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REVIEW

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Mechanisms of bunyavirus morphogenesis and egress

Jake Barker[1], Luis L. P. daSilva[2] and Colin M. Crump[1,*]

Abstract

Unlike many segmented negative-sense RNA viruses, most members of the *Bunyavirales* bud at Golgi membranes, as opposed to the plasma membrane. Central players in this assembly process are the envelope glycoproteins, Gn and Gc, which upon translation undergo proteolytic processing, glycosylation and trafficking to the Golgi, where they interact with ribonucleoprotein genome segments and bud into Golgi-derived compartments. The processes involved in genome packaging during virion assembly can lead to the generation of reassorted viruses, if a cell is co-infected with two different bunyaviruses, due to mismatching of viral genome segment packaging. This can lead to viruses with high pathogenic potential, as demonstrated by the emergence of Schmallenberg virus. This review focuses on the assembly pathways of tri-segmented bunyaviruses, highlighting some areas in need of further research to understand these important pathogens with zoonotic potential.

INTRODUCTION

Bunyavirales are an order of enveloped, negative-sense, single-stranded RNA [(−)ssRNA] viruses that contain predominantly tri-partite genomes (Fig. 1a). The taxonomic classifications for bunyaviruses have recently been updated, with the formation of the order *Bunyavirales* [1]. This reflects the enormous genetic and biological diversity of bunyaviruses and the related families that make up this new taxonomic order. *Bunyavirales* is currently subdivided into 14 virus families, including *Peribunyaviridae*, *Phenuiviridae*, *Nairoviridae*, *Hantaviridae*, *Tospoviridae* and *Arenaviridae*, of which all but the arenaviruses are thought to assemble virions at intracellular compartments. Intracellularly assembling bunyaviruses will form the focus of this review.

While the basic mechanisms for assembly appear conserved, there are undoubtedly differences between the virus families. These virus families are all composed of a small (S), medium (M) and large (L) genome segment. In general, the S segment encodes the nucleocapsid (N) protein, which wraps the RNA to form ribonucleoproteins (RNPs), the M segment encodes the glycoproteins Gn and Gc, and the L segment encodes the RNA-dependent RNA polymerase (RdRp). Along with this core set of proteins, some of these families of viruses also encode non-structural proteins from the S segment (NSs) and the M segment (NSm) (Fig. 1b) [2].

Most *Bunyavirales* are thought to assemble their virions by envelopment of genome segments at Golgi membranes, driven by interactions between the genome segments and the viral glycoprotein complex. The glycoprotein complex is a heterodimeric protein complex composed of the two individual glycoproteins, Gn and Gc. These glycoproteins are synthesized as an M-segment-derived polyprotein precursor (M polyprotein) at the endoplasmic reticulum (ER) and then proteolytically processed, glycosylated and assembled into heterodimers. Following this, the Gn–Gc heterodimer traffics to the Golgi, where interactions with the RNA genome segments and membrane budding occurs. This budding process produces virions within the lumen of Golgi-derived vesicles, which are then secreted from the cell, although the pathway by which virions are secreted is unclear (Fig. 2).

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Author affiliations: [1]Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK; [2]Departamento de Biologia Celular e Molecular, Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, University of São Paulo, State of São Paulo, Brazil.

***Correspondence:** Colin M. Crump, cmc56@cam.ac.uk

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Abbreviations: BUNV, Bunyamwera virus; CCHFV, Crimean-Congo haemorrhagic fever virus; ER, Endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartments; ESCRT, Endosomal sorting complex required for transport; FISH, Fluorescent in-situe hybridization; L, Large segment; LCV, La Crosse virus; Lrp1, Lipoprotein receptor-related protein 1; M, Medium segment; MLD, Mucin-like domain; N, Nucleocapsid; NSm, Non-structural protein medium segment; NSs, Non-structural protein small segment; OROV, Oropouche virus; p.f.u, Plaque formig units; PTV, Punta Toro virus; RdRp, RNA-dependent RNA polymerase; RNP, Ribonucleoprotein; RVFV, Rift Valley fever virus; S, Small segment; SBV, Schmallenberg virus; SFTSV, Sever fever with thrombocytopenia syndrome virus; SKI-1, Subtilisin kexin/isozyme-1; SNV, Sin Nombre virus; SP, Signal peptide; SPase, Signal peptidase; SPPase, Signal peptide peptidase; SSHV, Snowshoe hare virus; TMD, Trans-membrane domains; UTR, Untranslated region; UUKV, Uukuniemi virus; VLP, Virus-like particles.

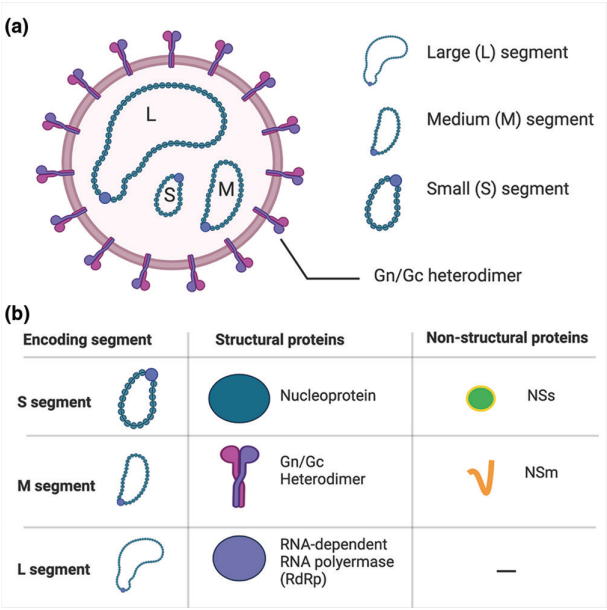

Fig. 1. Genome structure of a typical bunyavirus. Typical structure of a bunyavirus particle, with three nucleoprotein wrapped negative-sense, single-stranded RNA segments, capped by the RdRp. The virion contains three segments, small (S), medium (M) and large (L), and is coated with the Gn–Gc heterodimers (a). Each of the segments encodes its own set of proteins, with the core structural proteins common to all tri-segmented bunyaviruses being the nucleoprotein (N; encoded for by the S segment), Gn–Gc heterodimer (encoded for by the M segment) and the RdRp (encoded for by the L segment) (b). Non-structural proteins NSm and NSs are expressed by several bunyaviruses and are encoded by the M and S segments, respectively. Created in BioRender.com.

Having segmented genomes, co-infection of cells with two different bunyaviruses can lead to the generation of reassortant viruses, with recent sequencing evidence supporting the hypothesis that reassortment is a major factor driving bunyavirus genetic variability [3]. This can lead to the generation of new highly pathogenic viruses, such as has been described for Schmallenberg virus (SBV) [4], which is a reassortment between Sathuperi and Shimonda viruses [5], and also Ngari virus, which is a reassortment between Bunyamwera virus (BUNV) and Batai virus [6]. While there are many factors that can influence the chances of reassortment occurring, the molecular mechanisms behind how bunyaviruses assemble their virions may influence reassortment potential.

Members of the *Bunyavirales* cause a wide variety of zoonotic infections in humans, with clinical manifestations ranging from self-limiting symptoms such as fever, myalgia and arthralgia, as described for Oropouche virus (OROV) [7], to more debilitating symptoms such as haemorrhagic fever [Crimean–Congo haemorrhagic fever virus (CCHFV)] [8], acute pneumonia [Sin Nombre Virus (SNV)] [9] and fatal encephalitis [La Crosse Virus (LACV)] [10]. Transmission to humans primarily occurs through either arthropod vectors [arthropod-borne (arbo) viruses, e.g. peribunyaviruses] or rodents (e.g. hantaviruses). Climate change and urbanization are becoming increasingly responsible for the emergence and re-emergence of several members of the *Bunyavirales* [11].

This review provides an overview of the intracellular assembly and egress pathways for bunyaviruses, following recent advances that have furthered our understanding of bunyavirus morphogenesis.

GLYCOPROTEIN PROCESSING

The bunyavirus glycoproteins, Gn and Gc, are two of the three main structural protein components of bunyavirus particles (the other being the nucleocapsid protein). Gn and Gc are translated as an M polyprotein, which is subsequently cleaved in the ER membrane by cellular proteases to liberate the individual nascent glycoproteins [12]. The sizes and coding strategies of the polyproteins for each family of viruses differ considerably [10], and as a result, the proteolytic cleavage events can differ between virus families [13]. However, all appear to have a signal peptide (SP) at the M polyprotein N-termini, which is co-translationally cleaved by signal peptidase (SPase). One of the first experimental reports investigating M polyprotein cleavage came from the orthobunyavirus snowshoe hare virus (SSHV), whereupon a cleavage site was mapped to residue R299, predicted to be the final residue in Gn [14]. The authors suggested that cleavage at this residue was likely due to an enzyme with specificity for basic residues.

Further work indicated that M polyprotein cleavage for several members of the orthobunyaviruses, including SSHV, occurred after an R-(V/A)-A-R motif, conforming to the minimal consensus sequence cleavage site for the proprotein convertase enzyme furin. An R-(V/A)-A-R motif is found across members of the orthobunyaviruses [15, 16], and it was thought up until recently that furin

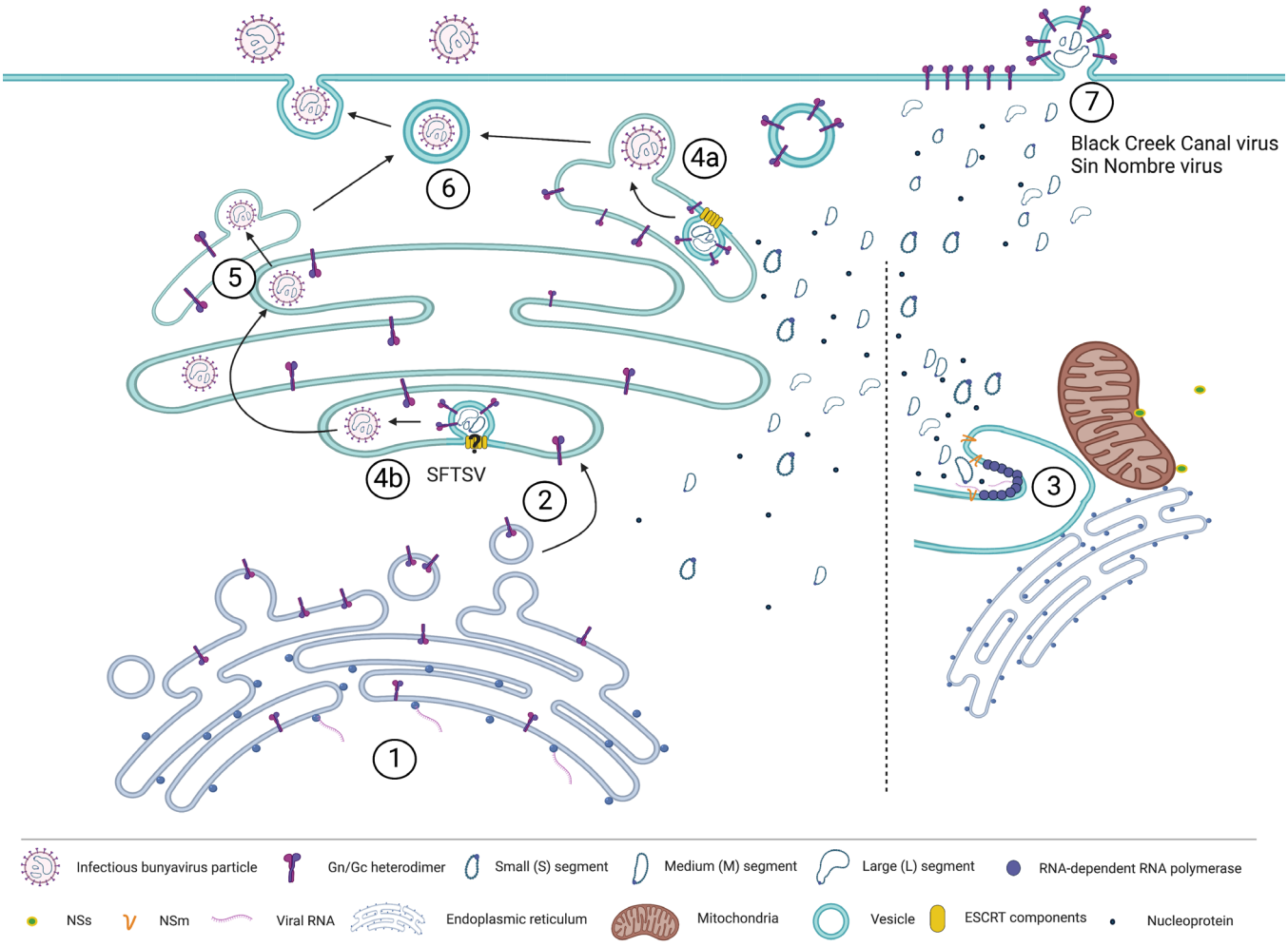

Fig. 2. Overview of bunyavirus assembly and egress. Bunyavirus assembly begins at the endoplasmic reticulum (ER), whereby the M polyprotein is translated, co-translationally cleaved into constituent glycoproteins Gn and Gc, and heterodimerizes to generate the Gn–Gc complex (1). Following this, the glycoprotein complexes are transported from the ER to the ERGIC/*cis*-Golgi, by what is assumed to be COP-II mediated transport, and will undergo further maturation as they transit through the Golgi (2). Nucleoprotein-wrapped RNA genome segments are produced and assembled in virus factories, which utilize Golgi membranes, mitochondria and the rough-ER; these virus factories are dependent on actin, and the virus NSm protein (3). The genome segments then assemble at membranes containing Gn–Gc heterodimers, assumed to be the *trans*-Golgi stacks for most bunyaviruses (4a), but may also occur at the ERGIC for some bunyaviruses such as SFTSV (4b). Budding of the virus particle into the Golgi lumen then occurs, a process shown to require ESCRT complexes for at least some bunyaviruses (4a). Virus particles that bud at ERGIC or *cis*-Golgi membranes then need to transit through the Golgi stack to reach the *trans*-Golgi cisternae (5). Once in the *trans*-Golgi, viruses are then secreted via the exocytic pathway (6). For some hantaviruses, such as Black Creek Canal virus and Sin Nombre virus, assembly is thought to occur directly at the plasma membrane via an uncharacterized mechanism (7). Created in BioRender.com.

was responsible for M polyprotein processing in these viruses. However, the fact that active furin is only found in *trans*-Golgi and post-Golgi compartments, along with the lack of R-(V/A)-A-R motif in some of the members of this genus (e.g. SBV and OROV) suggests that it is unlikely that furin or furin-like proteases are responsible for M polyprotein cleavage in the ER [17].

Recent work with BUNV demonstrated that M polyprotein cleavage is more likely due to the activity of SPase, wherein Gn can be liberated by cleavage between G331 and T332 and Gc can be liberated by cleavage between A477 and E478, with NSm consisting of T332-A447 (Fig. 3) [17]. After the cleavage event between G331 and T332, two transmembrane domains (TMDs) remain downstream of the Gn extracellular domain. Further data suggested that an additional cleavage event by signal peptide peptidase (SPPase) removes the terminal TMD resulting in Gn with a single TMD and a free cytoplasmic tail, although the precise location of this cleavage could not be confirmed [17].

SPPase is an ER resident protease that is normally responsible for intramembrane cleavage of some signal peptides after they have been cleaved by signal peptidase [18]. Interestingly, recent work on OROV suggests that only a single cleavage event is

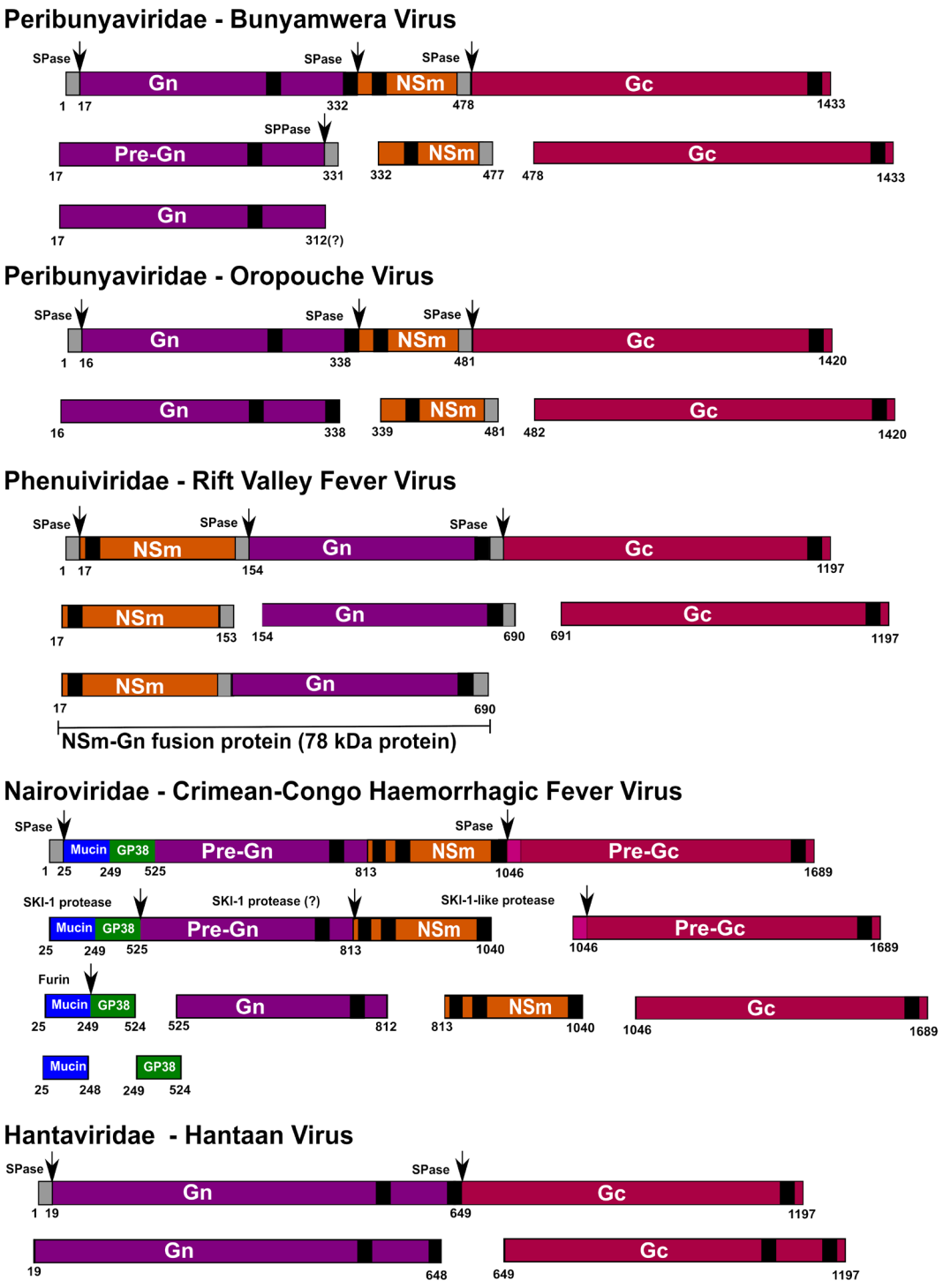

Fig. 3. Processing of M polyproteins for representative viruses of select bunyavirus families. Glycoprotein processing occurs co-translationally, with the first step for all viruses to remove the signal sequence via signal peptidase (SPase). Peribunyaviruses have two additional cleavages with SPase to liberate Gn and Gc from NSm, with Gn requiring a further cleavage with signal peptide peptidase (SPPase) to generate nascent Gn for Bunyamwera virus. Oropouche virus, however, only requires a single cleavage to liberate Gn. Phenuiviruses only utilize SPase for glycoprotein processing, but sometimes there is an NSm–Gn fusion protein (78 kDa protein/LGp) that is only incorporated into RVFV in insect cells. Nairoviruses have more complex glycoprotein processing, requiring multiple proteases. SPase liberates pre-Gc from Pre-Gn, before a series of SKI-1 and SKI-1-like proteases liberate nascent glycoproteins from the polyprotein precursor. Furin cleaves the mucin domain and GP38 domain from each other, although neither of these proteins are involved in assembly. Since hantaviruses do not encode NSm, they only require a single cleavage (following signal sequence cleavage) to liberate Gn from Gc. Black segments represent *trans*-membrane domains, grey segments represent the presence of a signal sequence and (?) indicates that the precise location/protease is not known, although the most likely is suggested. Schematics not to scale.

required to liberate Gn from NSm, resulting in Gn with two predicted TMDs and a cytosolic loop [19]. Phleboviruses also utilize a similar mechanism of glycoprotein processing, whereby SPase has been described to cleave the M polyprotein into nascent Gn and Gc [20]. This report also suggests that the Gn–Gc heterodimer can form prior to cleavage in the ER. For nairoviruses, with specific reference to CCHFV, the tetrapeptides RRLL and RKPL are located directly upstream of the cleavage sites for both Gn and Gc, respectively. These tetrapeptides are conserved amongst CCHFV strains, as well as having a high degree of similarity to motifs present in Lassa fever virus, an arenavirus [21]. This led to the hypothesis that CCHFV (and the wider nairovirus family) M polyprotein is cleaved by subtilisin kexin/isozyme-1 (SKI-1) proteases, whose consensus cleavage motif is (R/K)-(Xaa)n-R, where Xaa is any amino acid other than cysteine (n=0,2,4,6) [22]. Experimental analysis demonstrated that Gn processing was dependent on SKI-1, whereas Gc processing was not, being dependent instead upon SPase [23, 24]. To generate mature nairovirus Gn proteins, there needs to be a second cleavage site at a currently undetermined location in the C-terminus of Gn. As well as encoding for the canonical glycoproteins Gn and Gc, CCHFV also encodes for two further accessory proteins, GP38 and GP38 linked to a mucin-like domain (MLD-GP38/GP85) (Fig. 3). A study into the role of CCHFV accessory proteins during infection revealed functions for both GP38 and MLD-GP38 in virus assembly and infectivity. Deletion of the MLD does not impair the infectivity of CCHFV, but does reduce the incorporation of Gn and Gc into the viral envelope by 60% [25]. Additionally, deletion of GP38 abolished the production of infectious VLPs in a mini-genome system [25]. The importance of GP38 during viral infection is also further highlighted because GP38-targeting non-neutralizing antibodies protected against lethal CCHFV infection in mice [26]. Hantavirus glycoprotein processing is achieved via cleavage at a conserved pentapeptide, WAASA, by SPase [27]. Since hantaviruses do not encode an NSm protein (Fig. 3), only a single cleavage event is needed to liberate Gn and Gc into their constituent glycoproteins, with mature Gn predicted to have two TMs and a cytoplasmic loop, similar to OROV.

GLYCOPROTEIN MATURATION

Following processing of the M polyprotein into Gn and Gc, both individual glycoproteins must undergo structural maturation to traffic correctly to the Golgi. These maturation events typically involve post-translational modifications in the form of N-linked glycosylation events that ensure the Gn–Gc heterodimer is competent for Golgi trafficking.

N-linked glycosylation motifs mapped to Gn and Gc glycoproteins have been described for many members of the *Bunyavirales* [28–33]. Orthobunyaviruses contain two conserved N-linked glycosylation motifs (N-X-S/T), one on each glycoprotein; residue N60 on Gn, and residue N1169 on Gc, as described for BUNV. Due to the conservation of these residues, it was suggested they both play an essential function. However, only mutation of N60 resulted in any impairments in virus production, due to misfolding of both Gn and Gc along with failure for either protein to efficiently traffic to the Golgi [28]. The authors suggest the N60 glycan of Gn could act as a bridge/linker to promote formation of the Gn–Gc heterodimer, as Gc glycan mutants did not impair trafficking or folding [28]. Similar results were observed for CCHFV, whereby a Gn N557K mutant did not seem to affect Gn processing, but did result in mislocalization of Gn and Gc in the ER [29]. Rift Valley fever virus (RVFV) Gn also has predicted N-linked glycosylation motifs, although they have not been observed in the crystal structure of RVFV Gn [33]. Four N-linked glycosylation sites are conserved across the hantaviruses*:* N134, N347, N399 of Gn and N928 of Gc. However, only mutation of N134 within Gn was found to inhibit glycoprotein trafficking to the Golgi [31].

Although disruption of N-linked glycans on Gn can affect glycoprotein trafficking and folding, which indirectly affects virus assembly, it seems unlikely that glycan chains will have a direct role in virus assembly. Most of the literature suggests that N-linked glycans are primarily involved in receptor binding and entry. For example, high-mannose N-glycans facilitate entry via DC-SIGN for phleboviruses [34, 35], and similar work has suggested that tospoviruses [32, 36], and BUNV [28], utilize N-linked glycans for receptor binding. Despite this, recent experimental data for RVFV suggest that Gn glycosylation is dispensable for RVFV binding to its entry receptor lipoprotein receptor-related protein 1 (Lrp1) [37]. Lrp1 has also been identified as the entry receptor for OROV, although no investigations into the glycosylation state of the OROV Gn have been made [38]. These conflicting reports on bunyavirus glycoprotein glycosylation suggest that we only have a rudimentary understanding of the function of viral envelope protein glycosylation in bunyavirus infection.

GLYCOPROTEIN TRAFFICKING TO THE GOLGI

As previously mentioned, assembly of infectious bunyavirus particles occur at the Golgi membranes, and thus Gn–Gc heterodimers must be able to localize correctly there. The mechanisms by which this is achieved differ throughout the families in this order. Following the structural maturation of the Gn and Gc glycoproteins, they must dimerize and subsequently traffic to the Golgi, whereby they can encapsidate N-wrapped RNA segments to produce infectious virions. Interestingly, when Gn and Gc are expressed individually, they exhibit different sub-cellular localizations. For at least some orthobunyaviruses [39], phleboviruses [40], nairoviruses [41] and tospoviruses [42], Gc is retained in the endoplasmic reticulum, whereas Gn traffics to the Golgi when expressed individually. Only when they are both expressed together does Gc traffic to the Golgi for the studied viruses of these families, presumably in the form of Gn–Gc heterodimers. Phleboviruses have been described as containing a putative lysine-based ER-retrieval motif in the short cytoplasmic tail of Gc [43]. There is also a conserved lysine residue at position −3 relative to the Gc C-terminus across phlebo-, hanta- and

orthobunyaviruses. For some phleboviruses, this lysine is part of a KKXX ER-retrieval motif [43, 44]. Despite this, Punta Toro virus (PTV; *Phlebovirus*) Gc traffics to the plasma membrane irrespective of this conserved −3 lysine [45]. Tospoviruses and nairoviruses do not share this lysine at position −3 in Gc, suggesting that the mechanisms for ER retention are different for each virus family. Golgi localization motifs on Gn have been described in either the cytoplasmic tail (UUKV; *Phlebovirus*), the transmembrane domain (TMD) (BUNV; *Orthobunyavirus*), or both (PTV and RVFV; *Phleboviruses*) [40, 46–48].

As described above, most bunyaviruses appear to utilize a mechanism whereby Gn traffics to the Golgi on its own, and Gc is retained in the ER, and only when expressed together do they both traffic to the Golgi. For Puumala virus (*Hantavirus*), it is the reverse, when expressed individually Gc traffics to the Golgi and Gn is retained in the ER, and only when expressed together does the Gn–Gc heterodimer traffic to the Golgi [49]. For Hantaan virus (*Hantavirus*), Andes virus and SNV (*Nairovirusus*), and OROV (*Orthobunyavirus*), neither Gn nor Gc traffic to the Golgi when expressed individually but do so when expressed together [19, 50, 51]. Given the diversity between viruses, this suggests that structural conformation of the Gn–Gc heterodimer, rather than primary amino acid sequence motifs, are responsible for correct trafficking to the Golgi [50]. The basis for how this structural change is induced, along with the residues involved, are poorly understood on both a molecular and structural level for Gn and Gc. However, under normal cellular conditions trafficking to the Golgi from the ER requires multiple quality control checkpoints (reviewed in [52, 53]). An understanding of this process for bunyavirus Gn–Gc complexes will provide insight into the differences in glycoprotein trafficking, even within the same virus families.

GENOME PACKAGING

In general, unlike most (−)ssRNA viruses, members of the *Bunyavirales* do not express a matrix protein, with the exception of arenaviruses [54]. Matrix proteins typically act as an anchor between envelope glycoproteins and RNP segments to generate infectious virions. As a consequence of this, it has been proposed that heterodimerization of Gn–Gc promotes interactions between the cytoplasmic domains of Gn and Gc, with the resulting structure acting as a matrix surrogate (reviewed in [55]). This hypothesis has been tested in UUKV [56], whereby the use of a virus-like particle (VLP) system was employed to investigate interactions between RNPs and the viral glycoproteins. This UUKV VLP system was used to demonstrate that packaging of genome segments is dependent on the cytoplasmic tail of Gn, whereby residues M76/L79/T80/R81 are all essential for this interaction to occur [57]. The authors also demonstrate that no segment-specific interactions occur between the segments and Gn cytoplasmic tail, i.e. there was no preference over which segments were packaged. Similarly to UUKV, important contributions for the glycoprotein cytoplasmic domains were reported for BUNV, with comprehensive mutational analysis of the cytoplasmic tails of both Gn and Gc identifying specific protein sequences required for glycoprotein maturation and virion assembly [58]. While interactions of RNPs with the cytoplasmic domains of bunyavirus glycoproteins are important for incorporation of genome into virions, whether there is any requirement for RNPs as a structural component to form virions is less clear. Many VLP systems developed for bunyaviruses include minigenome reporter constructs, e.g. expressing *Renilla* luciferase, and the resulting reporter gene RNPs may facilitate VLP formation [58, 59]. However, there is also evidence that expression of Gn and Gc in the absence of other viral proteins leads to the release of mature glycoproteins and VLPs for at least some bunyaviruses, including UUKV, OROV and hantaviruses [19, 56, 60]. Whether such 'empty shell' VLPs non-specifically incorporate cellular RNA or proteins is unknown.

In order to package RNP segments into infectious virions, interactions must occur between the RNPs and the cytoplasmic tail of the glycoproteins [58, 61]. However, this packaging of RNPs seems to be a non-segment-specific process, as the generation of a recombinant BUNV L segment flanked by BUNV M segment untranslated regions (UTRs) (MLM segment) is packaged into virions [62]. The BUNV MLM virus did exhibit up to a 1000-fold growth defect in virus titre. However, it is unclear whether this was due to effects on virus genome packaging, genome replication, or perhaps both. To date, the genome packaging process has been best characterized for the phlebovirus RVFV. S and M segment RNP complexes are able to package into RVFV virions independently of other genome segments, but L segment RNP only packages in cells in which both S and M segment replication and translation occurs [63]. Two models of RVFV genome segments packaging were proposed: (i) M segment RNA acts as a central segment, recruiting S and L segments for packaging, or (ii) co-ordinated interactions between S and M segments induce conformational changes in the S segment RNA that promotes interaction with L segment RNA [63]. At around the same time, it was also observed that genomic RNA bound to Gn was required for efficient release of the virus, and packaging of genome segments was considered essential for efficient release of RVFV particles from the cell [64]. As well as packaging encapsidated genomic RNP segments, analysis of purified RVFV particles demonstrated the presence of anti-genomic RNA segments of all three segments, with the ambisense S segment providing the template for synthesis of the NSs mRNA [65]. This was later observed by an independent group, whereby NSs transcription was from an incoming anti-genomic S segment [66]. More recent work has further characterized that there is actually a preference for the packaging of this anti-genomic S segments into virion particles [67].

A recent review into *Bunyavirales* genome packaging suggests that viruses in this family support a stochastic process for packaging of genome segments, which has been best characterized for RVFV [61]. This hypothesis is further supported by the generation of a two-segmented RVFV, whereby the NSs protein coding sequence of RVFV S segment was replaced by full-length M polyprotein coding sequence, which was genetically stable following several passages in cell culture [68]. A four-segment RVFV was also generated,

whereby the M polyprotein coding sequence was separated so that two M segments each encode a single glycoprotein (Gn or Gc), and this four-segment virus could be maintained over 10 passages in mammalian cells, as well as causing disease in a mouse model [69]. This technology has now been translated into a potential live-attenuated vaccine strategy for RVFV, showing safety and efficacy in young sheep, cattle and goats [70], as well as preventing vertical transmission of RVFV in pregnant ewes [71]. The authors have further characterized the safety and efficacy of this type of vaccination strategy in primates [72]. The result of these studies, therefore, suggests that a four-segment RVFV may provide a safe and effective strategy for RVFV vaccination in humans.

Single-molecule fluorescent *in situ* hybridization (FISH) also demonstrates that RVFV exhibits predominantly non-segment-selective, stochastic genome packing, as genome segments do not co-localize in the cell, but suggests that this may not be true for all bunyaviruses [73]. The result is a heterogenous population of viruses that contain differing amounts and types of genome segments, with only virions containing a full complement of genome segments (or co-infection of cells with a full complement of genome segments) able to cause productive infection.

Although evidence so far suggests that members of the *Bunyavirales* exhibit stochastic genome packaging, a low particle-to-plaque forming units (p.f.u.) ratio of 2.6–7.2 for BUNV, which is increased by 100-fold for the MLM mutant [62], suggests some form of selectivity in genome packaging may occur. Completely stochastic genome packaging would be expected to have higher particle-to-p.f.u. ratios that would reflect the low probability of fully incorporating all three essential genome segments into each virion. These observations perhaps raise more questions than answers. Specifically, what are the molecular details that allow some members of the *Bunyavirales* to self-assemble into VLPs and some to seemingly not? And how does this lead to the events of reassortment between different bunyaviruses? For the majority of bunyaviruses, the particle-to-p.f.u. ratios are not known, and neither has it been investigated whether Gn–Gc heterodimers can self-assemble into VLPs in the absence of genome and N. A possible model could be that those viruses that have Gn–Gc heterodimers that can self-assemble into VLPs independently of genomic RNPs exhibit a stochastic mechanism of genome packaging along with higher particle-to-p.f.u. ratios (e.g. RVFV), whereas other viruses that may not be able to efficiently self-assemble in the absence of genomic RNPs exhibit lower particle-to--p.f.u. ratios, and perhaps employ a more selective form of genome packaging (e.g. BUNV).

Little is known about how newly synthesized genomes segments are recruited to the site of virus budding. Using electron microscopy, BUNV replication factories were observed as tubular structures that are associated with Golgi stacks, rough ER and mitochondria [74]. Both actin and NSm proteins are required for the formation of these replication complexes, with NSm hypothesized to play a role in trafficking RNP segments to the cytoplasmic tails of the Gn–Gc heterodimer. These virus factories are proposed to be essential for virus assembly and morphogenesis [74], with mutations in the N-terminal region of NSm found to impair virus assembly [75]. These virus factories have not been defined for other families of the *Bunyavirales* to date. In insect cells, however, these virus factories seem less extensive, and are instead found in peripheral Golgi stacks [76].

VIRUS BUDDING

Bunyaviruses are generally thought to bud at Golgi membranes, but there have been two reports of new world hantaviruses that bud at the plasma membrane; Black Creek Canal virus [77] and SNV [78]. Some bunyaviruses are also predicted to contain putative late domain motifs in their glycoproteins. Late domains were first described in retroviruses as being involved in virus budding through the recruitment of the endosomal sorting complex required for transport (ESCRT) machinery [79, 80]. These observations suggest that at least some bunyaviruses may also use the ESCRT machinery during virion assembly/budding. ESCRTs are a large class of proteins that predominantly function in membrane remodelling, in particular for budding topology away from the cytoplasm into the lumen of intracellular organelles (e.g. multi-vesicular bodies) or the extracellular environment. As a result, they can be hijacked by many viruses to aid in membrane budding and virus replication (reviewed in [81]). A role for ESCRT proteins in bunyavirus assembly has been demonstrated for OROV, whereby ESCRT machinery components were recruited to Golgi compartments to facilitate membrane remodelling [82], as well as for arenaviruses [54]. The importance of ESCRT function for OROV infectious particle production was shown using cells depleted of the late domain-binding ESCRT components Tsg101 or Alix that caused significantly reduced amounts of infectious particles to be released [82]. Consistently, a recent study identified that the OROV glycoproteins interact with the ESCRT protein CHMP6 and are sufficient to recruit ESCRT components to Golgi membranes [16]. These studies do not, however, demonstrate whether there is a late domain in OROV glycoproteins. Moreover, as these studies were only performed with orthobunyaviruses, questions arise as to whether other members of the bunyaviruses also use ESCRT components, and whether the presence of a late domain is important for this process, given that not all bunyaviruses contain predicted late domain motifs. An alternative mechanism for ESCRT recruitment, or ESCRT-independent mechanisms, could be utilized for virion budding by diverse bunyaviruses.

Interestingly, SFTSV traffics to the Golgi via ER–Golgi intermediate compartments (ERGICs), with SFTSV Gn–Gc heterodimers visualized in these compartments, simultaneously recruiting N and RdRp proteins, which do not localize to these compartments in the absence of SFTSV glycoproteins [83]. As a result, virus assembly of SFTSV may commence in the ERGIC, while the glycoproteins are still undergoing structural maturation. However, it was recently observed that SFTSV utilizes autophagosomes for their assembly mechanisms, whereby SFTSV triggers RB1CC1/FIP200-BECN1-ATG5-dependent classical autophagy flux

[84]. These autophagosome components were found within purified virus particles, and it has therefore been suggested that the virion membrane is directly derived from these autophagosomes. The authors suggest that autophagy inhibition may provide a novel therapeutic strategy for inhibiting the release of SFSTV. Related to these observations, ESCRT components can also regulate ERGIC assembly and autophagosome formation [85], suggesting a potential role for ESCRT proteins during SFTSV budding and assembly, be that at the ERGIC or autophagosomes.

EXOCYTOSIS OF BUNYAVIRUSES

After budding, bunyavirus particles go through further stages of maturation as they traffic through the Golgi (Fig. 2). This process has been best characterized for BUNV, whereby three distinct viral forms (intracellular immature, intracellular mature and extracellular) have been identified by electron microscopy. Type I intracellular viruses are less dense in appearance, described as including a Golgi-derived membrane, with spikes formed by Gn–Gc heterodimers with RNA segments encapsidated in N protein and bound to RdRp, and are assumed to be newly assembled immature virions [86]. Type II intracellular viruses are denser in appearance. They are thought to have gone through a maturation process, likely involving the processing of the glycan side chains within the *trans*-Golgi network, after which they become resistant to endoglycosidase H (EndoH) treatment. Growing BUNV in glycosylation-deficient cell lines indicated that correct folding of BUNV glycoproteins only occurs during acquisition of EndoH resistance [12, 86]. Type III (extracellular) viruses are thought to arise from type II viruses that are secreted from the cell, with the organization of glycoproteins on the envelope undergoing a conformational change, appearing to have a 'neater' coat of spikes with angular external contours that hint at icosahedral symmetry of infectious virions [86], later confirmed to possess T=12 icosahedral symmetry [87]. Similar results were also obtained by a second study [39], giving further confidence in the classifications defined.

Little is known about the process by which vesicles containing infectious bunyavirus are released from the cell, and whether mechanisms of release are conserved across bunyaviruses. It is generally assumed that bunyaviruses use the conventional secretory pathway as their primary mechanism to release from the cell, but the exact molecular mechanisms by which this occurs, as well as the specific cellular proteins involved, remain unknown. Recent studies into a recombinant BUNV expressing GFP at the C-terminus of the structural glycoprotein Gc point towards a propagation mechanism that involves cell-to-cell transmission [88], and similar conclusions were observed for SFTSV [89].

VIRUS ASSEMBLY IN ARTHROPOD HOST

As well as infecting the human host, it is pertinent to consider that bunyaviruses also infect their arthropod host (except for hantaviruses). The mechanisms and processes in which virions are assembled in arthropod hosts may differ from the mammalian hosts. These differences may lead to subtle changes in the composition or make up of infectious virions, which could facilitate the initial infection following transmission from the arthropod to the mammalian host, such as by altering cell tropism or helping the virus evade innate immune responses. For example, RVFV has been shown to incorporate a 78 kDa NSm–Gn fusion glycoprotein of unknown function into infectious virions, but only when grown in invertebrate-derived cell lines, suggesting potential roles in transmission from the mosquito into ruminant hosts, as well as aiding in replication in the mosquito host [90]. Despite the unknown biological function of the 78 kDa protein, it has recently been observed that viruses with this fusion glycoprotein replicate more efficiently in some cell lines (such as fibroblasts), but worse in other cell lines (such as PMA-stimulated THP-1 cells) [91]. Interestingly, it has also been observed for RVFV that genome packaging is more efficient in insect C6/36 cells than mammalian Vero E6 cells [92]. The 78 kDa protein has also been described as not contributing towards virulence within a mouse model, but instead being a crucial determinant for dissemination in the invertebrate host [93]

Although a minigenome system has been established for BUNV in mosquito cells [94], as has an optimized protocol for CCHFV Gn expression in insect cells [95], there has been little advancement in our understanding of the vector in disease biology, perhaps due to a lack of well-developed arthropod cell culture systems for bunyavirus replication and poorly annotated genomes of arthropod-derived cells. An understanding of the assembly process in arthropod cells will provide much needed insight into how bunyaviruses, and arboviruses in general, are able to circumvent innate immune responses to establish initial infection.

CONCLUDING REMARKS

While important advances have been made in our understanding of bunyavirus assembly and egress, there are still many open-ended questions that require further research. These include the following.

- How, exactly, do genome segments package into virions, and what are the cellular proteins/mechanisms involved? Several studies have suggested a stochastic means of packaging a genome during assembly. Yet, the low particle-to-p.f.u. ratios of some viruses, along with the apparent inability of some bunyavirus glycoproteins to self-assemble, appear to contradict this.
- What are the mechanisms that underpin bunyavirus budding and scission mechanisms, and can we begin to develop small-molecule inhibitors to these essential processes?

- What significance, if any, does the role of the arthropod host play in modulating the infectivity of virus particles during the initial infection of the mammalian host? How does the assembly pathway in the insect host differ from that in the human host, and how do these differences contribute to the pathogenicity and virulence of the virus?

The picture of bunyavirus assembly is not complete for any one virus, and thus increasing our understanding of these processes may help to predict how, and under what conditions, potentially fatal reassortment viruses arise.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Adams MJ, Lefkowitz EJ, King AMQ, Harrach B, Harrison RL, *et al*. Changes to taxonomy and the international code of virus classification and nomenclature ratified by the international committee on taxonomy of viruses. *Arch Virol* 2017;162:2505–2538.
2. Leventhal SS, Wilson D, Feldmann H, Hawman DW. A Look into *Bunyavirales* Genomes: Functions of Non-Structural (NS) Proteins. *Viruses* 2021;13:314.
3. Kapuscinski ML, Bergren NA, Russell BJ, Lee JS, Borland EM, *et al*. Genomic characterization of 99 viruses from the bunyavirus families *Nairoviridae, Peribunyaviridae, and Phenuiviridae*, including 35 previously unsequenced viruses. *PLoS Pathog* 2021;17:e1009315.
4. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, *et al*. Novel *Orthobunyavirus* in Cattle, Europe, 2011. *Emerg Infect Dis* 2012;18:469–472.
5. Yanase T, Kato T, Aizawa M, Shuto Y, Shirafuji H, *et al*. Genetic reassortment between Sathuperi and Shamonda viruses of the genus *Orthobunyavirus* in nature: implications for their genetic relationship to Schmallenberg virus. *Arch Virol* 2012;157:1611–1616.
6. Briese T, Bird B, Kapoor V, Nichol ST, Lipkin WI. Batai and Ngari viruses: M segment reassortment and association with severe febrile disease outbreaks in East Africa. *J Virol* 2006;80:5627–5630.
7. Sakkas H, Bozidis P, Franks A, Papadopoulou C. Oropouche fever: a review. *Viruses* 2018;10:1–16.
8. Ergonul O, Tuncbilek S, Baykam N, Celikbas A, Dokuzoguz B. Evaluation of serum levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha in patients with Crimean-Congo hemorrhagic fever. *J Infect Dis* 2006;193:941–944.
9. Netski D, Thran BH, St Jeor SC. Sin Nombre virus pathogenesis in *Peromyscus maniculatus*. *J Virol* 1999;73:585–591.
10. Thomson WH, Kalfayan B, Anslow RO. Isolation of California encephalitis group virus from a fatal human illness. *Am J Epidemiol* 1965;81:245–253.
11. Lorenz C, Azevedo TS, Virginio F, Aguiar BS, Chiaravalloti-Neto F, *et al*. Impact of environmental factors on neglected emerging arboviral diseases. *PLoS Negl Trop Dis* 2017;11:e0005959.
12. Walter CT, Barr JN. Recent advances in the molecular and cellular biology of bunyaviruses. *J Gen Virol* 2011;92:2467–2484.
13. Hulswit RJG, Paesen GC, Bowden TA, Shi X. Recent advances in Bunyavirus glycoprotein research:precursor processing, receptor binding and structure. *Viruses* 2021;13:1–30.
14. Fazakerley JK, Gonzalez-Scarano F, Strickler J, Dietzschold B, Karush F, *et al*. Organization of the middle RNA segment of snowshoe hare Bunyavirus. *Virology* 1988;167:422–432.
15. Briese T, Rambaut A, Lipkin WI. Analysis of the medium (M) segment sequence of Guaroa virus and its comparison to other *Orthobunyaviruses*. *J Gen Virol* 2004;85:3071–3077.
16. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002;3:753–766.
17. Shi X, Botting CH, Li P, Niglas M, Brennan B, *et al*. Bunyamwera orthobunyavirus glycoprotein precursor is processed by cellular signal peptidase and signal peptide peptidase. *Proc Natl Acad Sci U S A* 2016;113:8825–8830.
18. Weihofen A, Lemberg MK, Ploegh HL, Bogyo M, Martoglio B. Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. *J Biol Chem* 2000;275:30951–30956.
19. Barbosa NS, Concha JO, daSilva LLP, Crump CM, Graham SC. Oropouche virus Glycoprotein topology and cellular requirements for glycoprotein secretion. *J Virol* 2023;97:e0133122.
20. Gerrard SR, Nichol ST. Synthesis, proteolytic processing and complex formation of N-terminally nested precursor proteins of the Rift Valley fever virus glycoproteins. *Virology* 2007;357:124–133.
21. Sanchez AJ, Vincent MJ, Nichol ST. Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. *J Virol* 2002;76:7263–7275.
22. Seidah NG, Mowla SJ, Hamelin J, Mamarbachi AM, Benjannet S, *et al*. Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. *Proc Natl Acad Sci U S A* 1999;96:1321–1326.
23. Vincent MJ, Sanchez AJ, Erickson BR, Basak A, Chretien M, *et al*. Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J Virol* 2003;77:8640–8649.
24. Sanchez AJ, Vincent MJ, Erickson BR, Nichol ST. Crimean-congo hemorrhagic fever virus glycoprotein precursor is cleaved by Furin-like and SKI-1 proteases to generate a novel 38-kilodalton glycoprotein. *J Virol* 2006;80:514–525.
25. Freitas N, Enguehard M, Denolly S, Levy C, Neveu G, *et al*. The interplays between Crimean-Congo hemorrhagic fever virus (CCHFV) M segment-encoded accessory proteins and structural proteins promote virus assembly and infectivity. *PLOS Pathog* 2020;16:e1008850.
26. Golden JW, Shoemaker CJ, Lindquist ME, Zeng X, Daye SP, *et al*. GP38-targeting monoclonal antibodies protect adult mice against lethal Crimean-Congo hemorrhagic fever virus infection. *Sci Adv* 2019;5:eaaw9535.
27. Löber C, Anheier B, Lindow S, Klenk HD, Feldmann H. The Hantaan virus glycoprotein precursor is cleaved at the conserved pentapeptide WAASA. *Virology* 2001;289:224–229.
28. Shi X, Brauburger K, Elliott RM. Role of N-linked glycans on bunyamwera virus glycoproteins in intracellular trafficking, protein folding, and virus infectivity. *J Virol* 2005;79:13725–13734.

29. Erickson BR, Deyde V, Sanchez AJ, Vincent MJ, Nichol ST. N-linked glycosylation of Gn (but not Gc) is important for Crimean Congo hemorrhagic fever virus glycoprotein localization and transport. *Virology* 2007;361:348–355.
30. Kuismanen E. Posttranslational processing of Uukuniemi virus glycoproteins G1 and G2. *J Virol* 1984;51:806–812.
31. Shi X, Elliott RM. Analysis of N-linked glycosylation of hantaan virus glycoproteins and the role of oligosaccharide side chains in protein folding and intracellular trafficking. *J Virol* 2004;78:5414–5422.
32. Naidu RA, Ingle CJ, Deom CM, Sherwood JL. The two envelope membrane glycoproteins of tomato spotted wilt virus show differences in lectin-binding properties and sensitivities to glycosidases. *Virology* 2004;319:107–117.
33. Wu Y, Zhu Y, Gao F, Jiao Y, Oladejo BO, *et al*. Structures of phlebovirus glycoprotein Gn and identification of a neutralizing antibody epitope. *Proc Natl Acad Sci U S A* 2017;114:E7564–E7573.
34. Hofmann H, Li X, Zhang X, Liu W, Kühl A, *et al*. Severe fever with thrombocytopenia virus glycoproteins are targeted by neutralizing antibodies and can use DC-SIGN as a receptor for pH-dependent entry into human and animal cell lines. *J Virol* 2013;87:4384–4394.
35. Lozach P-Y, Kühbacher A, Meier R, Mancini R, Bitto D, *et al*. DC-SIGN as a receptor for *Phleboviruses*. *Cell Host Microbe* 2011;10:75–88.
36. Whitfield AE, Ullman DE, German TL. Expression and characterization of a soluble form of tomato spotted wilt virus glycoprotein GN. *J Virol* 2004;78:13197–13206.
37. Ganaie SS, Schwarz MM, McMillen CM, Price DA, Feng AX, *et al*. Lrp1 is a host entry factor for Rift Valley fever virus. *Cell* 2021;184:5163–5178.
38. Schwarz MM, Price DA, Ganaie SS, Feng A, Mishra N, *et al*. Oropouche orthobunyavirus infection is mediated by the cellular host factor Lrp1. *Proc Natl Acad Sci U S A* 2022;119:e2204706119.
39. Salanueva IJ, Novoa RR, Cabezas P, López-Iglesias C, Carrascosa JL, *et al*. Polymorphism and structural maturation of bunyamwera virus in Golgi and post-Golgi compartments. *J Virol* 2003;77:1368–1381.
40. Gerrard SR, Nichol ST. Characterization of the Golgi retention motif of Rift Valley fever virus G(N) glycoprotein. *J Virol* 2002;76:12200–12210.
41. Bertolotti-Ciarlet A, Smith J, Strecker K, Paragas J, Altamura LA, *et al*. Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J Virol* 2005;79:6152–6161.
42. Ribeiro D, Goldbach R, Kormelink R. Requirements for ER-arrest and sequential exit to the golgi of tomato spotted wilt virus glycoproteins. *Traffic* 2009;10:664–672.
43. Spiegel M, Plegge T, Pöhlmann S. The role of *Phlebovirus* glycoproteins in viral entry, assembly and release. *Viruses* 2016;8:202.
44. Nilsson T, Jackson M, Peterson PA. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* 1989;58:707–718.
45. Chen SY, Matsuoka Y, Compans RW. Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. *Virology* 1991;183:351–365.
46. Matsuoka Y, Chen SY, Compans RW. A signal for Golgi retention in the bunyavirus G1 glycoprotein. *J Biol Chem* 1994;269:22565–22573.
47. Matsuoka Y, Chen SY, Holland CE, Compans RW. Molecular determinants of Golgi retention in the Punta Toro virus G1 protein. *Arch Biochem Biophys* 1996;336:184–189.
48. Andersson AM, Melin L, Bean A, Pettersson RF. A retention signal necessary and sufficient for Golgi localization maps to the cytoplasmic tail of a Bunyaviridae (*Uukuniemi virus*) membrane glycoprotein. *J Virol* 1997;71:4717–4727.
49. Sperber HS, Welke R-W, Petazzi RA, Bergmann R, Schade M, *et al*. Self-association and subcellular localization of *Puumala hantavirus* envelope proteins. *Sci Rep* 2019;9:707.
50. Shi X, Elliott RM. Golgi localization of *Hantaan virus* glycoproteins requires coexpression of G1 and G2. *Virology* 2002;300:31–38.
51. Deyde VM, Rizvanov AA, Chase J, Otteson EW, St Jeor SC. Interactions and trafficking of Andes and Sin Nombre *Hantavirus* glycoproteins G1 and G2. *Virology* 2005;331:307–315.
52. Kopito RR. ER quality control: the cytoplasmic connection. *Cell* 1997;88:427–430.
53. Gomez-Navarro N, Miller E. Protein sorting at the ER-Golgi interface. *J Cell Biol* 2016;215:769–778.
54. Urata S, Yasuda J. Molecular mechanism of arenavirus assembly and budding. *Viruses* 2012;4:2049–2079.
55. Strandin T, Hepojoki J, Vaheri A. Cytoplasmic tails of Bunyavirus Gn glycoproteins-could they act as matrix protein surrogates? *Virology* 2013;437:73–80.
56. Overby AK, Popov V, Neve EPA, Pettersson RF. Generation and analysis of infectious virus-like particles of *Uukuniemi* virus (Bunyaviridae): a useful system for studying bunyaviral packaging and budding. *J Virol* 2006;80:10428–10435.
57. Overby AK, Pettersson RF, Neve EPA. The glycoprotein cytoplasmic tail of *Uukuniemi virus* (Bunyaviridae) interacts with ribonucleoproteins and is critical for genome packaging. *J Virol* 2007;81:3198–3205.
58. Shi X, Kohl A, Li P, Elliott RM. Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. *J Virol* 2007;81:10151–10160.
59. Rezelj VV, Mottram TJ, Hughes J, Elliott RM, Kohl A, *et al*. M segment-based minigenomes and virus-like particle assays as an approach to assess the potential of Tick-Borne *Phlebovirus* genome reassortment. *J Virol* 2019;93:e02068-18.
60. Acuña R, Cifuentes-Muñoz N, Márquez CL, Bulling M, Klingström J, *et al*. Hantavirus Gn and Gc glycoproteins self-assemble into virus-like particles. *J Virol* 2014;88:2344–2348.
61. Wichgers Schreur PJ, Kormelink R, Kortekaas J. Genome packaging of the Bunyavirales. *Curr Opin Virol* 2018;33:151–155.
62. Lowen AC, Boyd A, Fazakerley JK, Elliott RM. Attenuation of bunyavirus replication by rearrangement of viral coding and noncoding sequences. *J Virol* 2005;79:6940–6946.
63. Terasaki K, Murakami S, Lokugamage KG, Makino S. Mechanism of tripartite RNA genome packaging in Rift Valley fever virus. *Proc Natl Acad Sci U S A* 2011;108:804–809.
64. Piper ME, Sorenson DR, Gerrard SR. Efficient cellular release of Rift Valley fever virus requires genomic RNA. *PLoS One* 2011;6:e18070.
65. Ikegami T, Won S, Peters CJ, Makino S. Rift Valley fever virus NSs mRNA is transcribed from an incoming anti-viral-sense S RNA segment. *J Virol* 2005;79:12106–12111.
66. Brennan B, Welch SR, Elliott RM, Cherry S. The consequences of reconfiguring the ambisense S genome segment of Rift Valley fever virus on viral replication in mammalian and mosquito cells and for genome packaging. *PLoS Pathog* 2014;10:e1003922.
67. Tercero B, Narayanan K, Terasaki K, Makino S, Gallagher T. Characterization of the molecular interactions that govern the packaging of viral RNA segments into Rift Valley fever *Phlebovirus* particles. *J Virol* 2021;95.
68. Brennan B, Welch SR, McLees A, Elliott RM. Creation of a recombinant Rift Valley fever virus with a two-segmented genome. *J Virol* 2011;85:10310–10318.
69. Wichgers Schreur PJ, Oreshkova N, Moormann RJM, Kortekaas J. Creation of Rift valley fever viruses with four-segmented genomes reveals flexibility in bunyavirus genome packaging. *J Virol* 2014;88:10883–10893.
70. Wichgers Schreur PJ, Oreshkova N, van Keulen L, Kant J, van de Water S, *et al*. Safety and efficacy of four-segmented Rift valley fever virus in young sheep, goats and cattle. *NPJ Vaccines* 2020;5:65.
71. Wichgers Schreur PJ, Oymans J, Kant J, van de Water S, Kollár A, *et al*. A single vaccination with four-segmented Rift Valley fever virus prevents vertical transmission of the wild-type virus in pregnant ewes. *NPJ Vaccines* 2021;6:8.

72. Wichgers Schreur PJ, Mooij P, Koopman G, Verstrepen BE, Fagrouch Z, *et al*. Safety and immunogenicity of four-segmented Rift Valley fever virus in the common marmoset. *NPJ Vaccines* 2022;7:54.
73. Wichgers Schreur PJ, Kortekaas J, Palese P. Single-molecule FISH reveals non-selective packaging of Rift Valley fever virus genome segments. *PLoS Pathog* 2016;12:e1005800.
74. Fontana J, López-Montero N, Elliott RM, Fernández JJ, Risco C. The unique architecture of Bunyamwera virus factories around the Golgi complex. *Cell Microbiol* 2008;10:2012–2028.
75. Shi X, Kohl A, Léonard VHJ, Li P, McLees A, *et al*. Requirement of the N-terminal region of orthobunyavirus nonstructural protein NSm for virus assembly and morphogenesis. *J Virol* 2006;80:8089–8099.
76. López-Montero N, Risco C. Self-protection and survival of arbovirus-infected mosquito cells. *Cell Microbiol* 2011;13:300–315.
77. Ravkov EV, Nichol ST, Compans RW. Polarized entry and release in epithelial cells of Black Creek Canal virus, a new world hantavirus. *J Virol* 1997;71:1147–1154.
78. Goldsmith CS, Elliott LH, Peters CJ, Zaki SR. Ultrastructural characteristics of Sin Nombre virus, causative agent of hantavirus pulmonary syndrome. *Arch Virol* 1995;140:2107–2122.
79. Martin-Serrano J, Yarovoy A, Perez-Caballero D, Bieniasz PD. Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. *Proc Natl Acad Sci U S A* 2003;100:12414–12419.
80. Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, *et al*. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 2001;107:55–65.
81. Vietri M, Radulovic M, Stenmark H. The many functions of ESCRTs. *Nat Rev Mol Cell Biol* 2020;21:25–42.
82. Barbosa NS, Mendonça LR, Dias MVS, Pontelli MC, da Silva EZM, *et al*. ESCRT machinery components are required for *Orthobunyavirus* particle production in Golgi compartments. *PLoS Pathog* 2018;14:e1007047.
83. Lundu T, Tsuda Y, Ito R, Shimizu K, Kobayashi S, *et al*. Targeting of severe fever with thrombocytopenia syndrome virus structural proteins to the ERGIC (endoplasmic reticulum Golgi intermediate compartment) and Golgi complex. *Biomed Res* 2018;39:27–38.
84. Jin min Y*et al*. Bunyavirus SFTSV exploits autophagic flux for viral assembly and egress. *Autophagy* 2021;00:1–14.
85. Wang R, Miao G, Shen JL, Fortier TM, Baehrecke EH. ESCRT dysfunction compromises endoplasmic reticulum maturation and autophagosome biogenesis in *Drosophila*. *Curr Biol* 2022;32:1262–1274..
86. Novoa RR, Calderita G, Cabezas P, Elliott RM, Risco C. Key Golgi factors for structural and functional maturation of bunyamwera virus. *J Virol* 2005;79:10852–10863.
87. Overby AK, Pettersson RF, Grünewald K, Huiskonen JT. Insights into bunyavirus architecture from electron cryotomography of Uukuniemi virus. *Proc Natl Acad Sci U S A* 2008;105:2375–2379.
88. García-Serradilla M, Risco C. Light and electron microscopy imaging unveils new aspects of the antiviral capacity of silver nanoparticles in Bunyavirus-infected cells. *Virus Res* 2021;302:198444.
89. Silvas JA, Popov VL, Paulucci-Holthauzen A, Aguilar PV. Extracellular vesicles mediate receptor-independent transmission of novel tick-borne Bunyavirus. *J Virol* 2016;90:873–886.
90. Weingartl HM, Zhang S, Marszal P, McGreevy A, Burton L, *et al*. Rift Valley fever virus incorporates the 78 kDa glycoprotein into virions matured in mosquito C6/36 cells. *PLoS One* 2014;9:e87385.
91. Terasaki K, Kalveram B, Johnson KN, Juelich T, Smith JK, *et al*. Rift Valley fever virus 78kDa envelope protein attenuates virus replication in macrophage-derived cell lines and viral virulence in mice. *PLoS Negl Trop Dis* 2021;15:e0009785.
92. Bermúdez-Méndez E, Katrukha EA, Spruit CM, Kortekaas J, Wichgers Schreur PJ. Visualizing the ribonucleoprotein content of single bunyavirus virions reveals more efficient genome packaging in the arthropod host. *Commun Biol* 2021;4:1–13.
93. Kreher F, Tamietti C, Gommet C, Guillemot L, Ermonval M, *et al*. The Rift Valley fever accessory proteins NSm and P78/NSm-GN are distinct determinants of virus propagation in vertebrate and invertebrate hosts. *Emerg Microbes Infect* 2014;3:e71.
94. Kohl A, Hart TJ, Noonan C, Royall E, Roberts LO, *et al*. A Bunyamwera virus minireplicon system in mosquito cells. *J Virol* 2004;78:5679–5685.
95. Rahpeyma M, Samarbaf-Zadeh A, Makvandi M, Ghadiri AA, Dowall SD, *et al*. Expression and characterization of codon-optimized Crimean-Congo hemorrhagic fever virus Gn glycoprotein in insect cells. *Arch Virol* 2017;162:1951–1962.