**Do NSm virulence factors in the Bunyavirales order originate from Gn gene duplication?**

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**Abstract**

Several viral members of the large viral order of the Bunyavirales carry a NSm protein that acts as a virulence factor. Here we used AlphaFold to predict the structures of these NSm proteins and surprisingly found that the cytosolic domain of the *Nairoviridae* family NSm ($NSm^{cyto}$) is predicted to have a very similar fold to the cytosolic domain of glycoprotein N ($Gn^{cyto}$). This observation is particularly striking for CCHFV (a member of the Nairoviridae family), for which the NMR structure of the $Gn^{cyto}$ domain has already been described in the literature and shows a double zinc finger. We show that while the sequence identity between the $NSm^{cyto}$ and $Gn^{cyto}$ domains is generally weak, the strict conservation of the two zinc-finger forming CCCH motifs in CCHFV $NSm^{cyto}$ explains the full structural conservation predicted by AlphaFold. Interestingly, a similar observation is made for the predictions of NSm in another group of the order Bunyavirales, the family *Peribunyaviridae*, where the $Gn^{cyto}$ structures have not yet been described experimentally. Our findings suggest that NSm is the result of a gene duplication event in these viruses, and indicate that such events may indeed be common in the recent evolutionary history of RNA viruses. Importantly, our predictions provide a first insight into the long-unknown structure of NSm and its link to virulence.

## Introduction

The Bunyavirales are a large order of enveloped viruses with more than 300 members (Leventhal et al., 2021; Teng et al., 2022). They are diverse, but share a single-stranded negative-polarity RNA genome, often with the three segments L, M and S. They encode at least four structural proteins that make up the viral particle: the envelope glycoproteins Gn and Gc; the nucleoprotein (NP); and the viral RNA-dependent RNA polymerase (RdRp). In addition, other non-structural proteins may be present, typically NSs and NSm (Leventhal et al., 2021). Virus replication occurs in the cytoplasm of infected cells, and the virus particles mature by budding at the membranes of the Golgi apparatus. The viruses of this family recognize a wide variety of hosts, including humans, in which they can cause severe disease, such as that caused by Crimean-Congo hemorrhagic fever virus (CCHFV) (Hawman and Feldmann, 2023). They are increasingly considered a threat to human health and are relevant examples of emerging viral infections.

NSm is a non-structural protein (accessory protein) and virulence factor in the families *Peribunyaviridae*, *Nairoviridae*, and *Phenuiviridae* of the Bunyavirales order (Nakitare and Elliott, 1993). NSm has approximately 150-200 residues, depending on the family, and is predicted to be a two-membrane-spanning integral membrane protein in most viruses, localizing mainly to the Golgi in infected cells (Shi et al., 2006; Freitas et al., 2020). Deletions corresponding to the NSm open reading frame (ORF) do not affect viral growth in cell culture (Pollitt et al., 2006). However, studies of Bunyamwera virus-like particles (VLPs) found that M-segments lacking the N-terminal portion of NSm significantly reduced VLP production (Shi et al., 2006). In the Akabane virus, mutants lacking the entire NSm could not be rescued (Ishihara et al., 2016). In Rift Valley fever virus (RVFV), the role of NSm is unclear; deletion mutants show that it may be a virulence factor in mosquitoes (Kading et al., 2014). In Schmallenberg virus (SBV), viruses lacking NSm did not affect replication but showed reduced virulence (Kraatz et al., 2015). NSm was also found to be accessory in Oropouche virus infection (Tilston-Lunel et al., 2016). Thus, the importance of NSm in the bunyavirus life cycle is still not fully understood, and varies between different virus studies.

Structural studies of NSm proteins remain difficult due to their membrane-bound nature and small size. In addition, no successful protein production has been reported for these proteins, suggesting that they are not easily expressed. In contrast, two studies of the $Gn^{cyto}$ domain have been reported for the Hantavirus and Crimean-Congo hemorrhagic fever virus (CCHFV) (Estrada et al., 2009; Estrada and De Guzman, 2011). These showed that they belong to the class of zinc finger proteins, a large family that includes many nucleic acid-binding proteins. Indeed, zinc fingers can recognize and bind specific sequences on double-stranded nucleic acids without unwinding double helical structures, as has been shown for DNA (Park et al., 2011). The two protein structures were found to be virtually identical despite their low sequence conservation. This is due to the conservation of the 2xCCCH double zinc finger motif which is fully conserved in both viruses (Estrada et al., 2009; Estrada and De Guzman, 2011).

We show here that this motif is surprisingly equally conserved in NSm in CCHFV of the *Nairoviridae* family. Furthermore, we show that in the *Peribunyae* family, $Gn^{cyto}$ and $NSm^{cyto}$ share the double zinc finger motif, but only the second zinc finger is strictly conserved, while the first displays certain differences in its position and organization. We also show that the Phenuivirus NSm has a completely different predicted fold, devoid of zinc finger domains, and also not at all similar to $Gn^{cyto}$. Gene duplication thus appears to be an important strategy for increasing virulence that is widespread, but not common to all members, in the different viruses of the Bunyavirales order.

**Material and Methods**

An implementation of Alphafold (Jumper et al., 2021) software on a local server was used for predictions. Amino-acid sequences were entered and predictions were run in the monomer mode. Sequence alignments were produced using Clustal (Chenna et al., 2003).

**Results**

***AlphaFold predictions of CCHFV NSm***

We used AlphaFold to predict the structures of the protein for several members of the *Nairoviridae* family. The results for Dugbe virus (DUGV) and CCHFV are shown in Figure 1a,b respectively. The program predicts the presence of two transmembrane domains in accordance with the predictions, which are located where the grey box schematizes the lipid bilayer. The cytosolic domains of the proteins match structurally, and have a very similar fold. Surprisingly, AlphaFold predicts exactly the same structure for the NSm cytosolic domain ($NSm^{cyto}$) as for the experimentally-determined $Gn^{cyto}$ (PDB 2l7x (Estrada and De Guzman, 2011)), as shown in Figure 1c. Indeed, the $Gn^{cyto}$ has been determined before, by NMR, to display a double CCCH zinc-finger motif (Estrada and De Guzman, 2011), involving 6 cysteine and 2 histidine residues. The structure appears to be fully reproduced in the predicted $NSm^{cyto}$ model, despite only 32 % identity between the two proteins in the cytosolic domain. Considering that no experimental data could be obtained until now for the *Nairoviridae* NSm, this surprising result provides an intriguing first insight in a possible structure for the cytosolic domain of this protein. However, it is curious that AlphaFold predicts the structures to be identical up to this point, and so we next wanted to address the question of why, given the low sequence identity of the two proteins, the program reproduces exactly the same fold.

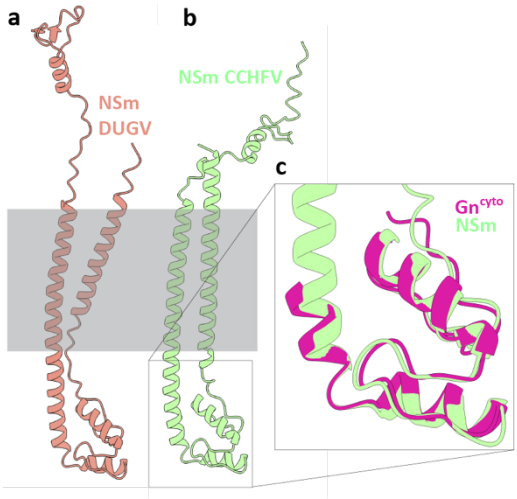


**Figure 1**. AlphaFold prediction of Nsm of DUGV and CCHFV, both from the *Nairoviridae* family. a,b) The predicted NSm protein structures for DUGV and CCHFV respectively, shown with the grey box roughly localizing the membrane, as deduced from the presence of mainly hydrophobic side chains in this portion. c) Overlay of the CCHFV model with the $Gn^{cyto}$ plus transmembrane domain model predicted from AlphaFold, but identical (with minor sequence differences) to the $Gn^{cyto}$ NMR structure (PDB 2l7x) reported by Guzman and coworkers (Estrada and De Guzman, 2011).

### *Nairoviridae NSm shows full conservation of the CCCH double zinc finger motifs of $Gn^{cyto}$*

The alignment of the two protein sequences reveals the rationale for the highly similar models: the two CCCH motifs of the double zinc finger in $Gn^{cyto}$ are completely conserved between the two proteins, as shown in Figure 2a, where nine different CCHFV sequences were aligned in the region of interest. It can be seen that besides the CCCH motifs, only few other amino acids are conserved, in particular a KRK motif, and a PY pair. Figure 2b shows the alignment of the cytosolic domains of two members of the Nairoviridae, DUGV and CCHFV, and Figure 2c the molecular details of the zinc finger.

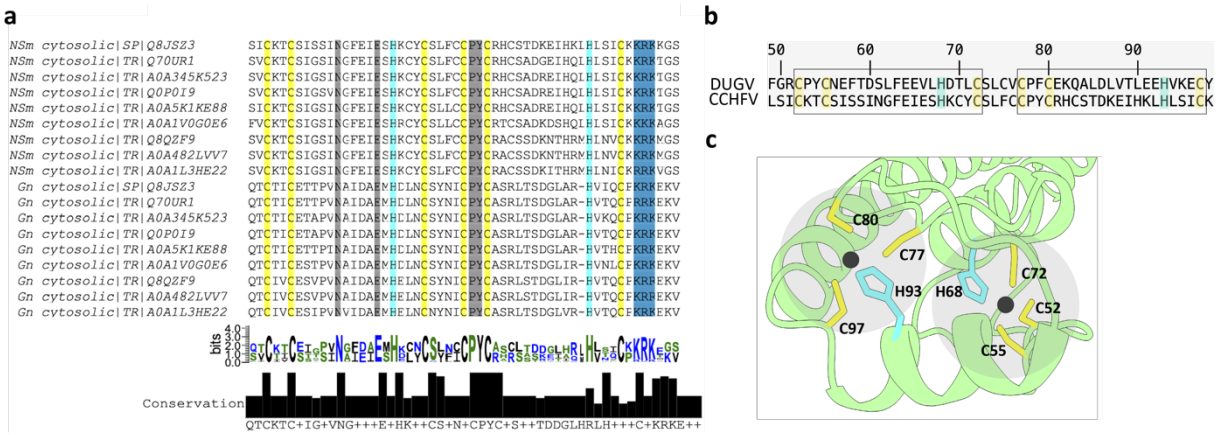


**Figure 2**. The two CCCH motifs of the double zinc finger are conserved between NSm and $Gn^{cyto}$. a) Sequence alignments of Gn and NSm cytosolic domains. The conserved residues are highlighted: yellow, Cys; cyan, His; grey, other conserved residues. b) Sequence alignments of DUGV and CCHFV regions of interest, showing that the CCCH motif is conserved between the two viruses. c) The double zinc finger in the structural model generated for CCHFV NSm.

### *NSm of the Peribunyaviridae family partially align to $Gn^{cyto}$*

Next, we tested sequence and structure alignments of the cytosolic domains of NSm and Gn from the *Peribunyaviridae* family, in order to assess whether the same similarities exist. Figures 3a,b show

the partial sequence alignments of $Gn^{cyto}$ and NSm from several members of this family. The alignments reveal the absolute conservation of 7 cysteine and 2 histidine residues within $Gn^{cyto}$, while in NSm, 6 cysteine and 2 histidine residues are conserved in all sequences. The pattern that could be postulated in $Gn^{cyto}$ as the second zinc finger is completely conserved when comparing the motif with NSm, resulting in a CCHC motif. The insertion of 12 residues in $Gn^{cyto}$ *versus* 11 in NSm between the second Cys and the His represents a minor difference between the two sequences. The situation is less obvious for the pattern corresponding to the first zinc finger, which in $Gn^{cyto}$ could be either CCCH, CCHC, or even CCCC, while in NSm, if there is a zinc finger, it must be CCHC. The positioning of this first motif is however quite different in this family between $Gn^{cyto}$ and NSm. AlphaFold predicts indeed a double zinc finger for both proteins, as shown in Figures 3c,d,f,g, and actually for both a CCHC motif is predicted. The coincidence of the models created by AlphaFold is good, and also consistent within most members of the family, as shown in the superpositions in Figures 3c,f for $NSm^{cyto}$ and $Gn^{cyto}$ respectively. However, Akabane virus (AKAV) represent an exception, since it displays a different model, which might be due to the lack of six residues in the NSm sequence when compared to the others.

Predictions for $Gn^{cyto}$ also yield consistent structures, but different from NSm. The second zinc finger is actually very close to that which has been determined for the CCHFV zinc fingers, resulting in a very similar motif containing an alpha-helix (compare Figures 2c and 3f). This was to be expected, since the spacing in the sequence is also similar, with an insertion of 12 residues between the second cysteine and the histidine (compare Figures 2b, 3a). However, the first zinc finger differs in both sequence and structure from the first CCHFV zinc finger. While AlphaFold finds several PDB structures that show a difference of 12 residues between CC-HC (*e.g.* PDBs 5AAZ (Thurston et al., 2016), 2JP9 (Stoll et al., 2007)), the first motif in NSm may be less canonical, and it is possible that no structural model exists in the database for this type of CCHC motif.

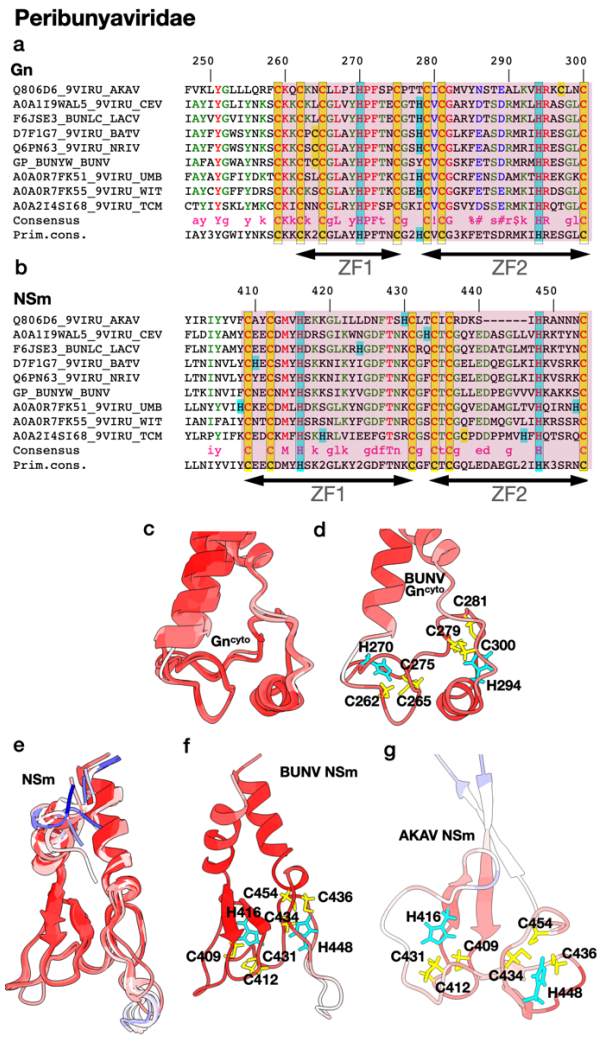


**Figure 3**. a,b) Sequence alignments of $Gn^{cyto}$ and NSm cytosolic domains of different members of the *Peribunyaviridae* family. AKAV, Akabane virus; CEV, California Encephalitis Virus; LACV, La Crosse Encephalitis Virus; BATV, Batai Virus; BUNV, Bunyamwera Virus; NRIV, Ngari Virus; UMBV, Umbre Virus; WITV, Witwatersrand Virus; TCMV, Tacaiuma Virus. Residues of interest are highlighted: Cys, yellow; His, cyan. c) Superposition of individual models predicted for BUNV $Gn^{cyto}$, and d) the details of the two zing-binding motifs at the example of BUNV. e) Overlay of the predicted model for NSm of the different members of the family, with exception of AKAV. f) Details of the zinc fingers in the models (at the example of BUNV) and the two CCHC motifs forming them. g) AKAV NSm, for which a different model from the other family members is predicted.

### *Models of NSm of the Phenuiviridae family are predicted differently from $Gn^{cyto}$*

The family Phenuiviridae is a recently (2017) established family of viruses that infect animals, plants, and fungi. They are a major threat to human health, livestock and agriculture. Rift Valley fever (RVF), the best-known member due to the associated severe fever with thrombocytopenia syndrome (SFTS) (Boushab et al., 2016), has been included in the World Health Organization's list of 11 emerging infectious diseases that urgently require accelerated research.

Some phenuiviruses transmitted by dipteran vectors encode a NSm protein, whose ORF is localized in front of the coding region of Gn. In this context, NSm has been shown to have an antiapoptotic role that is important for the infectivity in the vector (Terasaki et al., 2013). Looking at the cytoplasmic regions of the Gn and NSm proteins of the *Phenuiviridae* family, a first statement can be

made that neither belongs to the family of zinc-finger proteins, as indicated by the presence of only very few cysteine residues. An alignment of the NSm proteins of four members of the family (RVFV, Toscana virus (TOSV), Arumovot virus (AMTV) and Punoto Toro virus (PTPV)), is shown in Figure 4a. The identity in the alignment is less than 2%, and indeed one can see that only a few parts align well. Surprisingly, AlphaFold predictions actually led to very similar structural models of the proteins, as shown in Figure 4c,d, where NSm of TOSV is shown in isolation, or superimposed on the three other structural models. The fold shows two helices on one side, and a six-stranded beta-sheet on the other. Correspondingly, Figure 4b shows a corrected alignment of the proteins taking into account the secondary structural features of the predicted models.

In contrast to the two previous families, there seems to be no link to the cytosolic domain of Gn, or at least none that was recognized by AlphaFold. On the contrary, $Gn^{cyto}$ is predicted as three or four helices forming a not very well-defined 3D fold, as can be seen from the example of the RVFV $Gn^{cyto}$ in Figure 4e, and the superposition of all predicted models in Figure 4f. While the orientation of the helices with respect to each other is not maintained, the alpha-helical structures are consistent.

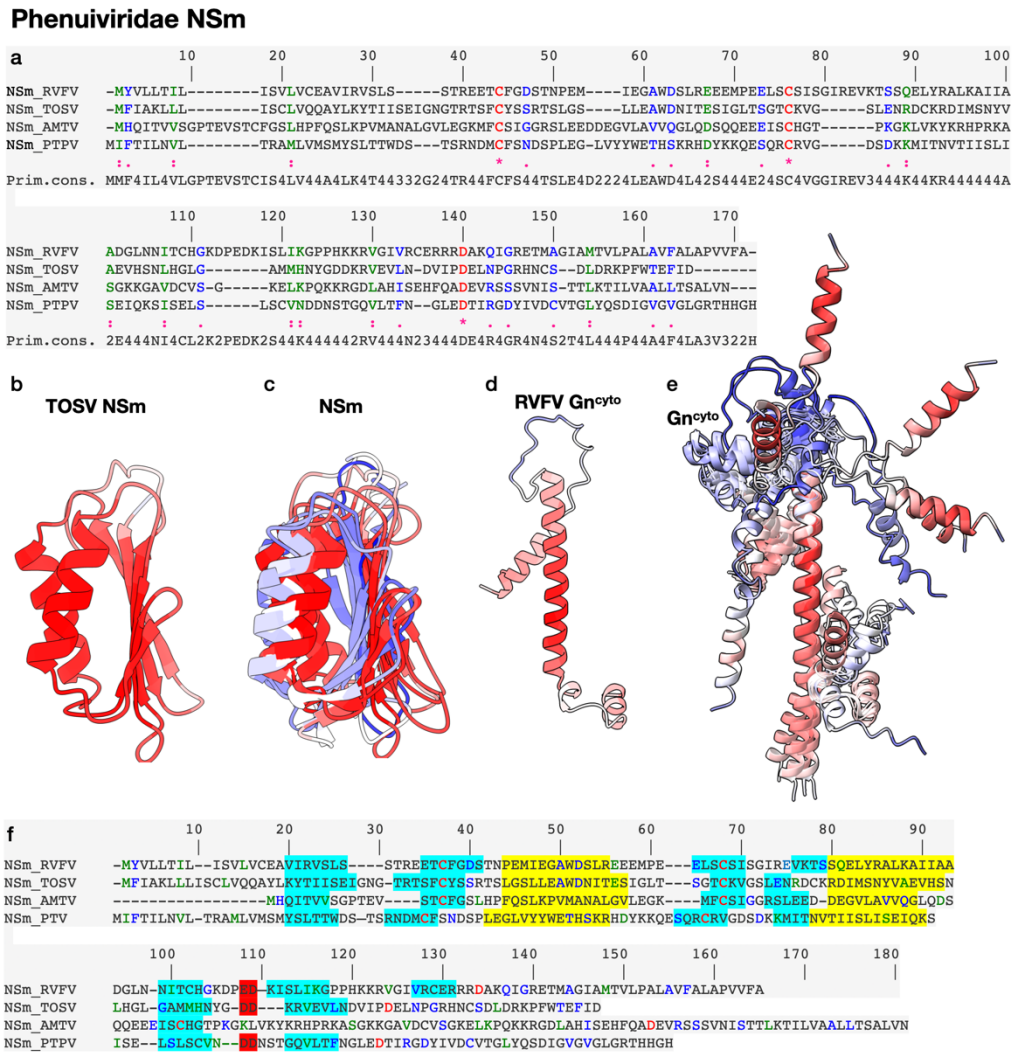

**Figure 4**. a) Sequence alignment of NSm cytosolic domains of different members of the *Phenuiviridae family*. RVFV, Rift Valley Fever Virus; TOSV, Toscana Virus; AMTV, Arumovot Virus; PTPV, Punta Toro Virus. b) Predicted model for NSm of TOSV. Color coding indicates reliability of the prediction, with red reliable, and blue poor. c) NSm AlphaFold predictions of all four viruses. d) RVFV $Gn^{cyto}$ structure predictions. e) Superposition of individual $Gn^{cyto}$ models of the four viruses. f) NSm sequences realigned taking into account the AlphaFold structure predictions, with residues in alpha-helical structure highlighted in yellow, and beta-strands highlighted in cyan.

## Discussion

Here we hypothesize, based on sequence alignments and AlphaFold structure predictions, that the NSm virulence factors in some members of the order Bunyavirales (families *Nairoviridae* and *Peribunyaviridae*) originate from a gene duplication from $Gn^{cyto}$. Our hypothesis is based on the high structural similarity of the NSm cytosolic domains of both families to the Gn cytosolic domains.

Gene duplication is a process by which a genetic sequence is copied, creating an additional gene (Ohno, 1970). This can occur naturally through mutations in DNA replication or through recombination. Gene duplications can result in the evolution of new functions of the duplicated gene, or to an increase in the amount of protein produced from the gene, which can be beneficial or harmful to an organism. Gene duplication is now recognized as an important mechanism of evolution (Taylor and Raes, 2004).

In viruses, the frequency of gene duplication can vary greatly depending on the specific virus and the conditions under which it replicates (Cisneros-Martínez et al., 2021). However, gene duplication is common in many viruses. In particular, RNA viruses can undergo frequent gene duplication due to their rapid replication cycles and error-prone replication mechanisms. For example, a gene duplication event from Gn has been proposed to have resulted in GP38, a virulence factor in CCHFV (Mishra et al., 2020), and an internal duplication within the CCHFV Gc glycoprotein has also been described (Hellert et al., 2019).

Gene duplication can increase the fitness of viruses in several ways, including gene amplification, the evolution of new functions for the duplicated gene, or diversification that allows the virus to infect new hosts. Identifying gene duplication events in viruses can be a complex and challenging task because sequence alignments can be inefficient when amino acid sequence conservation is poor. Protein tertiary structure comparisons are more appropriate in this case, but are not available for all viral proteins. With the advent of efficient prediction programs, more duplication events may be identified and gene duplications may be more common than previously thought.

In the Bunyavirales order, three families carry an NSm virulence factor: *Nairoviridae*, *Peribunyaviridae*, and *Phenuiviridiae*. NSm has been described to play a role in antagonizing the immune response (Hawman et al., 2021; Leventhal et al., 2021), promoting viral assembly and infectivity (Fontana et al., 2008), and even maintaining infection in host mosquito vectors (Engdahl et al., 2012). Most studies have shown that NSm proteins of the Bunyavirales order interfere with host innate immune responses. This suggests that these proteins have been conserved under common evolutionary pressures as the Bunyavirales species diverged from their most recent common ancestor.

Based on current knowledge of the phylogenetic tree of evolutionary relationships among different viral lineages of the order Bunyavirales (Leventhal et al., 2021), it is unclear how NSm

originated and evolved, making it difficult to trace its origin. Our analysis suggests that duplication events in the families *Nairoviridae* and *Peribunyaviridae* led to NSm. Considering that the similarity of the NSm zinc finger to the respective $Gn^{cyto}$ domains is greater than that between the $Gn^{cyto}$ and NSm of the different families, it can be concluded that NSm evolved by an independent duplication event in each family. For the Phenuiviridae, gene duplication is not the origin of NSm, and the protein, with a completely different structural organization than $Gn^{cyto}$, must have been acquired by another mechanism, possibly from the vector host, considering its possible role in maintaining infection in viral vectors (Herath et al., 2020).

Zinc fingers have been shown to play an important role in hepatocellular carcinoma (HCC) (Li et al., 2022). Indeed, CCHFV can cause severe liver injury in humans (Bayyurt et al., 2018; Liu et al., 2020); whether NSm and/or $Gn^{cyto}$ zinc fingers play a role in liver injury in CCHFV infection remains to be determined.

**Conclusion**

In conclusion, we have shown here that gene duplication could be a possible origin of the NSm gene in the *Nairoviridae* and *Peribunyaviridae* families. Gene duplication in viruses can lead to genetic novelty and redundancy, and is an important mechanism of evolutionary change also in other organisms. With gene duplication already reported for GP38 and Gc in CCHFV, our work adds NSm as a candidate product from such an event. Thus, while gene duplication has rarely been reported in RNA viruses in absence of structural information, this may be due to the high mutation rates in these viruses; as a consequence, the resulting low protein sequence homology can significantly complicate a conservative sequence search approach. Our work not only provides the first structural model for the enigmatic NSm proteins, but also contributes to the emerging understanding that comparisons of protein tertiary structure, rather than sequence data, may be a better means of identifying gene duplications in viruses.

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**Author contributions**

VL, RLFL and LC performed sequence analyses; VL, RLFL performed bioinformatics analyses and alignments; VL and AB did structure predictions; FLC advised on CCHFV virology; MLF and AB designed the research. AB and MLF wrote the paper, with input from all authors.

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