and Trafficking in Target Cells-Hijacking of Cell Signal
890 Pathways, Actin and Membrane Dynamics. *Viruses* **8**, doi:10.3390/v8110305 (2016).
- 891 38 Holland, S. J. *et al.* Bidirectional signalling through the EPH-family receptor Nuk and its
892 transmembrane ligands. *Nature* **383**, 722-725, doi:10.1038/383722a0 (1996).
- 893 39 Himanen, J. P., Henkemeyer, M. & Nikolov, D. B. Crystal structure of the ligand-binding domain
894 of the receptor tyrosine kinase EphB2. *Nature* **396**, 486-491, doi:10.1038/24904 (1998).
- 895 40 Bowden, T. A. *et al.* Structural Plasticity of Eph-Receptor A4 Facilitates Cross-Class Ephrin
896 Signaling. *Structure* **17**, 1679, doi:10.1016/j.str.2009.11.004 (2009).
- 897 41 Toth, J. *et al.* Crystal structure of an ephrin ectodomain. *Dev Cell* **1**, 83-92, doi:10.1016/s1534-
898 5807(01)00002-8 (2001).
- 899 42 Himanen, J. P. *et al.* Crystal structure of an Eph receptor-ephrin complex. *Nature* **414**, 933-938,
900 doi:10.1038/414933a
901 414933a [pii] (2001).

- 902 43 Himanen, J. P. *et al.* Ligand recognition by A-class Eph receptors: crystal structures of the
903 EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. *EMBO Rep* **10**, 722-728,
904 doi:10.1038/embor.2009.91 (2009).
- 905 44 Seiradake, E., Harlos, K., Sutton, G., Aricescu, A. R. & Jones, E. Y. An extracellular steric seeding
906 mechanism for Eph-ephrin signaling platform assembly. *Nat Struct Mol Biol* **17**, 398-402,
907 doi:10.1038/nsmb.1782 (2010).
- 908 45 Himanen, J. P. *et al.* Architecture of Eph receptor clusters. *Proc Natl Acad Sci U S A* **107**, 10860-
909 10865, doi:10.1073/pnas.1004148107
910 1004148107 [pii] (2010).
- 911 46 Nikolov, D. B., Xu, K. & Himanen, J. P. Eph/ephrin recognition and the role of Eph/ephrin
912 clusters in signaling initiation. *Biochim Biophys Acta* **1834**, 2160-2165,
913 doi:10.1016/j.bbapap.2013.04.020 (2013).
- 914 47 Singh, D. R., Kanvinde, P., King, C., Pasquale, E. B. & Hristova, K. The EphA2 receptor is activated
915 through induction of distinct, ligand-dependent oligomeric structures. *Commun Biol* **1**, 15,
916 doi:10.1038/s42003-018-0017-7 (2018).
- 917 48 Chen, Z. *et al.* Spatially modulated ephrinA1:EphA2 signaling increases local contractility and
918 global focal adhesion dynamics to promote cell motility. *Proc Natl Acad Sci U S A* **115**, E5696-
919 E5705, doi:10.1073/pnas.1719961115 (2018).
- 920 49 Singh, D. R., Pasquale, E. B. & Hristova, K. A small peptide promotes EphA2 kinase-dependent
921 signaling by stabilizing EphA2 dimers. *Biochim Biophys Acta* **1860**, 1922-1928,
922 doi:10.1016/j.bbagen.2016.06.004 (2016).
- 923 50 Ojosnegros, S. *et al.* Eph-ephrin signaling modulated by polymerization and condensation of
924 receptors. *Proc Natl Acad Sci U S A* **114**, 13188-13193, doi:10.1073/pnas.1713564114 (2017).
- 925 51 Gomez-Soler, M. *et al.* Engineering nanomolar peptide ligands that differentially modulate
926 EphA2 receptor signaling. *J. Biol. Chem.* **294**, 8791-8805, doi:10.1074/jbc.RA119.008213
927 (2019).
- 928 52 Su, C. *et al.* Molecular basis of EphA2 recognition by gHgL from gammaherpesviruses. *Nat*
929 *Commun* **11**, 5964, doi:10.1038/s41467-020-19617-9 (2020).
- 930 53 King, C., Stoneman, M., Raicu, V. & Hristova, K. Fully quantified spectral imaging reveals in vivo
931 membrane protein interactions. *Integr Biol (Camb)* **8**, 216-229, doi:10.1039/c5ib00202h
932 (2016).
- 933 54 Sahin, E. & Roberts, C. J. Size-exclusion chromatography with multi-angle light scattering for
934 elucidating protein aggregation mechanisms. *Methods Mol Biol* **899**, 403-423,
935 doi:10.1007/978-1-61779-921-1_25 (2012).
- 936 55 Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. The Phyre2 web portal for
937 protein modeling, prediction and analysis. *Nat Protoc* **10**, 845-858,
938 doi:10.1038/nprot.2015.053 (2015).
- 939 56 Meszaros, B., Erdos, G. & Dosztanyi, Z. IUPred2A: context-dependent prediction of protein
940 disorder as a function of redox state and protein binding. *Nucleic Acids Res* **46**, W329-W337,
941 doi:10.1093/nar/gky384 (2018).
- 942 57 Erdos, G. & Dosztanyi, Z. Analyzing Protein Disorder with IUPred2A. *Curr Protoc Bioinformatics*
943 **70**, e99, doi:10.1002/cpbi.99 (2020).
- 944 58 Backovic, M. *et al.* Structure of a core fragment of glycoprotein H from pseudorabies virus in
945 complex with antibody. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 22635-22640,
946 doi:10.1073/pnas.1011507107 (2010).
- 947 59 Vleck, S. E. *et al.* Structure-function analysis of varicella-zoster virus glycoprotein H identifies
948 domain-specific roles for fusion and skin tropism. *Proc Natl Acad Sci U S A* **108**, 18412-18417,
949 doi:10.1073/pnas.1111333108 (2011).
- 950 60 Hahn, A. S. & Desrosiers, R. C. Binding of the Kaposi's sarcoma-associated herpesvirus to the
951 ephrin binding surface of the EphA2 receptor and its inhibition by a small molecule. *J Virol* **88**,
952 8724-8734, doi:10.1128/JVI.01392-14 (2014).
- 953 61 Stoneman, M. R. *et al.* A general method to quantify ligand-driven oligomerization from
954 fluorescence-based images. *Nat Methods* **16**, 493-496, doi:10.1038/s41592-019-0408-9
955 (2019).

- 956 62 Adamkova, L. *et al.* Oligomeric Architecture of Mouse Activating Nkrp1 Receptors on Living
957 Cells. *Int J Mol Sci* **20**, doi:10.3390/ijms20081884 (2019).
- 958 63 Singh, D. R., Ahmed, F., Sarabipour, S. & Hristova, K. Intracellular Domain Contacts Contribute
959 to Ecadherin Constitutive Dimerization in the Plasma Membrane. *J Mol Biol* **429**, 2231-2245,
960 doi:10.1016/j.jmb.2017.05.020 (2017).
- 961 64 Ahmed, F., Zapata-Mercado, E., Rahman, S. & Hristova, K. The Biased Ligands NGF and NT-3
962 Differentially Stabilize Trk-A Dimers. *Biophys J* **120**, 55-63, doi:10.1016/j.bpj.2020.11.2262
963 (2021).
- 964 65 Pasquale, E. B. Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol*
965 **6**, 462-475, doi:10.1038/nrm1662 (2005).
- 966 66 Grosskopf, A. K. *et al.* A conserved Eph family receptor-binding motif on the gH/gL complex of
967 Kaposi's sarcoma-associated herpesvirus and rhesus monkey rhadinovirus. *PLoS Pathog* **14**,
968 e1006912, doi:10.1371/journal.ppat.1006912 (2018).
- 969 67 Akula, S. M. *et al.* Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) infection of
970 human fibroblast cells occurs through endocytosis. *J Virol* **77**, 7978-7990,
971 doi:10.1128/jvi.77.14.7978-7990.2003 (2003).
- 972 68 Himanen, J. P. *et al.* Repelling class discrimination: ephrin-A5 binds to and activates EphB2
973 receptor signaling. *Nat Neurosci* **7**, 501-509, doi:10.1038/nn1237 (2004).
- 974 69 Nikolov, D. B., Xu, K. & Himanen, J. P. Homotypic receptor-receptor interactions regulating Eph
975 signaling. *Cell adhesion & migration* **8**, 360-365, doi:10.4161/19336918.2014.971684 (2014).
- 976 70 Wykosky, J. *et al.* Soluble monomeric EphrinA1 is released from tumor cells and is a functional
977 ligand for the EphA2 receptor. *Oncogene* **27**, 7260-7273, doi:10.1038/onc.2008.328 (2008).
- 978 71 Beauchamp, A. & Debinski, W. Ephs and ephrins in cancer: ephrin-A1 signalling. *Semin Cell Dev*
979 *Biol* **23**, 109-115, doi:10.1016/j.semcdb.2011.10.019 (2012).
- 980 72 Miao, H. *et al.* EphA2 mediates ligand-dependent inhibition and ligand-independent
981 promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer Cell*
982 **16**, 9-20, doi:10.1016/j.ccr.2009.04.009 (2009).
- 983 73 Wang, X. *et al.* Male hormones activate EphA2 to facilitate Kaposi's sarcoma-associated
984 herpesvirus infection: Implications for gender disparity in Kaposi's sarcoma. *PLoS Pathog* **13**,
985 e1006580, doi:10.1371/journal.ppat.1006580 (2017).
- 986 74 Krey, T. *et al.* The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary
987 organization of the molecule. *PLoS Pathog* **6**, e1000762 (2010).
- 988 75 Hahn, A. *et al.* Kaposi's sarcoma-associated herpesvirus gH/gL: glycoprotein export and
989 interaction with cellular receptors. *J Virol* **83**, 396-407, doi:10.1128/JVI.01170-08 (2009).
- 990 76 Backovic, M. & Krey, T. Stable Drosophila Cell Lines: An Alternative Approach to Exogenous
991 Protein Expression. *Methods Mol. Biol.* **1350**, 349-358, doi:10.1007/978-1-4939-3043-2_17
992 (2016).
- 993 77 Fan, S. Q., Huang, W. & Wang, L. X. Remarkable transglycosylation activity of glycosynthase
994 mutants of endo-D, an endo-beta-N-acetylglucosaminidase from *Streptococcus pneumoniae*.
995 *J Biol Chem* **287**, 11272-11281, doi:10.1074/jbc.M112.340497 (2012).
- 996 78 Weber, P. *et al.* High-Throughput Crystallization Pipeline at the Crystallography Core Facility of
997 the Institut Pasteur. *Molecules* **24**, doi:10.3390/molecules24244451 (2019).
- 998 79 Kabsch, W. XDS. *Acta Crystallogr D Biol Crystallogr* **66**, 125-132 (2010).
- 999 80 The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **50**,
1000 760-763 (1994).
- 1001 81 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular
1002 structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221,
1003 doi:10.1107/S0907444909052925 (2010).
- 1004 82 Liebschner, D. *et al.* Macromolecular structure determination using X-rays, neutrons and
1005 electrons: recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75**, 861-877,
1006 doi:10.1107/S2059798319011471 (2019).
- 1007 83 Terwilliger, T. C. *et al.* Iterative model building, structure refinement and density modification
1008 with the PHENIX AutoBuild wizard. *Acta Crystallogr D Biol Crystallogr* **64**, 61-69,
1009 doi:10.1107/S090744490705024X (2008).

- 1010 84 Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D*
1011 *Biol Crystallogr* **60**, 2126-2132 (2004).
- 1012 85 BUSTER v. 2.8.0 (Global Phasing Ltd., Cambridge, United Kingdom, 2009).
- 1013 86 The PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA, 2002).
- 1014 87 Singh, D. R. *et al.* EphA2 Receptor Unliganded Dimers Suppress EphA2 Pro-tumorigenic
1015 Signaling. *J Biol Chem* **290**, 27271-27279, doi:10.1074/jbc.M115.676866 (2015).
- 1016 88 Singh, D. R., Kanvinde, P., King, C., Pasquale, E. B. & Hristova, K. The EphA2 receptor is activated
1017 through induction of distinct, ligand-dependent oligomeric structures. *Commun Biol* **1**, 15,
1018 doi:10.1038/s42003-018-0017-7 (2018).
- 1019 89 Sinha, B. *et al.* Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell* **144**,
1020 402-413, doi:10.1016/j.cell.2010.12.031 (2011).
- 1021 90 Biener, G. *et al.* Development and experimental testing of an optical micro-spectroscopic
1022 technique incorporating true line-scan excitation. *Int J Mol Sci* **15**, 261-276,
1023 doi:10.3390/ijms15010261 (2013).
- 1024 91 Sarabipour, S., King, C. & Hristova, K. Uninduced high-yield bacterial expression of fluorescent
1025 proteins. *Anal Biochem* **449**, 155-157, doi:10.1016/j.ab.2013.12.027 (2014).
- 1026 92 King, C., Raicu, V. & Hristova, K. Understanding the FRET Signatures of Interacting Membrane
1027 Proteins. *J Biol Chem* **292**, 5291-5310, doi:10.1074/jbc.M116.764282 (2017).
- 1028 93 Chen, L., Novicky, L., Merzlyakov, M., Hristov, T. & Hristova, K. Measuring the energetics of
1029 membrane protein dimerization in mammalian membranes. *J Am Chem Soc* **132**, 3628-3635,
1030 doi:10.1021/ja910692u (2010).
- 1031 94 Sarabipour, S., Del Piccolo, N. & Hristova, K. Characterization of membrane protein
1032 interactions in plasma membrane derived vesicles with quantitative imaging Forster
1033 resonance energy transfer. *Acc Chem Res* **48**, 2262-2269, doi:10.1021/acs.accounts.5b00238
1034 (2015).
- 1035 95 Fox, M. *Quantum optics: an introduction*. Vol. 15 (Oxford Univeristy Press, 2006).
- 1036

1037 Acknowledgements

1038 We thank Patrick Weber and Cédric Pissis the Crystallogenes core facility at the Institut Pasteur for
1039 assistance with crystallization trials, and to the staff at the beamlines Proxima 1 and Proxima 2 at the
1040 French national synchrotron facility (SOLEIL, St Aubin, France) in particular to Leo Chavas and Bill
1041 Shepard for help with data collection and processing. We are grateful to Ignacio Fernandez and Jan
1042 Hellert for reading the manuscript and for their suggestions. This project has been supported via the
1043 recurrent funding from Institut Pasteur (MB, FR, PGC, RP, DB) and grants from NIH GM068619 and NSF
1044 MCB 1712740 (TL, KH).

1045

1046 Contributions

1047 Conceived and designed the experiments (MB, KH, FR), produced reagents (MB, DB, AH), collected the
1048 data (MB, PGC, TL), performed the data analyses (TL, PGC, RP, KH, MB), wrote the paper (MB, KH, TL,
1049 FR, PGC, RP, AH, FN).

1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063

Figures and figure legends

Table of Contents

Table 1: Summary of the dimerization models fit to FRET data	35
Figure 1: Schematic representation of HHV-8 gH, gL and EphA2 and structure of the gH/gL-EphA2 LBD complex	36
Figure 2: The binding interface between EphA2 LBD and HHV-8 gH/gL.....	37
Figure 3: Structural mimicry between HHV-8 gH/gL and ephrin ligands	39
Figure 4: HHV-8 gH/gL induces constitutive EphA2 dimerization.	40
Figure 5: The gH/gL induced EphA2 dimers on cells engage the 'dimerization' interface. ..	41
Figure 6: HHV-8 gH/gL stimulates EphA2-induced cell contraction.	42
References	43

1064 Table 1: Summary of the dimerization models fit to FRET data

1065

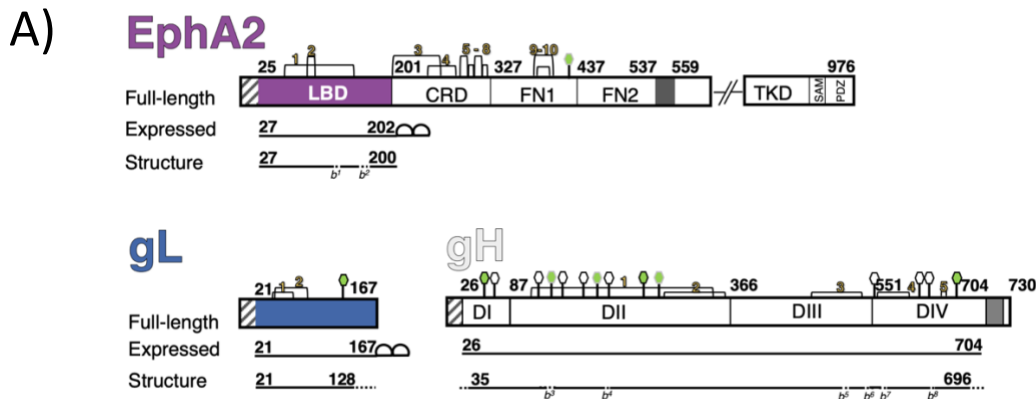
EphA2 construct	Soluble protein	K_{diss} (receptors/ μm^2)	Intrinsic FRET, \tilde{E}	distance, d (Å)
WT	-	302 ± 68	0.53 ± 0.02	53.6 ± 0.7
WT	gH/gL	100% dimer	0.31 ± 0.01	62.3 ± 0.2
WT	LBD	348 ± 130	0.50 ± 0.03	54.6 ± 1.2
WT	gH/gL-LBD	233 ± 103	0.55 ± 0.03	52.7 ± 1.2
WT	gH E52R/gL	300 ± 71	0.66 ± 0.03	48.9 ± 1.0
R103E	gH/gL	310 ± 124	0.76 ± 0.03	45.0 ± 1.5
G131Y	gH/gL	251 ± 103	0.58 ± 0.03	51.4 ± 1.2
L223R/L254R/V255R	gH/gL	17 ± 12	0.43 ± 0.02	57.1 ± 0.5

1066

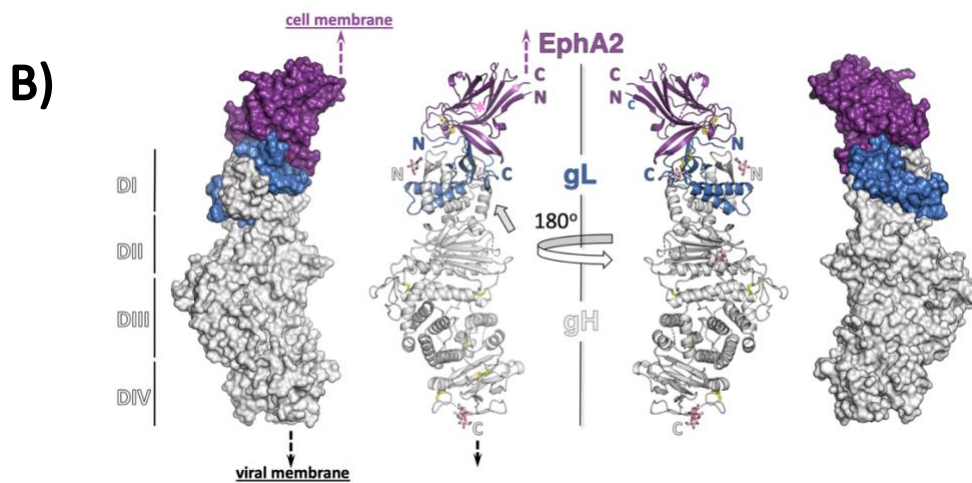
1067 Summary of the best-fit values for the dissociation constant (K_{diss}), the structural parameter Intrinsic
 1068 FRET (\tilde{E}), and the distance between fluorophores (d), obtained by fitting dimerization models to the
 1069 FRET data. K_{diss} and \tilde{E} are determined by a two-parameter fit using equation (6) and the distance d is
 1070 calculated using equation (4).

1071

1072 Figure 1: Schematic representation of HHV-8 gH, gL and EphA2
 1073 and structure of the gH/gL-EphA2 LBD complex



1074

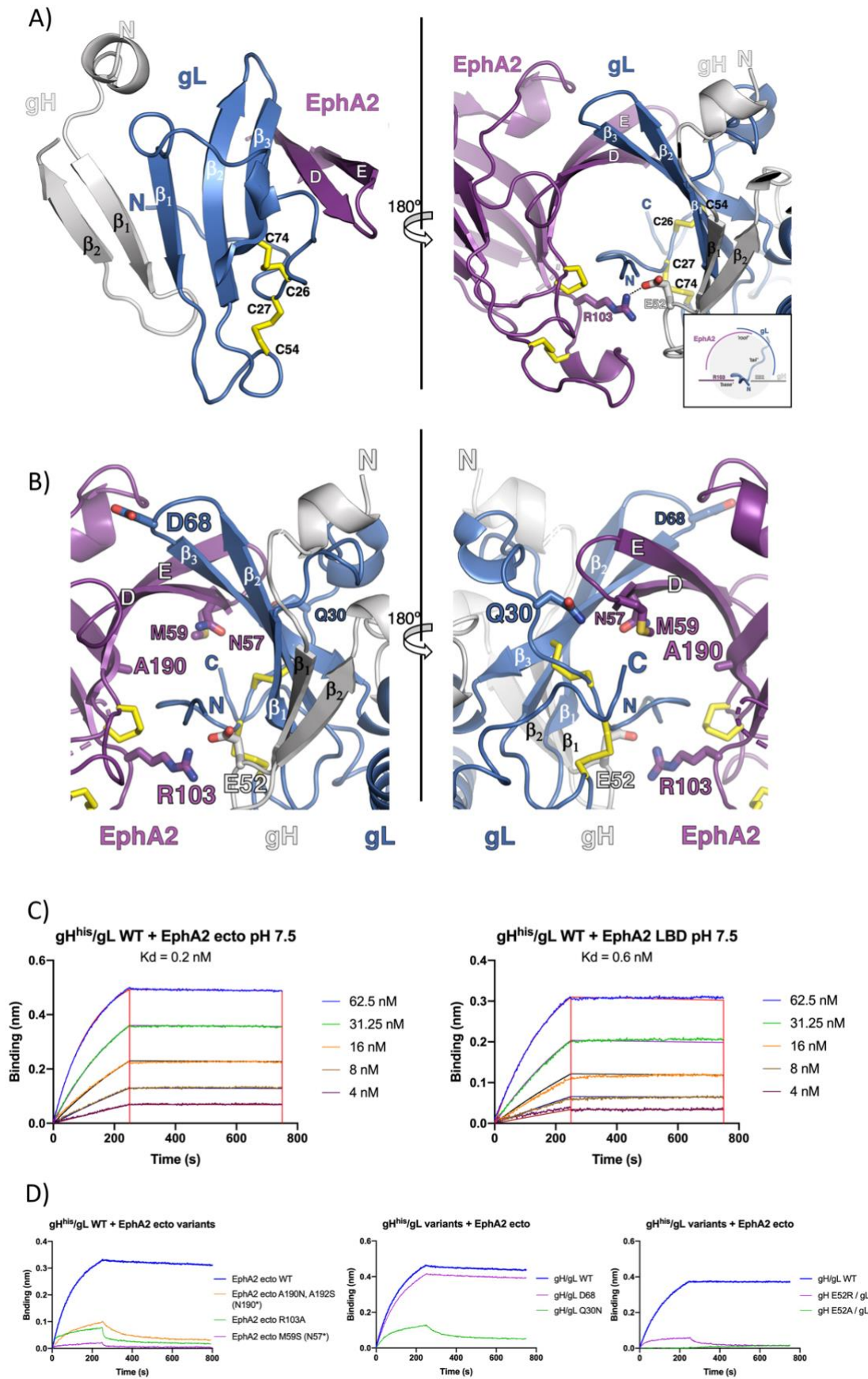


1075

1076 Figure 1 legend

1077 **A)** Schematic representation of the EphA2 receptor, HHV-8 gL and gH, highlighting the protein
 1078 segments that were expressed as recombinant proteins for crystallization, and the residues resolved
 1079 in the structure. The short fragments that could not be built in EphA2 LBD and gH because of the poor
 1080 electron density are marked with dotted lines and labeled as breaks (*b*) corresponding to the missing
 1081 residues: *b*¹ (111), *b*² (148-162) in EphA2 LBD, and *b*³(127-131), *b*⁴(212-216), *b*⁵(521-526), *b*⁶(547-550),
 1082 *b*⁷(558-559), *b*⁸(627-629) in gH. The disulfide bonds are indicated with yellow numbers, and N-linked
 1083 glycosylation sites with hexagons (green with black border – built in our structure; green with gray
 1084 border – built in the PDB accession code 7CZF [1]; white, with black borders – remaining, predicted
 1085 sites). Signal peptides at the start of each protein are represented as white boxes with grey lines,
 1086 transmembrane anchor domains in EphA2 and gH as dark grey boxes, and double strep tag for affinity
 1087 purification on gL and EphA2 LBD as half circles. **B)** The structure of the tertiary complex is represented
 1088 as molecular surface and cartoon model (EphA2 LBD in purple, gL in blue and gH in grey). The N- and
 1089 C- termini of each protein are labelled with letters “N” and “C”, respectively. The four domains of gH
 1090 are marked with roman numbers on the left side, and putative locations of the viral and cellular
 1091 membranes with dashed arrows (black and purple, respectively). The hinge / linker region on gH is
 1092 indicated with a grey arrow, and putative position of the unresolved J helix in the LBD with a pink *
 1093 symbol.

1094 Figure 2: The binding interface between EphA2 LBD and HHV-8 gH/gL



1095

1096

1097 Figure 2 legend

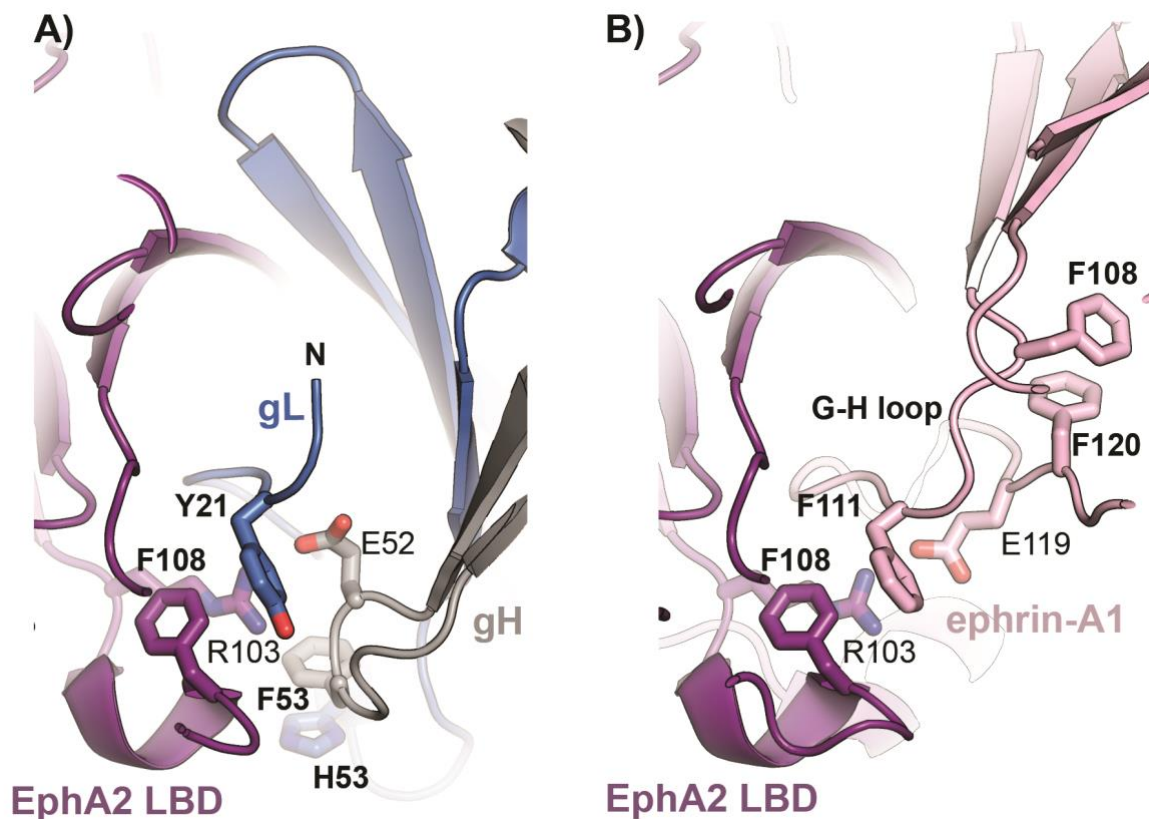
1098 **A)** The mixed β -sheet formed by strands of gH, gL and EphA2. The strands in gH and gL are labeled as
1099 β_{number} , while the EphA2 LBD strands are marked using the single-letter nomenclature assigned for the
1100 first solved structure of the EphB2 LBD 1KGY [2]. The same coloring scheme as in Figure 1 is applied
1101 (left panel). View are the gH/gL and EphA2 binding interface from the other side with the inlet
1102 illustrating the channel formed by the EphA2 and gL strands ('roof') that accommodates the gL N-
1103 terminal 'tail', reinforced by polar interactions between R103^{EphA2} and E52^{gH} ('base') (right panel).

1104 **B)** Locations of the point mutations introduced in EphA2 (R103A) and gH (E52A, E52R), and N-linked
1105 glycosylation sites in EphA2 (N57, A190N) and gL (Q30N, D68N) are indicated, and their side chains are
1106 shown as sticks. The same coloring scheme as in Figure 1B is applied.

1107 **C)** Sensorgrams recorded for WT EphA2 ectodomain of LBD binding to immobilized gH/gL by biolayer
1108 interferometry. A series of measurements using a range of concentrations for EphA2 ectodomain and
1109 LBD, respectively, was carried out to obtain the K_d for the WT proteins. Experimental curves (colored
1110 traces) were fit using a 1:1 binding model (black traces) to derive equilibrium K_d values.

1111 **D)** Sensorgrams recorded for EphA2 variants binding to immobilized gH/gL variants by biolayer
1112 interferometry. Single experimental curves obtained for EphA2 ectodomain concentration of 62.5 nM
1113 plotted to show the effect of the, respectively, EphA2 mutations, gL mutations, and gH mutations on
1114 binding.

1115 Figure 3: Structural mimicry between HHV-8 gH/gL and ephrin ligands



1116

ephrin-A1	T	L	K	G	E	F	K	E	G	123
ephrin-A2	S	L	G	F	E	F	R	P	G	146
ephrin-A3	S	L	G	Y	E	F	H	A	G	142
ephrin-A4	S	L	G	F	E	F	L	P	G	128
ephrin-A5	S	L	G	F	E	F	R	P	G	134
HHV-8 gH	S	I	E	L	E	F	N	G	T	56

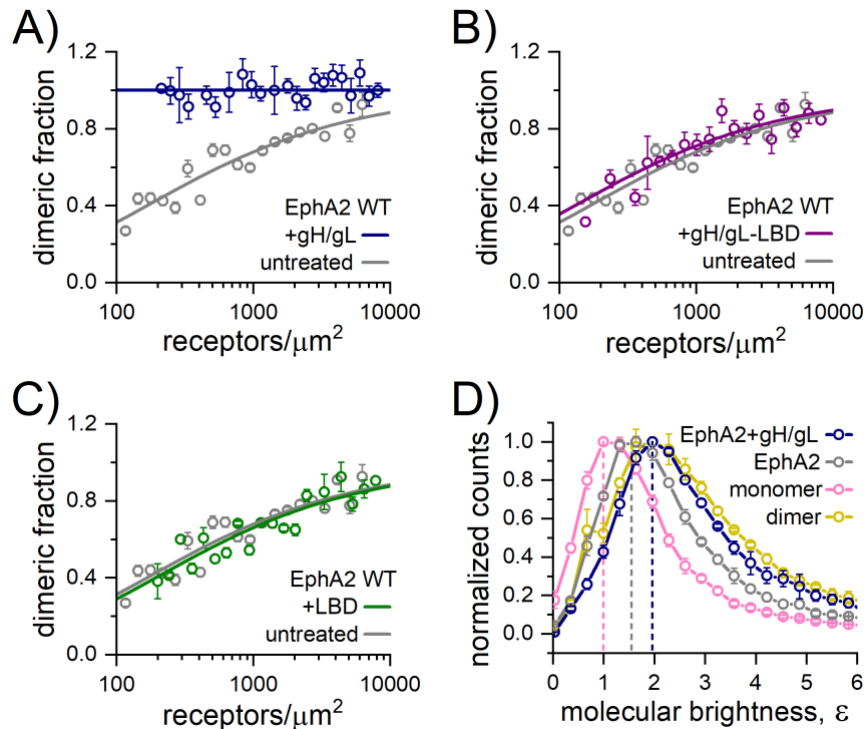
1117

1118

1119 Figure 3 legend

1120 The EphA2 LBD from the EphA2 LBD – ephrin-A1 complex structure (PDB 3HEI) was superimposed onto
 1121 the EphA2 LBD from our complex. The same coloring scheme for gH/gL and EphA2 is applied as in the
 1122 previous figures, with the GH^{ephrin-A1} loop shown in pink. For clarity, only the elements participating in
 1123 the interactions are shown. The E119^{ephrin-A1} is indicated. Sequence alignment of a GH loop segment of
 1124 ephrin-A ligands, and the HHV-8 gH sequence is displayed to highlight the conservation of the glutamic
 1125 acid that forms SBs with the EphA2^{R103} (E52^{gH} and E119^{ephrin-A1}).

1126 Figure 4: HHV-8 gH/gL induces constitutive EphA2 dimerization.



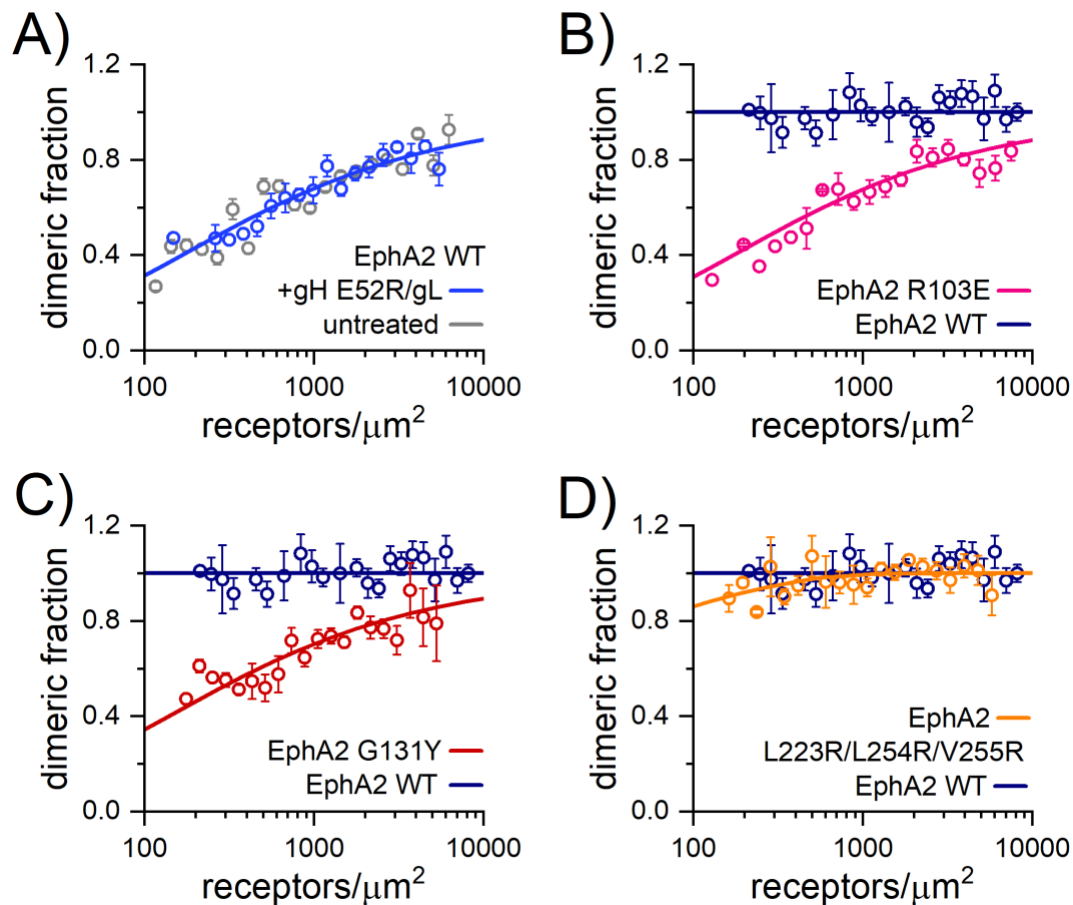
1127

1128

1129 Figure 4 legend

1130 The FSI-FRET data measured in HEK293T cells (Fig. S8) were fit to dimerization models to generate
 1131 dimerization curves by plotting the calculated dimeric fraction as a function of the total EphA2
 1132 concentration. The binned dimeric fractions are shown along with the best-fit curve. The data
 1133 measured for EphA2 WT in the presence of **(A)** 200 nM gH/gL, **(B)** 200 nM LBD, and **(C)** 200 nM gH/gL-
 1134 LBD are compared to EphA2 WT data in the absence of ligand, which was previously reported [3].
 1135 Soluble gH/gL induces constitutive EphA2 dimerization, as evidenced by the dimeric fraction of 1 at all
 1136 measured EphA2 concentrations. Little to no difference in the dimerization curves were observed
 1137 when in the presence of LBD and precomplexed gH/gL-LBD compared to untreated EphA2 WT which
 1138 suggests that EphA2 LBD blocks the effect of gH/gL on EphA2 dimerization. **(D)** Fluorescence Intensity
 1139 Fluctuation (FIF) measurements in HEK293T cells reporting on EphA2 WT-eYFP oligomer size in the
 1140 absence or presence of 200 nM gH/gL. Histograms of molecular brightness (ϵ), measured in small
 1141 regions of the basolateral membrane over all measured receptor concentrations, are compared to the
 1142 published FIF data for the monomer control (LAT) and dimer control (E-cadherin). The maximum of the
 1143 histogram for EphA2 WT in the presence of gH/gL shifts to higher brightness than for EphA2 WT in the
 1144 absence of ligand and is very similar to that of the E-cadherin dimer control, which suggests that EphA2
 1145 is a constitutive dimer in the presence of gH/gL, consistent with the FSI-FRET data.

1146 Figure 5: The gH/gL induced EphA2 dimers on cells engage the
1147 'dimerization' interface.
1148
1149

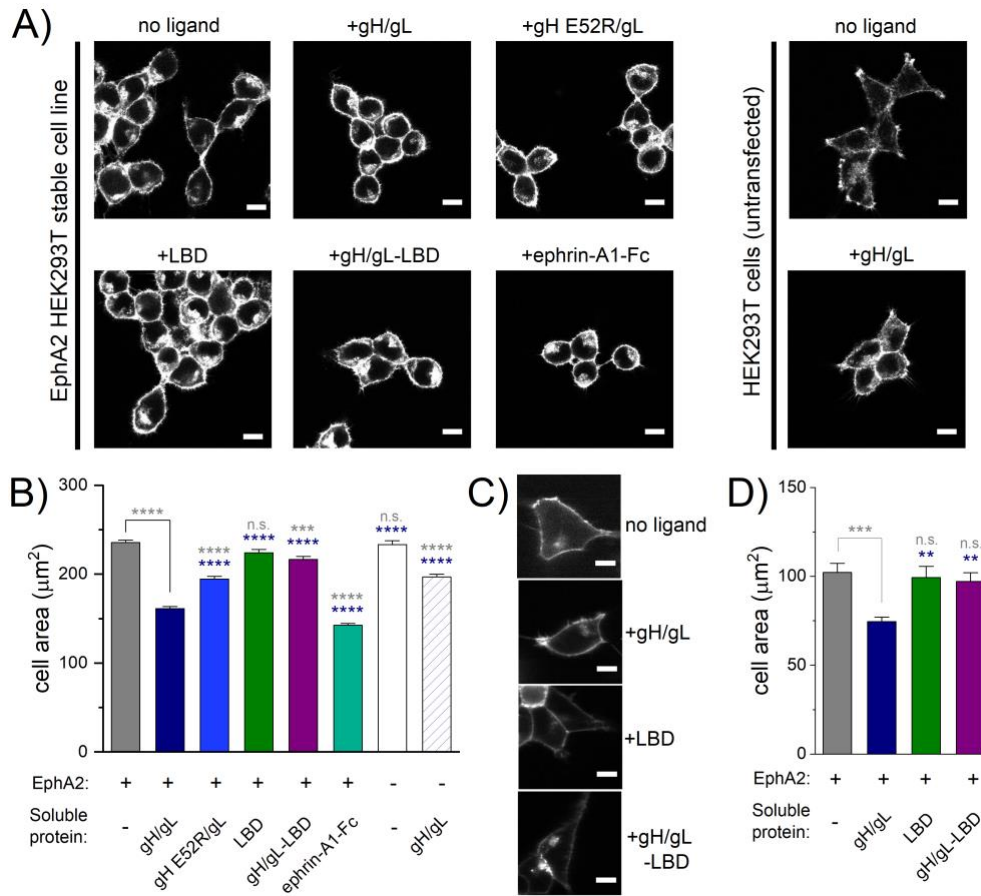


1150
1151
1152

Figure 5 legend

1153 Dimerization curves calculated from the FSI-FRET data for **(A)** EphA2 WT in the presence of 200 nM gH
1154 E52R/gL mutant with mutation in EphA2 binding, and for the EphA2 mutants **(B)** R103E^{EphA2} mutant
1155 impaired in ligand binding, **(C)** G131Y^{EphA2} mutant with mutation in DIN, and **(D)**
1156 L223R/L254R/V255R^{EphA2} mutant with mutations in CIN. The data in A are compared to EphA2 WT data
1157 in the absence of ligand. The data in B-D were collected in the presence of 200 nM gH/gL and are
1158 compared to EphA2 WT in the presence of gH/gL (Fig. S8). No difference in the dimerization curve is
1159 observed with the mutated gH E52R/gL and thus does not induce constitutive EphA2 dimers as gH/gL
1160 does, which suggests impaired binding to EphA2. Large differences in the dimerization curves are
1161 observed for the R103E^{EphA2} and G131Y^{EphA2} mutants, but the effect of the triple
1162 L223R/L254R/V255R^{EphA2} mutation is modest. This data indicates that gH/gL-bound EphA2 dimers
1163 interact mainly via the DIN (where G131 is engaged) but not via the CIN (where L223/L254/V255 are
1164 engaged) and that R103^{EphA2} is important for gH/gL binding.

1165 Figure 6: HHV-8 gH/gL stimulates EphA2-induced cell contraction.



1166

1167 Figure 6 legend

- 1168 **A)** Images of fixed HEK293T cells stained for actin with rhodamine-conjugated phalloidin. The six
1169 images on the left were collected with HEK293T cells stably expressing EphA2 WT-eYFP and the
1170 two images on the right with HEK293T cells which do not express EphA2. Cells were stimulated
1171 with PBS (no ligand), 200 nM gH/gL, 200 nM gH E52R/gL, 200 nM LBD, 200 nM gH/gL-LBD complex,
1172 or 500 ng/mL ephrin-A1-Fc for 10 min prior to fixing with paraformaldehyde. Scale bar is 10 μm .
- 1173 **B)** Histograms of the average cell areas and the standard errors determined from the images of fixed
1174 cells shown in panel A. In the presence of saturating gH/gL concentrations, the EphA2-eYFP-
1175 expressing cells are significantly smaller in size compared to the cases of no ligand, +gH E52R/gL,
1176 +LBD, and +gH/gL-LBD, but are larger than cells stimulated with ephrin-A1-Fc. Untransfected
1177 HEK293T cells experience a slight decrease in average cell area in the presence of gH/gL but not to
1178 the same extent as cells expressing EphA2. Statistical significance was determined by a one-way
1179 ANOVA and a Tukey's multiple comparison using the GraphPad Prism software ($P < 0.0001 = ****$,
1180 $P < 0.001 = ***$, $P < 0.01 = **$, $P < 0.1 = *$, $P \geq 0.1 = \text{n.s.}$). Statistics results in grey are compared to
1181 EphA2 no ligand and those in navy are compared to EphA2 + gH/gL.
- 1182 **C)** Images of live HEK293T cells transiently transfected with EphA2 WT-eYFP in the absence or
1183 presence of 200 nM gH/gL, 200 nM LBD, or 200 nM gH/gL-LBD. Scale bar is 10 μm .
- 1184 **D)** Histograms showing the average cell areas and the standard errors determined from the live cell
1185 images shown in panel C. In the presence of saturating gH/gL concentrations, the EphA2-eYFP-
1186 expressing cells are smaller in size compared to cells with no ligand, with LBD, and with gH/gL-LBD.
1187 Statistical significance was determined by a one-way ANOVA and a Tukey's multiple comparison
1188 using the GraphPad Prism software ($P < 0.0001 = ****$, $P < 0.001 = ***$, $P < 0.01 = **$, $P < 0.1 = *$,
1189 $P \geq 0.1 = \text{n.s.}$). Statistics results shown in gray when comparison is made compared to unliganded
1190 EphA2, and those in navy in comparison to EphA2 + gH/gL.

1191 References

1192

- 1193 1. Su, C., et al., *Molecular basis of EphA2 recognition by gHgL from gammaherpesviruses*. Nat
1194 Commun, 2020. **11**(1): p. 5964.
- 1195 2. Himanen, J.P., et al., *Crystal structure of an Eph receptor-ephrin complex*. Nature, 2001.
1196 **414**(6866): p. 933-8.
- 1197 3. Gomez-Soler, M., et al., *Engineering nanomolar peptide ligands that differentially modulate*
1198 *EphA2 receptor signaling*. Journal of Biological Chemistry, 2019. **294**(22): p. 8791-8805.
- 1199 4. Singh, D.R., et al., *The SAM domain inhibits EphA2 interactions in the plasma membrane*.
1200 Biochim Biophys Acta Mol Cell Res, 2017. **1864**(1): p. 31-38.
- 1201 5. Singh, D.R., et al., *Intracellular Domain Contacts Contribute to Ecadherin Constitutive*
1202 *Dimerization in the Plasma Membrane*. J Mol Biol, 2017. **429**(14): p. 2231-2245.
1203