A bioactive phlebovirus-like envelope protein in a hookworm endogenous virus

Monique Merchant^{1,2,†}, Carlos P. Mata^{1,2,†,‡}, Yangci Liu^{1,2}, Haoming Zhai^{1,2}, Anna V. Protasio^{3,4},
Yorgo Modis^{1,2*}

¹Molecular Immunity Unit, Department of Medicine, University of Cambridge, MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, United Kingdom

²Cambridge Institute of Therapeutic Immunology & Infectious Disease (CITIID), University of Cambridge School of Clinical Medicine, Cambridge CB2 0AW, United Kingdom

³Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom

⁴Christ's College, University of Cambridge, St Andrew's Street, Cambridge, CB2 3BU, United Kingdom

[†]These authors contributed equally to this work

[‡]Present address: Electron and Confocal Microscopy Unit, National Centre for Microbiology, 28220, Majadahonda, Madrid, Spain

*Corresponding author: ymodis@mrc-lmb.cam.ac.uk

Keywords

Transposable element; retrotransposon; endogenous retrovirus (ERV); envelope protein; membrane fusion; horizontal gene transfer (HGT); virus evolution; gene exaptation; nematode

Short title (50 characters)

A bioactive phlebovirus-like Env in a hookworm EVE

Abstract

Endogenous viral elements (EVEs), accounting for 15% of our genome, serve as a genetic reservoir from which new genes can emerge. Nematode EVEs are particularly diverse and informative of virus evolution. We identify Atlas virus – an intact retrovirus-like EVE in the human hookworm *Ancylostoma ceylanicum*, with an envelope protein genetically related to GN-GC glycoproteins from phleboviruses. A cryo-EM structure of Atlas GC reveals a class II viral membrane fusion protein fold not previously seen in retroviruses. Atlas GC has the structural hallmarks of an active fusogen. Atlas GC trimers insert into membranes with endosomal lipid compositions and low pH. When expressed on the plasma membrane, Atlas GC has cell-cell fusion activity. RNA-Seq data analysis detected transcripts mapping to Atlas virus at different stages of hookworm development. With its preserved biological activities, Atlas GC has the potential to acquire a cellular function. Our work reveals structural plasticity in reverse-transcribing RNA viruses.

Introduction

Retroviruses and other reverse-transcribing RNA viruses can frequently integrate their genome, reverse-transcribed from RNA into DNA, into the host-cell genome. Viral genomes integrated into germline cells are inherited by future generations as endogenous viral elements (EVEs). Most EVEs are endogenous retroviruses (ERVs) and account for approximately 15% of the human genome, seven times more than protein-coding genes (1, 2). EVEs and other transposons were initially viewed as parasitic DNA. It is now evident that they serve as a genetic reservoir, from which new genes and regulatory elements can emerge. Sequences of retroviral origin help control gene expression by serving as promoters, enhancers and other regulatory elements (3, 4). Genes coopted from EVEs have evolved to fulfill vital cellular functions (1). For example, syncytins, which drive cell-cell fusion of trophoblasts during placental development, are encoded by ERV envelope glycoprotein (*env*) genes (3, 5). Another recent example is the

Gag capsid protein encoded by the *Caenorhabditis elegans* Cer1 retrotransposon, which encapsidates small non-coding RNAs into nucleocapsids that can transfer RNAs conferring learned pathogen avoidance behavior from infected parents to naïve progeny (6).

The reduced mutation rate of host versus retrovirus genomes (10^{-9} vs. 10^{-3} mutations per site per year (7)) means that EVEs are windows to ancestral retroviral sequences – evolutionary fossils preserved from the time of integration (2). Some EVE genes are expressed in human tissues and retain their biological activities, such as membrane fusion activity in the case of Env proteins (Envs) (3, 5, 8). Aberrant expression of Envs is associated with disease (9, 10). With the biology of EVEs still largely uncharted, it is likely that many cellular functions of EVEs in health and disease remain undiscovered. Studying EVE genes with novel properties could therefore provide insights on the evolutionary history of reverse-transcribing RNA viruses and identify fundamental principles in host-virus coevolution.

Nematode EVEs are particularly diverse and informative of virus evolution. EVEs from the *Belpaoviridae* (BEL/Pao) family (11), related to retroviruses and widespread across metazoa, have revealing genetic features in nematodes (12). The presence in *C. elegans* EVEs of overlapping ORFs, otherwise unique to complex vertebrate retroviruses, suggests retroviruses originated in early metazoa with a common ancestor resembling belpaoviruses (13). Furthermore, nematode endogenous belpaoviruses encode Envs that are genetically unrelated to retrovirus Envs (14). Instead of a class I viral membrane fusion protein with a core fold of three bundled α -helices (15-18) – a defining feature of modern retroviruses (19) – belpaovirus Envs have sequence similarity to G_C (G_2) envelope glycoproteins from phleboviruses (family *Phenuiviridae*) (14). G_C proteins are class II membrane fusion proteins, with a three-domain β -strand architecture (20, 21) also found in alphaviruses (22), flaviviruses (23, 24) and Rubella virus (25) but structurally unrelated to class I fusion proteins. A series of conformational changes in class II fusion proteins, triggered by endosomal acidification,

catalyses fusion of the viral and endosomal membranes to deliver the viral genome into the cytosol (26-28). A hydrophobic fusion loop first inserts into the endosomal membrane. The proteins then form trimers and fold back on themselves, pulling the cell membrane (held by the fusion loop) and the viral membrane (held by a transmembrane anchor) together so they fuse (19, 21).

Class II fusion proteins are not limited to viruses: they also drive cell-cell fusion events of fundamental importance, including syncytial epithelia formation in *C. elegans* and other nematodes (29-32), and gamete fusion in protozoa, plants, algae and invertebrates (33-38). The identical topology and overall arrangement of the three domains of viral and eukaryotic class II fusion proteins, along with similarities in their membrane fusion mechanisms, makes it all but certain they evolved from a common ancestor (33). Although the evolutionary origin of the ancestral class II fusion protein remains unknown, the presence of class II fusion proteins in EVEs raises the provocative prospect that a gene transfer from a virus to a cell led to the advent of sexual reproduction (33, 39).

Here, we identify a novel, intact endogenous belpaovirus in the human hookworm *Ancylostoma ceylanicum* (a parasitic nematode) with an Env more similar than any other eukaryotic sequence to phlebovirus G_C protein sequences. We expressed and purified the G_C-homologous fragment from this EVE, henceforth Atlas virus. A cryo-electron microscopy (cryo-EM) structure of Atlas G_C reveals a class II viral fusion protein fold remarkably similar to phlebovirus G_C proteins and not seen in retroviruses, as predicted 20 years ago (12, 14). We show that Atlas G_C has all the hallmarks of an active class II membrane fusion protein. It undergoes a monomer-to-trimer transition and inserts into lipid membranes with a specific lipid composition in response to a low pH trigger. Our work provides biochemical validation for the hypothesis that acquisition of a fusion protein from an infectious virus, as exemplified by Atlas virus, represents a general paradigm for how retrotransposons can become retroviruses (14),

and indeed how ancestral reverse-transcribing viruses may have originated (11). The preserved biological activities of Atlas G_C, including membrane fusion activity, raise the question of whether these activities, and those of EVE gene products more broadly, have cellular functions or cause disease.

Results

An intact EVE with a phlebovirus-like Env in the hookworm *A. ceylanicum*

The bioinformatic discovery of nematode EVEs with phlebovirus G_C-like Env sequences not seen in retroviruses (12, 14), requires biochemical validation. To identify phlebovirus-like EVE Envs suitable for biochemical analysis, we performed PSI-BLAST searches (40) for protein sequences similar to biochemically characterized phlebovirus G_C proteins. A search with Rift Valley Fever virus (RVFV) G_C as the query identified the gene *Acey_s0020.g108* (UniProt A0A016UZK2) in the human hookworm *A. ceylanicum* as containing the most similar sequence outside infectious virus taxa (Expected value 10⁻²⁰). The homologous sequence lies within a 9,204-nucleotide element possessing all the features of an intact EVE, including 100% identical 271-nt LTRs and a coding sequence encoding a single 2,828-residue Gag-Pol-Env polyprotein without any stop codons or introns (**Fig. 1A and fig. S1A**). We refer to this element as *A. ceylanicum* Atlas virus. It is one of nine *A. ceylanicum* EVEs that encode complete Gag-Pol-Env polyproteins (**Fig. 1, B and C**). These EVEs have the distinguishing genomic features of belpaoviruses from other nematode species, including an atypical Env and an aspartate to asparagine substitution (Y[X]DD → YVDN) in the most conserved reverse transcriptase motif, the polymerase site (13, 41, 42) (**fig. S1B**). Phylogenetic classification of Atlas virus based on reverse-transcriptase (RT) sequences confirms the *Belpaoviridae* phylogeny of Atlas virus, with Cer13 from *C. elegans* (12) as the closest neighbor in the RT tree (**Fig. 1D**). With a Gag sequence less than 20% identical to its closest homolog (*Acey_s0020.g1106*), the Atlas EVE is

a candidate for classification as a member of the *Belpaoviridae* family (which contains a single genus, *Semotivirus*) (**fig. S1C**) (43).

The phlebovirus G_C-like sequence spans the last 498 residues of the Atlas virus polyprotein (residues 2330-2828). It contains a single predicted C-terminal transmembrane helix, like phlebovirus G_C proteins. Phleboviruses and other *Phenuiviridae* family members express a glycoprotein precursor that is cleaved by cellular proteases into two envelope glycoproteins, G_N and G_C (or G1 and G2). G_N is the receptor-binding protein required for cellular attachment and G_C is the membrane fusion protein required for cell entry. G_N is highly antigenic and more variable in sequence than G_C. Our analysis of the Atlas virus Env sequence detected a slight but statistically significant similarity in the 421 residues preceding the G_C-like sequence (residues 1909-2329) to G_N glycoproteins from *Phenuiviridae* family viruses (E -value $\geq 10^{-7}$). Moreover, the distribution of predicted transmembrane helices and proteolytic cleavage sites in and adjacent to the Atlas G_N- and G_C-like sequences is the same as in phlebovirus glycoproteins (**Fig. 1A**). Together, these sequence features suggest the Atlas virus Env contains tandem phlebovirus-like G_N and G_C glycoproteins instead of a retrovirus-like glycoprotein. With all the features of a recently active EVE and an apparently intact set of phlebovirus-like glycoproteins, Atlas virus is an excellent candidate for biochemical analysis.

Fig. 1. Atlas is an endogenous belpaovirus with a phlebovirus-like Env. (A) Gene

architecture of the Atlas endogenous viral element (EVE), gene *Acey_s0020.g108* from the

Ancylostoma ceylanicum human hookworm parasite. Inset: annotated closeup of the Atlas Env

region encoding phlebovirus G_N - and G_C -like glycoproteins. Red residue number refer to the G_C

sequence alone. **(B)** List of *A. ceylanicum* EVEs encoding complete Gag-Pol-Env polyproteins

with phlebovirus-like Envs. LTR sequence identities for two of the EVEs could not be calculated:

Acey_s0206.g1994 had degenerate LTRs, and one LTR of *Acey_s0025.g1106* was truncated in

the genome assembly. **(C)** Phylogenetic tree of G_C proteins from *Phenuiviridae* and G_C -like (G_2 -

like) sequences from EVEs. Ace, *A. ceylanicum*; Cel, *C. elegans*; Nam, *Necator Americanus*,

RVFV, Rift Valley Fever virus; DabieV, Dabie bandavirus (SFTSV); HRTV, Heartland virus. **(D)**

Phylogenetic classification of Atlas based on reverse-transcriptase (RT) sequences. Alu TasV,

Ascaris lumbricoides Tas virus; Dsi NinV, *Drosophila simulans* Ninja virus; Bmo PaoV, *Bombyx mori* Pao virus; Tru SuzV, *Takifugu rubripes* Suzu virus; Dme BelV, *D. melanogaster* Bel virus; Aga MooV, *Anopheles gambiae* Moose virus; RooV, Roo virus. Trees drawn with iTOL v6 (44).

Atlas G_C has a class II membrane fusion protein fold not seen in retroviruses

As the molecular structure of phlebovirus G_C proteins, and how they drive fusion of the viral and cellular membranes, are well established from previous studies (20, 28), we focused our biochemical analyses on the G_C-like sequence from Atlas virus. A recombinant ectodomain fragment of Atlas G_C (polyprotein residues 2330-2772) was expressed in *Drosophila melanogaster* D.mel-2 cells as a secreted protein. The purified protein was a soluble, folded homotrimer (**fig. S2**). The structure of the G_C trimer was determined by single-particle cryo-EM image reconstruction at an overall resolution of 3.76 Å (**Fig. 2, A and B; table S1; fig. S3**). The map was sufficiently detailed for an atomic model to be built and refined for Atlas G_C residues 2330-2769 using the crystal structure of RVFV G_C (28) as a starting model (see Methods; **fig. S4**). The structure reveals a three-domain class II membrane fusion protein fold (**Fig. 2C**). All previously described retroviral Env structures have a helical coiled-coil class I fusion protein fold (15-18), exemplified by the hemagglutinin of influenza virus (19). The structure of the Atlas G_C ectodomain fragment is remarkably similar to phlebovirus G_C structures, specifically the trimeric postfusion G_C structures from RVFV (28), Dabie bandavirus (DABV, formerly SFTS phlebovirus) (45) and Heartland virus (HRTV) (46) (**Fig. 2, D and E**). Domain I, a 10-stranded β-barrel augmented by a 3-stranded sheet, organizes the structure. Two insertions in domain I form the elongated, mostly β-stranded domain II. Domain III has the 7-stranded β-sandwich topology of fibronectin type III (FN3) domains also found in macroglobulin domains (35, 47), but the hydrophobic core and disulfide bonding pattern of domain III differ from these and other annotated domains from non-viral species. A 16-amino acid portion of the stem region, which links domain III to the C-terminal transmembrane anchor in class II fusion proteins, could be

modeled, spanning 5 nm from the end of domain III to within approximately 1 nm of the tip of domain II. The stem forms trimer contacts, adding a β -strand to domain II of a different subunit, as seen in RVFV G_C(28). The overall configuration bears strong similarity to other viral and cellular class II fusion proteins including, in order of decreasing similarity: alphavirus E1 proteins (22); EFF-1/AFF-1 cell-cell fusion proteins from *C. elegans* and other animals (29, 30); HAP2 gamete fusion proteins from protozoa (33-35, 48) and plants (34, 36); and flavivirus E proteins (49) (**Fig. 2E**). Despite these structural similarities, the only proteins or domains of known structure with detectable amino acid sequence similarity to Atlas G_C (*E*-value <1 in PSI-BLAST (40)) are the phlebovirus G_C proteins (22-24% sequence identity; **fig. S5**).

Atlas G_C has the same structural features that distinguish phlebovirus glycoproteins from other class II fusion proteins: a larger number of disulfide bonds, ten of which are conserved in phlebovirus G_C sequences but not in other class II proteins; N-linked glycosylation in domain III; and a more extensive and rigid interface between domains I and II (**Fig. 2, D and E, and fig. S5**). The most notable differences between Atlas virus and phlebovirus G_C structures are differences in the disulfide bonding pattern and in the composition of side chains lining the glycerophospholipid (GPL) headgroup binding pocket conserved in arboviral class II fusion proteins (28). We discuss these differences and their potential functional implications below. Atlas G_C also has a different glycosylation pattern, with a single predicted N-linked glycosylation site at Asn414 in domain III with a weak corresponding feature in the density map (**fig. S6**). In contrast, phlebovirus G_C proteins contain two N-linked glycans in domain III, at two different sites. One of these, Asn1035 in RVFV, covers the fusion loop in the prefusion conformation of RVFV G_C and stabilizes the prefusion dimer by forming contacts across the dimer interface (20), as also seen in flavivirus E proteins (23, 24). This glycosylation site is conserved in DABV and HRTV G_C but absent in Atlas G_C. Despite these minor differences, the striking overall structural similarity of Atlas G_C to phlebovirus G_C proteins in the postfusion conformation experimentally

validates the evolutionary link between nematode EVEs from the *Belpaoviridae* family and the fusion proteins of phleboviruses postulated based on previous genomic analyses (12, 14).

Fig. 2. Cryo-EM structure of a phlebovirus G_C-like Env fragment from Atlas virus. (A)

Domain organization of Atlas G_C. (B) Cryo-EM image reconstruction of a soluble ectodomain fragment of Atlas G_C at 3.76 Å overall resolution. The purified protein was a homotrimer (fig. S2) and threefold (C3) symmetry was imposed. The map is colored by domain as in (A). A representative cryo-EM micrograph is shown in fig. S4A. (C) Overview of the refined atomic

model of the Atlas G_C trimer. Disulfide bonds (green) and an N-linked glycan (blue) are shown as sticks. **(D)** Protein fold of an Atlas G_C protomer. **(E)** The structures most similar to Atlas G_C based on structure comparison with DALI (50), G_C glycoproteins from Rift Valley Fever virus (RVFV, Rmsd 2.6 Å, Z-score 29, PDB:6EGU (28)), Dabie bandavirus (DABV, formerly SFTS phlebovirus; Rmsd 2.6 Å, Z-score 28, PDB:5G47 (45)) and Heartland virus (HRTV, Rmsd 2.6 Å, Z-score 28, PDB:5YOW (46)). Other representative class II fusion proteins are shown for comparison: Semliki Forest virus (SFV) E1 (Z-score 19, PDB:1RER (27)), *C. elegans* EFF-1 (Z-score 19, PDB:4OJD (29)), *Chlamydomonas reinhardtii* HAP2 (Z-score 16; PDB:5MF1 (33)) and dengue virus (DENV) E (Z-score 15, PDB:4GSX (51)).

Structure of the putative lipid membrane anchor of Atlas G_C

Viral fusion proteins insert a membrane anchor – the fusion loop, in class II proteins – into the host cell membrane to initiate virus-cell membrane fusion. The putative fusion loop of Atlas G_C can be identified by analogy to phlebovirus G_C proteins as spanning residues 127-140. The local resolution of the cryo-EM density for this region is lower than for the rest of the map but the large number of structural constraints imposed by the positions of disulfide-bonded cysteines and other residues conserved in phleboviruses G_C proteins allowed an atomic model to be built unambiguously (**Fig. 3A and fig. S6, B and C**). Specifically, the fusion loop is constrained by four disulfide bonds conserved in phleboviruses, a fifth disulfide specific to Atlas G_C (Cys129-Cys138), a phenylalanine (Phe136) required in phleboviruses at the apex of the fusion loop for membrane binding and fusion (28, 45, 52), and two conserved glycines (Gly128 and Gly134) that provide the torsional flexibility necessary for the fusion loop's tightly folded conformation (**Fig. 3, A to C**).

The structure and chemical properties of the Atlas G_C fusion loop resemble phlebovirus fusion loops. Ile130, Phe136, Phe137 and the Atlas-specific disulfide (Cys129-Cys138) form a hydrophobic surface similar to phlebovirus and flavivirus fusion proteins (26, 28) (**Fig. 3C**). The

area of this surface is greater than in most viral class II fusion proteins. By analogy with other viral class II fusion proteins, the location and extent of the hydrophobic surface formed by the Atlas G_C fusion loop suggest it could function as a membrane anchor.

In addition to inserting nonpolar side chains into the hydrophobic region of the membrane, viral class II fusion proteins form polar contacts with lipid headgroups via the fusion loop and an adjacent glycerophospholipid (GPL) binding pocket (28). By selecting for headgroups with complementary electrostatic potential, polar contacts confer a degree of specificity to lipid binding. In phleboviruses, a set of conserved polar residues in the GPL binding pocket bind selectively to zwitterionic GPLs (28). The Atlas G_C structure reveals a putative GPL binding pocket with both conserved and novel features (**Fig. 3**). The arginine that forms bidentate hydrogen bonds with the GPL phosphate moiety in phleboviruses (28) is conserved in Atlas virus (Arg86). The disulfide bond and short-chain hydrophobic residue that bind the GPL glycerol moiety are also conserved (Cys87-Cys135, Ala90). However, an aspartate-arginine pair that binds choline and ethanolamine GPL moieties in phleboviruses is replaced in Atlas G_C by two histidines (His271 and His85). Moreover, Atlas virus has an extra residue in the fusion loop, Asp133, compared to phlebovirus G_C proteins. The Asp133 side chain points into the putative GPL binding pocket and is located near the position of the choline GPL moiety in the superimposed structure of RVFV G_C bound to a phosphatidylcholine ligand (28), suggesting that Asp133 could compensate for the lack of a conserved aspartate at position 271 (**Fig. 3, A to C**). Hence the putative GPL binding pocket of Atlas G_C appears to have the necessary physicochemical attributes to support GPL binding, with Arg86 binding the phosphate moiety, Cys87/Cys135/Ala90 binding the glycerol moiety, and His85/Asp133/His271 coordinating the end of the headgroup. We note the presence in the cryo-EM reconstruction of a bulge in the density around the GPL binding pocket that is unaccounted for by the atomic model (**fig. S6C**). Additionally, the absorbance at 260 nm of purified Atlas G_C was higher than

expected despite treatment with nucleases during purification (**fig. S2A**). These two observations would be consistent with lipid molecules with unsaturated acyl chains copurifying with Atlas G_C, but the local resolution of the map is insufficient to ascertain whether the GPL binding pocket contains a ligand.

Fig. 3. Structure of the putative fusion loop and lipid binding pocket of Atlas G_C. (A) Local sequence alignment of the fusion loops (orange boxes) of Atlas G_C, RVFV G_C, and DENV E. Green dumbbells indicate disulfides. Disulfides shown below the RVFV sequence are conserved in Atlas and RVFV. (B) Residues contributing to binding of the phosphate (Phos),

glycerol and choline (Chol) moieties of glycerophospholipid (GPL) molecules by RVFV G_C (28). Colors indicate side chain properties: blue, positive charge; pink, negative charge; magenta, hydrophobic; green, sulfhydryl; light blue, positive charge at endosomal pH. **(C)** Closeup of the fusion loop and GPL binding pockets of Atlas G_C, RVFV G_C and dengue E. A single protomer from the postfusion trimer is shown, with key residues shown in stick representation. Label colors indicate side chain properties: blue, positive charge; red, negative charge; orange, hydrophobic. **(D)** Closeup of the fusion loop and GPL binding pocket of the Atlas G_C trimer showing histidine residues in or near the pocket. Prime symbols following residue numbers denote the protomer to which the residue belongs. **(E)** Surface representation of the same view as in **(D)**, colored by side chain hydrophobicity, except for histidine residues shown in light blue.

Atlas G_C binds membranes with endosome-like lipid composition at low pH

A key step in viral membrane fusion is binding of the fusion protein to the host cell membrane. We assessed binding of Atlas G_C ectodomain to liposomes in density gradient centrifugation and dynamic light scattering (DLS) experiments. Viruses containing class II fusion proteins, like many retroviruses, undergo membrane insertion and fusion in endosomal compartments where the pH is acidic (53-56). We therefore assayed liposome binding at a range of pH values. In contrast to RVFV G_C ectodomain, Atlas G_C ectodomain did not bind liposomes containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), cholesterol and sphingomyelin (SM) at neutral or acidic pH (pH 4-8; **Fig. 4, A and B**). At neutral pH (pH 7.8), Atlas G_C ectodomain also failed to bind liposomes containing anionic lipids enriched in early or late endosomes: phosphatidylserine (PS) or bis(monoacylglycerol)phosphate (BMP, also known as lysobisphosphatidic acid or LBPA), respectively. At pH 4, however, Atlas G_C ectodomain bound tightly to liposomes containing PS or BMP, with weaker binding observed at pH 4.6 (**Fig. 4, A and B, and fig. S7**). Remarkably, Atlas G_C ectodomain bound only weakly to liposomes containing phosphatidylglycerol (PG) instead of BMP even though PG and BMP are

regioisomers with identical chemical composition and electrostatic charge (of -1). PG and BMP differ only in the position of the second acylglycerol linkage, resulting in a linear configuration for BMP instead of the usual branched configuration for PG. Our liposome binding data show that Atlas G_C binds to membranes containing specific GPLs that are enriched in the endosomal pathway in a pH-dependent manner. No other class II fusion protein has been reported to require low pH, PS or BMP for membrane insertion. However, phleboviruses, which require only PE or PC and cholesterol for membrane insertion (28), subsequently require BMP and low pH for fusion (57). Similarly, flaviviruses require BMP, PS or other anionic lipids and low pH for efficient fusion (53, 55, 58).

The optimal pH for membrane binding of Atlas G_C (pH 4-4.5) is similar to the optimal pH of hemifusion of Uukuniemi virus (57), a model phlebovirus, and would be consistent with membrane insertion in late endosomes, as is the case for phleboviruses (54). The side chains of His85 and His271, in the GPL binding pocket of Atlas G_C, would be fully protonated at pH 4-4.5. The resulting net positive charge of the His85/Asp133/His271 triad (+1/-1/+1), analogous to the Arg/Asp pair that coordinates the end of GPL headgroups in phleboviruses, mirrors the charge of the phosphoserine headgroup of PS (-1/+1/-1). Moreover, Atlas G_C contains four additional solvent-exposed histidines (residues 88, 94, 99 and 100) in the vicinity of the GPL binding pocket (**Fig. 3, D and E**). Protonation of these histidines at low pH may promote further interactions with anionic lipid headgroups. The presence of six histidines in and around the GPL binding pocket provides a possible explanation for the observed pH-dependent insertion of Atlas G_C into membranes containing PS and BMP. Consistent with a conserved role for the GPL binding pocket in determining lipid specificity of class II fusion proteins, mutations in alphaviruses at a position equivalent to His271, in the *ij* loop, determine the extent to which alphaviruses depend on cholesterol for membrane binding (59).

Fig. 4. Atlas G_C binds liposomes in a lipid- and pH-dependent manner. (A)

flotation lipid binding assay. A liposome- G_C ectodomain mixture in 40% OptiPrep density

gradient medium was overlaid with a 30% OptiPrep cushion and centrifuged at 100,000 g.

Flotation was defined as the amount of G_C ectodomain co-floating with liposomes in the top-half

fraction (T) divided by the total amount of G_C ectodomain in the top- and bottom-half (B)

fractions. G_C ectodomain was quantified by Coomassie-stained SDS-PAGE. Error bars show the standard deviation of three replicates except WT+PC:PE:SM:CH:PS (two replicates) and WT+PC:PE:SM:BMP (four replicates). See **fig. S8** for uncropped gels for all replicates. **(B)** Binding of G_C ectodomain (WT, F136A/F137A or R86A) to liposomes measured by dynamic light scattering (DLS) as differences in liposome diameter in the presence and absence of Atlas G_C ectodomain. Error bars show the standard deviation of three to seven replicates. Significance was determined by 2-way ANOVA of the mean change in liposome diameter, using Sidak's multiple comparisons test with a 95% confidence interval in GraphPad Prism 8 (see **fig. S7**). **, 0.001 < p < 0.01; *, p < 0.05; ns, not significant. See **dataset S1** for source data.

Atlas G_C membrane binding does not strictly require cholesterol and occurs via the fusion loop

In addition to GPLs, phleboviruses and alphaviruses (but not flaviviruses) require cholesterol for efficient membrane binding and subsequent fusion (28, 57, 59). Alphaviruses additionally require sphingolipids (such as SM) for efficient fusion (60, 61). We found that neither cholesterol nor SM were required for Atlas G_C to bind liposomes (**Fig. 4, A and B**). Removal of cholesterol reduced the fraction of G_C bound by 50% in the liposome flotation assay (**Fig. 4A**) though binding was still detected in the DLS assay (**Fig. 4B**). Hence, although cholesterol and SM are not strictly required for binding, they enhance binding, possibly by increasing membrane fluidity. Notably, the concentration of cholesterol in nematode cell membranes is approximately 20 times lower than in vertebrates (62, 63). This is insufficient for cholesterol to regulate the structure or fluidity of nematode membranes, in which cholesterol is thought to be instead a precursor for low-abundance metabolites (62-64). Likewise, *Drosophila* can grow indefinitely with only trace amounts of exogenous sterols (62), suggesting that arthropods, which are obligate vectors of the vast majority of viruses containing class II fusion proteins, rely on lipids other than cholesterol to regulate membrane fluidity.

To determine whether Atlas G_C binds membranes in a manner analogous to other viral class II fusion proteins – via the fusion loop and GPL binding pocket – we purified Atlas G_C ectodomain variants with mutations in the two phenylalanine residues in the fusion loop (F136A/F137A), or in the arginine predicted to bind the GPL phosphate moiety (R86A). The F136A/F137A mutant failed to bind liposomes containing PS and co-flotation with liposomes containing BMP at pH 4 and pH 4.6 was reduced to approximately one third of wild-type (**Fig. 4C**). The R86A mutation reduced binding to liposomes containing PS or BMP at pH 4, to one third and 75% of wild-type, respectively (**Fig. 4D**). For both variants, preparations contained trimers as the major species but a small monomeric fraction was also present (**fig. S2**), suggesting that the mutated lipid binding residues are required for efficient trimer assembly. We conclude that Atlas G_C binds to lipid membranes through insertion of hydrophobic fusion-loop residues and coordination of lipid headgroups in the GPL binding pocket, as in other viral class II fusion proteins.

Evidence for monomeric and trimeric states of Atlas G_C

Class II fusion protein ectodomains can be monomeric, dimeric or form icosahedral shells in their prefusion conformation, but the fusogenic conformational change is always accompanied by reorganization into trimers (26, 57, 65, 66). Fusion proteins from classes I and III, including retrovirus fusogens, remain trimeric throughout the fusion reaction, but no class II fusion proteins are known to be trimeric in their prefusion conformation. Having established that Atlas G_C can insert into membranes as a trimer with a postfusion-like conformation, we set out to determine whether it could undergo a conformational change as seen in the fusion reaction of class II proteins from infectious viruses. The Atlas G_C construct described above was expressed as a trimer with no trace of monomers or dimers (**Fig. 5A and fig. S2A**). However, we found that a construct with the stem region truncated, G_C(DI-III), spanning residues 2330-2751, was expressed as a mixture of monomers, trimers, and higher order oligomers (**Fig. 5B and fig.**

S2B). In contrast to G_C trimers, which were stable at different protein concentrations and pH values, G_C(DI-III) monomers were unstable over time. As noted above, monomeric fractions were also present in preparations of the fusion loop mutant (F136A/F137A) and GPL binding pocket mutant (R86A) (**fig. S2, C and D**). Whether these monomeric species are in a prefusion conformation remains to be determined, but the presence of metastable monomers and stable trimers recapitulates a key property of class II fusogens from infectious viruses.

pH-dependent stabilization of the Atlas G_C trimer by protonated histidine residues

The increase in positive charge resulting from histidine protonation is an important part of the pH sensing mechanism of viral class II fusion proteins. Protonation of conserved histidines at the domain I-domain III interface of alphavirus, flavivirus and phlebovirus glycoproteins promotes the fusogenic conformational change by destabilizing the prefusion conformation and stabilizing the postfusion conformation (20, 28, 67-70). For example, histidines in domain III of phlebovirus G_C proteins form interprotomer salt bridges with negatively charged side chains in the postfusion trimer and mutation of one such histidine in RVFV, His1087, renders the virus uninfecious (71). Similarly, in Atlas G_C His258, His333 and His336 form interprotomer or interdomain polar contacts (**Fig. 5C**). These histidine-dependent contacts would stabilize the trimeric postfusion-like conformation of Atlas G_C specifically in acidic endosomal compartments. The parallels in how Atlas G_C and class II fusogens from infectious viruses respond at the ultrastructural level to environmental cues support the hypothesis that Atlas G_C would have membrane fusion activity in late endosomes, like phleboviruses and many retroviruses.

Fifteen disulfide bonds stabilize Atlas G_C in its postfusion-like conformation

With 30 cysteine residues forming 15 disulfide bonds, Atlas G_C contains twice the average abundance of cysteines, more than has been found in any other class II protein. 20 of these cysteines form disulfides that are structurally conserved in phleboviruses (including RVFV, DABV and HRTV) but not in other class II proteins (**fig. S5**). An eleventh disulfide, in domain III,

is conserved in Atlas virus, DABV and HRTV but not RVFV. However, Atlas G_C contains four additional disulfides not conserved in phleboviruses: one in the fusion loop, two in domain II in the β -hairpin containing the *ij* loop (one of the cysteines forming these disulfides is conserved in phleboviruses but forms a disulfide with a cysteine in a different β -strand in domain II), and one in domain III (Figs. 2C and 3A, and fig. S5). As discussed above, the Atlas-specific disulfide in the fusion loop (Cys129-Cys138) extends the hydrophobic surface formed by conserved residues in the fusion loop that are required for membrane insertion (Fig. 5D). We note that due to the location of Cys129 and Cys138 close to the threefold symmetry axis of the trimer, the side chains of the two residues can be rearranged by torsional rotation, to form intermolecular disulfides across the trimer interface, thereby crosslinking all three protomers in the trimer.

Fig. 5. Oligomeric states and disulfide bonding pattern of Atlas G_C. (A) SEC-MALS of Atlas G_C ectodomain (residues 2330-2772). The protein formed trimers. (B) SEC of Atlas G_C(DI-III) (residues 2330-2751) expressed in a mixture of oligomeric states, including monomers, trimers, and larger aggregates (void peak). G_C(DI-III) monomers were unstable. (C) His258, His333 and His336 form interprotomer or interdomain polar contacts predicted to stabilize the G_C trimer specifically at pH <6, when the histidine side chains are charged. Residues from different

protomers are denoted with a prime symbol. **(D)** View along the threefold axis of the G_C trimer with intramolecular disulfides between the cysteines most proximal to the axis, Cys129 and Cys138.

Atlas G_C retains membrane fusion activity

Having identified the hallmarks of a fusion protein in Atlas G_C, we measured its membrane fusion activity in a cell-cell fusion assay. CHO cells were transfected with plasmids encoding Atlas G_C or Atlas G_N-G_C. To promote plasma membrane localization and minimize ER retention, we replaced the predicted transmembrane domain and cytosolic tail of G_C with the C-terminal transmembrane anchor and cytosolic tail from HLA-A2, known to localize to the plasma membrane (72). Plasmids encoding vesicular stomatitis virus (VSV) G, or no protein, were used as positive and negative controls, respectively. Since Atlas G_C trimers require low pH and BMP or PS to efficiently bind membranes, we treated transfected cells with exogenous BMP and then transferred them to pH 4.5 buffered medium to trigger fusion. Similar treatments have been used previously to measure cell-cell fusion activity of flavi- and alphaviruses (53). Confocal light microscopy with nuclear and plasma membrane stains showed that cells with three or more nuclei were common in cells expressing Atlas G_C following treatment with BMP and pH 4.5, though less abundant than in cells expressing VSV G, with or without treatment (**Fig. 6, A and B, and movie S1**). Cells with more than three nuclei were rare in cells expressing Atlas G_N-G_C. To quantify cell-cell fusion we counted nuclei and multinuclear cells in micrographs. We found that in cells expressing Atlas G_C, the fraction of multinuclear cells, defined as cells containing two or more nuclei, was 25 ± 3 % following treatment with BMP and pH 4.5, versus 10 ± 0.2 % without treatment (**Fig. 6C**; 95% confidence intervals). Transfection of the G_C fusion loop mutant F136A/F137A resulted in essentially identical multinucleation fractions as with empty vector, with or without treatment with BMP and pH 4.5 (**Fig. 6C**), underpinning the importance of the fusion loop in fusion activity. By comparison, in cells expressing VSV G, 73 ± 8 % of treated

cells were multinuclear. 8 ± 1 % of cells transfected with empty vector were binuclear with or without treatment, but none contained more than two nuclei (**fig. S9**). Incubation of transfected cells with CDK4, which arrests the cell cycle at G1 phase, had no measurable effect on the fraction of multinuclear cells (**fig. S9D**), indicating that cytokinesis effects do not significantly contribute to this cell-cell fusion assay readout. We conclude that Atlas G_C has approximately one third of the membrane fusion activity of VSV G under the treatment conditions tested, which is significant given that VSV G is considered highly fusogenic and widely used as a model fusogen.

The Atlas G_N-G_C construct tested had little fusion activity (**Fig. 6C**). To investigate this, we measured G_C expression on the surface of cells transfected with the G_N-G_C plasmid. Cell-surface proteins were biotinylated, affinity-purified and analyzed by immunoblot and liquid chromatography with tandem mass spectrometry (LC-MS/MS). When expressed as part of a G_N-G_C construct, only trace levels of cell-surface G_C were detected by Western blot (**Fig. 6D**). Moreover, no G_N peptides were observed by LC-MS/MS, and the LC-MS/MS signal for G_C was significantly weaker than in cells transfected with G_C alone (**Fig. 6E**), consistent with the lack of fusion activity in cells transfected with G_N-G_C. We conclude that the G_N was degraded or failed to be secreted in CHO cells, which in turn prevented efficient secretion of G_C.

Fig. 6. Cell-cell fusion assay. (A) Confocal micrographs of CHO cells expressing Atlas G_C fused to the transmembrane anchor from HLA-A2, or VSV G. Cells were treated with pH 4.5

buffer containing BMP, or pH 7.4 buffer without lipid. Blue, Hoechst 33342 nuclear stain; Magenta, CellBrite Red plasma membrane dye. Micrographs were selected to show fusion events – see **fig. S9** for representative raw micrographs. Scale bars, 10 μ m. **(B)** Confocal micrograph of a multinucleated CHO cell expressing Atlas G_C following treatment with pH 4.5 and BMP. Blue, Hoechst 33342; magenta, CellBrite Red; green, α -HA antibody for Atlas G_C detection. Scale bar, 10 μ m. A composite Z-stack of this cell is shown in **movie S1**. **(C)** The fraction of multinuclear cells transfected with plasmids encoding Atlas G_C, Atlas G_N-G_C, VSV G or pHLsec empty vector was calculated by counting mono- and multi-nucleated cells using the Hoechst and CellBrite stains. Error bars represent SD between measurements from three distinct experiments, with 135-407 nuclei counted per replicate. *P*-values were calculated by 2-way ANOVA (Tukey's multiple comparisons test, GraphPad Prism 9). **(D and E)** G_C cell-surface expression analysis. Cells were biotinylated, cell-surface proteins were affinity-purified with NeutrAvidin agarose, and the cell-surface G_C fraction was quantified by Western blot, **(D)**, and LC-MS/MS mass spectrometry, **(E)**. See **dataset S2** for source data.

Transcriptional activity of Atlas virus based on RNA-Seq data

Transcription of endogenous viruses is tightly regulated to prevent harmful gene expression and genome damage from transposition events. However, some EVEs escape repression and are highly transcribed, including a subset of belpaoviruses from the parasitic trematode *Schistosoma mansoni* (blood fluke) (73). We analyzed transcript levels of Atlas and other complete belpaoviruses in previously published RNA-Seq data for *A. ceylanicum* (74). Transcript abundance of the belpaovirus EVEs encoding complete polyproteins varied, ranging from undetectable to above the median value for all annotated genes (**Fig. 7 and fig. S10**). For Atlas virus, transcript levels varied across developmental stages: expression was above the median for annotated genes at the larval L3 stage, below the first quartile at stage L4, and near the median in adult hookworms (**Fig. 7A**). Notably, Atlas virus transcript expression increased

15-fold from the L4 to adult (mixed sex) stages (adjusted p -value = 0.0021, see **dataset S3** for differential expression calculations).

Fig. 7. RNA-Seq signal from Atlas virus and other *A. ceylanicum* genes. (A) Box plots of RNA-Seq Reads Per Kilobase of transcript per Million mapped reads (RPKM) for all annotated *A. ceylanicum* genes, at different developmental stages (L3 and L4 larva, adult male and adult mixed sex). RNA-Seq data are from Bernot *et al.* (74). Bars in each box represent the median. Box boundaries represent the first and third quartiles. RPKM values are shown for Atlas virus and the complete belpaovirus EVEs with the highest and lowest RNA-Seq signals, s0025.g1106 and s0203.g1846, respectively. (B) Distribution of RNA-Seq reads over the Atlas virus coding sequence for the adult (mixed sex) and L4 developmental stages. Read coverage was greater in adults. See **fig. S10** for a snapshot of this region from Integrative Genomics Viewer with unmerged BAM files.

Discussion

Here, we identify Atlas virus as an endogenous belpaovirus containing intact *gag*, *pol* and *env* genes with novel features in the human hookworm *A. ceylanicum*. The cryo-EM structure of the Atlas Env reveals a class II viral fusion protein fold remarkably similar to that of the G_C glycoprotein from Rift Valley Fever virus. Atlas G_C has the hallmarks of an active class II

membrane fusion protein: a stable trimeric assembly, a putative fusion loop, membrane insertion triggered by low pH with specificity for late endosomal lipid composition, and membrane fusion activity. Moreover, Atlas virus mRNA is expressed at specific stages of hookworm development.

Our work supports the model first proposed based on phylogenetic studies that the nematode belpaoviruses acquired their Env by horizontal gene transfer from a virus from the *Phenuiviridae* family or a phlebovirus-like ancestor (14). It remains unclear whether RNA encoding the phlebovirus-like glycoprotein integrated into the belpaovirus ancestor as mRNA in a splicing event, or by first becoming a substrate for the reverse transcriptase with subsequent genomic integration as dsDNA. The envelope proteins from retroviruses, including ERVs, that have been biochemically characterized were all found to be class I fusion proteins with an α -helical coiled-coil as the core fold. Viral class II fusion proteins have so far been found only in non-integrating RNA viruses. Our discovery of an endogenous belpaovirus with a potentially functional, phlebovirus-like class II fusion protein that is structurally unrelated to retrovirus Envs reveals an unexpected degree of structural and genetic plasticity in reverse-transcribing RNA viruses. More generally, the presence of the class II fusion protein fold in EVEs and across many phyla suggests this fold is derived from a common ancestor that could have been viral or cellular in origin (33-36, 75).

While rare, horizontal gene transfer of atypical fusogens into retroelements is not unique to the belpaoviruses. The Tas element from the nematode *Ascaris lumbricoides*, a common parasitic worm in humans, has an *env* gene with weak genetic similarity to herpesvirus gB proteins (14), which have a class III fusion protein fold (also found in *Rhabdoviridae* and *Baculoviridae*). Taken together, these findings lead us to hypothesize that acquisition of a fusion protein from an infectious virus represents a general paradigm for how retrotransposons can

become retroviruses, and indeed how ancestral reverse-transcribing viruses may have originated.

The *env* gene is often the first element to be lost in EVEs, as it is not required for intracellular proliferation, so it is notable that the G_N-G_C *env* module is intact in Atlas virus. With its identical LTRs, and no stop codons or frameshift mutations, Atlas virus shows all the signs of being intact and recently active. This supports the notion that the envelope may be functional (12). The preserved biological activities of Atlas G_C suggest these activities could have cellular functions in health and disease, as reported for a small but increasing number of ERV *env* and *gag* gene products (1, 3, 5, 76). Further studies are required to determine the full extent to which protein expression from transposable elements – and its dysregulation – contribute to basic cellular functions, embryonic development, and disease outcomes. This work provides a blueprint for such efforts.

Materials and Methods

Genomic analyses of *A. ceylanicum* Atlas virus. A PSI-BLAST search (40) for protein sequences similar to biochemically characterized phlebovirus fusion proteins identified the gene *Acey_s0020.g108* (UniProt A0A016UZK2; Genbank JARK01001356.1, genomic translation EYC20859.1) in the human hookworm *Ancylostoma ceylanicum* as the most similar sequence outside infectious virus taxa, with an Expected value (E-value) of 10⁻²⁰ against the Rift Valley Fever virus (RVFV) G_C sequence (UniProt P03518). A second iteration performed using position-specific scoring matrix based on an alignment of sequences identified in the first iteration gave an E-value of 10⁻¹⁴⁴. The *Acey_s0020.g108* gene is referred to here as Atlas virus.

Phylogenetic analysis of the phlebovirus G_C-like protein from Atlas virus and other G_C or G_C-like proteins with similar protein sequences was performed as follows. A reference multiple sequence alignment was initially generated by aligning the Atlas G_C protein sequence with the

sequences of reference protein RVFV G_C and other viral G_C proteins identified as having structural similarity in structure comparisons with DALI (50), namely the G_C proteins from Dabie bandavirus (DABV, formerly SFTSV phlebovirus, GenBank:AGM33042.1) and Heartland virus (HRTV, GenBank:AFP33394.1). For each G_C protein, the sequence range present in the corresponding Protein Data Bank (PDB) entry (7A4A, 6EGU, 5G47, or 5YOW, respectively) was defined as the G_C ectodomain sequence and used to generate the reference alignment with MAFFT in SnapGene v5.1.7. The sequences of the following EVEs with detectable Env protein sequence similarity were then added to the alignment: *Necator americanus* NECAM_13468 (GenBank:XM_013440329.1), *C. elegans* Cer13 virus (GenBank: Z81510.2; WormBase WBTransposon00000728, gene Y75D11A.5), and seven *A. ceylanicum* EVEs encoding complete Gag-Pol-Env polyproteins with predicted phlebovirus-like Envs (**Fig. 1B**; GenBank EYC27361.1, EYC19962.1, EYC20099.1, EYC18998.1, EYC07469.1, EYB91703.1, EYB80846.1). The tree was calculated with IQ-TREE v1.6.12 and drawn with iTOL v6 (44).

The reverse transcriptase (RT) phylogenetic tree was calculated based on the protein sequences of Atlas virus, Cer13 virus, the seven *A. ceylanicum* EVEs listed above, and seven other belpaoviruses (GenBank AF060859.1, Z29712.1, L09635.1, U23420.1, AY180917.1, D83207.1, AF537216.1). RT domains (Pfam:PF00078, RVT_1) were identified with PfamScan in five of the sequences (GenBank Z81510.2, EYC27361.1, EYC19962.1, EYC07469.1, EYB80846.1). The protein sequences of these five RTs were extracted and aligned with MAFFT and Clustal Omega v1.2.4. The tree was generated with IQ-TREE v1.6.12 and iTOL v6 (44).

The Atlas virus LTRs were identified with REPuter (77). Sequence identity matrices (**fig. S1**) were calculated with SDT (78).

Protein expression and purification. Synthetic genes encoding soluble ectodomain fragments of the Env of endogenous retrovirus Y032_0020g108 from *A. ceylanicum* were subcloned into

the pMT/BiP/V5-His vector (ThermoFisher) in frame with the BiP signal sequence and the C-terminal V5 and six-histidine tags. The constructs referred to here as Atlas G_C and Atlas G_C(DI-III) span amino acids 2330-2772 and 2330-2751 from UniProt A0A016UZK2, respectively. Atlas G_C mutants were generated by Dpn I-based site-directed mutagenesis. D.mel-2 insect cells (ThermoFisher) were co-transfected with the expression construct and blasticidin resistance marker pCoBlast (ThermoFisher) at a 20:1 molar ration and cultured for 6 weeks in 0.5 µg ml⁻¹ blasticidin to obtain a population of expressor cells. Expression was induced in a shaking cell suspension at 27°C with 0.5 mM CuSO₄ at a cell density of 5 x 10⁶ cells ml⁻¹. The cell culture medium was harvested 4 to 5 days after induction, centrifuged to remove cells (2,000 g) and cell debris (17,000 g), filtered with a 0.2 µm filter, concentrated by tangential-flow filtration, and buffer exchanged into 20 mM Tris pH 7.8, 0.3 M NaCl, 5% glycerol, 20 mM imidazole, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine). Atlas G_C was purified by nickel-affinity chromatography with a HisTrap Excel column (Cytiva), followed by anion-exchange chromatography with a MonoQ or Resource Q column (Cytiva) using 20 mM Tris pH 8.0, 50 mM NaCl, 5% glycerol, 0.5 mM TCEP as the binding buffer and binding buffer plus 1 M NaCl as the elution buffer. Peak fractions were concentrated and further purified by size-exclusion chromatography (SEC) with a Superdex 200 Increase (10/300) column (Cytiva) in 20 mM Tris pH 7.8-8.0, 0.15 M NaCl, 5% glycerol and 0.5 mM TCEP. The C-terminal V5 and histidine tags were optionally cleaved by incubation with carboxypeptidase A (CPA) for 3 h at 4°C (1:500 CPA:G_C molar ratio).

Liposome binding assay. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-snglycero-3-phosphoethanolamine (PE), egg sphingomyelin (SM), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (PG), (S,R) Bis(Monoacylglycero)Phosphate (BMP) (Avanti Polar Lipids), and 1-cholesterol (Sigma-Aldrich) were dissolved in chloroform. 25 mM lipid solutions were mixed at various molar ratios and dried under nitrogen gas for over 4 h. The lipid film was resuspended in Liposome

Buffer (20 mM Tris pH 7.8, 0.15 M NaCl, 5% glycerol, 0.5 mM TCEP, 2 mM MgCl₂, 2 mM CaCl₂, 2 mM KCl) and subjected to five cycles of freeze-thawing in liquid nitrogen, followed by 25 cycles of extrusion through two 0.2 µm polycarbonate filter membranes (Whatman). Purified Atlas G_C ectodomain was added in a 1:771 protein:lipid molar ratio and incubated at 37°C for 5 min. The pH was reduced by adding a 2 M stock solution of sodium acetate pH 4.6 or 4.0 to a final concentration of 0.2 M. Following a 2 h incubation at 37°C the pH of the suspension was neutralized with 1 M Tris pH 8. OptiPrep density gradient medium (Sigma-Aldrich) was added to a concentration of 40%, maintaining 0.15 M NaCl throughout. Approximately 0.5 ml of the liposome suspension was placed in a centrifuge tube, overlaid with a 2.5 ml cushion of 30% OptiPrep solution and centrifuged at 100,000 g for 1 h at 4°C in a TLA100.3 rotor (Beckman Coulter). Top (T) and bottom (B) fractions (approximately 1.5 ml each) were collected from the top meniscus with a micropipette. Atlas G_C was quantified by densitometry of the absorbance at 700 nm of bands in Coomassie-stained SDS-PAGE gels with an Odyssey scanner (LI-COR). Flotation was defined as the amount of Atlas G_C ectodomain in the top fraction divided by the total amount of Atlas G_C ectodomain in both fractions.

For measurement of liposome diameter by dynamic light scattering (DLS), the liposome suspensions were diluted ten-fold in Liposome Buffer prior to the addition of 40% OptiPrep solution. Following centrifugation liposome diameters were measured in 384-well clear-bottomed optical imaging plates (Corning) with a DynaPro Plate Reader III (Wyatt Technologies). The mean diameter was calculated as the average of three independent measurements, each consisting of fifteen two-second acquisitions. Protein-free acidified liposome controls were treated and measured in parallel, with Liposome Buffer instead of Atlas G_C solution.

Size-exclusion chromatography and multi-angle scattering (SEC-MALS) analysis. 100 µl samples containing 1.6 – 2.5 mg ml⁻¹ Atlas G_C ectodomain were analysed by size-exclusion

chromatography (SEC) at 293 K on a Superdex 200 (10/300) column (Cytiva) in 20 mM Tris pH 7.8, 0.15 M NaCl, 5% glycerol and 0.5 mM TCEP with a flow rate of 0.5 ml min⁻¹. The SEC system was coupled to both multi-angle light scattering (MALS) and quasi-elastic light scattering (QELS) modules (DAWN-8+, Wyatt Technology). The protein was also detected as it eluted from the column with a differential refractometer (Optilab T-rEX, Wyatt Technology) and a UV detector at 280 nm (Agilent 1260 UV, Agilent Technology). Molar masses of peaks in the elution profile were calculated from the light scattering and protein concentration, quantified using the differential refractive index of the peak assuming a dn/dc of 0.1860, with ASTRA6 (Wyatt Technology).

Cryo-EM sample preparation and data collection. Purified Atlas G_C ectodomain trimer (3 µl at a concentration of 0.025 mg ml⁻¹) in 20 mM Tris pH 7.8, 0.15 M NaCl, 5% glycerol and 0.5 mM TCEP, was applied onto glow-discharged R1.2/1.3 400 mesh copper grids (Quantifoil Micro Tools, Germany). The grids were blotted for 4 s and plunge-frozen in liquid ethane with a Vitrobot Mark IV (ThermoFisher) at 4°C and 100% humidity. Preliminary sample screening and initial datasets were acquired on a FEI Tecnai F20 microscope operated at 200 kV equipped with Falcon II direct electron detector (ThermoFisher) at -4 µm defocus. High-resolution cryo-EM dataset collection was performed on a Titan Krios microscope (ThermoFisher) operated at 300 kV equipped with a 20 eV slit-width GIF quantum energy-filtered Gatan K2-Summit direct electron detector in counting mode. A total of 3,027 movies were recorded at a calibrated magnification of 130,000x leading to a magnified pixel size of 1.047 Å on the specimen. Each movie comprised 36 frames with an exposure rate of 1.28 e⁻ Å⁻² per frame, with a total exposure time of 8 s and an accumulated exposure of 46.18 e⁻ Å⁻². Data acquisition was performed with EPU Automated Data Acquisition Software for Single Particle Analysis (ThermoFisher) with three shots per hole at -1.3 µm to -3.5 µm defocus.

Image processing. Micrographs from initial datasets allowed us to obtain a consistent model at ~19 Å resolution from 3,790 particles selected after 2D and 3D classification, and consequent auto-refinement. All movies from high-resolution datasets were motion-corrected and dose-weighted with MOTIONCOR2 (79). Aligned, non-dose-weighted micrographs were then used to estimate the contrast transfer function (CTF) with GCTF (80). All subsequent image processing steps were performed using RELION 3.0 (81, 82). 2D references from initial datasets were used to auto-pick the micrographs. One round of reference-free 2D classification was performed to produce templates for better reference-dependent auto-picking, resulting in a total of 987,570 particles. After a first round of 2D classification, 595,011 particles were selected to perform a second 2D classification, resulting in a final number of 320,041 selected particles. Then, a 3D classification imposing C3 symmetry was performed using the model from the initial datasets filtered at 40 Å resolution as initial model. The best class, containing 197,145 particles, was selected, and subjected to 3D auto-refinement imposing C3 symmetry, yielding a map with an overall resolution at 4.11 Å based on the gold-standard (FSC = 0.143) criterion. After refinement, the CTF refinement (per-particle defocus fitting and beam tilt estimation) and Bayesian polishing (83) routines implemented in RELION 3.0 were performed, yielding a final map with an overall resolution at 3.76 Å. Local resolution was estimated with RELION.

Model building and refinement. The most similar sequence to Atlas G_C with a structure available was glycoprotein G_C from Rift Valley Fever virus (RVFV). The crystal structure of RVFV G_C in the postfusion conformation (PDB:6EGU (28)) was used as template to build a homology model with the sequence of Atlas G_C using the Swiss-Model server (<https://swissmodel.expasy.org>). The output model was docked as a rigid body into the density with UCSF Chimera (84). Initial docking was performed manually and was followed by real space fitting with the Fit in Map routine. A preliminary step of real space refinement was performed on the three-subunit model, with Phenix 1.13 (85), with global minimization, atomic

displacement parameter (ADP), simulated annealing and morphing options selected. The model was then rebuilt in Coot (86) to optimize the fit to the density. Due to low resolution information in the fusion loop region, the density was converted to .mtz file using CCP-EM software package tools and blurring of the density allowed us to localize bulky residues and disulphide bonds, and thus use them as a guide to build the entire fusion loop. A final step of real space refinement was performed with Phenix 1.15, with global minimization and ADP options selected. The following restraints were used in the real space refinement steps: secondary structure restraints, non-crystallographic symmetry (NCS) restraints between the protein subunits, side chain rotamer restraints, and Ramachandran restraints. Key refinement statistics are listed in **table S1**.

Model validation and analysis. The FSC curve between the final model and full map after post-processing in RELION, Model vs Map, is shown in **fig. S3**. Cross-validation against overfitting was performed as described (87). The atoms in the final atomic model were displaced by 0.5 Å in random directions with Phenix. The shifted coordinates were then refined against one of the half-maps generated in RELION, the “work set”. This test refinement was performed in Phenix using the same procedure as for the refinement of the final model (see above). The other half-map, the “test set” was not used in refinement for cross-validation. FSC curves of the refined shifted model against the work set, FSCwork, and against the test set, FSCtest, are shown in **fig. S3**. The FSCwork and FSCtest curves are not significantly different, consistent with the absence of overfitting in our final models.

The quality of the atomic models, including basic protein geometry, Ramachandran plots, clash analysis, was assessed and validated with Coot, MolProbity (88) as implemented in Phenix 1.15, and with the Worldwide PDB (wwPDB) OneDep System (<https://deposit-pdbe.wwpdb.org/deposition>).

Cell-cell fusion assay. Chinese hamster ovary (CHO) Lec3.2.8.1 cells were transfected with pHL-Sec plasmids encoding ectodomain fragments of Atlas G_N-G_C (polyprotein residues 1909-2795) or Atlas G_C (residues 2330-2795) fused to the C-terminal transmembrane domain from HLA-A2 (residues 288-345) and cloned in frame with the vector's secretion signal and a C-terminal HA tag. Empty pHL-Sec plasmid, and pcDNA encoding vesicular stomatitis virus (VSV) G, were used as negative and positive controls, respectively. 16-20 h post-transfection, cells were transferred to PBS supplemented with 2.5 mM BMP (18:1(S,S) Bis(Monoacylglycero)Phosphate (BMP); Avanti Polar Lipids). To obtain a homogeneous BMP suspension, the mixture was freeze-thawed five times using liquid nitrogen and a water bath, followed by a 3 min incubation in a sonicating water bath. Cells were incubated in the BMP suspension (or PBS for the untreated control) at 37°C for 5 min, shown previously to be sufficient for anionic lipid incorporation into the plasma membrane (53). Cells were transferred to pH 4.5 complete medium (DMEM adjusted to pH 4.5 with HCl supplemented with 10% foetal bovine serum), or pH 7.4 complete medium for the untreated control, and centrifuged at 2,500 g at 37°C for 2 min. Cells were immediately resuspended in complete media and plated out. Following reattachment (4-6 h after treatment), cells were stained with Hoechst 33342 (Bio-Rad) and CellBrite Red cytoplasmic membrane dye (Biotium, cat. no. 30023) and imaged on a Nikon iSIM Swept Field inverted confocal microscope with a 60x/1.2NA water objective. Cluster analysis of the Hoechst channel with Fiji (89) was used to count single nuclei and identify polynuclear clusters. Nuclei within polynuclear clusters were counted by visual inspection. The plasma membrane stain was used to confirm polynuclear clusters and count the number of multinuclear cells, defined as cells with two or more nuclei, by visual inspection. The fraction of multinucleated cells (F) was calculated using the formula:

$$F = 1 - (n. mononucleated cells) / (total n. nuclei)$$

For testing the effect of inhibiting the cell cycle on the fraction of multinucleated cells, cells were transfected with plasmid pEGFP-N1, and treated on the following day with 10 μ M CDK4 (Cayman Chemical, Cat. No. 17648), or PBS in the negative control, for 6 h at pH 7.4.

Cell surface biotinylation and mass spectrometry. Proteins on the cell surface were biotinylated and isolated with the PierceTM Cell Surface Protein Biotinylation and Isolation Kit (ThermoFisher, A44390). HEK293T cells were transfected with 3 μ g pHLsec-G_NG_C, or pHLsec-G_C. One day later, cells were washed with PBS and incubated with 0.25 g/l Sulfo-NHS-SS-Biotin on ice for 10 min. Then cells were washed with PBS and suspended in 0.5 ml of ThermoFisher Lysis Buffer supplemented with cComplete EDTA-free Protease Inhibitor Cocktail (Merck). Cells were lysed on ice for 30 min (with 5s vortexing before and after lysis) and centrifuged at 15,000 x g for 5 min at 4°C. The lysate supernatant was incubated with 0.25 ml NeutrAvidin Agarose resin for 30 min at 20°C on an end-over-end rotator. The resin was washed with Wash Buffer, and bound proteins eluted Elution Buffer from the kit

Samples were prepared for mass spectrometry with the EasyPep Mini MS Sample Prep Kit (ThermoFisher, A40006) following the manufacturer's instructions. Briefly, proteins were extracted, reduced, alkylated, and digested with trypsin/Lys-C protease. Hydrophilic and hydrophobic contaminants were removed with a peptide clean-up step. LC-MS/MS analysis of peptide samples was performed on an Ultimate 3000 rapid separation liquid chromatography (RSLC) system connected to a Q-Exactive plus mass spectrometer (ThermoFisher). The acquired raw files were processed with MaxQuant v1.6.6.0.

Western blotting. The cell surface biotinylated protein samples purified from HEK293T cells as described above were heated to 95°C in SDS sample buffer for 10 min. 15 μ l of each sample was run on a polyacrylamide gel. Gels were blotted onto PVDF membranes (Merck). Blots were blocked in 5% milk in PBS, 0.2% Tween-20 and incubated overnight with anti-HA rabbit

monoclonal antibody (Cell Signaling Technology, 3724, RRID:AB_1549585) diluted 1:1,000 in blocking solution. Blots were imaged with the 800 nm channel of a LI-COR Odyssey fluorescent scanner after incubation with anti-rabbit DyLight 800-conjugated secondary antibody (Cell Signaling Technology, 5151, RRID:AB_10697505) at 1:5,000 dilution for 30 min at room temperature.

RNA-Seq analysis. We analysed data published by Hawdon and colleagues (74).

ArrayExpress run accession numbers and corresponding life cycle stages were: SRR6359160, L3; SRR6359161 and SRR6359163, L4; SRR6359164 and SRR6359165, Adult mixed pooled worms; SRR6359162 and SRR6359166, Adult male pooled worms. Our mapping and counting strategy was modified as recommended for repetitive genomic features (90). Reads were mapped against the reference *A. ceylanicum* genome (91) with STAR v2.7.5a (92) with parameters: "--outSAMtype BAM SortedByCoordinate --runMode alignReads --outFilterMultimapNmax 1000 --outSAMmultNmax 1 --outFilterMismatchNmax 3 --outMultimapperOrder Random --winAnchorMultimapNmax 1000 --alignEndsType EndToEnd --alignIntronMax 1 --alignMatesGapMax 350". FeatureCounts v2.0.1 (93) with parameters "-M -F GFF -s 0 -p -t exon -g gene_id" was used to count reads over a modified annotation file based on the original annotation (91) and downloaded from Wormbase Parasite (94). The original annotation file in GFF format was slightly modified to contain a "gene_id" feature in the ninth column and hence facilitate the calculation of aggregate reads by gene (for more details see header of the GFF file, **dataset S4**). The resulting table of counts was imported into R (95) and processed for differential expression analysis using DESeq2 v1.26.0 (96). Plots were generated using ggplot2 (97) and karyoploteR (98). RNA-Seq analysis scripts and instructions for how to use them are provided in the Supplementary Materials (**dataset S5**) and are additionally available on Github [https://github.com/annaprotasio/Merchant_et_al_2020].

Statistics. Error bars represent the standard deviation or standard error –as indicated in the respective figure legend– of 2-7 replicates conducted across at least two independent experiments. SDS-PAGE gels and DLS data shown are representative of at least two independent experiments. Significance and *P*-values were determined by 2-way ANOVA. For DLS data, Sidak’s multiple comparisons test was used with a 95% confidence interval, in Prism 8 (GraphPad). For cell-cell fusion data, Tukey’s multiple comparisons test was used in Prism 9 (GraphPad). Source data are provided in **datasets S1 and S2**. No statistical methods were used to predetermine sample size, experiments were not randomized, and the investigators were not blinded to experimental outcomes.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The atomic coordinates were deposited in the Protein Data Bank with accession code 7A4A [<https://doi.org/10.2210/pdb7A4A/pdb>]. The cryo-EM density was deposited in the EM Data Bank with code EMD-11630 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-11630>]. The raw electron micrographs were deposited in EMPIAR with code EMPIAR-10266 [<https://doi.org/10.6019/EMPIAR-10266>]. RNA-Seq analysis scripts and instructions for how to use them are provided in the Supplementary Materials (**dataset S5**) and are additionally available on Github [https://github.com/annaprotasio/Merchant_et_al_2020].

References

1. J. A. Frank, C. Feschotte, Co-option of endogenous viral sequences for host cell function. *Curr Opin Virol* **25**, 81-89 (2017).
2. A. Katzourakis, R. J. Gifford, Endogenous viral elements in animal genomes. *PLoS genetics* **6**, e1001191 (2010).

3. M. Friedli, D. Trono, The developmental control of transposable elements and the evolution of higher species. *Annu Rev Cell Dev Biol* **31**, 429-451 (2015).
4. E. B. Chuong, N. C. Elde, C. Feschotte, Regulatory evolution of innate immunity through co-option of endogenous retroviruses. *Science* **351**, 1083-1087 (2016).
5. A. Dupressoir, C. Lavialle, T. Heidmann, From ancestral infectious retroviruses to bona fide cellular genes: role of the captured syncytins in placentation. *Placenta* **33**, 663-671 (2012).
6. R. S. Moore *et al.*, The role of the Cer1 transposon in horizontal transfer of transgenerational memory. *Cell* **184**, 4697-4712 e4618 (2021).
7. T. Gojobori, S. Yokoyama, Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and of its cellular homologues. *Proc Natl Acad Sci U S A* **82**, 4198-4201 (1985).
8. K. R. McCarthy *et al.*, The ancient fusogen EnvP(b)1 is expressed in human tissues and its structure informs the evolution of gammaretrovirus envelope proteins. *bioRxiv*, 2020.2004.2022.056234 (2020).
9. D. Kremer *et al.*, Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann Neurol* **74**, 721-732 (2013).
10. W. Li *et al.*, Human endogenous retrovirus-K contributes to motor neuron disease. *Sci Transl Med* **7**, 307ra153 (2015).
11. M. Krupovic *et al.*, Ortervirales: New Virus Order Unifying Five Families of Reverse-Transcribing Viruses. *J Virol* **92**, (2018).
12. I. G. Frame, J. F. Cutfield, R. T. Poulter, New BEL-like LTR-retrotransposons in *Fugu rubripes*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. *Gene* **263**, 219-230 (2001).
13. N. J. Bowen, J. F. McDonald, Genomic analysis of *Caenorhabditis elegans* reveals ancient families of retroviral-like elements. *Genome Res* **9**, 924-935 (1999).

14. H. S. Malik, S. Henikoff, T. H. Eickbush, Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res* **10**, 1307-1318 (2000).
15. D. Fass, S. C. Harrison, P. S. Kim, Retrovirus envelope domain at 1.7 angstrom resolution. *Nat Struct Biol* **3**, 465-469 (1996).
16. D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263-273 (1997).
17. W. Weissenhorn, A. Dessen, S. C. Harrison, J. J. Skehel, D. C. Wiley, Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426-430 (1997).
18. B. Kobe, R. J. Center, B. E. Kemp, P. Pountourios, Crystal structure of human T cell leukemia virus type 1 gp21 ectodomain crystallized as a maltose-binding protein chimera reveals structural evolution of retroviral transmembrane proteins. *Proc Natl Acad Sci U S A* **96**, 4319-4324 (1999).
19. S. C. Harrison, Viral membrane fusion. *Virology* **479-480**, 498-507 (2015).
20. M. Dessau, Y. Modis, Crystal structure of glycoprotein C from Rift Valley fever virus. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 1696-1701 (2013).
21. Y. Modis, Relating Structure to Evolution in Class II Viral Membrane Fusion Proteins. *Curr Opin Virol*, DOI:10.1016/j.coviro.2014.1001.1009 (2014).
22. J. Lescar *et al.*, The Fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. *Cell* **105**, 137-148 (2001).
23. F. A. Rey, F. X. Heinz, C. Mandl, C. Kunz, S. C. Harrison, The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291-298 (1995).
24. Y. Modis, S. Ogata, D. Clements, S. C. Harrison, A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci U S A* **100**, 6986-6991 (2003).
25. R. M. DuBois *et al.*, Functional and evolutionary insight from the crystal structure of rubella virus protein E1. *Nature* **493**, 552-556 (2013).

26. Y. Modis, S. Ogata, D. Clements, S. C. Harrison, Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313-319 (2004).
27. D. L. Gibbons *et al.*, Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature* **427**, 320-325 (2004).
28. P. Guardado-Calvo *et al.*, A glycerophospholipid-specific pocket in the RVFV class II fusion protein drives target membrane insertion. *Science* **358**, 663-667 (2017).
29. J. Perez-Vargas *et al.*, Structural basis of eukaryotic cell-cell fusion. *Cell* **157**, 407-419 (2014).
30. O. Avinoam *et al.*, Conserved eukaryotic fusogens can fuse viral envelopes to cells. *Science* **332**, 589-592 (2011).
31. A. Sapir *et al.*, AFF-1, a FOS-1-regulated fusogen, mediates fusion of the anchor cell in *C. elegans*. *Dev Cell* **12**, 683-698 (2007).
32. B. Podbilewicz *et al.*, The *C. elegans* developmental fusogen EFF-1 mediates homotypic fusion in heterologous cells and in vivo. *Dev Cell* **11**, 471-481 (2006).
33. J. Fedry *et al.*, The Ancient Gamete Fusogen HAP2 Is a Eukaryotic Class II Fusion Protein. *Cell* **168**, 904-915 e910 (2017).
34. J. Fedry *et al.*, Evolutionary diversification of the HAP2 membrane insertion motifs to drive gamete fusion across eukaryotes. *PLoS Biol* **16**, e2006357 (2018).
35. J. Feng *et al.*, Fusion surface structure, function, and dynamics of gamete fusogen HAP2. *eLife* **7**, (2018).
36. C. Valansi *et al.*, Arabidopsis HAP2/GCS1 is a gamete fusion protein homologous to somatic and viral fusogens. *J Cell Biol* **216**, 571-581 (2017).
37. T. Mori, H. Kuroiwa, T. Higashiyama, T. Kuroiwa, GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. *Nat Cell Biol* **8**, 64-71 (2006).

38. K. von Besser, A. C. Frank, M. A. Johnson, D. Preuss, Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development* **133**, 4761-4769 (2006).
39. T. Clark, HAP2/GCS1: Mounting evidence of our true biological EVE? *PLoS Biol* **16**, e3000007 (2018).
40. S. F. Altschul *et al.*, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402 (1997).
41. O. Poch, I. Sauvaget, M. Delarue, N. Tordo, Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J* **8**, 3867-3874 (1989).
42. H. Felder *et al.*, Tas, a retrotransposon from the parasitic nematode *Ascaris lumbricoides*. *Gene* **149**, 219-225 (1994).
43. M. Krupovic, E. V. Koonin, Homologous Capsid Proteins Testify to the Common Ancestry of Retroviruses, Caulimoviruses, Pseudoviruses, and Metaviruses. *J Virol* **91**, (2017).
44. I. Letunic, P. Bork, Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* **47**, W256-W259 (2019).
45. S. Halldorsson *et al.*, Structure of a phleboviral envelope glycoprotein reveals a consolidated model of membrane fusion. *Proc Natl Acad Sci U S A* **113**, 7154-7159 (2016).
46. Y. Zhu *et al.*, The Postfusion Structure of the Heartland Virus Gc Glycoprotein Supports Taxonomic Separation of the Bunyaviral Families Phenuiviridae and Hantaviridae. *J Virol* **92**, (2018).
47. D. J. Leahy, W. A. Hendrickson, I. Aukhil, H. P. Erickson, Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. *Science* **258**, 987-991 (1992).
48. J. F. Pinello *et al.*, Structure-Function Studies Link Class II Viral Fusogens with the Ancestral Gamete Fusion Protein HAP2. *Curr Biol* **27**, 651-660 (2017).

49. M. Kielian, F. A. Rey, Virus membrane-fusion proteins: more than one way to make a hairpin. *Nat. Rev. Microbiol.* **4**, 67-76 (2006).
50. L. Holm, P. Rosenstrom, Dali server: conservation mapping in 3D. *Nucleic Acids Res* **38**, W545-549 (2010).
51. D. E. Klein, J. L. Choi, S. C. Harrison, Structure of a dengue virus envelope protein late-stage fusion intermediate. *J Virol* **87**, 2287-2293 (2013).
52. S. Murakami, K. Terasaki, S. I. Ramirez, J. C. Morrill, S. Makino, Development of a novel, single-cycle replicable rift valley Fever vaccine. *PLoS neglected tropical diseases* **8**, e2746 (2014).
53. E. Zaitseva, S. T. Yang, K. Melikov, S. Pourmal, L. V. Chernomordik, Dengue virus ensures its fusion in late endosomes using compartment-specific lipids. *PLOS Pathog.* **6**, e1001131 (2010).
54. P. Y. Lozach *et al.*, Entry of bunyaviruses into mammalian cells. *Cell Host Microbe* **7**, 488-499 (2010).
55. A. M. Nour, Y. Li, J. Wolenski, Y. Modis, Viral membrane fusion and nucleocapsid delivery into the cytoplasm are distinct events in some flaviviruses. *PLoS Pathog* **9**, e1003585 (2013).
56. Y. Kubo, H. Hayashi, T. Matsuyama, H. Sato, N. Yamamoto, Retrovirus entry by endocytosis and cathepsin proteases. *Adv Virol* **2012**, 640894 (2012).
57. D. Bitto, S. Halldorsson, A. Caputo, J. T. Huiskonen, Low pH and Anionic Lipid-dependent Fusion of Uukuniemi Phlebovirus to Liposomes. *J Biol Chem* **291**, 6412-6422 (2016).
58. D. L. Esposito, J. B. Nguyen, D. C. DeWitt, E. Rhoades, Y. Modis, Physico-chemical requirements and kinetics of membrane fusion of flavivirus-like particles. *J Gen Virol* **96**, 1702-1711 (2015).
59. M. Umashankar *et al.*, Differential cholesterol binding by class II fusion proteins determines membrane fusion properties. *J Virol* **82**, 9245-9253 (2008).

60. P. K. Chatterjee, C. H. Eng, M. Kielian, Novel mutations that control the sphingolipid and cholesterol dependence of the Semliki Forest virus fusion protein. *J Virol* **76**, 12712-12722 (2002).
61. J. M. Smit, R. Bittman, J. Wilschut, Low-pH-dependent fusion of Sindbis virus with receptor-free cholesterol- and sphingolipid-containing liposomes. *J Virol* **73**, 8476-8484 (1999).
62. T. V. Kurzchalia, S. Ward, Why do worms need cholesterol? *Nat Cell Biol* **5**, 684-688 (2003).
63. M. Merris, J. Kraeft, G. S. Tint, J. Lenard, Long-term effects of sterol depletion in *C. elegans*: sterol content of synchronized wild-type and mutant populations. *J Lipid Res* **45**, 2044-2051 (2004).
64. M. Ruiz *et al.*, Membrane fluidity is regulated by the *C. elegans* transmembrane protein FLD-1 and its human homologs TLCD1/2. *eLife* **7**, (2018).
65. K. Stiasny, S. L. Allison, J. Schlich, F. X. Heinz, Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J. Virol.* **76**, 3784-3790 (2002).
66. D. L. Gibbons, A. Ahn, P. K. Chatterjee, M. Kielian, Formation and characterization of the trimeric form of the fusion protein of Semliki Forest Virus. *J Virol* **74**, 7772-7780 (2000).
67. R. Fritz, K. Stiasny, F. X. Heinz, Identification of specific histidines as pH sensors in flavivirus membrane fusion. *J Cell Biol* **183**, 353-361 (2008).
68. V. Nayak *et al.*, Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. *J. Virol.* **83**, 4338-4344 (2009).
69. Z. L. Qin, Y. Zheng, M. Kielian, Role of conserved histidine residues in the low-pH dependence of the Semliki Forest virus fusion protein. *J Virol* **83**, 4670-4677 (2009).
70. Y. Zheng, C. Sanchez-San Martin, Z. L. Qin, M. Kielian, The domain I-domain III linker plays an important role in the fusogenic conformational change of the alphavirus membrane fusion protein. *J Virol* **85**, 6334-6342 (2011).

71. S. M. de Boer *et al.*, Acid-activated structural reorganization of the Rift Valley fever virus Gc fusion protein. *J Virol* **86**, 13642-13652 (2012).
72. M. A. Vega, J. L. Strominger, Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. *Proc Natl Acad Sci U S A* **86**, 2688-2692 (1989).
73. R. DeMarco *et al.*, Saci-1, -2, and -3 and Perere, four novel retrotransposons with high transcriptional activities from the human parasite *Schistosoma mansoni*. *J Virol* **78**, 2967-2978 (2004).
74. J. P. Bernot *et al.*, Transcriptomic analysis of hookworm *Ancylostoma ceylanicum* life cycle stages reveals changes in G-protein coupled receptor diversity associated with the onset of parasitism. *Int J Parasitol* **50**, 603-610 (2020).
75. D. Moi *et al.*, Archaeal origins of gamete fusion. *bioRxiv*, 2021.2010.2013.464100 (2021).
76. R. S. Moore *et al.*, Horizontal and vertical transmission of transgenerational memories via the *Cer1* transposon. *bioRxiv*, 2020.2012.2028.424563 (2020).
77. S. Kurtz *et al.*, REPuter: the manifold applications of repeat analysis on a genomic scale. *Nucleic Acids Res* **29**, 4633-4642 (2001).
78. B. M. Muhire, A. Varsani, D. P. Martin, SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS One* **9**, e108277 (2014).
79. S. Q. Zheng *et al.*, MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* **14**, 331-332 (2017).
80. K. Zhang, Gctf: Real-time CTF determination and correction. *J Struct Biol* **193**, 1-12 (2016).
81. S. He, S. H. W. Scheres, Helical reconstruction in RELION. *J Struct Biol* **198**, 163-176 (2017).
82. S. H. Scheres, RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J Struct Biol* **180**, 519-530 (2012).

83. J. Zivanov, T. Nakane, S. H. W. Scheres, A Bayesian approach to beam-induced motion correction in cryo-EM single-particle analysis. *IUCrJ* **6**, 5-17 (2019).
84. E. F. Pettersen *et al.*, UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**, 1605-1612 (2004).
85. P. D. Adams *et al.*, PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221 (2010).
86. P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**, 2126-2132 (2004).
87. A. Amunts *et al.*, Structure of the yeast mitochondrial large ribosomal subunit. *Science* **343**, 1485-1489 (2014).
88. V. B. Chen *et al.*, MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12-21 (2010).
89. J. Schindelin *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
90. A. Teissandier, N. Servant, E. Barillot, D. Bourc'his, Tools and best practices for retrotransposon analysis using high-throughput sequencing data. *Mob DNA* **10**, 52 (2019).
91. E. M. Schwarz *et al.*, The genome and transcriptome of the zoonotic hookworm *Ancylostoma ceylanicum* identify infection-specific gene families. *Nat Genet* **47**, 416-422 (2015).
92. A. Dobin, T. R. Gingeras, Mapping RNA-seq Reads with STAR. *Curr Protoc Bioinformatics* **51**, 11.14.11-11.14.19 (2015).
93. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).
94. K. L. Howe, B. J. Bolt, M. Shafie, P. Kersey, M. Berriman, WormBase ParaSite - a comprehensive resource for helminth genomics. *Molecular and biochemical parasitology* **215**, 2-10 (2017).

95. R. C. Team. (R Foundation for Statistical Computing, Vienna, Austria, 2019).
96. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
97. H. Wickham, *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag, New York, 2016).
98. B. Gel, E. Serra, karyoploteR: an R/Bioconductor package to plot customizable genomes displaying arbitrary data. *Bioinformatics* **33**, 3088-3090 (2017).

Acknowledgements

We thank Sew-Yeu Peak-Chew at the MRC Laboratory of Molecular Biology (LMB) Mass Spectrometry Facility for assistance with mass spectrometry data collection and analysis. We acknowledge the MRC-LMB Electron Microscopy (EM) Facility and UK national electron Bio-Imaging Centre (eBIC) at Diamond Light Source (DLS) for access and support of EM sample preparation and data collection. We acknowledge DLS for access and support of eBIC under proposals EM17434 and EM20121 funded by the Wellcome Trust, MRC and BBSRC. We are grateful to Kyle Dent for aiding in using the microscopes at eBIC. We are grateful for access to the MRC-LMB Scientific Computing facilities. This work was supported by Senior Research Fellowships 101908/Z/13/Z and 217191/Z/19/Z from the Wellcome Trust and NIH grant R01 GM102869-01 to Y.M.

Author contributions

MM and YM conceived the experiments. MM purified the proteins and performed the biochemical assays. CPM collected and processed the cryo-EM data and performed the image reconstruction. CPM and YM built and refined the atomic model. YL and HZ carried out the cell-cell fusion assay. AVP performed the RNA-Seq analysis. All authors contributed to the figures. YM wrote the manuscript. Project supervision, administration, and funding acquisition, YM.

Competing interests

1025 YM is a consultant for Related Sciences LLC and has profits interests in Danger Bio LLC. The
1026 authors declare no other competing interests.

1027 **Materials & Correspondence**

1028 Correspondence and material requests should be addressed to Y.M. (ymodis@mrc-
1029 lmb.cam.ac.uk).

1030 **A bioactive phlebovirus-like envelope protein in a hookworm**
 1031 **endogenous virus**

1032 Monique Merchant, Carlos P. Mata, Yangci Liu, Haoming Zhai, Anna V. Protasio,
 1033 Yorgo Modis*

1034 *Corresponding author. Email: ymodis@mrc-lmb.cam.ac.uk

1035

1036 **Supplementary Materials**

1037 **Figures S1 to S10**

1038 **Table S1**

1039 **Movie S1**

1040 **Datasets S1 to S5**

1041

1042

Fig. S1. Atlas is an endogenous belpaovirus with a phlebovirus-like Env. (A) Nucleotide sequence of the Atlas LTRs, predicted by REPuter (76). The LTRs are 100% identical. (B) Aligned protein sequences of reverse transcriptase (RT) polymerase site from Atlas and other representative belpao- and retroviruses. *A. ceylanicum* belpaoviruses have an Asp to Asn substitution (Y[X]DD to YVDN) in the polymerase site, the most conserved RT motif (Motif V, or Motif C). GenBank accession numbers are listed after virus names. MoMLV, Moloney murine leukemia virus; HERVK, human ERV-K; *Dme*, *Drosophila melanogaster*; *Sce*, *Saccharomyces cerevisiae*; *Bmo*, *Bombyx mori*; *Cel*, *C. elegans*; *Ace*, *A. ceylanicum*; *Alu*, *Ascaris lumbricoides*; *Ncr*, *Neurospora crassa*; *Cre*, *C. reinhardtii*; *Tbr*, *Trypanosoma brucei*. (C) Sequence identity matrix for Gag protein sequences from representative belpaoviruses and *A. ceylanicum* belpaoviruses with intact protein coding regions. *Amy*, *Antheraea mylitta*; *Dsi*, *Drosophila simulans*; *Aga*, *Anopheles gambiae*; *Tru*, *Takifugu rubripes*. (D) Sequence identity matrix for phlebovirus-like Env protein sequences from nematode belpaoviruses. Identity matrices were calculated with SDT (77).

Fig. S2. Size-exclusion chromatography of Atlas G_c following ion-exchange chromatography. Samples were analysed on a Superdex 200 Increase (10/300) column (Cytiva). The $A_{260}:A_{280}$ ratios for selected peaks are shown in bold. **(A)** The Atlas G_c fragment used for cryo-EM image reconstruction (residues 2330-2772). The protein had higher absorbance at 260 nm (A_{260}) than expected for pure protein. The elution volume was consistent with a homotrimer. **(B)** Atlas G_c(DI-III), maintained at pH > 7 throughout expression and purification. Multiple oligomeric states were present including peaks with elution volumes consistent with monomers, trimers, and higher order oligomers. **(C)** Atlas G_c fusion loop mutant F136A/F137A. **(D)** Atlas G_c GPL binding pocket mutant R86A.

Fig. S3. Image reconstruction quality assessment and workflow. (A) Fourier Shell Correlations (FSC) of the reconstruction of Atlas Gc from two independently refined half-maps (map2map, in blue); and of the reconstruction from the whole dataset versus a map calculated from the refined atomic model (model2map, in red). The gold-standard cutoff (FSC = 0.143) and the FSC = 0.5 level are marked with dashed lines. The resolution values of each curve at these levels are indicated. (B) FSC plots for cross-validation as described by Amunts *et al.* (86). FSCwork (magenta), FSC of refined test model versus work set (half-map used in test refinement). FSCfree (green), FSC of refined test model versus test set (half-map not used in test refinement). The FSC = 0.5 level is indicated by a dashed line. (C) Flow chart showing the workflow pipeline for cryo-EM image processing, classification, and model refinement, as described in the Materials and Methods.

Fig. S4. Representative Cryo-EM micrograph and density with local resolution estimation
 (A) Representative cryo-EM micrograph of Atlas G_C trimers. (B) Local resolution estimation for the cryo-EM volume, calculated in RELION 3.0 (82). (C) Representative samples of local cryo-EM density from the structure with fitted and refined atomic models. The deposited density map was contoured at 3 σ in Pymol (Schrodinger, LLC).

Fig. S5. Protein sequence alignment of Atlas G_c and its closest orthologs
Amino acid sequence alignment of Atlas G_c and the most similar sequences from infectious viruses: Rift Valley Fever virus (RVFV), Dabie bandavirus (DABV, formerly SFTSV phlebovirus) and Heartland virus (HRTV). Domains and structural features are marked with lines above the alignment. GPL, residues in or near the glycerophospholipid headgroup binding pocket. N-glyc., N-glycosylation site. Cyt. tail, cytoplasmic tail. Conserved disulfide bonds are numbered in green. Disulfide bonds specific to Atlas virus are denoted with an "A". An RVFV-specific disulfide is denoted with an "R". A phlebovirus-specific disulfide is denoted with a "P". The disulfide marked 11' is conserved in Atlas, DABV and HRTV but not RVFV.

Fig. S6. Cryo-EM density for N-linked glycan and GPL pocket of Atlas G_c. (A) Cryo-EM density and atomic model of the N-linked glycan at Asn414. The deposited density map was contoured at 2.2 σ in Pymol (Schrodinger, LLC). (B) Cryo-EM density and atomic model for the fusion loop region of the Atlas G_c trimer. A B-factor blurring correction of +150 $\text{\AA}^2$ was applied to the deposited density map. The resulting map was contoured at 1 σ in Pymol. (C) Cryo-EM density in the glycerophospholipid (GPL) headgroup binding pocket unaccounted for by the atomic model. Shown in grey stick representation is the phosphatidylcholine (PC) headgroup bound to RVFV G_c structure (28) superimposed on the Atlas G_c structure. A B-factor blurring correction of +163 $\text{\AA}^2$ was applied to the deposited density map. The resulting map was contoured at 1.2 σ in Pymol.

Fig. S7. Dynamic light scattering of Atlas G_c-liposome complexes. Liposome diameter dynamic light scattering (DLS) data from **Fig. 4B** are shown as the mean change in liposome diameter upon binding Atlas G_c ectodomain (WT, F136A/F137A or R86A). Error bars show the standard error of the mean (s.e.m.) of three to seven replicates (see **dataset S1** for source data). Significance was determined by 2-way ANOVA analysis using Sidak's multiple comparisons test with a 95% confidence interval, in Prism 8 (GraphPad). **, 0.001 < p < 0.01; *, p < 0.05; ns, not significant.

Fig. S8. Uncropped gels. Gels shown for all liposome flotation replicates (Fig. 4A), along with immunoblot from Fig. 6D.

Fig. S9. Cell-cell fusion assay with CHO cells. (A) Representative confocal micrographs of CHO cells transfected with plasmid encoding Atlas G_C, Atlas G_N-G_C, VSV G, or no protein (pHLsec empty vector). Cells were treated with pH 4.5 buffer containing BMP, or pH 7.4 buffer without exogenous lipid. Blue, Hoechst 33342 nuclear stain; Magenta, CellBrite Red plasma membrane dye. (B) Permeabilized and fixed cells transfected with plasmid encoding G_C stained with anti-HA antibody, to detect the C-terminal HA tag on G_C. Blue, Hoechst 33342; green, anti-HA antibody. The boxed region is also shown in Fig. 6B and Movie S1. (C) Left, confocal micrograph of cells transfected with pHLsec plasmid stained with Hoechst 33342. Right, masks from cluster analysis of the Hoechst channel with Fiji (88). All scale bars are 10 μ m. (D) Effect of cell cycle inhibitor CDK4 on the fraction of di- or multinucleated cells. Cells were transfected with a plasmid encoding eGFP (pEGFP-N1) and incubated with 10 μ M CDK4 (right) or with PBS (left) for 6 h at pH 7.4. *n*, number of nuclei counted. See dataset S2 for source data.

Fig. S10. RNA-Seq analysis of intact *A. ceylanicum* belpaovirus EVEs. (A) Box plots of RNA-Seq expression in Reads Per Kilobase of transcript per Million mapped reads (RPKM) for all annotated genes, and for nine intact *A. ceylanicum* belpaovirus EVEs including Atlas virus, at the L3, L4 and adult (male and mixed sex) developmental stages. RNA-Seq data are from Bernot *et al.* (71). (B) Integrative Genomics Viewer snapshot showing the distribution of raw RNA-Seq reads over the Atlas virus (*Acey_s0020.g108*) coding sequence in mixed sex adult worms and L4 larvae with individual unmerged BAM files.

Table S1. Cryo-EM data collection, structure determination, model building and refinement parameters and statistics.

Atlas Gc	
Data Collection and Processing	
Microscope	FEI Titan Krios
Voltage (kV)	300
Electron exposure (electrons Å ⁻²)	46.12
Exposure per frame (electrons Å ⁻²)	1.28
Defocus range (μm)	-1.3 to -3.5
Pixel size (Å)	1.047
N. initial particles	987,570
N. final particles	197,145
Map resolution (Å)	3.76
Map resolution range (Å)	3.50 - 5.20
FSC threshold for resolution limit	0.143
Model refinement	
Map sharpening B factor (Å ²)	-180
Symmetry imposed	C3
Mask correlation coefficient	0.78
Model composition	
N. non-hydrogen atoms	10,159
Protein residues (chains A, B, C)	1,320
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.694
Planarity (Å)	0.004
B-factors/ADPs*	
Minimum	35
Maximum	176
Mean	82
Validation (Phenix 1.15)	
MolProbity overall score	2.23
MolProbity all-atom clashscore	5.86
Rotamer outliers (%)	2.28
Ramachandran plot	
% favored	86.2
% allowed	13.8
% outliers	0.0
PDB code	7A4A
EMDB code	EMD-11630
EMPIAR code	10266

ADPs, atomic displacement parameters.

Movie S1. Confocal Z-stack of the multinucleated cell shown in Fig. 6B (separate file).

CHO cell expressing Atlas G_C following treatment with pH 4.5 and BMP. Blue, Hoechst 33342; magenta, CellBrite Red; green, a-HA antibody for Atlas G_C detection. Scale bar, 10 μ m.

Dataset S1. (Separate file)

Source data file for Fig. 4 – liposome binding assays.

Dataset S2. (Separate file)

Source data file for Fig. 6 and Fig. S9 – cell-cell fusion assays.

Dataset S3. (Separate file)

Source data file for Fig. 7 – differential RNA-Seq expression calculations.

Dataset S4. (Separate file)

Source data file for Fig. 7 – original RNA-Seq annotation (GFF) file.

Dataset S5. (Separate file)

RNA-Seq analysis scripts and instructions (also available on Github [https://github.com/annaprotasio/Merchant_et_al_2020]).