Neuropilin 1 is an entry factor that promotes EBV infection of nasopharyngeal epithelial cells

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Epstein–Barr virus (EBV) is implicated as an aetiological factor in B lymphomas and nasopharyngeal carcinoma. The mechanisms of cell-free EBV infection of nasopharyngeal epithelial cells remain elusive. EBV glycoprotein B (gB) is the critical fusion protein for infection of both B and epithelial cells, and determines EBV susceptibility of non-B cells. Here we show that neuropilin 1 (NRP1) directly interacts with EBV gB23–431. Either knockdown of NRP1 or pretreatment of EBV with soluble NRP1 suppresses EBV infection. Upregulation of NRP1 by overexpression or EGF treatment enhances EBV infection. However, NRP2, the homologue of NRP1, impairs EBV infection. EBV enters nasopharyngeal epithelial cells through NRP1-facilitated internalization and fusion, and through macropinocytosis and lipid raft-dependent endocytosis. NRP1 partially mediates EBV-activated EGFR/RAS/ERK signalling, and NRP1-dependent receptor tyrosine kinase (RTK) signalling promotes EBV infection. Taken together, NRP1 is identified as an EBV entry factor that cooperatively activates RTK signalling, which subsequently promotes EBV infection in nasopharyngeal epithelial cells.
EBV infection of B cells consists of at least two distinct mechanistic steps. EBV targets to the attached cells through the interaction of EBV glycoprotein gp350/220 with CD21 (the B cell complement receptor, CR2) or CD35 (refs 8,9). Subsequently, EBV fuses and penetrates into B cells, triggered by the interaction of gp42 (an additional EBV glycoprotein) with HLA class II, in the presence of EBV gB and gHgL (the core fusion machinery)10. However, the binding receptors CD21 and CD35, and the fusion receptor HLA class II, are expressed at low or undetectable levels in epithelial cells11,12. Therefore, EBV gp42 and gp350 were not essential in EBV infection of epithelial cells, suggesting different mechanisms contributing to EBV infection of epithelial cells12.

EBV gB is the most highly conserved glycoprotein required for membrane fusion in herpesviruses, but its cellular mediator involved in EBV fusion has not been identified so far13. EBV strains with higher expression of gB exhibit an increased capacity to infect cells that are normally refractory to EBV infection14. EBV gB contains a consensus furin cleavage site15,16. After cleavage by furin, EBV gB exhibited as a N-terminal peptide with 78 kDa, and a C-terminal peptide with 58 kDa. Both full-length and furin-cleaved gB are moderately abundant potential fusogens in mature EBV envelopes16. Deletion of the consensus furin cleavage site of gB, which is speculated to be a potential cryptic CendR motif, results in the suppression of cell-cell fusion, indicating the importance of this site to EBV infection15. Peptides that expose the CendR motif with the consensus sequence R/K/XXR/K at the C-terminus bind to Neuropilin 1 (NRP1) and are internalized into the cell17,18.

NRP1, as a co-receptor for class III semaphorins and multiple growth factors, such as EGF, VEGF, PDGF, HGF, TGF-β and FGF, cooperatively enhances the activity of the receptor tyrosine kinases (RTKs)19. In addition, NRP1 mediates the penetration of iRGD conjugated nanoparticles into tissue and cells through functioning as a receptor for CendR motif, the proteolytic cleavage products of iRGD after binding to integrins17,20. Multiple viruses possess CendR motifs within their capsid proteins and may undergo proteolytic cleavage to expose the CendR motif to be infective18. Human T-cell lymphotropic virus type 1 (HTLV-1) is one of such virus that bind to and internalize into immune cells via the interaction with NRP1 and its surface subunit (SU) containing a CendR motif (KPRX)21,22.

Together, these observations led us to deduce that NRP1 might serve as an unidentified entry factor or a cellular mediator for gB during EBV infection. Here, we demonstrate that NRP1 interacts with EBV gB and promotes EBV infection of epithelial cells by coordinating the RTK signalling pathway and macrophagic events.
about twofold (Fig. 2a,b and Supplementary Fig. 5). Pre-incubation of EBV with the soluble ectodomain of NRP1 (NRP1ABC) inhibited EBV infection by about 50%, whereas an anti-NRP2 antibody increased EBV infection by about threefold (Fig. 2c).

Next, we examined the role of overexpression of NRP1 and NRP2 on EBV infection. The efficiency of EBV infection was significantly enhanced by an increase in NRP1 expression, whereas overexpression of NRP2 inhibited EBV infection (Fig. 2d,e and Supplementary Fig. 6). Epidermal growth factor (EGF) is a known cytokine that induces NRP1 expression in multiple cancer cells29. To examine the effect of EGF on the expression of NRP1 and EBV infection, HNE1 cells were incubated with EGF for 24 h. EGF significantly enhanced the expression of NRP1 in HNE1 cells, but did not change the level of NRP2 (Fig. 2f). Consistently, the efficiency of EBV infection was significantly enhanced by EGF (Fig. 2g, Supplementary Figs 6 and 7). In addition, EGF promotes EBV infection and the expression of NRP1 in a dose-dependent manner (Supplementary Fig. 8). Furthermore, knockdown of NRP1 led to a significantly decreased EBV infection in EGF-treated HNE1 and NPEC1-Bmi1 cells maintained in KSF medium supplemented with EGF (Fig. 2h,i and Supplementary Fig. 9), suggesting that EGF-induced uptake of EBV at least partially depended on the induction of NRP1.

NRP1 is a receptor for a number of ligands (for example, semaphorins, VEGF-A)30,31. To investigate whether the binding of NRP1 and its ligands would affect EBV infection, HNE1 cells were incubated with the indicated doses of NRP1 ligands (SEMA3A, SEMA3F or VEGFA) for 1 h before EBV infection. SEMA3A, SEMA3F or VEGFA had no effect on EBV infection (Supplementary Fig. 10); however, whether other ligands for NRP1 affect EBV infection remains to be further investigated.

NRP1 co-localizes with EBV and binds to EBV-gB. To investigate whether NRP1 could directly mediate EBV infection, we examined the localization of EBV and NRP1 in NRP1-over-expressing HNE1 cells. Confocal microscopy revealed that both Alexa Fluor 594-labelled EBV and anti-gp350 antibody-stained EBV co-localized with NRP1-EGFP on the cell membrane or at vesicular structures (Fig. 3a and Supplementary Fig. 11). Similarly, the co-localized signals of NRP2 and EBV could also be detected (Fig. 3b).

The binding of the purified EBV gB23–431 and GST-NRP1 or GST-NRP2 was then analysed by ELISA. The apparent affinity constant for binding of gB and NRP2 was higher than that for gB and NRP1 (Fig. 3c,d).

As NRP1 and NRP2 played opposite effects on EBV infection, we therefore evaluated whether NRP2 would affect the binding of NRP1 to gB. Co-immunoprecipitation indicated that the interaction of NRP1 and gB was obviously reduced in the presence of NRP2 (Fig. 3e).

NRP1 facilitates EBV internalization and fusion. As mentioned above, EBV entry comprises at least two steps, including binding...
(attachment) and penetration (fusion). We then investigated in which step NRP1 played a role. Upregulation of NRP1 by either overexpression or EGF treatment increased EBV internalization by about 2.5-fold, while knockdown of NRP1 by siRNA caused a decrease to about 50% of the control. In contrast, overexpression of NRP2 impaired EBV internalization, while knockdown of NRP2 enhanced EBV internalization. Both gain- and loss-of-function experiments revealed that neither NRP1 nor NRP2 exerted any effect on EBV binding (Fig. 4a–c). To determine the role of NRP1 or NRP2 in the efficiency of cell-cell fusion, NRP1- or NRP2-overexpressing HEK-293FT cells were co-cultured with EBV glycoproteins (gB and gH/gL)-overexpressing HEK-293FT or NRP2-overexpressing HEK-293FT cells. Overexpression of NRP1 significantly promoted cell-cell fusion while overexpression of NRP2 had no effect on cell fusion (Fig. 4d).

Collectively, these data suggested that NRP1 may facilitate EBV internalization and fusion, but not binding.

**EBV enters cells via endocytosis.** It has been reported that NRP1 mediates endocytosis via different pathways, depending on its ligands. We thus examined the mechanistic basis for EBV endocytosis. Enveloped viruses penetrate into the cytosol directly through caveole-mediated endocytosis (for particles with size of

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**Figure 2 | NRPI enhances EBV infection, while NRPII suppresses EBV infection.** (a,b) Downregulation of NRPI impaired, whereas knockdown of NRPII promoted EBV infection. HNE1 cells were transfected with siRNA duplexes targeting NRPI or NRPII for 48 h, followed by NRPI expression analysis by real-time PCR (a) or analysis for the efficiency of EBV infection (b); n = 3. (c) EBV infection was blocked by soluble NRPIABC, but enhanced by antibody against NRPII. For NRPIABC protein-blocking experiment, HNE1 cells were infected with EBV, which was pre-incubated with purified NRPIABC for 1 h. For antibody against NRPII-blocking experiment, HNE1 cells were pre-incubated with an anti-NRP2 antibody (100 μg ml−1) or goat IgG (control) at 4 °C for 1 h and then were exposed to EBV at an MOI of 5 × 10^3 for 3 h at 4 °C. (d,e) Overexpression of NRPI enhanced EBV infection, while NRPII suppressed EBV infection. HNE1 cells were transiently transfected with the expression plasmid for NRPI, NRPII or the empty vector (pMSCV) for 24 h, followed by analysis for the expression of NRPI and NRPII by western blotting (d) or were exposed to EBV (e). (f,g) EGF upregulated NRPI expression and enhanced EBV infection. HNE1 cells cultured with 10 ng ml−1 EGF for 24 h were analysed for the expression of NRPI and NRPII by western blotting (f) or were exposed to EBV (g). (h,i) EGF-enhanced EBV infection was partially dependent on NRPI. After transfected with siRNA against NRPI for 48 h, EGF-treated HNE1 and NPEC-Bmi1 cells maintained in KSF medium supplemented with EGF were analysed for NRPI expression by western blotting (h) or were exposed to EBV (i). For (b), (c), (e), (g) and (i), HNE1 or NPEC1-Bmi1 cells were exposed to EBV at an MOI of 2.5 × 10^3 for 2 h at 37 °C, unless otherwise indicated. The percentage of GFP-positive infected cells was analysed by FACS 48 h post infection, with controls (empty vector-transfected cells or vehicle treated-cells) set to 100%. Data represent three to five independent experiments. Values in all graphs are means ± s.e.m. ***P < 0.001; **P < 0.01; *P < 0.05; Student’s t-test. For (d), (f) and (h), GAPDH served as an internal control.
50–80 nm), clathrin-mediated endocytosis (CME, for particles with size of 85–180 nm) and macropinocytosis (for particles with size of 0.5–10 μm)33. EBV is an enveloped virus with a diameter of 120–220 nm. Therefore, EBV infection is unlikely to be dependent on caveola-mediated endocytosis. Confocal analysis showed that both EBV-Alexa fluor 594 and NRP1 co-localized with SNX5 (marker of macropinocytosis), but not with CLCa (marker of CME) (Fig. 5a–d), suggesting that NRP1-mediated EBV internalization may be dependent on macropinocytosis, but not CME. To further verify this observation, EGF-treated HNE1 cells and NPEC1-Bmi1 cells were pre-incubated with the inhibitors of macropinocytosis (5-(N-ethyl-N-isopropyl)-amiloride, EIPA), lipid raft-dependent endocytosis (Methyl-

\[ \text{GST-NRP1 (nM)} \]

| molarity | GST-NRP1 | Relative OD450 value (max OD450 = 100%) |
|---------|---------|--------------------------------------|
| 2^{-5}  | 0       | 0                                    |
| 2^{-4}  | 0       | 0                                    |
| 2^{-3}  | 0       | 0                                    |
| 2^{-2}  | 0       | 0                                    |
| 2^{-1}  | 0       | 0                                    |
| 2^{0}   | 0       | 0                                    |
| 2^{1}   | 0       | 0                                    |
| 2^{2}   | 0       | 0                                    |
| 2^{3}   | 0       | 0                                    |
| 2^{4}   | 0       | 0                                    |
| 2^{5}   | 0       | 0                                    |
| 2^{10}  | 0       | 0                                    |

**Figure 3** | NRP1 and NRP2 co-localize with EBV and bind to EBV-gB^{23–431}. (a,b) NRP1 and NRP2 co-localize with EBV. HNE1 cells transiently transfected with the indicated expression vectors for 24 h were infected with Alexa fluor 594-labelled EBV (EBV-Alexa 594). Expressions of NRP1-EGFP and NRP2-EGFP were visualized as green. EBV-Alexa fluor 594 was imaged as red. Nuclei were stained with DAPI (blue). The images of the boxed areas labelled as 1 and 2 were enlarged and shown. (c,d) The affinity constant of the interaction between gB and NRP1 or NRP2. Microtitre plates were coated with 200 ng purified FLAG-gB^{23–431} and incubated with various concentrations of GST-NRP1 or GST-NRP2, followed by the rabbit anti-GST and the HRP-conjugated anti-rabbit IgG secondary antibodies. The binding of purified EBV-gB^{23–431} and GST-NRP1 (c) or GST-NRP2 (d) were analysed by ELISA. Values are means ± s.e.m. of three independent experiments. (e) NRP2 influences the binding of NRP1 to EBV-gB. HEK-293FT cells transfected with the indicated doses of expression vector for 36 h. The cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody, followed by immunoblotting (IB) analysis with an anti-GFP, anti-HA or anti-FLAG antibody, as indicated. The experiments were performed three times with similar results.
cultured for 48 h. EBV infection was dose-dependently suppressed by M\textsubscript{b}CD and EIPA, but not by CPZ in both types of cells, with preserved cell viability (Fig. 5e–g and Supplementary Fig. 12). These data demonstrated that EBV entered epithelial cells via macropinocytosis and lipid raft-dependent endocytosis, but not clathrin-mediated endocytosis.

EBV activates NRP1-dependent EGFR signalling pathways. Macropinocytosis and lipid raft-dependent endocytosis can be induced by RTKs\textsuperscript{34,35}. As a co-receptor of RTKs, NRP1 enhances the affinity of multiple growth factors, such as EGF, HGF, VEGF, PI GF and PDGF-BB, to RTKs and thus augments RTKs signalling\textsuperscript{36–40}. EGFR, the prototypical RTK aberrantly expressed in NPC\textsuperscript{41}, as well as its critical downstream signalling components AKT and ERK, were rapidly phosphorylated at 10 min post EBV infection, and the phosphorylation increased and persisted for at least 120 min (Fig. 6a). To exclude that the activation of EGFR signalling pathways was caused by the virus debris, HNE1 cells were infected with EBV purified by high-speed centrifugation in dextran T-10 density gradients for 1 h. EGFR, AKT and ERK were phosphorylated by purified EBV (Fig. 6b), indicating that the EGFR signalling pathway was indeed activated by EBV. Knockdown of NRP1 partially suppressed the phosphorylation of EGFR, AKT and ERK on EBV infection (Fig. 6c), suggesting that NRP1 was associated with EBV activation of EGFR/AKT and EGFR/ERK pathways.

To confirm the role of RTK signalling pathways in EBV infection, EGF-treated HNE1 and NPEC1-Bmi1 cells pre-incubated with inhibitors of tyrosine kinases (Genistein), MEK1/MEK2 (U0126), PI3K/AKT (LY294002), EGFR (Gefitinib) and VEGFR2/PDGFR/Raf signalling cascades (Sorafenib), were infected with EBV. Genistein, Gefitinib, Sorafenib and U0126 partially eliminated EBV entry, whereas LY294002 did not affect EBV infection (Fig. 6d,e). These data suggested that the activation of multiple RTKs and the downstream signalling Ras/Raf/MEK/ERK rather than PI3K may enhance EBV infection.

As Genistein and Sorafenib partially impaired EBV infection, we further investigated whether other signalling pathways besides EGF/EGFR were also important for EBV infection. HNE1 cells were transfected with siRNA duplexes targeting EGFR or c-Met (receptor for HGF), followed by EBV infection. The expression of EGFR and c-Met was nearly diminished in HNE1 cells transfected with siEGFR or siMET. Knockdown of either EGFR

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**Figure 4 | NRP1 facilitates EBV internalization and cell–cell fusion, while NRP2 inhibits EBV entry.** (a) NRP1 promoted EBV internalization, but did not affect EBV binding. HNE1 cells transfected with overexpression plasmids for NRP1 or NRP2 were infected with EBV at 37 °C to allow virus internalization, followed by real-time PCR analysis for EBV copy number; n = 3. (b) EGF-enhanced EBV internalization. EGF-treated HNE1 cells were subjected to analysis for EBV binding and internalization; n = 6. (c) Downregulation of NRP1 impaired, whereas knockdown of NRP2 promoted EBV internalization in EGF-treated HNE1 cells. EGF-treated HNE1 cells transfected with siRNA against NRP1 or NRP2 were subjected to analysis for EBV binding and internalization; n = 3. (d) NRP1, but not NRP2, promoted cell–cell fusion. HEK-293FT cells transfected with expression vectors for T7 polymerase, gB and gH/gL were co-cultured with HEK-293FT cells co-transfected with pT7EMCLuc, pRL-SV40, the expression plasmid for NRP1, NRP2 or the empty vector (pMSCV). The relative fusion activity was calculated as the ratio of firefly to Renilla luciferase activity analysed 24 h after co-culture, with the empty vector controls set to 100%; n = 3. Graphs show mean ± s.e.m. **P < 0.01; Student’s t-test.
or c-Met impaired EBV infection by about 50% (Fig. 6f,g), indicating that there may be indeed other RTKs contributing to EBV infection.

Furthermore, activated Ras (HRas V12) partially rescued the suppressive effect of Gefitinib on EBV infection, confirming that HRas mediates EGFR-dependent EBV entry (Fig. 6h). Knockdown of NRP1 even suppressed EBV infection in HNE1 cells with persistently activated Ras (Fig. 6i), suggesting that the activated Ras signalling was associated with but insufficient for EBV infection, and highlights the role of NRP1 in facilitating EBV entry into nasopharyngeal epithelial cells.

Discussion

Epstein–Barr virus (EBV), an ubiquitous human herpesvirus, has been classified as a group 1 carcinogen. It is aetiologically associated with lymphoid and epithelial tumours, suggesting its primary tropism for these cells. The mechanism contributing to EBV infection of B cells has been well documented, while the mechanisms of cell-free EBV infection of epithelial cells remain elusive. Here, we established a cell model relatively susceptible to cell-free EBV infection, and highlighted the important role of NRP1 in mediating cell-free EBV infection of nasopharyngeal epithelial cell lines and EBV activated the RTK signalling pathway.

In addition to cell-free EBV infection, cell-to-cell contact is supposed to be an efficient mode of EBV infection of diverse human epithelial cells. Like cell-free EBV infection, EGF promotes the transmission of EBV from infected Akata cells to uninfected HNE1 cells, partially depending on the expression of NRP1 on the host cells (Supplementary Fig. 13), suggesting an important role of NRP1 and EGF in facilitating not only cell-free EBV infection, but also cell-to-cell contact-mediated infection.

EBV gB, the most highly conserved glycoprotein, is necessary for EBV infection. It mainly presented as the trimeric form, consistently with the previous report about the crystal analysis of...
EBV gB, EBV gB23–431, gB23–683 (the ectodomain of EBV-gB) and gB23–853 (the almost full-length EBV-gB) showed interaction with NRP1. In addition to the CendR motif, gB23–88 is another important element contributing to the interaction between NRP1 and gB. Reimer et al.24 revealed that, although linker insertions at position 88 did not affect cell-surface expression of gB, it abrogated the ability of the variant protein to mediate fusion. Backovic et al.23 reported residues 88 was a hydrophilic residue. Therefore, gB23–88 may define novel binding sites for ligands, such as a gB receptor or other EBV envelope glycoproteins.
involved in EBV infection\textsuperscript{23}. We demonstrated that NRP1 and NRP2 interacted with the glycoprotein gB, but had opposite effect on EBV infection. Overexpression of NRP1 significantly promoted cell-cell fusion, while overexpression of NRP2 had no effect on cell fusion. Both being as co-receptors for RTKs, NRP1 and NRP2 bind to different ligands. NRP1 binds to Sema3A and initiates plexin signalling, which activates CRMP, ERK and Rac1, whereas NRP2 binds to Sema3F and activates Rac GTPase-activating protein (GAP) β2-Chimaerin\textsuperscript{30,44,45}. Although the exact mechanism underlying the discrepancy of NRP1 and NRP2 on the susceptibility of EBV infection remains to be determined, it could be attributed to the different ligands they bind, and the distinct downstream signalling pathways they activated. However, we found that the ligands for NRP1 (SEMA3A, SEMA3F and VEGF-A) had no effect on EBV infection, whether other ligands for NRPs affect EBV infection of nasopharyngeal epithelial cells remain further investigation.

It has been reported that the interaction between epithelial integrins (for example, αvβ6, αvβ8 or α5β1) and EBV envelope proteins (gHgL or BMRF2) is required for EBV infection of epithelial cells originated from tongue, nasopharyngeal and gastric carcinoma\textsuperscript{46,47}. We found integrins αvβ1 and β6, but not integrins α5 and β8, may contribute to EBV infection of nasopharyngeal epithelial cells (Supplementary Fig. 14).

Integrins bind the RGD/KGD motif-containing peptide, which is cleaved by cell surface-associated proteases to expose the cryptic CendR element, RXXK/R, at the C terminus. The CendR element then mediates binding to NRP1, resulting in the penetration of cells and tissues\textsuperscript{18}. Both the full-length and cleaved forms of gB are present in the purified EBV (Supplementary Fig. 2). We therefore suppose the model that EBV entry into epithelial cells: for the full-length gB-containing EBV, EBV binds to nasopharyngeal epithelial cells through the interaction between epithelial integrins (αv, β1 and β6) and other unknown factors and envelope proteins (gHgL or BMRF2). Then, EBV gB was cleaved by furin to expose the CendR motif. For cleaved gB containing EBV, the CendR motif may be already exposed\textsuperscript{16,23}. Followed by the interaction between the CendR motif on the cleaved EBV gB and NRP1, EBV enters into epithelial cells.

In addition to Gefitinib, Sorafenib and Genistein partially impaired EBV infection, suggesting there are multiple RTKs and the downstream signalling pathways other than EGFR contributing to EBV infection. This point was further confirmed by the evidence that knockdown of c-Met attenuated EBV infection. Therefore, various RTKs signalling pathway may promote EBV infection, and the detailed mechanisms are deserved to be further investigated.

Multiple viruses, such as HTLV-1, possess CendR motifs within their capsid proteins. Similarly, NRP1 also serves as an entry factor for HTLV-1 through interaction with the KPXR element (a CendR motif) exposed on its surface subunit (SU). Whether NRP1-mediating RTK signalling pathways also play important role in HTLV-1 infection remains to be further investigated.

EBV gB is critical to mediate virus cell fusion\textsuperscript{48,49}. We found that NRP1 serves as an entry factor for EBV infection of nasopharyngeal epithelial cells. However, whether NRP1 also serves a similar function for EBV entry into B cells has not been explored and deserves further investigation, although the expression of NRP1 on B cells is quite low (Supplementary Fig. 15). NRP1 not only functioned as an entry factor for EBV to entry into epithelial cells, but was also associated with EBV-activated EGFR/RAS/ERK signalling, which in turn potentiated EBV infection (Fig. 7). As multiple inhibitors of receptor tyrosine kinase signalling pathways impaired EBV infection, further investigations are required both to investigate whether receptor tyrosine kinases beyond EGFR are also involved in promoting EBV infection, and to elucidate the detailed mechanism by which NRP1 contributes to viral entry. Such findings would assist in the development of anti-EBV agents that target this crucial stage in virus infection.

**Methods**

**Reagents.** The reagents used were as follows: mouse monoclonal antibodies against FLAG (F1804, Sigma-Aldrich, 1:2,000 dilution), α-tubulin (T6074,
dilution) was gifted from R&D Systems. Alexa Fluor 594-conjugated goat-anti-
1:3,000 dilution). HRP-conjugated donkey-anti-sheep secondary antibody (1:2,000
gap polyclonal antibody against NRP2 (AF2215, R&D Systems, 1:500 dilution);
the horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antibodies (Fibbiotech,
13,000 dilution). HRP-conjugated donkey-anti-sheep secondary antibody (1:2,000
dilution) was performed in a humidified 5% CO2 incubator at 37
medium. The cells grown out from the biopsies were propagated and stained with
5% FBS. Cells were grown in a humidified 5% CO2 incubator at 37
NPECs-Bmi1 cells and NPECw cells, grown in keratinocyte-serum-free
be thawed immediately before infection.
Cell culture. NPECs-Bmi1 cells and NPC cells, grown in keratinocyte-serum-free
and 20% FBS. Cells were grown in a humidified 5% CO2 incubator at 37
Plasmids. To construct expression vectors for NRPI and NRPII, the full-length
complementary DNA (cDNA) sequences of NRPI and NRPII were PCR-amplified
using cDNA from NPC-Bmi1 cells and cloned into the pCEP-his vector.
for the activated Ras was gifted from Hammerschmidt, Department of Gene Vectors, Helmholtz Zentrum München.
To investigate whether gB interacted with NRPI, the plasmids (pCEP-his-
egB23–683, eG B23–431, eG B89–431 and eG B89–427 were PCR-amplified from the
EBV gp175 glycoprotein expression plasmid p2670 (gifted from Professor W.
Hammerschmidt, Department of Gene Vectors, Helmholtz Zentrum München) and cloned into the pCEP-his vector.
To determine the fusion efficiency, plasmids pCAGGT7 encoding T7 RNA
polymerase, and pTTEMCLuc carrying the firefly luciferase gene under the control of
the T7 promoter (gifted from Professor R. Longnecker, Northwestern
University) were used.
To investigate whether gB interacted with NRPI, the plasmids (pCEP-his-
egFLAG-gB23–683, pCEP-his-FLAG-gB23–431, pCEP-his-FLAG-gB89–427, and
pCEP-his-FLAG-gB23–683 were constructed. The fragments
were amplified from pGEX-NRP2ABC, respectively.
NRP1-Mcherry. The full-length cDNA sequence of SNX5 was PCR-amplified using
the cDNA from NPEC1-Bmi1 cells and cloned into the pEGFP-C2 vector
complementary DNA (cDNA) sequences of NRPI and NRPII were PCR-amplified
using cDNA from NPEC1-Bmi1 cells, and then integrated into the XhoI/EcoRI
cloning sites in pMSCV-puro vector (Clontech) and into the HindIII/SalI sites in pLncx2-
neutral (Clontech), naming as pEGFP-NRP1, or inserted into
pMSCV-HRAS-V12 for the activated Ras was gifted
from Professor Vimla Band (the University of Nebraska Medical Center). Primers for cloning are listed in Supplementary Table 1.
siRNA oligoribonucleotides. ON-TARGET plus SMART pool siRNA duplexes
 targeting ITGAV (Cat# M-004565-03), ITGBl (Cat# M-004506-00), ITGB6 (Cat# M-008012-01), ITGB8 (Cat# M-008014-02) and the
gene was targeted by double stranded RNA. The siRNA duplexes targeting two distinct sites of
human NRPI mRNA (NCBI, NM_003873.5, Gene ID: 8829) were denoted as
siNRPI-1# and siNRPI-2#, while the siRNAs duplexes targeting two distinct sites of
human NRPII RNA (NCBI, NM_003872.2, Gene ID: 8828) were named as
siNRPI-1# and siNRPI-2#. The siRNA duplexes against NRPI and NRPII were
synthesized by GenePharma (Shanghai). All siRNA duplexes are listed in
Supplementary Table 2.
Cell transfection. Cell transfection was performed with FuGene HD (Roche),
Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) as
indicated, according to the manufacturer’s instructions. For overexpression experiments, HNE1 cells were plated at a density of 4–6 × 10^4 cells per well in
24-well plates. Sixteen hours after seeding, cells were grown to about 40% confluence, and each well received 1.5 μl Fugene HD and 0.75 μl of the indicated
plasmids. For immunoprecipitation assay, HEK-293FT cells were plated in six-well plates at a density of 4 × 10^5 cells per well and grown to about 80% confluence.
Sixteen hours after seeding, each well was co-transfected with 1 μg of pCEP-his-
egFLAG-gB23–431 and 1 μg of pEGFP-NRP1 or pEGFP-NRP2 with 5 μl Lipofectamine
2000 for 36 h. For siRNA experiments, a final concentration of 20 nM siRNA
duplex was reversely transfected with Lipofectamine RNAiMAX, unless otherwise indicated.

Virus production. Akata cells carrying EBV, in which the thymidine kinase gene
was interrupted with a double cassette expressing GFP and neomycin resistance
gene, were resuspended in FBS-free RPMI 1640 medium at a concentration of
2–3 × 10^6 cells/ml, followed by induction with 0.75% (v/v) of goat anti-human
immunoglobulin G serum (Shuangliu Zhenglong Biochem.Lab) for 6 h at 37°C. After culture in fresh RPMI1640 medium supplemented with 4% FBS for 3 days,
virus from the supernatant was collected under sterile conditions, passed through
two Millipore filters (0.8 and 0.45 μm), 100-fold concentrated by high-speed
centrifugation with 50,000 g, and then resuspended in fresh FBS-free RPMI1640
(ref. 30). The virus was stored at −80°C and thawed immediately before infection.

Cell-free EBV infection. EBV-negative NPEC/NPC cells were plated at a density of
4–6 × 10^5 cells per well in 24-well plates and grown to about 60% confluence.
Cells were briefly washed with Hanks solution twice and were infected with 200 μl
EBV at a multiplicity of infection (MOI) of about 2.5 × 10^3 for 3 h at 37°C, unless indicated. After brief wash with Hanks solution twice to remove unbound virus,
cells were cultured in the fresh medium for 48 h. The GFP-positive infected cells
were determined by fluorescence microscopy (Olympus) and/or flow cytometry
(Beckman Coulter FC500) at the indicated times post infection.

 Determination of MOI. To determine the MOI (a multiplicity of infection) of
EBV, TaqMan real-time PCR was used to detect the BamHI-W fragment region of
the EBV genome 15. A calibration curve was performed, using DNA extracted from
the EBV-positive cell line Namalwa, which contains two integrated viral genomes
cell as a standard.
To evaluate the infection efficiency of the newly purified EBV, 1 × 10^5 HNE1
cells plated in 24-well plate were infected with serial dilutions of EBV. The
percentage of infected cells was analysed by flow cytometry 48 h post infection.
EBV infection increased along with the virus titre. About the MOI of 2 × 10^3 was
required for 20–30% of HNE1 cells to be infected, whereas an MOI of 1 × 10^4 was
required for >80% of HNE1 cells to be infected. Therefore, EBV infection of
epithelial cells was mediated in a virus-titer-dependent manner.

Virus binding and internalization. Cells were seeded at the density of 5 × 10^4
cells per well in 24-well plates for 24 h. To measure virus binding, cells were washed
with ice-cold Hanks solution twice and then were incubated with EBV for 3 h at
4°C in the presence of cell surface binding fluorescence. The cells were then washed three times with
ice-cold Hanks solution to remove unbound virus. To measure virus internalization,
cells were washed with Hanks solution twice and then were incubated with EBV for
2 h at 37°C to allow internalization, then washed three times with Hanks solution
to remove surface-bound virus. The cell was resuspended in 200 μl trypan blue
(GIBCO) and proteinase K for 5 min. The cell pellets were then washed three times with
Hanks solution.

Cell fusion assay. HEK-293FT cells co-transfected with the expression vectors
(pCAGGT, p2670, pCAGGS-gB and pCAGGS-gE) expressing T7 polymerase, gB,
gB or gl, respectively) were used as effector cells. HEK-293FT cells

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co-transfected with pMSCV-NRP1 (or the empty vector pMSCV as control), a reporter plasmid pTET-MCLuc encoding the luciferase gene driven by the T7-polymerase and an internal control plasmid pRLE-40 encoding the Renilla luci-
ferase gene driven by the SV40 promoter served as target cells. Twenty-four hours
post transfection, the effector and target cells were detached using trypsin-EDTA and
co-cultured in a 24-well plate at a density of 2 × 10⁴ cells for 24 h. Firefly and
Renilla luciferase activities were assayed by using the dual-luciferase reporter assay
system (Promega) with the Ventis luminometer (Promega). The relative fusion
activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity. The mean
value for pMSCV vector-transfected cells was normalized to 100% relative fusion activity.

Preparation of recombinant soluble NRPs protein. For blocking assays, soluble
NRP1ABC protein was prepared. The plasmid (pET32a-NRP1ABC) was transformed into E. coli strain BL21 (DE3). A single colony was inoculated into 5 ml LB-Amp (LB medium containing 100 μg ml⁻¹ ampicillin) and grown at 37°C with shaking. The seed culture was then inoculated into 500 ml LB-Amp medium. When the OD₆₀₀ reached 0.6–0.7, induction was initiated by the addition of 50 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG), followed by an overnight culture. The culture was then centrifuged, resuspended in 50 ml cold NTA-buffer (50 mM sodium phosphate at pH 8.0 and 0.3 M NaCl) containing 1 mM PMSF, and then sonicated on ice and
clarified by centrifugation at 12,000 g for 10 min. The 6×His-FLAG-tagged
NRP1ABC protein in the precipitate was purified by Ni-NTA agarose (Fisher Scientific).
After elution with buffer (50 mM sodium phosphate at pH 8.0 and 0.3 M NaCl, 6 M urea and 150 mM imidazole), soluble NRP1ABC protein was elutriated with RPLM1640, concentrated with Amicon Ultra-4 (Millipore) cen-
trifugation tubes with a 10-kDa molecular mass cutoff and was stored at −20°C.

To determine the affinity constant, recombinant soluble GST-NRP1ABC and
GST-NRP2ABC proteins were prepared. pETG-GST-NRP1ABC or pGEX-GST-NRP2ABC, expressing soluble GST-NRP1ABC or GST-NRP2ABC, was transformed into E. coli strain Rosetta. A single colony was grown in the LB-Amp medium. When the OD₆₀₀ reached 0.6–0.7, induction was initiated by 200 μM IPTG, followed by an overnight shaking at 18°C before collection. The culture was centrifuged and homogenized with high pressure on ice and then centrifuged at 50,000 g for 120 min. The GST-NRP1ABC and GST-NRP2ABC proteins were purified by Glutathione agarose (Fisher Scientific). After elution with 10 mM GSH buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 10 mM GSH), the protein was
extensively dialyzed with PBS buffer, and then stored at −20°C.

Determination of affinity constant by ELISA. Microtiter plates were coated with
200 ng Flag-gp overnight at 4°C in a 0.5% -w/v gelatin solution. After blocking with 5% BSA in PBS for 2 h at RT, and then incubated with the various concentrations of
GST-NRP1 and GST-NRP2 proteins for 2 h at RT. After washing with PBS
(0.05% Tween-20 in PBS), the plate was incubated with a Rabbit anti-GST antibody (1:2,000 dilution) for 2 h at RT. The plate was then washed and incubated at 37°C for
1 h with HRP-conjugated Goat anti-Rabbit antibody. After addition of the
substrate tetramethyl-benzidine, absorbance was measured at a wavelength of
450 nm using the Spectramax M5 (Molecular Devices).

Blocking assays. Blocking assays were performed with soluble NRP1ABC or
antibody against NRP2. To investigate whether soluble NRP1ABC would block EBV infection, EBV pre-incubated with soluble NRP1ABC or BSA (as a negative control) at 250 μg ml⁻¹ for 1 h, infected of HNE1 cells for 2 h at 37°C. To determine whether antibody against NRP2 would promote EBV infection, HNE1 cell were pre-incubated with 25 μg anti-NRP2 antibody or goat IgG (as a negative control) for 1 h and then were infected with EBV in the presence of an anti-NRP2 antibody (100 μg ml⁻¹) for 3 h at 4°C. Cells were collected at 48 h post infection. The percentages of GFP-positive infected cells were determined by a fluorescence microscope and/or flow cytometry analysis.

Immunoprecipitation. The transfected cells were lysed in radio-in-
munoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂, 5% glycerol and 0.5% Triton X-100) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and Roche Complete protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany). After centrifugation at 15,000 g for 20 min at 4°C, the lysate was preincubated with protein G-Sepharose beads (GE Healthcare) to preclear and then centrifuged at 15,000 g for 5 min to remove the beads. The supernatant was incubated with 2.5 μg (5 μg ml⁻¹) of the indicated antibody at 4°C overnight, and the immune complexes were captured by protein G-Sepharose beads for 2 h at 4°C. After washing three times in RIPA buffer and twice in PBS to remove unbound proteins, the sample was suspended in 2 × SDS-sample buffer and boiled for 5 min. The complex proteins were then analysed by western blotting, using specific detection antibodies.

Pull-down assay. Soluble NRP10-363 (nNRP1) and NRP20-366 was expressed by Trx Quick
Coupled Transcription/Translation System (promega), in the presence of canine pancreatic microsomal membranes, according to the manufacturer’s instructions.

EBV labelling with Alexa Fluor 594. Labelling of EBV was performed with Alexa Fluor 594 (Amine- Reactive probe; MP) according to the manufacturer’s instructions and published procedure52,53. In brief, 20 μl of 5 mg ml⁻¹ Alexa Fluor 594 dissolved in dimethyl sulfoxide (DMSO) was mixed with 2 ml of 200-fold
concentrated EBV in FBS-free RPMI1640 and 200 μl of 0.5 M carbonate
bicarbonate buffer at pH 9.0 for 1 h in the dark at room temperature. To separate the labelled EBV from the free dye, labelled EBV was diluted 10 times with cold RPMI1640, centrifuged at 50,000 × g for 90 min at 4°C, and then resuspended in 1.5 ml fresh FBS-free RPMI1640. The concentrated labelled virus was purified in dextran T-10 gradients, followed by re-suspension in 2 ml of fresh FBS-free RPMI1640. The purified Alexa Fluor 594-labelled EBV was subsequently dialysed four times with 500 ml RPMI1640 per time, and then stored at −80°C until use.

Immunofluorescence confocal microscopy. HNE1 cells were seeded on covers-
lips in 24-well plates at a density of 5 × 10⁴ cells per well for 12–16 h, and then
transfected with the indicated plasmids for 24 h. Transfected cells infected with the
unlabelled EBV and one at an MOI of 10–15 for 1 h at 37°C were briefly washed with PBS
twice, fixed with 3% paraformaldehyde in PBS for 20 min, and then permeabilized
with 0.1% Triton X100 in PBS for 5 min. After blocking with 5% BSA in PBS, EBV-
infected cells were stained with an antibody against gp350 (72A1, 1:200 dilution) for
4°C overnight, and washed with PBS three times, followed by incubation with
Alexa Fluor 594-labeled goat antibody to mouse IgG (1:2,000 dilution). After
washing with PBS three times, cells were mounted with ProLong Gold mounting medium (Invitrogen) containing 0.2 μg ml⁻¹ DAPI, which stains nuclei. Transfected cells inoculated with Alexa Fluor 594-labelled EBV were examined for 500 ml RPMI1640 per time, and then stored at −80°C until use.

Western blotting. Western blotting analysis was performed as previously
reported54. In brief, cells were lysed in RIPA buffer containing a protease inhibitor
mixture (Roche) and incubated on a rocker at 4°C for 15 min. The protein
concentration of the lysates were measured using the BCA protein assay kit (Pierce) and
were normalized to equal amounts of protein, separated by 9%
SDS/PAGE, transferred to PVDF and probed with the indicated primary
antibodies. After probed with the indicated antibodies, the blot was incubated with species-specific HRP-conjugated secondary antibodies, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Pierce). The same membranes were then stripped and reprobed with mouse monoclonal antibodies
against GAPDH or z-tubulin to confirm equal loading of the samples. Full scans of
all western blots are included in Supplementary Fig. 16–19.

RNA extraction, EBV DNA extraction and real-time PCR. Total cellular RNA
was extracted from cultured cells using the TRizol reagent (Invitrogen) according
to the manufacturer’s instructions. cDNA was synthesized from 2 μg of the total
RNA using a reverse transcriptase kit (Invitrogen). The mRNA level was evaluated by
qRT–PCR, using the Power SYBR Green qPCR SuperMix-UDG (Invitrogen) and
was analysed on Roche Lightcycler 480. All the gene expressions were normalized
to the housekeeping gene GAPDH, used as an internal standard. Primers are listed in
Supplementary Table 3. EBV DNA was extracted from EBV-infected cells using
Omega tissue DNA Mini Kit (Omega) as recommended by the manufacturer. The
copy number of EBV bound to the cell surface or internalized into HNE1 cells was
measured using TaqMan real time PCR for detection of the BamHI-W fragment
region of the EBV genome. Real-time PCR for the GAPDH DNA was used for cell
normalization. A calibration curve was performed with each analysis, using
dNA extracted from the EBV-positive cell line Namalva, which contains two
tegrated viral genomes per cell, as a standard. The EBV copy number
was expressed as a ratio of the copy number of the EBV genome to the copy number
of the GAPDH DNA.

Statistical analyses. The data are expressed as the mean ± s.e.m. from at least three independent experiments. Statistical analyses were performed with Graphpad
Prism 5 (GraphPad Software, San Diego, CA, USA).
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Author contributions
H.-B.W. and M.-S.Z. designed the study, H.-B.W and H.Z. performed the key experiments and analysed the data. Y.D. and Y.L. performed real-time PCR experiments, respectively.
pull-down assay and cell culture. G.-K.F., B.Z., D.X., Q.Z., W.-L.L. provided helpful comments. H.-B.W. and J.-P.Z. prepared figures and graphs. H.D. prepared the recombinant protein. M.-Z.L assisted with cell culture. W.-L.H, S.W.T., L.H.F., Y.-X.Z. and E.K. contributed key reagents and provided import comments for the study design. H.-B.W., J.-P.Z. and M.-S.Z. wrote the manuscript. M.-S.Z. supervised the project.

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