Lipid membrane fusion is an essential function in many biological processes. Detailed mechanisms of membrane fusion and the protein structures involved have been mainly studied in eukaryotic systems, whereas very little is known about membrane fusion in prokaryotes. Haloarchaeal pleomorphic viruses (HRPVs) have a membrane envelope decorated with spikes that are presumed to be responsible for host attachment and membrane fusion. Here we determine atomic structures of the ectodomains of the 57-kDa spike protein VP5 from two related HRPVs revealing a previously unreported V-shaped fold. By Volta phase plate cryo-electron tomography we show that VP5 is monomeric on the viral surface, and we establish the orientation of the molecules with respect to the viral membrane. We also show that the viral membrane fuses with the host cytoplasmic membrane in a process mediated by VP5. This sheds light on protein structures involved in prokaryotic membrane fusion.
lipid membrane fusion is usually driven by membrane-associated proteins that confer specificity and overcome the energy barriers related to the fusion process. There is a wealth of information about the mechanisms and the protein structures involved in eukaryotic membrane fusion. In particular, extensive studies of enveloped viruses infecting eukaryotes have led to their assignment into three structural classes I–III. In contrast, very few examples of enveloped viruses that infect prokaryotes have been characterized. A notable example is the enveloped bacteriophage φ6, which fuses with the outer membrane of its Gram-negative host by a structurally unknown mechanism.

Haloarchael pleomorphic viruses (HRPVs) infect archaean hosts living in hypersaline environments. The newly established family Pleolipoviridae includes several HRPVs with Halorubrum pleomorphic virus 1 (HRPV-1) as the typical member. The virions contain two major protein species, a spike protein (VP4 and VP4-like proteins) protruding from the membrane surface and a smaller membrane associated protein (VP3 and VP3-like proteins) residing mostly inside the virion. The genomes of pleolipoviruses consist of either single- or double-stranded DNA molecules which are not associated with any protein. The VP4 spike protein of HRPV-1 is a type I transmembrane protein (with an N-terminal signal peptide sequence of ~33 amino acids), N-glycosylated with a pentasaccharide containing legionaminic acid as the terminal residue and suggested to be involved in the host recognition whereas for other pleolipovirus spike proteins, glycan modifications have not been detected.

In the absence of atomic structures for the spike proteins from enveloped viruses infecting prokaryotes, it is unknown whether they contain fusion proteins that are similar to those characterized in eukaryotic viruses. Pleolipovirus VP4-like surface spike proteins are good candidates to be involved in membrane fusion, however their structure and function remains to be established. The structure on the native virion and the distribution of the VP4-like proteins on the virion envelope also remain unknown.

Here we describe crystallographic structures of VP4-like spike proteins from two pleolipoviruses, HRPV-2 and HRPV-6, showing that these proteins have a unique fold, different from class I–III fusion proteins. By cryo-electron tomography utilizing Volta phase plates we show that the spike protein of HRPV-6 is monomeric on the surface of the virion. Fluorescence-based assays demonstrate that the spikes induce fusion of the viral membrane with the host cell membrane. In addition, we provide preliminary evidence that the membrane fusion may be preceded by a conformational change in the spikes. Together, our structural and functional analysis of the VP4-like spike proteins and virions allows us to suggest a model of prokaryotic virus-cell membrane fusion.

Results
Pleolipovirus spike structures reveal a V-shaped fold. In order to characterize the VP4-like fusion proteins (termed VP5 in these viruses) from HRPV-2 and HRPV-6, we purified them from infection-competent virions using either detergent (HRPV-2) or protease digestion (HRPV-6). After protein purification, we determined the structures of the HRPV-2 and HRPV-6 VP5 ectodomains (residues 1-498 out of 533 residues and 9-498 out of 537 residues, Supplementary Figure 1) at 2.5 and 2.7 Å resolution, respectively, by X-ray crystallography (Table 1). The two proteins, which are 67% identical in amino acid sequence (Supplementary Figure 1), share a highly conserved V-shaped fold (r.m.s.d. for 453 corresponding Ca atoms: 1.6 Å) (Fig. 1a). The structure is composed of two elongated domains roughly equal in size and linked by a single residue (Q258 in HRPV-2 and Q262 in HRPV-6) (Fig. 1a). The N-terminal domain comprises two subdomains (N1 and N2) while the C-terminal domain includes three subdomains (C1–C3) (Fig. 1b).

The N-terminal subdomain, N1, contains a bundle of four α-helices, one of which, a3, spans more than 40 residues (Fig. 1b). Interacting with helix a3 of the helix bundle, subdomain N2 is composed of three small β-sheets of two or three strands each. Subdomain N2 is the least conserved between HRPV-2 and HRPV-6 and amongst other pleolipoviruses (Fig. 1c; Supplementary Figure 1).

Previously we have determined the N-terminal sequences of the ectodomains of both HRPV-2 and HRPV-6 VP5 proteins. The first twenty N-terminal residues of both VP5s are hydrophobic and predicted, by several programs for analysing hydrophobic regions of proteins (see Methods), to form a transmembrane helix, indicating the preference of this region to partition into membranes. Interestingly, this hydrophobic segment is shielded from the solvent in both crystal structures by the C-terminal domain. Such protection is typical for fusion peptides or fusion loops in the pre-fusion forms of class I and II fusion proteins, where the shielding is conferred by another chain, either of the same or different protein species.

One of the C-terminal subdomains is α-helical and the other two are formed of β-strands (Fig. 1b). Surprisingly, the entire C-terminal domain is disordered in one of the two molecules of the asymmetric unit of HRPV-6 VP5. Although there is space in the crystal lattice to accommodate this disordered domain, the bulk of adjacent molecules prevents it from adopting the same orientation with respect to the N-terminal domain seen in the other copies of VP5. It appears that the N- and C-terminal domains can flex with respect to each other in the face of crystal packing forces.

The last 20 residues of both HRPV-2 and HRPV-6 VP5s are predicted to form a transmembrane helix. Analysis of peptidase K treated HRPV-6 particles by LC-MS/MS resulted in the identification of several peptides with core sequence FGVPGE-VAV representing part of the predicted C-terminal...
Fig. 1 The overall fold of VP5. 

a Cartoon representation of HRPV-2 VP5 (left) and HRPV-6 VP5 (right) coloured from the N-terminal (blue) to the C-terminal (red). Residues Q258 and Q262 connecting the N- and C-terminal domains are shown as green spheres.

b Cartoon and topology representation of HRPV-2 VP5 coloured by domains.

c Structural differences between the N2 domains of superposed HRPV-2 VP5 (green) and HRPV-6 VP5 (pink).

d Localization of the potential fusion peptide depicted as magenta sticks within HRPV-2 VP5 shown as grey cartoon.
transmembrane domain (Supplementary Figure 2). In contrast, HRPV-2 VP5 was released intact from the virion by detergent treatment. However, the last 35 residues are not visible in the electron density.

VP5 is monomeric on the virion surface. To determine the low-resolution structure and oligomeric state of VP5 on the HRPV-6 surface, we used cryo-electron tomography and sub-tomogram averaging (Fig. 2a, b; Supplementary Figure 3; Supplementary Table 1). This was facilitated by Volta phase plate technology[^10^], which provided sufficient contrast in the tomographic reconstructions for identification and alignment of the relatively small VP5 (~57 kDa) spikes (Figs. 2a and 3). Sub-tomogram averaging of 8953 manually picked spikes resolved the VP5 structure in situ at 16-Å resolution, as estimated by Fourier shell correlation (0.143 threshold). Each virion ($N = 247$) had 35 ± 7 VP5 spikes on the surface and each spike is a monomer with the characteristic triangular shape reminiscent of that seen in the high-resolution X-ray structures (Fig. 2c, d). In cryo-electron microscopy, high concentrations of salt surrounding the particles diminish the contrast, hence HRPV-6 virions had been quickly diluted before vitrification. In control experiments, HRPV-6 data collected without reducing the salt concentration showed that the radial profile of the cylindrically averaged spikes has the same height in high and low salt conditions (Fig. 3). Since there is robust agreement between the high-salt X-ray structure and the low-salt tomographic data, transient exposure to low-salt conditions has no major effect on the spike conformation.

We fitted the VP5 X-ray structure into the 16-Å resolution VP5 tomographic density as a rigid body (Fig. 2c). Although the fitting is generally robust (cross-correlation coefficient 0.84), there is additional density in the groove between the N- and C-terminal domains, which slightly protrudes from the EM density (Fig. 2c), suggesting that VP5 attached to the viral membrane may adopt a more compact conformation than in the crystal. The C-terminus is proximal to the viral membrane, consistent with the predicted C-terminal transmembrane region anchoring VP5 to the membrane. Density corresponding to the last 39 residues of HRPV-6 VP5 is not observed: the 23 residues preceding the very last two C-terminal asparagine residues correspond to the TM domain, and the preceding 19 missing residues (glycine rich; Supplementary Figure 1) would act to link the ectodomain to the TM domain. In this model, rigid body fitting placed Val461 close to the membrane but this assertion remains to be validated experimentally. The variable, putative receptor binding N2 subdomain is located at the top of the spike. The accurate localization of the putative fusion peptide was hampered by the relatively low resolution (16 Å) of the tomographic reconstruction. Further studies at higher resolution are required to determine how the putative fusion peptide is shielded from solvent in this pre-fusion conformation on the virion surface.

![Fig. 2 Cryo-electron tomography studies of HRPV-6.](image)
VP5-spikes induce fusion with host cell membranes. With the aim to test whether pleomorphic archaeal viruses fuse their membrane envelope with the host cytoplasmic membrane of cells we established a lipid mixing assay by using haloarchaeal pleomorphic virus HRPV-6 labelled with a lipophilic dye, octadecyl rhodamine B chloride (R18). Labelled viruses were added to the host cell culture for fluorescence microscopy. Cells displayed areas of diffuse fluorescence, indicating that the dye had spread from the viral membrane into the cell membrane (Fig. 4). In some cells, punctate fluorescent signals were also observed. These signals most likely corresponded to virions that were bound to the cell surface, but had not yet made their way through the S-layer and reached the cell membrane (Fig. 4c). As a control, we used labelled HRPV-6 particles, which were treated with proteinase K to proteolytically cleave and remove the spikes, but not the smaller (14.5 kDa) membrane associated protein termed VP4 in this virus. When these spikeless particles were used in equal numbers (as measured by the total R18 fluorescence signal), no fluorescent signal was observed in the cells (Fig. 4b). This verified that viral surface spikes are required for transfer of fluorescence from virions to the host cell membrane.

In vitro time resolved fusion assays based on fluorescent lipid probes have been widely used to characterize fusion kinetics of eukaryotic viruses. To establish such an assay for haloarchaeal viruses, we mixed R18-labelled HRPV-6 particles with cells and monitored R18 de-quenching over time. When we incubated HRPV-6 with its host cells Halorubrum sp. SS7-4, lipid mixing over 10% could be observed at 37 °C, revealing a half-time for fusion of 18.1 ± 1.8 min (n = 3, Fig. 5a, Supplementary Table 2). To corroborate the specificity of the lipid mixing assay, various control experiments were performed (Fig. 5a). Lipid mixing did not occur when HRPV-6 was incubated with the host cells at 4 °C, confirming the arrestment of membrane fusion. We further found that lipid mixing did not occur with proteinase K digested spikeless HRPV-6 particles, hence indicating that fusion was induced by the viral VP5 spike protein (Fig. 5a). Finally, we found that lipid mixing also did not occur when HRPV-6 was incubated at 37 °C with Halorubrum sp. strain PV6, the host of HRPV-1, suggesting the requirement of specific host factors to induce membrane fusion at 37 °C.

After having established the specificity of the kinetic lipid mixing assay between HRPV-6 and host cells, we proceeded to develop a cell-free fusion assay. To this end, we prepared liposomes from the Halorubrum sp. SS7-4 host cells and monitored HRPV-6-liposome fusion at 37 °C. As expected from the inherent removal of host proteins during lipid extraction, no lipid mixing could be observed under this condition (Fig. 5b). Lipid mixing could be readily detected when we increased the temperature to 55 °C, reported to decrease HRPV-1 infectivity by partial denaturation, with a half-time for fusion of 12.1 ± 4 min (n = 3, Fig. 5b, Supplementary Table 2). This was not the case when spikeless HRPV-6 particles were incubated at 55 °C with the liposomes, confirming that VP5 protein was required for the merger of membranes. To further characterize the temperature of heat-induced fusion, we examined fusion temperatures between 37 °C and 55 °C. Lipid mixing was not detected in the temperature range from 37°C to 45°C while at 50°C some membrane fusion occurred, albeit at a much lower level than at 55°C (Fig. 5c). These data together confirm that HRPV-6 infects cells by virus-cell membrane fusion induced by its VP5 spike protein. Although host cell infection and fusion seems to depend on stringent host cell factors in vivo, VP5 protein fusion activation can be triggered through heating to 55 °C.

In order to test whether the heat treatment could trigger a conformational change of the spikes occurring during fusion, we analysed HRPV-6 particles heated at 55°C for 30 min by cryo-
EM. As shown in Fig. 6a, b, we can perceive extended spikes on heat-treated HRPV-6 particles whilst such structures are not observed on particles incubated at room temperature. Because these particles were imaged at high salt, the contrast remains low. Still, a conformational change was observed with spikes of one virion in the sample imaged at low salt conditions (Fig. 6c). The reason for this very rare event of spontaneous triggering is not known. Thus, future studies are required to confirm and further characterise such a conformational change.

**Discussion**

A wealth of information describing the mechanisms and the protein structures involved in eukaryotic membrane fusion exists for both cellular processes and infection by enveloped viruses\(^1\). In contrast, prokaryotic membrane fusion has been studied only in a few cases. One of these is the enveloped bacteriophage \(\phi 6\) which fuses its membrane with the outer membrane (OM) of the Gram-negative host bacterium *Pseudomonas syringae*\(^2\). The viral protein involved in this fusion process is embedded in the membrane and as such represents an atypical fusion protein with as yet uncharacterized atomic structure. Another class of prokaryotic viral membrane fusion proteins are spanins which are periplasmic proteins and have been suggested to fuse the Gram-negative host cytoplasmic membrane (CM) with the OM consequently facilitating the lysis and exit of the virus progeny from the infected cell\(^12\). While atomic structures of spanins are lacking, functional analysis of phage lambda spanin suggests similarities to type I fusion proteins\(^13\).

Our structural analysis of the pleolipovirus spike protein revealed a V-shaped fold dividing the molecule into two
elongated domains roughly equal in size. The structure is highly conserved between the two VP5 proteins from two different viruses and released from the virions using two different dissociation methods. HRPV-2 and HRPV-6 VP5 proteins share an average 67% amino acid sequence identity with the N2 subdomain being the least conserved between the two spike proteins. HRPV-2 and -6 infect different host strains suggesting that domain N2 may play a role in host recognition. Furthermore, fitting of the X-ray structure into the HRPV-6 VP5 tomographic density positions N2 subdomain exposed to the surface of the monomer. Thus, we hypothesize that the N2 subdomain is responsible for the host receptor recognition. That question will be the subject of future studies.

Using fluorescence microscopy we were able to show that the lipophilic fluorescent label incorporated in the viral membrane was spreading to the host cell membrane only during infection with the wild-type particles and not with spikeless particles containing the intact smaller structural protein VP4. In concordance with this result, we demonstrated that lipid mixing occurs during the infection of cells by HRPV-6 and that membrane fusion can be induced with host-lipid derived liposomes when HRPV-6 is incubated with liposomes at 55 °C. Such a heat-induced activation based on partial protein denaturation has previously been reported only for class I fusion proteins14,15. In most known haloarchaeal species the cell wall consists of the cell membrane covered by proteinaceous paracrystalline S-layer16, a rigid layer which, however, has to be able to accommodate the dynamics of transfer of molecules to and from the cell for cellular responses and maintenance. The pleolipovirus in vivo fusion mechanism may require an active interplay with the host S-layer which most probably harbours the receptor for this archaeal virus. The identity of the host receptor and the trigger for fusion at physiological temperature remain an active area of investigation.

On the basis of the present results, we propose a model for the infection mechanism of haloarchaeal pleomorphic viruses (Fig. 6d). Upon interaction of VP5 with the receptor on the host cell surface, possibly via the N2 subdomain, the α-helical bundle of N1 subdomain would open and extend ~150 Å due to interactions with components of the glycoprotein S-layer. This might further lead to opening of the surface layer allowing projection of the putative fusion peptide towards the host cell membrane (Fig. 6d), reminiscent of the large-scale conformational changes observed in class I viral fusion proteins. The extended conformation would allow VP5 to reach across the ~120 Å thick S-layer16. This hypothesis is supported by the evidence of HRPV-6 particles heated at 55 °C where the spikes seem to have switched in a concerted fashion to an extended conformation (Fig. 6a, b). The estimated maximum length of the filament-like spikes (190 Å) also matches our predictions of the maximum length of the extended conformation of the spike (200 Å; Fig. 6d). This preliminary observation of extended conformation of the spikes is intriguing and will be addressed in the future by tomography and sub-tomogram averaging.

Although many aspects of pleolipovirus fusion remain uncharacterized, our results unequivocally highlight the role of VP5 mediating fusion to enter its host cell. Only particles with intact VP5 spikes could fuse their membranes with the host cell and liposome, whereas VP5-free particles could not. VP5 is thus a bona fide fusion protein. The monomeric fold of VP5 on the viral particle surface is unlike all other known viral fusion proteins which fall into one of three classes, I–III8. Other likely exceptions to this classification are the still poorly understood and unclassified fusion proteins of hepadnaviruses, poxviruses, pesti-
hepaciviruses17–20. We therefore propose that VP5 and related pleiopivirus spike proteins are a different type of fusion proteins, and may be the representatives of a previously unreported class of viral fusion proteins. The unique features of these proteins may reflect their similarity to a very ancient fusion mechanism maintained in Archaea, the evolutionary pressure imposed by the very high salt environment, and the presence of the thick pro-oteaceous S-layer on the host surface. Further studies on membrane fusion proteins of viruses infecting prokaryotes and eukaryotes may illuminate the evolutionary connections between the fusogens of prokaryotic and eukaryotic viruses.

**Methods**

**Haloarchaeal host strains and viruses and growth conditions.** Haloarchaeal pleiopivirus HRPV-2 and HRPV-6 as well as their host strains Halorubrum sp. SS5-4 and SS7-4 were initially isolated from samples collected in a solar saltern samples in Samoth Saloon, Thailand19,22 and the non-pigmented derivative of SS7-4 (see below), respectively, were used in this study. H. halorubrum sp. PV6 was initially isolated from samples collected in a solar saltern in Trapani, Sicily, and used as a non-host strain. Haloarchaeal strains were grown aerobically in Modified Growth Media23 (MGM) at 37 °C. MGM broth, plates and soft agar contained 20, 20 and 18% (wt vol−1) artificial salt water (SW, HalobacGuidance [http://www.halobacGuidance.com/resources/halobacGuidance)].) respectively. Viruses were propagated on lawns of host cells grown in MGM soft agar. HRPV-6 and HRPV-2 virus solutions were prepared by incubating the transparent semi-confluent plates and incubating it together with MGM (2 ml for each plate) used at 37 °C with shaking for 2 h. The debris was removed by centrifugation (Sorvall F12, 11,000 × g for 40 min at 4 °C) and the clarified lysate was stored at 4 °C. 12% SW-HEPES was diluted from the 30% SW-HEPES stock solution in which Tris-HCl buffer (pH 7.2) was replaced by HEPES buffer of the same concentration and pH.

**Production of non-pigmented mutant strain.** Ethyl methane sulphonate (EMS) was used to mutate cells of Halorubrum sp. SS7-4 and screen for non-pigmented cells conducted as described by Mevarech and Werzberger24. Briefly, cells were grown to exponential growth phase and collected by centrifugation (Sorvall SA600, 9700 × g at ambient temperature) followed by resuspension in an equal volume of buffer containing 50 mM Tris-HCl pH 7.2 and 3 M NaCl. EMS (Sigma M0880) was added to the final concentration of 100 µg ml−1. Cells were incubated with EMS at 37 °C with aeration for 2 h after which cells were washed twice with the buffer. Finally cells were resuspended in equal volume of MGM broth and grown until they reached the density of ~1 × 109 cfu ml−1. Suspension was diluted and plated on MGM media plates and grown at 37 °C until colonies appeared.

**Purification of viruses and spike proteins.** HRPV-6 and HRPV-2 virions were purified from the storage solutions first by concentrating them with 10% polyethylene glycol 6000 (PEG6000) at 4 °C for one hour with gentle stirring. Precipitated viral particles were collected by centrifugation (Sorvall F12, 11,000 × g for 40 min at 4 °C) and the clarified lysate was stored at 4 °C. 12% SW-HEPES was diluted from the 30% SW-HEPES stock solution in which Tris-HCl buffer (pH 7.2) was replaced by HEPES buffer of the same concentration and pH.

**Labelling of HRPV-6.** Highly purified (2×-purified) HRPV-6 virions produced in the non-pigmented SS7-4 host were labelled using R18 (O-246-Molecular Probes). Approximately 1.7 × 1013 plaque forming units per millilitre (PFU ml−1) were mixed with 45 µg ml−1 of the lipophilic dye and divided in equal volume. After one hour-incubation at 37 °C in dark, proteinase K (Fermentas) was added to one aliquot at final concentration of 100 µg ml−1 and both virions were incubated by rate zonal centrifugation (Sorvall TH641, 134,000 × g for 3 h 30 min at 15 °C). Light scattering zones containing the virions were collected, concentrated and washed by ultrafiltration (Amicon Ultra Centrifugal Filter Devices, Millipore, 100,000 nominal molecular weight limit). The buffer of the final protein sample contained 20 mM Tris-HCl pH 7.2, 1.6 M NaCl and 10 mM MgCl2.

**Adsortion tests.** The host culture to be tested was diluted to ∼5 × 109 cells ml−1 for the wild-type HRPV-6 particles and 8.0 × 10−2 PFU cm−2 for the spikeless particles. Analysis of the viral particles in SDS-PAGE showed that most of the spike protein was removed by the proteinase K digestion (Supplementary Figure 4).

**Virus-cell and virus-liposome fusion assay.** Virus fusion with target membranes was monitored by fluorescence quenching of R18-labelled virions by standard techniques25. To this end, purified (1×-purified) HRPV-6 virions produced in the non-pigmented H. halorubrum sp. SS7-4 host were labelled with R18 at a self-quenching concentration as described above. R18 labelled HRPV-6 virions were separated from excess probe by using a sephadex G-75 column (GE Healthcare). A negative control, R18 labelled and purified HRPV-6 particles pre-treated with proteinase K at 200 µg ml−1 for 1 h at 37 °C to eliminate any exposed membrane proteins.

**Fluorescence microscopy.** Non-pigmented haloarchaeal SS7-4 host cells produced in this study (see Production of non-pigmented mutant strain) were grown as described for the adsorption test. To a solution of cells at their logarithmic growth phase (A5415D = 0.4–0.5, ~1–2 × 108 CFU ml−1) labelled viruses were added at an approximate M.O.I. of 10–50 (virions per proteinase K treatment) and approximately the same number of proteinase K-treated viral particles as determined by released fluorescence. Suspensions were incubated at 37 °C for 15 min with shaking after which cells from 1 ml of sample were collected by centrifugation (Eppendorf Centrifuge 5415D, 5000 × g, 5 min, RT) and the supernatant and the cell pellet, resuspended in 200 µl of fresh growth medium, were titrated in 100 µl aliquots with host cells as described before26.
resuspended in HRPV buffer. Fresh liposomes were prepared by the freeze-thaw and extrusion method27 using a polycarbonate filter with a pore size of 0.2 mm (Avanti Polar Lipids, Alabaster, AL). Lipid thickness of Triton X-100 (2%) after the lipid mixing of each condition had concluded. After data collection, the lipid mixing kinetic was fitted to a single exponential fit using Eq. (1).

\[
\text{Lipid mixing} = \text{Lipid mixing}_{\text{max}} \times (1 - \exp(-k \times t))
\]

where \(t\) is the time constant and Lipid mixing \(_{\text{max}}\) corresponds to the maximum lipid mixing value at infinite time.

**LC-MS/MS.** Peptides were quenched with 10% trifluoroacetic acid (TFA) and purified with C18 microspin columns (Harvard Apparatus, USA) eluting the samples to 0.1% TFA in 50% acetonitrile (ACN). The dried peptides were reconstituted in 50% ACN/5% TFA (buffer A). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was carried out on an EASY-nLC 1000 (Thermo Fisher Scientific, Germany) connected to a Velos Pro-Orbitrap Elite/Q Exactive hybrid mass spectrometer (Thermo Fisher Scientific, Germany), with nano-electrospray source (Thermo Fisher Scientific, Germany). LC-MS/MS samples were separated using a two-column setup consisting of a 2 cm C18 Pepmap column (#164946 Thermo Fisher Scientific, Germany), followed by 15 cm C18 Pepmap analytical column (#164940 Thermo Fisher Scientific, Germany). The linear separation gradient consisted of 5% buffer B in 5 min, 35% buffer B in 60 min, 80% buffer B in 5 min and 100% buffer B in 10 min at a flow rate of 0.3 µl min\(^{-1}\) (buffer A: 0.1% TFA in 1% acetonitrile; buffer B: 0.1% TFA acid in 98% acetonitrile). 4 µl of sample was injected per LC-MS/MS run and analysed. Full MS scan was acquired with a resolution of 60,000 at normal mass range in the orbitrap analyzer the method was set to fragment the 20/10 (QE 10 and OQ top 20) most intense precursor ions with CID (Elite OE/HCD with QE) (energy 35). Data were acquired using Xcalibur software.

Acquired MS2 scans were searched against home-made protein database using the Sequest search algorithms in Thermo Proteome Discoverer. Allowed mass error for the precursor ions was 15 ppm. For the fragment in 0.8 Da or 0.05 Da (Q Exactive). A static residue modification parameter was set for carbamidomethyl (+57.021 Da) (C) of cysteine residue. Methionine oxidation was set as dynamic modification +15.995 Da (M). The analysis was carried out at the Protein Science Chemistry Core Laboratory, Institute of Biotechnology, Helsinki Institute of Life Science HiLIFE, University of Helsinki.

**Cryostatization and data collection.** Both HRPV-2 VPs and HRPV-6 VP proteins were cryostatized in 96-well plates (Greiner Bio-One Ltd, Stonehouse, England) using the same vapour-diffusion method as described at 21 °C with a 70% humidified atmosphere and a 30% humidified atmosphere with 5% humidity. A 400 µl drop of the protein solution containing 12% (w/v) acetone to 50% acetonitrile was applied and the vial was immersed in the reservoir solution but containing 30% (w/v) acetone to 50% acetonitrile. 4 µl of sample was injected per LC-MS/MS run and analysed. Full MS scan was acquired with a resolution of 60,000 at normal mass range in the orbitrap analyzer the method was set to fragment the 20/10 (QE 10 and OQ top 20) most intense precursor ions with CID (Elite OE/HCD with QE) (energy 35). Data were acquired using Xcalibur software.

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**Cryo-electron microscopy data collection.** Cryo-EM data were collected at the national Electron Bio-Imaging Center (eBIC), at the Diamond Light Source on a FEI Titan Krios transmission electron microscope operating at 300 kV. The Krios was equipped with an energy filter (GIF Quantum, Gatan) operating in zero-loss mode with a 20 eV slit and a Volta phase plate (VPP: Thermo Fisher). Single axis tilt series (from −45° to +45° with angular increments of 3°) were collected using EPU software (FEI) on a direct electron camera (K2 Summit, Gatan). At each tilt, images were recorded as a movie consisting of eight frames with a total exposure of 1.6 s per tilt and at a calibrated magnification of ×22,222 in a single electron counting mode, corresponding to a pixel size of 2.25 Å. The datasets were collected in focus and the standard autofocus routine implemented in EPU was used at every tilt angle. Cumulative electron dose was kept constant in all datasets and the irradiated area on the VPP was changed after each tomogram. Images of heat-treated particles were collected at the University of Helsinki cryo-EM facility on a Tecnai 12 electron microscope operating at 200 kV and using Falcon 3D detector (Thermo Fisher).

**Image pre-processing and tomogram generation.** Drift correction22 was used to correct for the electron beam induced motion by averaging eight frames for each tilt. 25 tilt series were aligned by patch tracking in IMOD package45. The six best tilt series, which showed consistent contrast, were reconstructed into tomograms with a final pixel size of 2.25 Å. Altogether 247 HRPV-6 VPs were picked in Bbox44 and extracted from the tomograms using Isontomos5 for further analysis.

**Sub-tomogram averaging.** For initial template generation, extracted virion volumes were low pass filtered to 80 Å using Bsoft44. Spikes (8953 in total) were manually picked from the volumes of HRPV-6 using Dynamo46. All picked spikes were extracted from the unfiltered tomograms into boxes of 128 × 128 × 128 voxels. Sub-tomogram averaging was carried out in Dynamo46, following protocols we have described earlier47,48. In the first stage of refinement, both the locations and directions of the picked particles were allowed to change. The angle around the spike long axis (azimuth) was kept fixed. The refinement was carried out while restricting the resolution to 36 Å, during which a large spherical mask (radius 63 pixels) and full cylindrical symmetrization were applied to roughly align the spikes. At the second stage, a customised post-processing plugin47 was designed to carry out gold-standard refinements where only the azimuth angle was refined. The dataset was randomly split into two datasets consisting of 4502 and 4451 particles and each dataset was averaged to produce a template for refinement. Independent refinements were carried out on the two datasets. At the end of each iteration, the Fourier shell correlation (FSC) between the two averages was computed and the resolution estimated using a criterion of 0.143. Two shells in Fourier space were subtracted from the estimated resolution value, and the difference was used as the threshold for a low-pass filter for the next iteration. A spherical mask (radius 32 pixels), encompassing only the spike ectodomain was used, and no symmetry was assumed. After this stage, particles were refined using the FSC at 25% overlap. Averaging the remaining particles produced an ellipsoidal-shaped spike density, which was connected with the membrane by one, possibly two small stumps.

**Protein in silico analyses.** The hydrophobic regions of HRPV-2 and HRPV-6 proteins were predicted using TMpred server2, Phobius40 and MEGA41.

**Sample preparation for electron microscopy.** VPs spikes on HRPV-6 are very sensitive to ionic strength and only stable in 1.6 M NaCl or above. At lower concentrations of NaCl the spikes fall apart. As a result a careful optimisation of NaCl concentration and incubation time was performed. For cryo-EM, samples were prepared by diluting the purified virus (10%) in the buffer containing 250 mM NaCl immediately prior vitrification. Aliquots of 4 µl were added onto a glow-discharged holey carbon copper grid (C-flat, CF-2/1-2C; Protoc bios). Grids were blotted for 3 s, in 90% relative humidity, and vitrified in liquid ethane with a plunging device (Vitrobot; FEI). In order to keep the spikes intact and in native form, grids were plunge-cooled for up to 30 s of dilution in low salt buffer. To assess the salt concentration effect on the virions and spike, HRPV-6 in 1.5 M NaCl buffer were plunge-frozen on the grids as control.

To study heat-treated particles of HRPV-6, 100 µl of highly purified particles (~1 × 10^7 pfu ml^-1) were split into two 50 µl aliquots. One aliquot was incubated at 65°C for 30 min in HEPES buffer and the other aliquot was added onto glow-discharged copper grid sample. The final concentration of NaCl in the protein buffer, NaCl was assumed. After this stage, 1436 particles were removed from the dataset due to 2 VP5 (62% sequence identity to HRPV-6 VP5) was solved by molecular replacement using the asymmetric unit, although no density could be seen for half of one of them. HRPV-2 VPs (62% sequence identity to HRPR-6 VPs) was solved by molecular replacement using the asymmetric unit, although no density could be seen for half of one of them. HRPV-2 VPs (62% sequence identity to HRPR-6 VPs) was solved by molecular replacement using the asymmetric unit, although no density could be seen for half of one of them. HRPV-2 VPs (62% sequence identity to HRPR-6 VPs) was solved by molecular replacement using the asymmetric unit, although no density could be seen for half of one of them.
In the third stage, an ellipsoidal mask (semi-principal axes of length 40, 24 and 24 pixels in the x, y and z direction), encompassing only the spike ectodomain was used. In this stage, the azimuth angle was further refined with small angular search steps. After this stage, 629 particles were removed from the dataset due to low cross-correlation. The coordinates of the remaining particles were plotted and 226 particles located obviously outside of the virus were further removed.

In the fourth stage, all six parameters (three location coordinates and three Euler angles) were allowed to change simultaneously. After this stage, 741 particles were removed due to low cross-correlation or overlap, giving finally 312 and 2945 particles for the two datasets. The final map resolution of 1.6 Å was estimated by FSC, using 0.143 as threshold. To assess the salt concentration effect on the virions and spike, 1200 spikes from HRPV-6 in 1.5 M NaCl buffer were cylindrically aligned and averaged.

X-ray structure fitting. X-ray structure of HRPV-6 VP5 was fitted as a rigid body into the segmented EM density of the VP5 spike in ucsm using BUSTER.

The source data underlying Figs. 3d, 4, 5, Supplementary Figure 2 and 3b are provided as a Source Data file. Coordinates and structure factors of HRPV-2 VP5 and HRPV-6 VP5 have been deposited in the Protein Data Bank under ID codes 6G0G and 6G0H, respectively. The accession ID for the EM map reported in this paper is EMD-9779 and the PDB-code for fitted HRPV-6 VP5 is 60TV. The EM/MS data was submitted to PeptideAtlas with identifier PASSO124.

Received: 11 February 2018 Accepted: 22 January 2019

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Acknowledgements

We acknowledge Sari Korhonen and Tarja Grundström for excellent technical assistance. We thank Diamond Light Source for beamtime (proposal mx10827), and the staff of beamlines 102 and 124 for assistance with crystal testing and data collection. We also acknowledge Diamond for access and support of the Cryo-EM facilities at the UK National Electron Bio-imaging Centre (eBIC) funded by the Wellcome Trust, MRC and BBSRC (EM14865). The use of the facilities and expertise of the Instruct-HiLIFE CryoEM unit, member of Biocenter Finland and Instruct-IL, are acknowledged. This work was supported by the Medical Research Council (MRC, grant G1000099 and MR/N00065X/1), the Wellcome Trust by providing administrative support (grant 090532/Z/09/Z), Academy of Finland (Academy Professor funding grants 283072 and 255342 to D. H.B.), by the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (649053 to J.T.H.), CONICYT (Chile) through Fondo Nacional de Desarrollo Científico y Tecnológico FONDECYT 1181799 and Programa de Apoyo a Centros con Financiamiento Basal AFB 170004 to Fundación Ciencia & Vida (N.D.T.), and Helsinki University 3-year grant and the Protein Production Platform (P-cube) funding to E.R. The use of the facilities and expertise of the Instruct-HiLIFE Biocomplex unit, member of Biocenter Finland and Instruct-IL, is acknowledged. Academy of Finland support (grant 1306833) for Instruct-HiLIFE Biomolecular Complex Purification unit is also acknowledged.

Author contributions

K.E.O., P.S., D.H.B., N.D.T., E.A.B., J.T.H., D.I.S. and E.R. planned the study. E.R. purified protein and produced viruses. E.A.B., F.H., N.D.T., P.S., M.L., M.M. and E.R. participated in adsorption and membrane fusion studies. K.E.O., T.S.W., K.H. and E.R. crystallized protein, and K.E.O., T.S.W., K.H. and J.M.G. acquired X-ray crystallography data. K.E.O solved and refined X-ray crystallography structures. A.K. prepared and optimized samples for cryo-EM and acquired cryo-EM data. A.K., K.E.O., F.D.H., D.K.C. and S.L. collected the data, S.L. and J.T.H. processed cryo-EM data and conducted the subtomogram averaging and fitting. K.E.O., D.B.H., N.D.T., J.T.H., D.I.S. and E.R. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-08728-7.

Competing interests: The authors declare no competing interests.

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