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Alphavirus nsP2: A Multifunctional Regulator of Viral Replication and Promising Target for Anti-Alphavirus Therapies

Sainan Wang[1] | Suresh Mahalingam[2,3,4] | Andres Merits[1]

[1]Institute of Bioengineering, University of Tartu, Tartu, Estonia | [2]Institute for Biomedicine and Glycomics, Griffith University, Gold Coast, Australia | [3]Global Virus Network (GVN) Centre of Excellence in Arboviruses, Griffith University, Gold Coast, Australia | [4]School of Pharmacy and Medical Sciences, Griffith University, Gold Coast, Australia

Correspondence: Andres Merits (andres.merits@ut.ee) | Suresh Mahalingam (s.mahalingam@griffith.edu.au)

ABSTRACT

Alphaviruses are re-emerging vector-born pathogens that cause arthralgia or encephalitic diseases on a global scale. While a vaccine against chikungunya virus was recently approved, no vaccines currently exist for other alphaviruses, nor are there antiviral drugs for the treatment of alphavirus infections. Alphaviruses have positive-strand RNA genomes, and their RNA replication is coordinated by activities of the multifunctional nonstructural protein 2 (nsP2), a helicase-protease and a subunit of viral RNA replicase. We provide a comprehensive overview of nsP2 functions and inhibitors of its activities for their potential as effective antivirals. Furthermore, analysis of nsP2 activities suggests that it could be targeted to develop advanced live attenuated vaccines and strategies for controlling alphavirus transmission by mosquito vectors.

1 | Introduction

Alphaviruses (genus *Alphavirus*, family Togaviridae) are enveloped viruses with positive-strand RNA genomes. Currently, there are more than 30 recognized alphavirus species, most of which are transmitted between vertebrate hosts via invertebrate vectors, primarily mosquitoes, though ticks, lice and cliff swallow bugs can also play a role in transmission. Alphaviruses can infect humans, domestic animals such as pigs and equids, and wild mammals, including non-human primates and rodents, as well as birds, reptiles, amphibians and fish [1–5]. Arthropod-transmitted

members of the genus are divided into eight phylogenetic complexes [1]. Human infections with arthritogenic alphaviruses, such as chikungunya virus (CHIKV), Sindbis virus (SINV), Ross River virus (RRV), Mayaro virus (MAYV) and oʻnyongʻnyong virus (ONNV), primarily result in fever and polyarthritis. Currently, CHIKV is the most medically important alphavirus, with approximately 480,000 human cases and 190 deaths reported in 2024. Its healthcare burden is further increased by its ability to cause chronic arthritic symptoms that can last months or even years in approximately 30%–60% of patients [6–9]. As their names suggest, Venezuelan, Eastern and Western equine encephalitis

viruses (VEEV, EEEV and WEEV) cause encephalitis in humans and domestic animals [10, 11]. The fatality rate for symptomatic human infections with EEEV reaches 30%. Table 1 lists medically relevant alphaviruses, their geographical distribution, vectors, reservoir hosts and disease symptoms in humans.

Alphaviruses are known to cause periodic large-scale outbreaks, such as the CHIKV epidemic in the Indian Ocean region from 2004 to 2011, with millions of human cases [43, 44]. Increased international travel, expanded distribution of mosquito vectors, and adaptive changes of alphaviruses to the new vectors [12, 45] have made these viruses a potential 'Disease X' threat. Currently, only one live-attenuated vaccine has been approved to prevent CHIKV infection [46]; however, there are no vaccines for other alphaviruses or specific antiviral drugs available. Treatment of alphavirus infections primarily aims to relieve disease symptoms and involves the use of analgesics and/or NSAIDs (non-steroidal anti-inflammatory drugs). Understanding the replication mechanisms of alphaviruses is crucial for developing effective countermeasures.

Alphavirus RNA genome has a 5′ methylguanylate cap and a 3′ polyadenylate tail, with two open reading frames (ORFs) [47]. The first ORF encodes the nonstructural (ns) polyproteins P123 (90%) and P1234 (10%) [48], which are precursors to 4 ns proteins (nsP1-4). The second ORF encodes structural polyproteins, translated from the subgenomic (SG) RNA produced in infected cells (Figure 1) [10]. RNA replication of alphaviruses is highly regulated by the ns polyprotein processing; both processing intermediates and mature nsPs are important for RNA replication. nsP1 is the membrane anchor of the replication complex (RC) [50, 51] and is essential for capping newly synthesised genomic and SG RNAs [52, 53]. nsP2 is a cysteine protease that orchestrates P1234 polyprotein processing [54, 55] and has RNA helicase, nucleoside triphosphatase (NTPase) and RNA triphosphatase (RTPase) activities [56–59]. nsP3 acts as an interaction hub for host factors and is crucial for negative-strand and SG RNA synthesis [60–66]. It assembles into a crown-like structure associated with RC and forms bundles of tubular structures in infected cells [67]. nsP4 is the RNA-dependent RNA polymerase and terminal adenylyltransferase; it is also shown to counteract antiviral effects of the unfolded protein response (UPR) [68–71].

2 | Organisation of nsP2 and Its Enzymatic Activities

In this review, we focus on alphavirus nsP2, a ~90 kDa protein with multiple interconnected functions that play a central role in RNA replication. For arthritogenic alphaviruses, nsP2 also counteracts the host antiviral response by shutting down cellular transcription and acting as a critical determinant of viral pathogenesis [72–74]. Reflecting its multiple functions, nsP2 has a complex structure: its N-terminal RNA helicase region (nsP2h) is connected to C-terminal protease region (nsP2p) via a short flexible linker [49]. nsP2h belongs to a superfamily 1 (SF1) of helicases and consists of N-terminal domain (NTD), stalk, superfamily 1 accessory domain 1B and two similar Rec-A-like domains (RecA1 and RecA2). The conserved helicase motifs

establish polar contacts with the RNA backbone. The two aromatic residues of domain 1B (Y161 and F164) form stacking interactions with RNA bases and are crucial for CHIKV viability [59]. A recombinant nsP2h has NTPase and RTPase activities, but the presence of nsP2p increases nsP2h's NTPase activity by 5–7 fold, and only the full-length nsP2 demonstrates RNA helicase activity [75]. The nsP2p region consists of two domains. A papain-like cysteine protease domain contains a catalytic dyad formed by cysteine and histidine residues [55, 76, 77]. A S-adenosyl-L-methionine (SAM)-dependent RNA methyltransferase-like (MTL) domain that lacks RNA methyltransferase activity (Figure 1). This domain contains numerous positively charged amino acid residues, binds RNA at sequence-independent manner and regulates enzymatic activities of nsP2 [78–80]. nsP2 recognises and cleaves three cleavage sites within the P1234 polyprotein: the sites between nsP1 and nsP2 (1/2 site), nsP2 and nsP3 (2/3 site) and nsP3 and nsP4 (3/4 site). In cell-free reaction nsP2p processes 3/4 site more efficiently than 1/2 site. Studies have shown that the amino acid residues at positions P4–P1′ (nomenclature of Schechter and Berger is used here and below) in the 3/4 site play an essential role in cleavage efficiencies [81]. However, apart from the invariable P2 Gly residue, the P4–P1′ sequences typically differ across sites, partially accounting for varying cleavage efficiencies. These differences alone, however, do not fully explain the order of cleavage events, suggesting the existence of additional recognition mechanisms [82]. This is particularly evident for the 2/3 site, which can be processed only by full-length nsP2. Furthermore, its cleavage requires the presence of the first domain (macro-domain) of nsP3, which functions in precisely positioning the 2/3 site within nsP2's protease active site in the substrate [83, 84].

Taken together, the two seemingly different regions of nsP2 (nsP2h and nsP2p) are functionally coupled. This also reflects the crucial role of the linker connecting these regions, as deletion of 3 or 5 linker residues is lethal for CHIKV [49]. This functional coupling also extends to nsP3, which was found to regulate nsP2p activity directly. Specifically, nsP3's macro-domain mediated de-mono-ADP-ribosylation (de-MARylation) activity is required to remove MARylation (cellular modification inactivating protease activity of nsP2) from nsP2, thereby reactivating its proteolytic function [85]. Thus, the various functions of nsP2 and its interactions with other nsPs must be well coordinated to support its essential role in viral RNA replication. However, the precise mechanism(s) underlying these coordinated activities remain unclear.

3 | nsP2 Regulates the Formation of Alphavirus RCs

Alphavirus genome replication is initiated and coordinated through the processing of P1234 polyprotein (Figure 2) [54]. First, P1234 polyprotein is rapidly cleaved at the 3/4 site. In infected cells, the cleavage occurs in cis; however, as the site is easily accessible for nsP2, cleavage in trans is also possible. Regardless of the mode of cleavage, the release of nsP4 results in the formation of a short-lived early replicase complex (P123 polyprotein + nsP4) that synthesises negative-strand RNA using the genomic RNA as a template, thereby forming dsRNA. The physical structure and

TABLE 1 | Vectors, hosts and symptoms of pathogenic alphaviruses.

Alphavirus	Confirmed or putative mosquito vectors	Confirmed or putative reservoir hosts	Incubation period	Symptoms in humans	Occurrence
Chikungunya virus (CHIKV)	*Aedes aegypti*; *Aedes albopictus* [12]	Nonhuman primates [13]	2–12 days	Main symptoms include fever, myalgia, polyarthralgia, rash, headache and chronic symptoms in up to 80% of patients. Other symptoms include nausea, vomiting, tenosynovitis conjunctivitis, occasional bleeding gums, and epistaxis [14, 15]. Rare atypical symptoms include encephalitis, meningitis, and Guillain-Barré syndrome [16, 17].	Americas, Africa, Asia, Europe and the Indian and Pacific Oceans
o'nyong'nyong virus (ONNV)	*Anopheles funestus*; *Anopheles gambiae* [18]	Unknown	8 days	Similar to CHIKV in major symptoms (usually milder) with the addition of 71% pruritus and 45% cervical lymphadenitis [19].	Africa
Barmah Forest virus (BFV)	*Culex annulirostris* [20, 21]	Not established, but it is likely that macropods and marsupials are the hosts	7–9 days	Fever, myalgia, rash, headache, and lethargy [22, 23].	Australia
Mayaro virus (MAYV)	*Haemagogus janthinomys* [24]; *A. aegypti* [25]	Nonhuman primates [26]	7–12 days	Main symptoms are similar to CHIKV (mostly milder); however, about 50% of patients develop oedema, retroocular pain, and 10%–20% of patients have swollen lymph nodes and diarrhoea [27].	Latin America, Caribbean
Ross River virus (RRV)	*Culex annulirostris*; *Aedes vigilax* [28]	Marsupials [29]	3–21 days	Fever, myalgia, rash, headache, photophobia, and rarely encephalitis [30].	Australia, new Guinea, Fiji, the Solomon Islands, American Samoa, South Pacific islands
Sindbis virus (SINV)	*Culex pipiens*; *Culex univittatus* [31]	Thrush family (Turdidae) [32]	2–10 days	Fever, rash, myalgia, arthralgia [33, 34].	Europe, Africa, Australia, Asia, Philippines
Venezuelan equine encephalitis virus (VEEV)	*Ochlerotatus taeniorhynchus*; *Culex melanoconion*; mammalophilic mosquitoes [35, 36]	Equines and sylvatic rodent [37]	2–5 days	Usually mild flu-like illness with rarely fatal (less than 1%) encephalitis [36, 38].	Latin America and the USA

(Continues)

TABLE 1 | (Continued)

Alphavirus	Confirmed or putative mosquito vectors	Confirmed or putative reservoir hosts	Incubation period	Symptoms in humans	Occurrence
Western equine encephalitis virus (WEEV)	*Culex tarsalis*; *Aedes albifasciatus* [39]	Birds and lagomorphs [40]	2–7 days	From mild febrile flu-like illness to severe or fatal (3%–7%) encephalitis [36].	The USA, Canada and the southern cone region of South America
Eastern equine encephalitis virus (EEEV)	*Culiseta melanura*; [41] *Culex erraticus* [42]	Passeriformes birds [41]	4–10 days	Similar to WEEV, however, the fatality can be 50%–70% [36].	Mainly in the eastern coastal areas of the USA and Canada, the Caribbean and Argentina
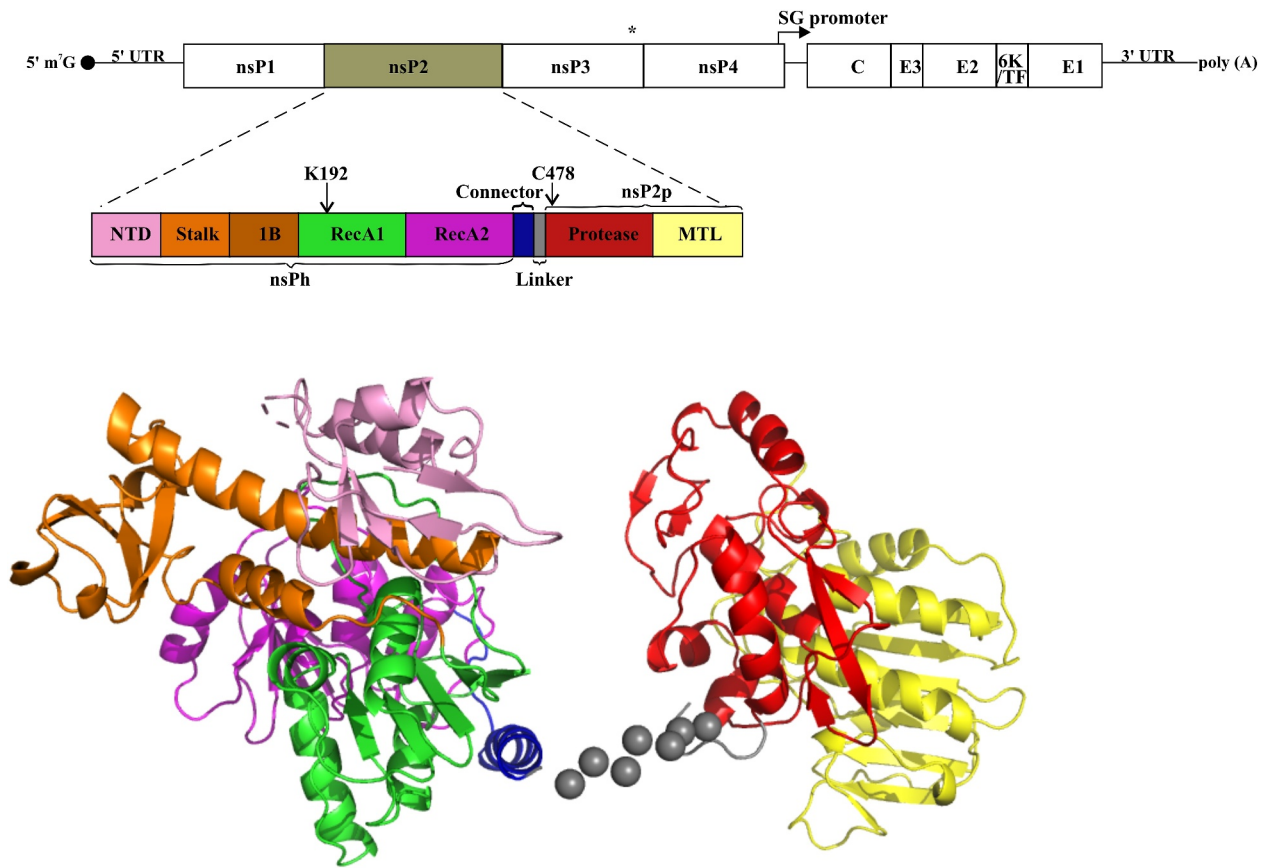
FIGURE 1 | The organisation of the alphavirus genome and domain organisation of nsP2 protein of CHIKV. * designates an opal stop codon that is present at the end of nsP3 region of the majority of alphaviruses. nsP2 consists of nsP2h and nsP2p regions connected by flexible linker; the sub-domains of nsP2h and nsP2p are colour-coded. The 3D structure of CHIKV nsP2 is kindly provided by Yee–Song Law [49]. NTD (residues 1–26), N-terminal domain; Stalk (residues 27–109); 1B (residues 110–174), superfamily 1 accessory domain 1B; RecA1 (residues 175–308) and RecA2 (residues 309–439), conserved Rec-A-like domains; connector and linker (residues 440–464); protease (residues 465–604) and MTL (residues 605–798), S-adenosyl-L-methionine (SAM)-dependent RNA methyltransferase-like domains are shown. Arrows point to the active site residues of NTPase/helicase (K192) and protease (C478).

precise composition of this early replicase are not yet known. In Semliki Forest virus (SFV) and SINV, the 1/2 site is cleaved slowly and exclusively, or almost exclusively, in cis [87]; acceleration of its processing results in attenuated or non-viable viruses [88], suggesting that this cleavage is purposefully delayed. The resulting nsP1 + P23 + nsP4 complex has a very short half-life because the formed P23 polyprotein is rapidly cleaved into nsP2 and nsP3. This cleavage occurs *in trans*, as in the P23 polyprotein, the 2/3 site is over 40 Å from the active site of nsP2p and cannot be cleaved by the same molecule [80]. The late RNA replicase (nsP1 + nsP2 + nsP3 + nsP4) then uses the negative strand of dsRNA as a template to synthesise large amounts of genomic and SG RNAs. This synthesis occurs in a bulb-like membrane invaginations, or spherules, approximately 50 nm in diameter, which initially form at the plasma membrane of infected cells (Figure 2) [86, 89]. In SFV-infected cells, spherules are
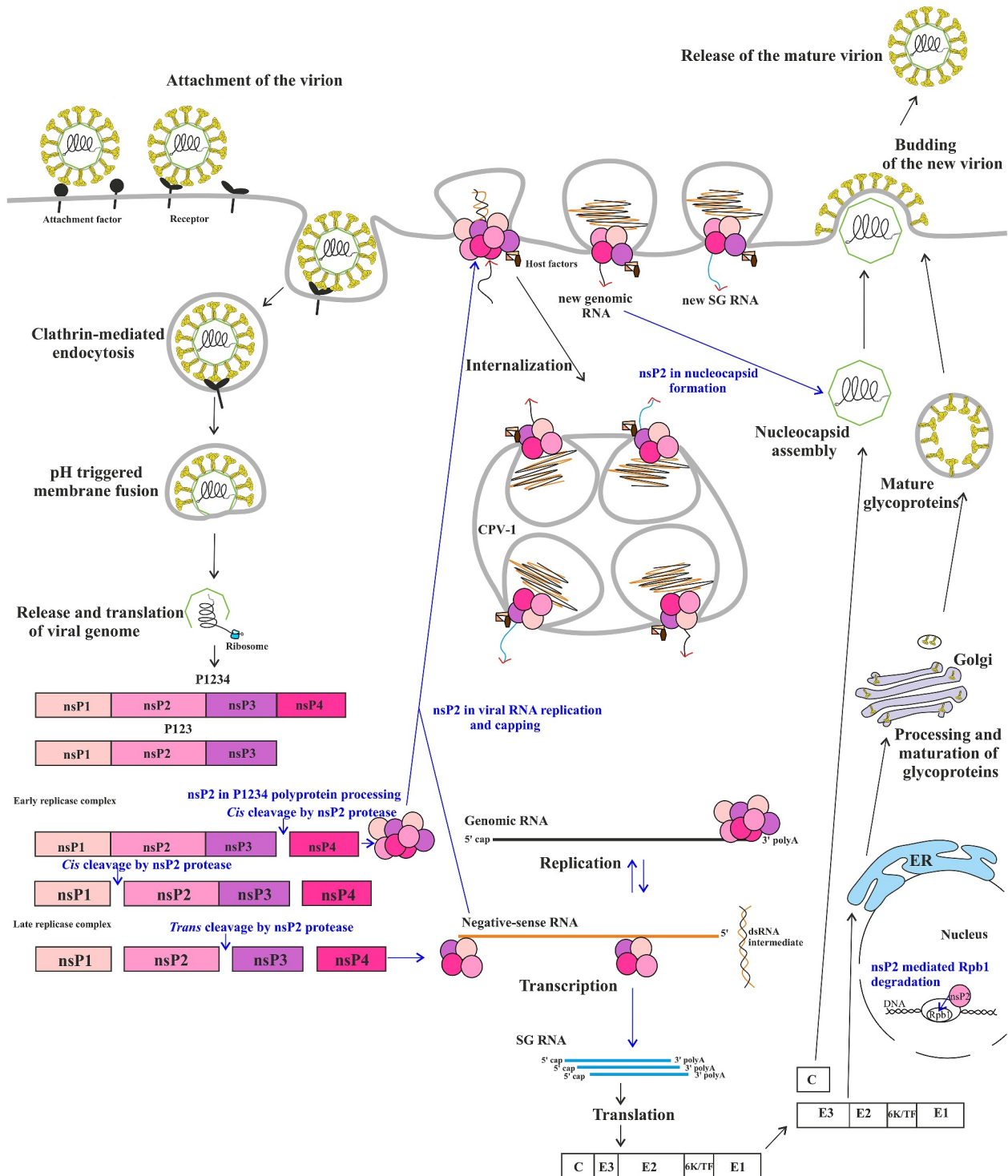
FIGURE 2 | Alphavirus infection cycle. Infection is initiated by the binding of virions to cellular receptor(s) and their entry by clathrin-dependent endocytosis. At low pH, the virion envelope fuses with endosomal membrane, releasing the nucleocapsid into the cytoplasm. The genomic RNA is then uncoated and translated. Step-by-step processing of P1234 polyprotein results in the formation of an early replicase complex and its conversion to late replicase complex; this co-insides with the formation of spherules at the plasma membrane. For SFV and RRV, spherules are internalized and, at later stages of infection, localise on the internal surface of modified endosomes and lysosomes, forming so-called cytopathic vacuoles (CPV-1) [86]. The dsRNA molecule localises inside of spherule and is used by the late replicase to produce new genomic and SG RNAs. The SG RNA serves as a mRNA for translation of structural proteins required for assembly and release of progeny virions. Spheres indicate ns-proteins included in the active replicase complexes (stoichiometry and exact localization of replicase subunits is shown on Figure 3). Blue arrows indicate the key activities of nsP2: processing of P1234 polyprotein, assisting of viral RNA replication, participation in the capping of positive strand RNAs, involvement in formation of nucleocapsids, and (for arithrogenic alphaviruses) suppression of cellular transcription by degradation of Rpb1. Red arrowheads indicate the direction of RNA transport in the neck of spherule.

subsequently internalized using phosphatidylinositol-3-kinase-, actin- and microtubule-dependent transport and finally localise on the internal surface of modified endosomes and lysosomes forming so-called cytopathic vacuoles (CPV-1) [86]. Disruption of this endocytic process by treatment with blebbistatin, wortmannin, or other inhibitors has little to no impact on the viral RNA synthesis [86, 90]. In contrast, in CHIKV-infected cells, the majority of spherules remain associated with the plasma membrane. Different phenotypes of SFV and CHIKV are due to differences in activation of phosphatidylinositol-3-kinase-Akt-mTor pathway mediated by nsP3 proteins of these viruses [91]. Furthermore, size of the SFV spherules is uniform and determined by the length of replicating viral RNA [92]. However, in CHIKV-infected human cells, spherules that remain associated with the plasma membrane are unexpectedly heterogeneous [92]. The large diameter of these compartments, which remain dynamically active in viral RNA synthesis, suggests a continuous growth of these organelles beyond the replication of a single RNA genome [93]. Do these differences have an impact on other properties of these viruses remains currently unknown.

Thus, all key events in alphavirus RNA synthesis depend on the protease activity of nsP2. However, only cleavage at the 3/4 site is absolutely required for RNA replication. In contrast, blocking processing at the 2/3 site or at both the 1/2 and 2/3 sites is not lethal; SINV mutants with two blocked cleavage sites can still replicate in interferon-defective cells to levels comparable to the wild-type virus [94, 95].

4 | nsP2: A Part of Core-Structure of Alphavirus RNA Replicase

The core of alphavirus late replicase consist of a ring structure formed by 12 molecules of nsP1 [96, 97], with a single nsP4 molecule situated in the central pore of the nsP1 ring and 1 nsP2 molecule positioned on the cytosolic side of the complex (Figure 3). Within this complex, the NTD and stalk sub-domains of nsP2h interact with the palm sub-domain of nsP4. Notably, nsP1 and nsP4 complexes can be formed from purified recombinant proteins [51]. Adding recombinant nsP2 to these complexes increases their RNA polymerase activity and enables the terminal adenylyltransferase activity, indicating cooperative interactions between nsP4 and nsP2.

Only a fraction of RNA replicase cores formed in infected cells are associated with spherules, and localise at the neck region of these structures (Figure 3). While the precise localization of nsP2p remains unclear, it is thought to be confined to an amorphous cytoplasmic ring structure associated with spherules [51]. Cytoplasmic ring, composed of 11 subunits of nsP3, incorporates host factors such as G3BPs (Ras-GTPase activating SH3 domain-binding proteins) and FHL1 (four-and-a-half LIM domain protein 1) [67]. Interestingly, majority of replicase cores are not associated with spherules, lack cytoplasmic ring structures [51], and are presumably not involved in the viral RNA synthesis. Their potential functions remain unknown. What is known is that nsP2 also plays a role in regulating the formation of RCs; excessive nsP2 levels lead to the cleavage of P1234 polyprotein into P12 and P34, which cannot form functional RCs [98].

One of the least understood aspects of alphavirus RNA replication is the mechanisms of the template RNA recognition and interactions between replicase proteins and genomic RNA (and dsRNA). Although nsP2 can bind RNA, evidence for sequence-specific binding is indirect. Changes in the 5′ cis-acting RNA elements of the viral genome result in the compensatory mutations in nsP2h [99, 100], and temperature-sensitive mutations in nsP2h have been shown to influence efficiency of SG RNA synthesis [101, 102]. As alphavirus nsP2 helicase translocates along ssRNA in a 5′ to 3′ direction [103], it could be assumed that nsP2 guides the 3′ end of genomic RNA into the spherule via the putative RNA channel formed by nsP1 and nsP4. However, recombinant nsP2 possesses low RNA helicase activity and very limited processivity. Interestingly, despite its designation as a nonstructural protein, nsP2 of SINV has been shown to be present in its virions [104]. While the exact reason for nsP2's inclusion into virions remains unclear, it is hypothesised that nsP2, the viral genome, capsid proteins and host factors such as G3BPs are interconnected [105], suggesting a link between alphavirus RNA synthesis and its encapsidation. Supporting this, mutations introduced into nsP2 of SFV result in the potential compensatory changes in the capsid protein [88], and mutations introduced into capsid protein of VEEV were shown to cause compensatory mutations in nsP2 [106].

5 | nsP2 Interacts With Host Proteins, Triggers Host Cell Shutoff and Enables Immune Evasion

nsP2 plays a critical role in alphavirus-host interactions. In infected cells, numerous host proteins are pulled down via nsP2 [107]. However, many of these proteins interact with RCs of alphavirus and not necessarily with nsP2 itself as they can also be pulled-down via nsP3 [108, 109]. nsP2 can also be found outside of the RCs or membrane-bound nsP1:nsP4:nsP2 complexes and can directly interact with host proteins. High-throughput yeast two-hybrid (HT-Y2H) assays identified heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and ubiquitin 4 (UBQLN4) as critical interactors of CHIKV nsP2, essential for viral replication. The interaction between hnRNP-K and nsP2 has also been demonstrated for SINV [108, 110].

While free nsP2 localises diffusely in the cytoplasm or binds to ribosomes [107, 111, 112], a significant fraction of it accumulates in the nucleus [113–115]. In SFV-infected vertebrate cells, approximately 50% of nsP2 resides in the nucleus, driven by nuclear localization signals (NLSs) [115–117]. For SFV, mutations in these NLSs prevent nuclear localization of nsP2, reducing RNA replication, cytotoxicity [118], and neurovirulence in mice [72]. However, for SINV and CHIKV, mutations in putative NLSs have little or no impact on the nuclear localization of nsP2 [119, 120].

Although the precise mechanism of nuclear transport remains controversial, nuclear accumulation of nsP2 is clearly critical for arithrogenic alphaviruses [74, 113]. In vertebrate cells, alphavirus infection induces host cell shutoff and prominent cytopathic effects [121]. For arithrogenic alphaviruses, these effects are mediated by nsP2. Mutations in the MTL domain of nsP2 reduce cytopathicity, enabling persistent replication [120, 122,
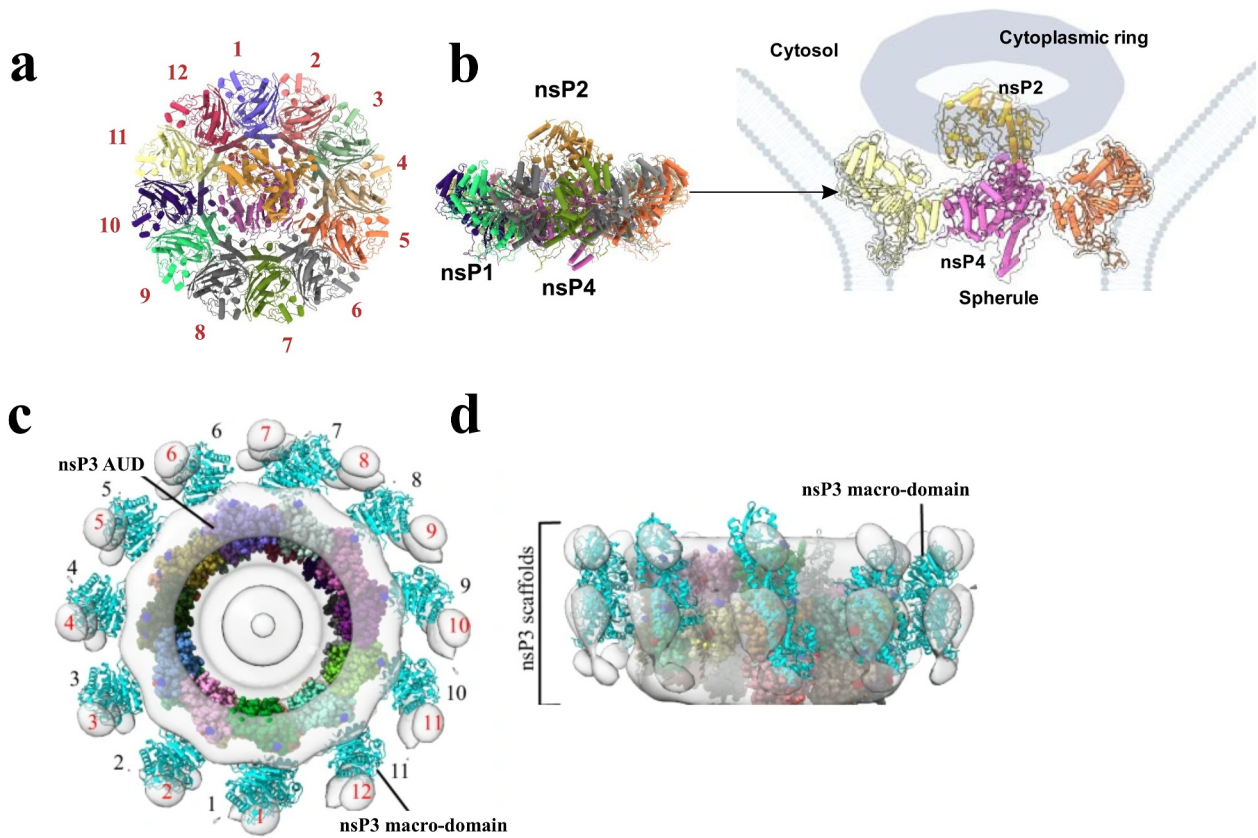
FIGURE 3 | Structure of alphavirus replicase complex. (a), a top view, and (b), a side view of the replicase core reconstructed from recombinant nsP1, nsP2, and nsP4 proteins. In the core structure of replicase complex one molecule of nsP4 sits within the central pore of the dodecameric ring formed by nsP1. The nsP4 interacts with the NTD and Stalk sub-domains of nsP2h. Image of the complex located in the neck of spherule is shown on right; the nsP2p is considered to be confined to a cytoplasmic ring structure. (c), a top view, and (d), a side view of the cytoplasmic ring composed of 11 nsP3 molecules. AUD, alphavirus unique domain of nsP3. Images are adapted from Tan et al., 2022 (a, b) and Kril et al., 2024 (c, d) [51, 67].

123]. The absence of host cell shutoff increases interferon (IFN) production, thereby inhibiting virus replication and spread in IFN-competent cell cultures [113]. The nsP2 of SINV, SFV, CHIKV and MAYV inhibits host cell transcription by inducing the degradation of the catalytic subunit of DNA-dependent RNA polymerase II (Rpb1) [73, 124]. This process is mediated by nsP2-induced Rpb1 ubiquitination rather than nsP2's protease activity [123] and depends on the integrity of the helicase and S-adenosylmethionine (SAM)-dependent methyltransferase-like domains. nsP2 induced transcriptional shutoff occurs before other virus-induced changes, such as apoptosis, autophagy and inhibition of STAT1 phosphorylation, thereby allowing the virus to evade innate immune responses [73]. Functions of nsP2 in suppression of antiviral immune response are, however, not limited to the general shutoff of host cell transcription. For example, CHIKV nsP2 can specifically inhibit the IFN-stimulated Janus kinase–signal transducer and activator of transcription (JAK-STAT) signalling pathway [125]. These effects are, at least to an extent, also controlled by a negative feedback loop. For example, CHIKV nsP2 interacts with the human autophagy receptor NDP52. This interaction limits the amount of nsP2 in the nucleus, which reduces host shutoff and cytopathic effects and prolongs CHIKV replication in human cells [126]. NTPase activity of nsP2 also contributes to the translational shutoff of host cells through the cAMP-PKA-eEF2K signalling pathway [127]. Interestingly, for encephalitic alphaviruses such as VEEV and EEEV, transcriptional shutoff is mediated by the capsid protein [128], while nsP2 of VEEV plays a crucial role in the shutoff of host cell translation [129].

In summary, nsP2 plays a central role in various stages of alphavirus infection including RNA replication and packaging, with its multifunctional nature influencing replication fidelity, template recognition, and the coordination of RNA synthesis with viral assembly. Furthermore, it interacts with other viral and host components and has an important role for virus-host interactions. The key activities of nsP2 of alphaviruses are summarised in Table 2.

6 | nsP2 as a Target for Antiviral Compounds

Alphaviruses cause acute infections and can lead to the development of chronic arthritic symptoms. The mechanism behind chronic disease caused by CHIKV infection remains poorly understood; however, studies suggest that macrophages may harbour persistently replicating virus, contributing to debilitating arthropathy [130]. Compounds targeting viral proteases are used clinically to treat both acute diseases, such as COVID-19 with nirmatrelvir and chronic diseases, such as human immunodeficiency virus type 1 infection with lopinavir/ritonavir, darunavir, and others or hepatitis C with simeprevir, grazoprevir, and others [131–134]. Alphavirus nsP2, which is crucial

TABLE 2 | Main functions of alphavirus nsP2.

Activity of alphavirus nsP2	Functions	Importance
RNA helicase	Unwinding dsRNAs [56, 59, 75].	Crucial for viral RNA replication [59].
Nucleoside triphosphatase (NTPase)	Hydrolysis of NTPs [57].	Energy source of RNA helicase, important for viral RNA replication, contributes to the host cell translational shutoff [127].
RNA triphosphatase (RTPase)	Removal of γ-phosphate from the 5′ end of genomic and subgenomic RNAs [58].	Crucial for viral RNA capping, essential for protection of viral RNAs from degradation and recognition by the host immune system [58].
Cysteine protease	Processing of non-structural polyproteins [55].	Regulation of the replicase complex formation and viral RNA replication [55, 90].
Non-enzymatic activities	Degradation of cellular DNA-dependent RNA polymerase II (Rpb1) [73, 124].	Shut-off of host cell transcription. Blocking of antiviral responses [73].
	Translocation to the nucleus and nucleolus of vertebrate cells [113–115].	Influencing -cytotoxicity and virulence of SFV [118].
	Interacting with components of RC, capsid, and host proteins [51, 105, 126].	Important for viral RNA replication and encapsidation of genomic RNA [51, 105, 108].
	Inhibition of IFN-stimulated Janus kinase–signal transducer and activator of transcription (JAK-STAT) signalling pathway [125].	Important in viral pathogenesis [125].

for RC formation and functioning as well as for suppression of the antiviral responses (Table 2), is, therefore, a promising target for antiviral drug development. However, the complex structure and interconnected functions of nsP2 have made it a challenging target.

To date, the majority of inhibitors of alphavirus nsP2 target its protease activity. This is partly due to the availability of high-resolution 3D-structures of nsP2p from several alphaviruses, including CHIKV (3TRK) and VEEV (2HWK) [78, 79], which facilitate structure-based in silico drug design approaches [135–139]. The known 3D structures can also be used to predict the structures of proteases from other alphaviruses, expanding the list of potential targets. For example, Bassetto and colleagues utilised the structure of VEEV nsP2p to predict the 3D structure of CHIKV nsP2p, resulting in a model that closely resembles the actual 3D structure of CHIKV nsP2p, which became available several years later. The virtual screening identified hits that were shown to inhibit CHIKV replication in vitro with an effective concentration of 50 (EC_{50}) of 5 μM [140]. A combination of in silico screening and a cell culture virus-yield reduction assay was also employed to develop inhibitors of VEEV nsP2 protease [141]. However, this method lacks biological validation for target specificity, and later studies revealed that the compound identified by Bassetto and colleagues did not inhibit CHIKV nsP2 protease in a cell-free assay [142]. To address this, an improved method was developed that integrates in silico screening, cell-free protease assay using recombinant nsP2p and cell-based virus-yield reduction assay [142].

The cell-free protease assay is based on the ability of nsP2p, a shorter and more easily expressed and purified region of the nsP2, to cleave in trans substrates corresponding to 1/2 or 3/4 sites in P1234 polyprotein. Due to the higher cleavage efficiency

at the 3/4 site, a corresponding cleavable linker is used in both recombinant proteins and synthetic peptide-based substrates. In the latter case, fluorescence resonance energy transfer (FRET) is used to detect processing [75, 142–144], enabling the use of high-throughput screening (HTS) assays [141, 145–149]. Compounds reported to inhibit nsP2 are summarized in Table 3. However, it is important to note that results from cell-free and cell-based assays often differ. Some compounds that do not inhibit nsP2 protease activity in cell-free assays have been shown to inhibit virus infection, while others that effectively inhibit nsP2 protease activity demonstrate little or no antiviral effects [142, 155, 159]. Such discrepancies are hardly surprising, as cell-free assays do not account for the ability of compounds to penetrate the plasma membrane or their stability within cells. Furthermore, in the processing cascade leading to RC formation, both the 1/2 and 3/4 sites in P1234 polyprotein are cleaved in cis, and therefore, the requirements for their processing are likely different from those of substrates used in cell-free reactions. In addition, as described above, the cleavage site requirements of nsP2 are complex. For instance, the 2/3 site cannot be cleaved by nsP2p alone and requires the presence of the nsP3 macro-domain in the substrate. Finally, in infected cells, the consequences of lack of cleavage at different processing sites are drastically different: only cleavage at the 3/4 site is mandatory, whereas a slowdown in the cleavage of the 1/2 site (and possibly the 2/3 site) may even positively impact virus RNA replication. This latter property raises concerns regarding the sub-optimal efficiency of nsP2 inhibitors, which, until recently, have demonstrated activities only in the micromolar range (Table 3). An important breakthrough was achieved in 2024 with the development of the first CHIKV nsP2 protease inhibitor with an EC_{50} of approximately 10 nM, using a covalent fragment-based screening approach [146]. This achievement underscores the potential for clinically applicable nsP2 protease

TABLE 3 | Compounds targeting alphavirus nsP2 protease.

Compound	Structure	Targeted virus	EC$_{50}$ (µM)	CC$_{50}$ (µM)	Selectivity index (SI = CC$_{50}$/EC$_{50}$)	Cell line	Ref.
(E)-3-(4-tert-butyl phenyl)-N-[(E)-(3,4-diethoxyphenyl) methylideneamino] prop-2-enamide (compound 25)		CHIKV	3.2 ± 1.8	101 ± 50	32	Vero	[140, 150, 151]
5-chloro-N-[4-[C-methyl-N-[(2-phenylcyclopropanecarbonyl) amino] carbonimidoyl] phenyl] thiophene-2-carboxamide (compound 8)		CHIKV	1.5	> 200	> 133.3	BHK21	[142]
ID1452-2		CHIKV	31	> 31	Not specified	HEK293	[152]
Novobiocin		CHIKV	20	Not specified	Not specified	Vero	[153]
Telmisartan		CHIKV	45	Not specified	Not specified	Vero	[153]
MBZM-N-IBT		CHIKV	38.68	> 800	> 21	Vero	[154]
1,3-thiazolidin-4-one (compound 7)		CHIKV	0.42	> 100	> 238	Vero	[155]
Peptidomimetic 3a		CHIKV	8.76	Not specified	Not specified	Vero	[156]
β-Aminomethyl vinyl sulfone RA-0002034		CHIKV	0.01	> 10 µM	> 1000	MRC5	[146]
LMQ330		CHIKV	5.2 ± 0.52	165.3 ± 2.7	31.78	Vero	[149]
NCGC00488909-01		VEEV	1.76	> 50	> 28	A549	[141]
Vinyl sulfone-based inhibitor (compound 11)		VEEV	2.4	30	12	HeLa	[157]
CA-074		VEEV	Not specified	Not specified	Not specified	HEK293 vero A549	[158]

inhibitors and highlights the need for improved screening methods and advanced cell-based assays (compatible with HTS) to identify and characterize these inhibitors.

Compounds targeting other nsP2 functions are rare. No inhibitors of nsP2 RNA helicase, NTPase, or RNA triphosphatase activities have been reported. However, with the published

structures of nsP2h and its complex with nsP4 [51, 160], the in silico design and cell-based analysis of such compounds has become feasible. A cell-based *trans*-reporter assay, measuring the induction of luciferase gene expression by an artificial transcription factor, has been developed and used to identify compounds that block nsP2-induced transcriptional shutoff and inhibit CHIKV replication [108, 152]. The use of mutations in nsP2 that permit persistent replication of alphavirus replicons (modified genomes in which ORF2 is replaced with a sequence encoding a reporter and/or selection marker) has led to the development of stable replicon cell lines. These cell lines serve as valuable tools for screening antiviral compounds targeting the functions of ns proteins. Stable cell lines harbouring non-cytopathic CHIKV replicons have been used for antiviral screening, leading to the identification of β-d-N4-hydroxy-ycytidine (NHC), as well as 5,7-dihydroxyflavones apigenin, chrysin, naringenin and silybin. Some of these compounds, among other mechanisms, may also target nsP2 functions and have been shown to suppress CHIKV replication [161, 162].

Alphavirus RNA replication is error-prone; hence these viruses are expected to develop resistance against directly acting anti-virals, including nsP2 inhibitors. As truly active nsP2 inhibitors are just starting to emerge, no data concerning virus resistance to such compounds is available. However, the resistance-associated mutations have been used to identify nsP2 as the target of quinazolinone compound CID15997213, an inhibitor of VEEV and WEEV, as well as radicicol, a heat shock protein 90 (Hsp90) inhibitor that effectively suppresses CHIKV replication [163, 164]. Resistance-associated mutations in nsP2, along with those in nsP4, were also obtained during the passaging of VEEV in the presence of benzamidine ML336 [165].Furthermore, mutations both in nsP2 and nsP4 emerged in response to treatment of CHIKV infected cells with mutagenic agent riba-virin [144] or with 4′-fluorouridine, a chain terminator targeting nsP4 [166]. In both cases, it was observed that, on their own, mutations in nsP2 had little impact; however, they did slightly increase the impact of mutations located in nsP4. A similar situation was observed with the CHVB series, a novel class of CHIKV inhibitors that target the viral capping machinery. In this case, mutations in nsP1 were coupled with mutation located in the MTL domain of nsP2. Though the mutations in nsP1 were necessary and sufficient to achieve the resistance, it was suggested that mutation in nsP2 has some impact, possibly due to the interaction of MTL with nsP1 during the viral RNA capping [167]. Combinations or resistance-associated mutations in nsP1 and nsP2 or in nsP4 and nsP2 may also reflect the molecular architecture of the alphavirus replicase core (Figure 3), where these proteins interact with each other and function in a coordinated manner.

It should also be taken into account that alphavirus nsP2 is a multifunctional fine-tune instrument involved in many activities, from viral replication to shutoff of hosts' antiviral responses. Therefore, it is also reasonable to expect that mutations, allowing resistants to nsP2 inhibitors, likely also affect other important functions of the protein. Thus, the resistance to nsP2 inhibitors is likely associated with high fitness costs. This may generate high genetic barriers for resistance, making the development of nsP2 inhibitors especially attractive.

7 | nsP2 Can be Exploited for Alphavirus Attenuation and as a Potential Tool for Vector Control

The critical role of nsP2 in the host-cell shutoff, counteraction of the IFN response, and the pathogenesis of alphavirus infections offers numerous opportunities to develop advanced alphavirus-based gene expression systems, including improved self-replicating RNAs and rationally attenuated viruses. These applications require precise knowledge of nsP2 functions. For example, selecting alphaviruses incapable of inducing transcriptional shutoff led to the identification of a short, highly variable peptide loop (VLoop) in nsP2 of SINV and CHIKV. Mutations in the VLoop enable the corresponding viruses to establish persistent, noncytopathic replication in mammalian cells [168] and attenuate CHIKV replication in vivo [169]. In another study, a mutation affecting the P4 residue of the 1/2 cleavage site in CHIKV, paired with a mutation in the corresponding S4 residue of substrate-binding pocket of nsP2p [170], was found to strongly attenuate virus replication in IFN competent cells. This mutant virus was avirulent in vivo, and mice immunized with it were fully protected against challenge with wild-type CHIKV [171]. A manipulation that slowed cleavage at the 1/2 site of RRV produced similar phenotypic effects [172]. These and other attenuated alphaviruses represent promising candidates for highly effective live attenuated vaccines to prevent infections of RRV, ONNV, MAYV and other alphaviruses pathogenic for humans.

In alphavirus-infected cells, the accumulation of free nsP2 halts RC formation by cleaving P1234 polyprotein into P12 and P34. This mechanism is also employed by some of alphaviruses to establish superinfection exclusion (SIE), a phenomenon where a preexisting viral infection prevents secondary infection by the same or closely related virus [173–180]. For instance, SINV infection inhibits replication of superinfecting SINV, SFV and RRV in mosquito (*Aedes albopictus*) cell lines [178]. Similarly, prior infection of *Aedes aegypti* mosquitoes with the insect-specific Eilat virus delays the dissemination of CHIKV by 3 days [181]. The expression of nsP2 induces a phenotype that mimics SIE, offering a potential strategy for creating alphavirus-refractory systems in mosquitoes [182–184]. As a proof of concept, Reitmayer and colleagues developed transgenic *Aedes aegypti* mosquitoes expressing nsP2 of CHIKV or SINV, demonstrating reduced replication and dissemination of the respective viruses in these mosquitoes [184]. This approach highlights the potential of developing nsP2-expressing transgenic mosquitoes that restrict the dissemination of multiple pathogenic alphaviruses. Reduction of vector competence of a mosquito is, therefore, a promising arbovirus control strategy that can be applied to reduce cases of human infection as well as to limit the spread of alphaviruses to new geographical locations.

8 | Perspectives

Significant progress has been made in identifying host factors as targets for treating alphavirus disease; however, targeting viral factors remains equally important [185–188]. Targeting alphavirus-encoded proteins, such as nsP2, with directly acting

antivirals represents the most straightforward approach towards the development of specific treatments of diseases caused by alphaviruses. Despite numerous studies, many nsP2 functions remain poorly understood; furthermore, characterisation of nsP2 functions has been mostly performed using CHIKV, SFV, SINV, and, to a lesser extent, VEEV. Consequently, studies are needed to confirm whether the obtained findings apply equally across all alphaviruses. Moreover, many important activities of nsP2 are still unknown. In particular, there is a lack of information concerning the role of nsP2 in the early replicase complexes, including its interactions with viral genome and the exact role of its NTPase/RNA helicase activities. It is especially attractive to target the early stages of infection when the virus is not yet fully established and is most vulnerable. Further studies are needed to uncover these and other unknown facets of nsP2 in alphavirus infection.

As multi-functional protein nsP2 is difficult to study and represents a challenging target. Furthermore, an individual nsP2 may not be the most promising target; instead, it could be nsP2 included to P1234 and P123 polyproteins, that are indispensable for alphavirus replicase complex formation. As of now, the development of drugs to prevent replicase complex formation is hampered by the lack of information about the structural organisation of P123 and P1234 polyproteins. The ability of nsP2 to process P1234 polyprotein into P123 and nsP4—a critical event for replicase complex formation—both in cis and *in trans* represents an additional hurdle. Studies of functions of nsP2 functions using advanced methods of structural biology, single molecule trafficking, etc, would offer new possibilities for targeting this protein with antiviral compounds. As of now, compared to targeting the protease activity of nsP2, targeting its other functions has been clearly under-explored. Recent advances, especially resolving the structure of the core of alphavirus replicase, open perspective to apply rational drug development approaches to target helicase part of nsP2 and/or inhibit its interaction with nsP4. Such approaches could be combined with the development of high throughput screening compatible cell-based assays using P1234 polyprotein. The combination of these assays holds a key for the identification of potent broad-spectrum inhibitors that could serve as leads for the development of direct-acting antivirals to treat alphavirus infections.

Increasing our understanding of nsP2′s structure, functions, and interactions could also lead to broadly applicable strategies for alphavirus attenuation, supporting the development of novel live attenuated vaccines. It could be expected that these vaccines will, similar to IxChiq (the licenced vaccine against CHIKV), create long-lasting protection. The combination of efficient vaccines with potent, directly acting antivirals represents a unique and powerful combination allowing new treatment/prevention strategies as well as unique opportunities to counter problems caused by rare side effects of live attenuated vaccines.

Author Contributions

S.W. and A.M. conceptualised and draughted the manuscript. S.M. critically reviewed the manuscript. All authors approved the final version of the manuscript.

Conflicts of Interest

Andres Merits is listed as an inventor on patent number 11201907272Y, titled 'Alphavirus nsP Mutants as Vaccines' (March 27, 2024). Suresh Mahalingam is an inventor on patent number WO/2017/201579, titled 'Arthrogenic Alphavirus Vaccine'. Suresh Mahalingam serves as an editorial board member for Reviews in Medical Virology.

Data Availability Statement

The authors have nothing to report.

References

1. R. Chen, S. Mukhopadhyay, A. Merits, et al., "ICTV Virus Taxonomy Profile: Togaviridae," *Journal of General Virology* 99, no. 6 (2018): 761–762, https://doi.org/10.1099/jgv.0.001072.

2. O. W. Lwande, J. Lutomiah, V. Obanda, et al., "Isolation of Tick and Mosquito-Borne Arboviruses From Ticks Sampled From Livestock and Wild Animal Hosts in Ijara District, Kenya," *Vector Borne and Zoonotic Diseases* 13, no. 9 (2013): 637–642, https://doi.org/10.1089/vbz.2012.1190.

3. R. O. Hayes, D. B. Francy, J. S. Lazuick, G. C. Smith, and E. P. J. Gibbs, "Role of the Cliff Swallow Bug (Oeciacus Vicarius) in the Natural Cycle of a Western Equine Encephalitis-Related Alphavirus," *Journal of Medical Entomology* 14, no. 3 (1977): 257–262, https://doi.org/10.1093/jmedent/14.3.257.

4. B. Li, H. Wang, and G. Liang, "Getah Virus (Alphavirus): An Emerging, Spreading Zoonotic Virus," *Pathogens* 11, no. 8 (2022): 945, https://doi.org/10.3390/pathogens11080945.

5. M. La Linn, J. Gardner, D. Warrilow, et al., "Arbovirus of Marine Mammals: A New Alphavirus Isolated From the Elephant Seal Louse, Lepidophthirus Macrorhini," *Journal of Virology* 75, no. 9 (2001): 4103–4109, https://doi.org/10.1128/JVI.75.9.4103-4109.2001.

6. D. Sissoko, D. Malvy, K. Ezzedine, et al., "Post-Epidemic Chikungunya Disease on Reunion Island: Course of Rheumatic Manifestations and Associated Factors Over a 15-Month Period," *PLoS Neglected Tropical Diseases* 3, no. 3 (2009): e389, https://doi.org/10.1371/journal.pntd.0000389.

7. P. Gérardin, A. Fianu, D. Malvy, et al., "Perceived Morbidity and Community Burden After a Chikungunya Outbreak: The TELECHIK Survey, a Population-Based Cohort Study," *BMC Medicine* 9, no. 1 (2011): 5, https://doi.org/10.1186/1741-7015-9-5.

8. S. P. Manimunda, P. Vijayachari, R. Uppoor, et al., "Clinical Progression of Chikungunya Fever During Acute and Chronic Arthritic Stages and the Changes in Joint Morphology as Revealed by Imaging," *Transactions of the Royal Society of Tropical Medicine and Hygiene* 104, no. 6 (2010): 392–399, https://doi.org/10.1016/j.trstmh.2010.01.011.

9. A. Suhrbier, M. C. Jaffar-Bandjee, and P. Gasque, "Arthritogenic Alphaviruses—An Overview," *Nature Reviews Rheumatology* 8, no. 7 (2012): 420–429, https://doi.org/10.1038/nrrheum.2012.64.

10. J. H. Strauss and E. G. Strauss, "The Alphaviruses: Gene Expression, Replication, and Evolution," *Microbiological Reviews* 58, no. 3 (1994): 491–562, https://doi.org/10.1128/mr.58.3.491-562.1994.

11. A. M. Powers, A. C. Brault, Y. Shirako, et al., "Evolutionary Relationships and Systematics of the Alphaviruses," *Journal of Virology* 75, no. 21 (2001): 10118–10131, https://doi.org/10.1128/JVI.75.21.10118-10131.2001.

12. K. A. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, and S. Higgs, "A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential," *PLoS Pathogens* 3, no. 12 (2007): e201, https://doi.org/10.1371/journal.ppat.0030201.

13. L. A. Silva and T. S. Dermody, "Chikungunya Virus: Epidemiology, Replication, Disease Mechanisms, and Prospective Intervention Strategies," *Journal of Clinical Investigation* 127, no. 3 (2017): 737–749, https://doi.org/10.1172/JCI84417.

14. M. C. Jaffar-Bandjee, D. Ramful, B. A. Gauzere, et al., "Emergence and Clinical Insights into the Pathology of Chikungunya Virus Infection," *Expert Review of Anti-infective Therapy* 8, no. 9 (2010): 987–996, https://doi.org/10.1586/eri.10.92.

15. K. P. Vijayakumar, T. S. Nair Anish, B. George, T. Lawrence, S. C. Muthukkutty, and R. Ramachandran, "Clinical Profile of Chikungunya Patients During the Epidemic of 2007 in Kerala, India," *Journal of Global Infectious Diseases* 3, no. 3 (2011): 221–226, https://doi.org/10.4103/0974-777X.83526.

16. B. V. Tandale, P. S. Sathe, V. A. Arankalle, et al., "Systemic Involvements and Fatalities During Chikungunya Epidemic in India, 2006," *Journal of Clinical Virology* 46, no. 2 (2009): 145–149, https://doi.org/10.1016/j.jcv.2009.06.027.

17. M. L. Brito Ferreira, M. de F. P. Militão de Albuquerque, C. A. A. de Brito, et al., "Neurological Disease in Adults With Zika and Chikungunya Virus Infection in Northeast Brazil: A Prospective Observational Study," *Lancet Neurology* 19, no. 10 (2020): 826–839, https://doi.org/10.1016/S1474-4422(20)30232-5.

18. G. Rezza, R. Chen, and S. C. Weaver, "O'nyong-nyong Fever: A Neglected Mosquito-Borne Viral Disease," *Pathogens and Global Health* 111, no. 6 (2017): 271–275, https://doi.org/10.1080/20477724.2017.1355431.

19. D. L. Posey, T. O'rourke, J. T. Roehrig, R. S. Lanciotti, M. Weinberg, and S. Maloney, "O'Nyong-Nyong Fever in West Africa," *American Journal of Tropical Medicine and Hygiene* 73, no. 1 (2005): 32, https://doi.org/10.4269/ajtmh.2005.73.1.0730032.

20. R. L. Doherty, J. G. Carley, B. H. Kay, C. Filippich, E. N. Marks, and C. L. Frazier, "Isolation of Virus Strains From Mosquitoes Collected in Queensland, 1972-1976," *Australian Journal of Experimental Biology & Medical Science* 57, no. 5 (1979): 509–520, https://doi.org/10.1038/icb.1979.52.

21. I. D. Marshall, G. M. Woodroofe, and S. Hirsch, "Viruses Recovered From Mosquitoes and Wildlife Serum Collected in the Murray Valley of South-Eastern Australia, February 1974, During an Epidemic of Encephalitis," *Australian Journal of Experimental Biology & Medical Science* 60, no. Pt 5 (1982): 457–470, https://doi.org/10.1038/icb.1982.51.

22. J. P. Flexman, D. W. Smith, J. S. Mackenzie, et al., "A Comparison of the Diseases Caused by Ross River Virus and Barmah Forest Virus," *Medical Journal of Australia* 169, no. 3 (1998): 159–163, https://doi.org/10.5694/j.1326-5377.1998.tb116019.x.

23. E. Wishart, K. A. O 'Grady, J. Passmore, and R. Moran, "An Outbreak of Barmah Forest Virus Disease in Victoria," *Communicable Diseases Intelligence Quarterly Report* 26, no. 4 (2020), https://doi.org/10.3316/ielapa.511388563985454.

24. I. M. Mackay and K. E. Arden, "Mayaro Virus: A Forest Virus Primed for a Trip to the City?," *Microbes and Infection* 18, no. 12 (2016): 724–734, https://doi.org/10.1016/j.micinf.2016.10.007.

25. K. C. Long, S. A. Ziegler, S. Thangamani, et al., "Experimental Transmission of Mayaro Virus by *Aedes aegypti*," *American Journal of Tropical Medicine and Hygiene* 85, no. 4 (2011): 750–757, https://doi.org/10.4269/ajtmh.2011.11-0359.

26. A. J. Auguste, J. Liria, N. L. Forrester, et al., "Evolutionary and Ecological Characterization of Mayaro Virus Strains Isolated During an Outbreak, Venezuela, 2010," *Emerging Infectious Diseases* 21, no. 10 (2015): 1742–1750, https://doi.org/10.3201/eid2110.141660.

27. R. S. S. Azevedo, E. V. P. Silva, V. L. Carvalho, et al., "Mayaro Fever Virus, Brazilian Amazon," *Emerging Infectious Diseases* 15, no. 11 (2009): 1830–1832, https://doi.org/10.3201/eid1511.090461.

28. M. S. Shocket, S. J. Ryan, and E. A. Mordecai, "Temperature Explains Broad Patterns of Ross River Virus Transmission," *Elife* 7 (2018): e37762, https://doi.org/10.7554/eLife.37762.

29. E. B. Stephenson, A. J. Peel, S. A. Reid, C. C. Jansen, and H. McCallum, "The Non-Human Reservoirs of Ross River Virus: A Systematic Review of the Evidence," *Parasites & Vectors* 11, no. 1 (2018): 188, https://doi.org/10.1186/s13071-018-2733-8.

30. D. Harley, A. Sleigh, and S. Ritchie, "Ross River Virus Transmission, Infection, and Disease: A Cross-Disciplinary Review," *Clinical Microbiology Reviews* 14, no. 4 (2001): 909–932, https://doi.org/10.1128/cmr.14.4.909-932.2001.

31. R. M. Taylor, H. S. Hurlbut, T. H. Work, J. R. Kingston, and T. E. Frothingham, "Sindbis Virus: A Newly Recognized Arthropod-Transmitted Virus," *American Journal of Tropical Medicine and Hygiene* 4, no. 5 (1955): 844–862, https://doi.org/10.4269/ajtmh.1955.4.844.

32. S. Harding, S. Sewgobind, and N. Johnson, "JMM Profile: Sindbis Virus, a Cause of Febrile Illness and Arthralgia," *Journal of Medical Microbiology* 72, no. 3 (2023): 001674, https://doi.org/10.1099/jmm.0.001674.

33. S. Kurkela, T. Manni, J. Myllynen, A. Vaheri, and O. Vapalahti, "Clinical and Laboratory Manifestations of Sindbis Virus Infection: Prospective Study, Finland, 2002-2003," *Journal of Infectious Diseases* 191, no. 11 (2005): 1820–1829, https://doi.org/10.1086/430007.

34. M. Laine, R. Luukkainen, and A. Toivanen, "Sindbis Viruses and Other Alphaviruses as Cause of Human Arthritic Disease," *Journal of Internal Medicine* 256, no. 6 (2004): 457–471, https://doi.org/10.1111/j.1365-2796.2004.01413.x.

35. P. V. Aguilar, J. G. Estrada-Franco, R. Navarro-Lopez, C. Ferro, A. D. Haddow, and S. C. Weaver, "Endemic Venezuelan Equine Encephalitis in the Americas: Hidden Under the Dengue Umbrella," *Future Virology* 6, no. 6 (2011): 721–740, https://doi.org/10.2217/FVL.11.5.

36. M. A. Zacks and S. Paessler, "Encephalitic Alphaviruses," *Veterinary Microbiology* 140, no. 3–4 (2009): 281–286, https://doi.org/10.1016/j.vetmic.2009.08.023.

37. S. C. Weaver, C. Ferro, R. Barrera, J. Boshell, and J. C. Navarro, "Venezuelan Equine Encephalitis," *Annual Review of Entomology* 49, no. 1 (2004): 141–174, https://doi.org/10.1146/annurev.ento.49.061802.123422.

38. B. Kumar, A. Manuja, B. R. Gulati, N. Virmani, and B. N. Tripathi, "Zoonotic Viral Diseases of Equines and Their Impact on Human and Animal Health," *Open Virology Journal* 12, no. 1 (2018): 80–98, https://doi.org/10.2174/1874357901812010080.

39. G. Avilés, M. S. Sabattini, and C. J. Mitchell, "Transmission of Western Equine Encephalomyelitis Virus by Argentine Aedes Albifasciatus (Diptera: Culicidae)," *Journal of Medical Entomology* 29, no. 5 (1992): 850–853, https://doi.org/10.1093/jmedent/29.5.850.

40. A. S. Campos, A. C. Franco, F. Godinho, et al., "Molecular Epidemiology of Western Equine Encephalitis Virus in Brazil, 2023–2024," *medRxiv*, (2024), https://doi.org/10.1101/2024.04.15.24305848. Published April 18, 2024.

41. J. Soghigian, T. G. Andreadis, and G. Molaei, "Population Genomics of Culiseta Melanura, the Principal Vector of Eastern Equine Encephalitis Virus in the United States," *PLoS Neglected Tropical Diseases* 12, no. 8 (2018): e0006698, https://doi.org/10.1371/journal.pntd.0006698.

42. S. Mukherjee, E. E. Moody, K. Lewokzco, et al., "Eastern Equine Encephalitis in Tennessee: 2002-2008," *Journal of Medical Entomology* 49, no. 3 (2012): 731–738, https://doi.org/10.1603/me11151.

43. O. Schwartz and M. L. Albert, "Biology and Pathogenesis of Chikungunya Virus," *Nature Reviews Microbiology* 8, no. 7 (2010): 491–500, https://doi.org/10.1038/nrmicro2368.

44. K. A. Tsetsarkin, R. Chen, M. B. Sherman, and S. C. Weaver, "Chikungunya Virus: Evolution and Genetic Determinants of Emergence," *Current Opinion in Virology* 1, no. 4 (2011): 310–317, https://doi.org/10.1016/j.coviro.2011.07.004.

45. H. Cordel, I. Quatresous, C. Paquet, and E. Couturier, "Imported Cases of Chikungunya in Metropolitan France, April 2005 - February 2006. Weekly Releases," *Weekly releases (1997–2007)* 11, no. 16 (2006): 2944, https://doi.org/10.2807/esw.11.16.02944-en.

46. Commissioner O of the, *FDA Approves First Vaccine to Prevent Disease Caused by Chikungunya Virus* (FDA, 2024)https://www.fda.gov/news-events/press-announcements/fda-approves-first-vaccine-prevent-disease-caused-chikungunya-virus.

47. E. G. Strauss, C. M. Rice, and J. H. Strauss, "Complete Nucleotide Sequence of the Genomic RNA of Sindbis Virus," *Virology* 133, no. 1 (1984): 92–110, https://doi.org/10.1016/0042-6822(84)90428-8.

48. E. G. Strauss, C. M. Rice, and J. H. Strauss, "Sequence Coding for the Alphavirus Nonstructural Proteins Is Interrupted by an Opal Termination Codon," *Proceedings of the National Academy of Sciences of the United States of America* 80, no. 17 (1983): 5271–5275, https://doi.org/10.1073/pnas.80.17.5271.

49. Y. S. Law, S. Wang, Y. B. Tan, et al., "Interdomain Flexibility of Chikungunya Virus nsP2 Helicase-Protease Differentially Influences Viral RNA Replication and Infectivity," *Journal of Virology* 95, no. 6 (2021), https://doi.org/10.1128/jvi.01470-20.

50. J. Peränen, P. Laakkonen, M. Hyvönen, and L. Kääriäinen, "The Alphavirus Replicase Protein nsP1 Is Membrane-Associated and Has Affinity to Endocytic Organelles," *Virology* 208, no. 2 (1995): 610–620, https://doi.org/10.1006/viro.1995.1192.

51. Y. B. Tan, D. Chmielewski, M. C. Y. Law, et al., "Molecular Architecture of the Chikungunya Virus Replication Complex," *Science Advances* 8, no. 48 (2022): eadd2536, https://doi.org/10.1126/sciadv.add2536.

52. T. Ahola and L. Kääriäinen, "Reaction in Alphavirus mRNA Capping: Formation of a Covalent Complex of Nonstructural Protein nsP1 With 7-Methyl-GMP," *Proceedings of the National Academy of Sciences of the United States of America* 92, no. 2 (1995): 507–511, https://doi.org/10.1073/pnas.92.2.507.

53. S. Mi and V. Stollar, "Expression of Sindbis Virus nsP1 and Methyltransferase Activity in *Escherichia coli*," *Virology* 184, no. 1 (1991): 423–427, https://doi.org/10.1016/0042-6822(91)90862-6.

54. W. R. Hardy and J. H. Strauss, "Processing the Nonstructural Polyproteins of Sindbis Virus: Nonstructural Proteinase Is in the C-Terminal Half of nsP2 and Functions Both in Cis and in Trans," *Journal of Virology* 63, no. 11 (1989): 4653–4664, https://doi.org/10.1128/JVI.63.11.4653-4664.1989.

55. K. Rausalu, A. Utt, T. Quirin, et al., "Chikungunya Virus Infectivity, RNA Replication and Non-structural Polyprotein Processing Depend on the nsP2 Protease's Active Site Cysteine Residue," *Scientific Reports* 6, no. 1 (2016): 37124, https://doi.org/10.1038/srep37124.

56. M. Gomez de Cedrón, N. Ehsani, M. L. Mikkola, J. A. García, and L. Kääriäinen, "RNA Helicase Activity of Semliki Forest Virus Replicase Protein NSP2," *FEBS Letters* 448, no. 1 (1999): 19–22, https://doi.org/10.1016/s0014-5793(99)00321-x.

57. M. Rikkonen, J. Peränen, and L. Kääriäinen, "ATPase and GTPase Activities Associated With Semliki Forest Virus Nonstructural Protein nsP2," *Journal of Virology* 68, no. 9 (1994): 5804–5810, https://doi.org/10.1128/JVI.68.9.5804-5810.1994.

58. L. Vasiljeva, A. Merits, P. Auvinen, and L. Kääriäinen, "Identification of a Novel Function of the Alphavirus Capping Apparatus. RNA 5'-triphosphatase Activity of Nsp2," *Journal of Biological Chemistry* 275, no. 23 (2000): 17281–17287, https://doi.org/10.1074/jbc.M910340199.

59. Y. S. Law, A. Utt, Y. B. Tan, et al., "Structural Insights into RNA Recognition by the Chikungunya Virus nsP2 Helicase," *Proceedings of the National Academy of Sciences of the U S A* 116, no. 19 (2019): 9558–9567, https://doi.org/10.1073/pnas.1900656116.

60. Y. F. Wang, S. G. Sawicki, and D. L. Sawicki, "Alphavirus nsP3 Functions to Form Replication Complexes Transcribing Negative-Strand RNA," *Journal of Virology* 68, no. 10 (1994): 6466–6475, https://doi.org/10.1128/JVI.68.10.6466-6475.1994.

61. M. W. LaStarza, J. A. Lemm, and C. M. Rice, "Genetic Analysis of the nsP3 Region of Sindbis Virus: Evidence for Roles in Minus-Strand and Subgenomic RNA Synthesis," *Journal of Virology* 68, no. 9 (1994): 5781–5791, https://doi.org/10.1128/JVI.68.9.5781-5791.1994.

62. Y. Gao, N. Goonawardane, J. Ward, A. Tuplin, and M. Harris, "Multiple Roles of the Non-Structural Protein 3 (nsP3) Alphavirus Unique Domain (AUD) During Chikungunya Virus Genome Replication and Transcription," *PLoS Pathogens* 15, no. 1 (2019): e1007239, https://doi.org/10.1371/journal.ppat.1007239.

63. I. M. Cristea, J. W. N. Carroll, M. P. Rout, C. M. Rice, B. T. Chait, and M. R. MacDonald, "Tracking and Elucidating Alphavirus-Host Protein Interactions," *Journal of Biological Chemistry* 281, no. 40 (2006): 30269–30278, https://doi.org/10.1074/jbc.M603980200.

64. M. Amaya, T. Brooks-Faulconer, T. Lark, et al., "Venezuelan Equine Encephalitis Virus Non-Structural Protein 3 (nsP3) Interacts With RNA Helicases DDX1 and DDX3 in Infected Cells," *Antiviral Research* 131 (2016): 49–60, https://doi.org/10.1016/j.antiviral.2016.04.008.

65. M. Neuvonen, A. Kazlauskas, M. Martikainen, A. Hinkkanen, T. Ahola, and K. Saksela, "SH3 Domain-Mediated Recruitment of Host Cell Amphiphysins by Alphavirus nsP3 Promotes Viral RNA Replication," *PLoS Pathogens* 7, no. 11 (2011): e1002383, https://doi.org/10.1371/journal.ppat.1002383.

66. W. H. Ng, X. Liu, Z. L. Ling, et al., "FHL1 Promotes Chikungunya and O'Nyong-Nyong Virus Infection and Pathogenesis With Implications for Alphavirus Vaccine Design," *Nature Communications* 14, no. 1 (2023): 6605, https://doi.org/10.1038/s41467-023-42330-2.

67. V. Kril, M. Hons, C. Amadori, et al., "Alphavirus nsP3 Organizes into Tubular Scaffolds Essential for Infection and the Cytoplasmic Granule Architecture," *Nature Communications* 15, no. 1 (2024): 8106, https://doi.org/10.1038/s41467-024-51952-z.

68. M. K. Pietilä, K. Hellström, and T. Ahola, "Alphavirus Polymerase and RNA Replication," *Virus Research* 234 (2017): 44–57, https://doi.org/10.1016/j.virusres.2017.01.007.

69. A. P. S. Rathore, M. L. Ng, and S. G. Vasudevan, "Differential Unfolded Protein Response During Chikungunya and Sindbis Virus Infection: CHIKV nsP4 Suppresses eIF2α Phosphorylation," *Virology Journal* 10, no. 1 (2013): 36, https://doi.org/10.1186/1743-422X-10-36.

70. Y. B. Tan, L. S. Lello, X. Liu, et al., "Crystal Structures of Alphavirus Nonstructural Protein 4 (nsP4) Reveal an Intrinsically Dynamic RNA-Dependent RNA Polymerase Fold," *Nucleic Acids Research* 50, no. 2 (2022): 1000–1016, https://doi.org/10.1093/nar/gkab1302.

71. S. Tomar, R. W. Hardy, J. L. Smith, and R. J. Kuhn, "Catalytic Core of Alphavirus Nonstructural Protein nsP4 Possesses Terminal Adenylyltransferase Activity," *Journal of Virology* 80, no. 20 (2006): 9962–9969, https://doi.org/10.1128/JVI.01067-06.

72. J. K. Fazakerley, A. Boyd, M. L. Mikkola, and L. Kääriäinen, "A Single Amino Acid Change in the Nuclear Localization Sequence of the nsP2 Protein Affects the Neurovirulence of Semliki Forest Virus," *Journal of Virology* 76, no. 1 (2002): 392–396, https://doi.org/10.1128/jvi.76.1.392-396.2002.

73. I. Akhrymuk, S. V. Kulemzin, and E. I. Frolova, "Evasion of the Innate Immune Response: The Old World Alphavirus nsP2 Protein

Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II," *Journal of Virology* 86, no. 13 (2012): 7180–7191, https://doi.org/10.1128/jvi.00541-12.

74. R. Gorchakov, E. Frolova, and I. Frolov, "Inhibition of Transcription and Translation in Sindbis Virus-Infected Cells," *Journal of Virology* 79, no. 15 (2005): 9397–9409, https://doi.org/10.1128/JVI.79.15.9397-9409.2005.

75. P. K. Das, A. Merits, and A. Lulla, "Functional Cross-Talk Between Distant Domains of Chikungunya Virus Non-Structural Protein 2 Is Decisive for Its RNA-Modulating Activity," *Journal of Biological Chemistry* 289, no. 9 (2014): 5635–5653, https://doi.org/10.1074/jbc.M113.503433.

76. A. Golubtsov, L. Kääriäinen, and J. Caldentey, "Characterization of the Cysteine Protease Domain of Semliki Forest Virus Replicase Protein nsP2 by In Vitro Mutagenesis," *FEBS Letters* 580, no. 5 (2006): 1502–1508, https://doi.org/10.1016/j.febslet.2006.01.071.

77. E. G. Strauss, R. J. De Groot, R. Levinson, and J. H. Strauss, "Identification of the Active Site Residues in the nsP2 Proteinase of Sindbis Virus," *Virology* 191, no. 2 (1992): 932–940, https://doi.org/10.1016/0042-6822(92)90268-T.

78. J. Cheung, "Structure of the Chikungunya Virus nsP2 Protease," Unpublished work (2011), https://www.rcsb.org/structure/3TRK.

79. A. T. Russo, M. A. White, and S. J. Watowich, "The Crystal Structure of the Venezuelan Equine Encephalitis Alphavirus nsP2 Protease," *Structure* 14, no. 9 (2006): 1449–1458, https://doi.org/10.1016/j.str.2006.07.010.

80. G. Shin, S. A. Yost, M. T. Miller, E. J. Elrod, A. Grakoui, and J. Marcotrigiano, "Structural and Functional Insights Into Alphavirus Polyprotein Processing and Pathogenesis," *Proceedings of the National Academy of Sciences of the U S A* 109, no. 41 (2012): 16534–16539, https://doi.org/10.1073/pnas.1210418109.

81. A. Lulla, V. Lulla, K. Tints, T. Ahola, and A. Merits, "Molecular Determinants of Substrate Specificity for Semliki Forest Virus Nonstructural Protease," *Journal of Virology* 80, no. 11 (2006): 5413–5422, https://doi.org/10.1128/JVI.00229-06.

82. V. Lulla, L. Karo-Astover, K. Rausalu, A. Merits, and A. Lulla, "Presentation Overrides Specificity: Probing the Plasticity of Alphaviral Proteolytic Activity Through Mutational Analysis," *Journal of Virology* 87, no. 18 (2013): 10207–10220, https://doi.org/10.1128/JVI.01485-13.

83. A. Lulla, V. Lulla, and A. Merits, "Macromolecular Assembly-Driven Processing of the 2/3 Cleavage Site in the Alphavirus Replicase Polyprotein," *Journal of Virology* 86, no. 1 (2012): 553–565, https://doi.org/10.1128/JVI.05195-11.

84. L. Vasiljeva, L. Valmu, L. Kääriäinen, and A. Merits, "Site-specific Protease Activity of the Carboxyl-Terminal Domain of Semliki Forest Virus Replicase Protein nsP2," *Journal of Biological Chemistry* 276, no. 33 (2001): 30786–30793, https://doi.org/10.1074/jbc.M104786200.

85. S. Krieg, F. Pott, L. Potthoff, et al., "Mono-ADP-ribosylation by PARP10 Inhibits Chikungunya Virus nsP2 Proteolytic Activity and Viral Replication," *Cellular and Molecular Life Sciences* 80, no. 3 (2023): 72, https://doi.org/10.1007/s00018-023-04717-8.

86. P. Spuul, G. Balistreri, L. Kääriäinen, and T. Ahola, "Phosphatidylinositol 3-Kinase-Actin-and Microtubule-Dependent Transport of Semliki Forest Virus Replication Complexes From the Plasma Membrane to Modified Lysosomes," *Journal of Virology* 84, no. 15 (2010): 7543–7557, https://doi.org/10.1128/jvi.00477-10.

87. L. Vasiljeva, A. Merits, A. Golubtsov, V. Sizemskaja, L. Kääriäinen, and T. Ahola, "Regulation of the Sequential Processing of Semliki Forest Virus Replicase Polyprotein," *Journal of Biological Chemistry* 278, no. 43 (2003): 41636–41645, https://doi.org/10.1074/jbc.M307481200.

88. V. Lulla, L. Karo-Astover, K. Rausalu, S. Saul, A. Merits, and A. Lulla, "Timeliness of Proteolytic Events Is Prerequisite for Efficient Functioning of the Alphaviral Replicase," *Journal of Virology* 92, no. 14 (2018): e001511–18, https://doi.org/10.1128/JVI.00151-18.

89. K. Hellström, K. Kallio, A. Utt, et al., "Partially Uncleaved Alphavirus Replicase Forms Spherule Structures in the Presence and Absence of RNA Template," *Journal of Virology* 91, no. 18 (2017), https://doi.org/10.1128/jvi.00787-17.

90. E. I. Frolova, R. Gorchakov, L. Pereboeva, S. Atasheva, and I. Frolov, "Functional Sindbis Virus Replicative Complexes Are Formed at the Plasma Membrane," *Journal of Virology* 84, no. 22 (2010): 11679–11695, https://doi.org/10.1128/jvi.01441-10.

91. B. Thaa, R. Biasiotto, K. Eng, et al., "Differential Phosphatidylinositol-3-Kinase-Akt-mTOR Activation by Semliki Forest and Chikungunya Viruses Is Dependent on nsP3 and Connected to Replication Complex Internalization," *Journal of Virology* 89, no. 22 (2015): 11420–11437, https://doi.org/10.1128/JVI.01579-15.

92. K. Kallio, K. Hellström, G. Balistreri, P. Spuul, E. Jokitalo, and T. Ahola, "Template RNA Length Determines the Size of Replication Complex Spherules for Semliki Forest Virus," *Journal of Virology* 87, no. 16 (2013): 9125–9134, https://doi.org/10.1128/JVI.00660-13.

93. J. Girard, O. Le Bihan, J. Lai-Kee-Him, et al., "In Situ Fate of Chikungunya Virus Replication Organelles," *Journal of Virology* 98, no. 7 (2024): e003688–e003724, https://doi.org/10.1128/jvi.00368-24.

94. R. Gorchakov, E. Frolova, S. Sawicki, S. Atasheva, D. Sawicki, and I. Frolov, "A New Role for ns Polyprotein Cleavage in Sindbis Virus Replication," *Journal of Virology* 82, no. 13 (2008): 6218–6231, https://doi.org/10.1128/JVI.02624-07.

95. K. H. Kim, T. Rümenapf, E. G. Strauss, and J. H. Strauss, "Regulation of Semliki Forest Virus RNA Replication: A Model for the Control of Alphavirus Pathogenesis in Invertebrate Hosts," *Virology* 323, no. 1 (2004): 153–163, https://doi.org/10.1016/j.virol.2004.03.009.

96. R. Jones, G. Bragagnolo, R. Arranz, and J. Reguera, "Capping Pores of Alphavirus nsP1 Gate Membranous Viral Replication Factories," *Nature* 589, no. 7843 (2021): 615–619, https://doi.org/10.1038/s41586-020-3036-8.

97. K. Zhang, Y. S. Law, M. C. Y. Law, Y. B. Tan, M. Wirawan, and D. Luo, "Structural Insights Into Viral RNA Capping and Plasma Membrane Targeting by Chikungunya Virus Nonstructural Protein 1," *Cell Host & Microbe* 29, no. 5 (2021): 757–764.e3, https://doi.org/10.1016/j.chom.2021.02.018.

98. A. Merits, L. Vasiljeva, T. Ahola, L. Kääriäinen, and P. Auvinen, "Proteolytic Processing of Semliki Forest Virus-Specific Non-Structural Polyprotein by nsP2 Protease," *Journal of General Virology* 82, no. Pt 4 (2001): 765–773, https://doi.org/10.1099/0022-1317-82-4-765.

99. R. Fayzulin and I. Frolov, "Changes of the Secondary Structure of the 5' End of the Sindbis Virus Genome Inhibit Virus Growth in Mosquito Cells and Lead to Accumulation of Adaptive Mutations," *Journal of Virology* 78, no. 10 (2004): 4953–4964, https://doi.org/10.1128/jvi.78.10.4953-4964.2004.

100. G. Michel, O. Petrakova, S. Atasheva, and I. Frolov, "Adaptation of Venezuelan Equine Encephalitis Virus Lacking 51-nt Conserved Sequence Element to Replication in Mammalian and Mosquito Cells," *Virology* 362, no. 2 (2007): 475–487, https://doi.org/10.1016/j.virol.2007.01.009.

101. V. Lulla, A. Merits, P. Sarin, L. Kääriäinen, S. Keränen, and T. Ahola, "Identification of Mutations Causing Temperature-Sensitive Defects in Semliki Forest Virus RNA Synthesis," *Journal of Virology* 80, no. 6 (2006): 3108–3111, https://doi.org/10.1128/JVI.80.6.3108-3111.2006.

102. J. Suopanki, D. L. Sawicki, S. G. Sawicki, and L. Kääriäinen, "Regulation of Alphavirus 26S mRNA Transcription by Replicase Component nsP2," *Journal of General Virology* 79, no. Pt 2 (1998): 309–319, https://doi.org/10.1099/0022-1317-79-2-309.

103. M. E. Fairman-Williams, U. P. Guenther, and E. Jankowsky, "SF1 and SF2 Helicases: Family Matters," *Current Opinion in Structural Biology* 20, no. 3 (2010): 313–324, https://doi.org/10.1016/j.sbi.2010.03.011.

104. R. Schuchman, A. Kilianski, A. Piper, et al., "Comparative Characterization of the Sindbis Virus Proteome From Mammalian and Invertebrate Hosts Identifies nsP2 as a Component of the Virion and Sorting Nexin 5 as a Significant Host Factor for Alphavirus Replication," *Journal of Virology* 92, no. 14 (2018): e006944–18, https://doi.org/10.1128/JVI.00694-18.

105. P. Yin, X. Jian, Y. Liu, et al., "Elucidating Cellular Interactome of Chikungunya Virus Identifies Host Dependency Factors," *Virologica Sinica* 38, no. 4 (2023): 497–507, https://doi.org/10.1016/j.virs.2023.05.007.

106. D. Y. Kim, S. Atasheva, E. I. Frolova, and I. Frolov, "Venezuelan Equine Encephalitis Virus nsP2 Protein Regulates Packaging of the Viral Genome Into Infectious Virions," *Journal of Virology* 87, no. 8 (2013): 4202–4213, https://doi.org/10.1128/JVI.03142-12.

107. S. Atasheva, R. Gorchakov, R. English, I. Frolov, and E. Frolova, "Development of Sindbis Viruses Encoding nsP2/GFP Chimeric Proteins and Their Application for Studying nsP2 Functioning," *Journal of Virology* 81, no. 10 (2007): 5046–5057, https://doi.org/10.1128/jvi.02746-06.

108. M. Bouraï, M. Lucas-Hourani, H. H. Gad, et al., "Mapping of Chikungunya Virus Interactions With Host Proteins Identified nsP2 as a Highly Connected Viral Component," *Journal of Virology* 86, no. 6 (2012): 3121–3134, https://doi.org/10.1128/JVI.06390-11.

109. E. Frolova, R. Gorchakov, N. Garmashova, S. Atasheva, L. A. Vergara, and I. Frolov, "Formation of nsP3-Specific Protein Complexes During Sindbis Virus Replication," *Journal of Virology* 80, no. 8 (2006): 4122–4134, https://doi.org/10.1128/jvi.80.8.4122-4134.2006.

110. A. J. Burnham, L. Gong, and R. W. Hardy, "Heterogeneous Nuclear Ribonuclear Protein K Interacts With Sindbis Virus Nonstructural Proteins and Viral Subgenomic mRNA," *Virology* 367, no. 1 (2007): 212–221, https://doi.org/10.1016/j.virol.2007.05.008.

111. S. A. Montgomery, P. Berglund, C. W. Beard, and R. E. Johnston, "Ribosomal Protein S6 Associates With Alphavirus Nonstructural Protein 2 and Mediates Expression From Alphavirus Messages," *Journal of Virology* 80, no. 15 (2006): 7729–7739, https://doi.org/10.1128/JVI.00425-06.

112. M. Ranki, I. Ulmanen, and L. Kääriäinen, "Semliki Forest Virus-Specific Nonstructural Protein Is Associated With Ribosomes," *FEBS Letters* 108, no. 1 (1979): 299–302, https://doi.org/10.1016/0014-5793(79)81232-6.

113. E. I. Frolova, R. Z. Fayzulin, S. H. Cook, D. E. Griffin, C. M. Rice, and I. Frolov, "Roles of Nonstructural Protein nsP2 and Alpha/Beta Interferons in Determining the Outcome of Sindbis Virus Infection," *Journal of Virology* 76, no. 22 (2002): 11254–11264, https://doi.org/10.1128/jvi.76.22.11254-11264.2002.

114. M. Rikkonen, "Functional Significance of the Nuclear-Targeting and NTP-Binding Motifs of Semliki Forest Virus Nonstructural Protein nsP2," *Virology* 218, no. 2 (1996): 352–361, https://doi.org/10.1006/viro.1996.0204.

115. M. Rikkonen, J. Peränen, and L. Kääriäinen, "Nuclear and Nucleolar Targeting Signals of Semliki Forest Virus Nonstructural Protein nsP2," *Virology* 189, no. 2 (1992): 462–473, https://doi.org/10.1016/0042-6822(92)90570-f.

116. J. Peränen, M. Rikkonen, P. Liljeström, and L. Kääriäinen, "Nuclear Localization of Semliki Forest Virus-Specific Nonstructural Protein nsP2," *Journal of Virology* 64, no. 5 (1990): 1888–1896, https://doi.org/10.1128/jvi.64.5.1888-1896.1990.

117. M. Rikkonen, J. Peränen, and L. Kääriäinen, "Nuclear Targeting of Semliki Forest Virus nsP2," *Archives of Virology, Supplement* 9 (1994): 369–377, https://doi.org/10.1007/978-3-7091-9326-6_37.

118. K. Tamm, A. Merits, and I. Sarand, "Mutations in the Nuclear Localization Signal of nsP2 Influencing RNA Synthesis, Protein Expression and Cytotoxicity of Semliki Forest Virus," *Journal of General Virology* 89, no. Pt 3 (2008): 676–686, https://doi.org/10.1099/vir.0.83320-0.

119. I. Frolov, N. Garmashova, S. Atasheva, and E. I. Frolova, "Random Insertion Mutagenesis of Sindbis Virus Nonstructural Protein 2 and Selection of Variants Incapable of Downregulating Cellular Transcription," *Journal of Virology* 83, no. 18 (2009): 9031–9044, https://doi.org/10.1128/jvi.00850-09.

120. A. Utt, P. K. Das, M. Varjak, V. Lulla, A. Lulla, and A. Merits, "Mutations Conferring a Noncytotoxic Phenotype on Chikungunya Virus Replicons Compromise Enzymatic Properties of Nonstructural Protein 2," *Journal of Virology* 89, no. 6 (2015): 3145–3162, https://doi.org/10.1128/JVI.03213-14.

121. D. E. Griffin, "Alphavirus Pathogenesis and Immunity," in *The Togaviridae and Flaviviridae*, eds. S. Schlesinger and M. J. Schlesinger (Springer, 1986), 209–249, https://doi.org/10.1007/978-1-4757-0785-4_8.

122. E. V. Agapov, I. Frolov, B. D. Lindenbach, B. M. Prágai, S. Schlesinger, and C. M. Rice, "Noncytopathic Sindbis Virus RNA Vectors for Heterologous Gene Expression," *Proceedings of the National Academy of Sciences* 95, no. 22 (1998): 12989–12994, https://doi.org/10.1073/pnas.95.22.12989.

123. N. Garmashova, R. Gorchakov, E. Frolova, and I. Frolov, "Sindbis Virus Nonstructural Protein nsP2 Is Cytotoxic and Inhibits Cellular Transcription," *Journal of Virology* 80, no. 12 (2006): 5686–5696, https://doi.org/10.1128/jvi.02739-05.

124. R. Ishida, J. Cole, J. Lopez-Orozco, et al., "Mayaro Virus Non-Structural Protein 2 Circumvents the Induction of Interferon in Part by Depleting Host Transcription Initiation Factor IIE Subunit 2," *Cells* 10, no. 12 (2021): 3510, https://doi.org/10.3390/cells10123510.

125. J. J. Fros, W. J. Liu, N. A. Prow, et al., "Chikungunya Virus Nonstructural Protein 2 Inhibits Type I/II Interferon-Stimulated JAK-STAT Signaling," *Journal of Virology* 84, no. 20 (2010): 10877–10887, https://doi.org/10.1128/JVI.00949-10.

126. D. Judith, S. Mostowy, M. Bourai, et al., "Species-Specific Impact of the Autophagy Machinery on Chikungunya Virus Infection," *EMBO Reports* 14, no. 6 (2013): 534–544, https://doi.org/10.1038/embor.2013.51.

127. E. E. Treffers, A. Tas, F. E. M. Scholte, et al., "The Alphavirus Nonstructural Protein 2 NTPase Induces a Host Translational Shut-Off Through Phosphorylation of eEF2 via cAMP-PKA-eEF2K Signaling," *PLoS Pathogens* 19, no. 2 (2023): e1011179, https://doi.org/10.1371/journal.ppat.1011179.

128. N. Garmashova, R. Gorchakov, E. Volkova, S. Paessler, E. Frolova, and I. Frolov, "The Old World and New World Alphaviruses Use Different Virus-Specific Proteins for Induction of Transcriptional Shutoff," *Journal of Virology* 81, no. 5 (2007): 2472–2484, https://doi.org/10.1128/jvi.02073-06.

129. N. Bhalla, C. Sun, L. K. Metthew Lam, C. L. Gardner, K. D. Ryman, and W. B. Klimstra, "Host Translation Shutoff Mediated by Nonstructural Protein 2 Is a Critical Factor in the Antiviral State Resistance of Venezuelan Equine Encephalitis Virus," *Virology* 496 (2016): 147–165, https://doi.org/10.1016/j.virol.2016.06.005.

130. J. J. Hoarau, M. C. Jaffar Bandjee, T. P. Krejbich, et al., "Persistent Chronic Inflammation and Infection by Chikungunya Arthritogenic Alphavirus in Spite of a Robust Host Immune Response," *Journal of Immunology* 184, no. 10 (2010): 5914–5927, https://doi.org/10.4049/jimmunol.0900255.

131. Y. Duan, H. Zhou, X. Liu, et al., "Molecular Mechanisms of SARS-CoV-2 Resistance to Nirmatrelvir," *Nature* 622, no. 7982 (2023): 376–382, https://doi.org/10.1038/s41586-023-06609-0.

132. D. R. Owen, C. M. N. Allerton, A. S. Anderson, et al., "An Oral SARS-CoV-2 Mpro Inhibitor Clinical Candidate for the Treatment of

COVID-19," *Science* 374, no. 6575 (2021): 1586–1593, https://doi.org/10.1126/science.abl4784.

133. S. Venkatraman, "Discovery of Boceprevir, a Direct-Acting NS3/4A Protease Inhibitor for Treatment of Chronic Hepatitis C Infections," *Trends in Pharmacological Sciences* 33, no. 5 (2012): 289–294, https://doi.org/10.1016/j.tips.2012.03.012.

134. J. M. Llibre, "First-line Boosted Protease Inhibitor-Based Regimens in Treatment-Naive HIV-1-Infected Patients--Making a Good Thing Better," *AIDS Reviews* 11, no. 4 (2009): 215–222.

135. T. Agarwal, S. Asthana, and A. Bissoyi, "Molecular Modeling and Docking Study to Elucidate Novel Chikungunya Virus nsP2 Protease Inhibitors," *Indian Journal of Pharmaceutical Sciences* 77, no. 4 (2015): 453–460, https://doi.org/10.4103/0250-474x.164769.

136. N. Khan, R. Bhat, A. K. Patel, and P. Ray, "Discovery of Small Molecule Inhibitors of Chikungunya Virus Proteins (nsP2 and E1) Using In Silico Approaches," *Journal of Biomolecular Structure and Dynamics* 39, no. 4 (2021): 1373–1385, https://doi.org/10.1080/07391102.2020.1731602.

137. M. K. Meena, D. Kumar, K. Kumari, et al., "Promising Inhibitors of Nsp2 of CHIKV Using Molecular Docking and Temperature-Dependent Molecular Dynamics Simulations," *Journal of Biomolecular Structure and Dynamics* 40, no. 13 (2022): 5827–5835, https://doi.org/10.1080/07391102.2021.1873863.

138. D. Montes-Grajales, H. Puerta-Guardo, D. A. Espinosa, et al., "In Silico Drug Repurposing for the Identification of Potential Candidate Molecules Against Arboviruses Infection," *Antiviral Research* 173 (2020): 104668, https://doi.org/10.1016/j.antiviral.2019.104668.

139. KhD. Singh, P. Kirubakaran, S. Nagarajan, et al., "Homology Modeling, Molecular Dynamics, e-Pharmacophore Mapping and Docking Study of Chikungunya Virus nsP2 Protease," *Journal of Molecular Modeling* 18, no. 1 (2012): 39–51, https://doi.org/10.1007/s00894-011-1018-3.

140. M. Bassetto, T. De Burghgraeve, L. Delang, et al., "Computer-Aided Identification, Design and Synthesis of a Novel Series of Compounds With Selective Antiviral Activity Against Chikungunya Virus," *Antiviral Research* 98, no. 1 (2013): 12–18, https://doi.org/10.1016/j.antiviral.2013.01.002.

141. X. Hu, E. Morazzani, J. R. Compton, et al., "In Silico Screening of Inhibitors of the Venezuelan Equine Encephalitis Virus Nonstructural Protein 2 Cysteine Protease," *Viruses* 15, no. 7 (2023): 1503, https://doi.org/10.3390/v15071503.

142. P. K. Das, L. Puusepp, F. S. Varghese, et al., "Design and Validation of Novel Chikungunya Virus Protease Inhibitors," *Antimicrobial Agents and Chemotherapy* 60, no. 12 (2016): 7382–7395, https://doi.org/10.1128/AAC.01421-16.

143. X. Hu, J. R. Compton, D. H. Leary, et al., "Kinetic, Mutational, and Structural Studies of the Venezuelan Equine Encephalitis Virus Nonstructural Protein 2 Cysteine Protease," *Biochemistry* 55, no. 21 (2016): 3007–3019, https://doi.org/10.1021/acs.biochem.5b00992.

144. K. A. Stapleford, K. Rozen-Gagnon, P. K. Das, et al., "Viral Polymerase-Helicase Complexes Regulate Replication Fidelity to Overcome Intracellular Nucleotide Depletion," *Journal of Virology* 89, no. 22 (2015): 11233–11244, https://doi.org/10.1128/JVI.01553-15.

145. A. Ghoshal, K. H. Asressu, M. A. Hossain, et al., "Structure Activity of β-Amidomethyl Vinyl Sulfones as Covalent Inhibitors of Chikungunya nsP2 Cysteine Protease With Anti-Alphavirus Activity," *Journal of Medicinal Chemistry* 67, no. 18 (2024): 16505–16532, https://doi.org/10.1101/2024.06.12.598722.

146. E. M. Merten, J. D. Sears, T. M. Leisner, et al., "Discovery of a Cell-Active Chikungunya Virus nsP2 Protease Inhibitor Using a Covalent Fragment-Based Screening Approach," *Proceedings of the National Academy of Sciences of the United States of America* 121, no. 42 (2024): e2409166121, https://doi.org/10.1101/2024.03.22.586341.

147. A. Saha, B. N. Acharya, R. Priya, et al., "Development of nsP2 Protease Based Cell Free High Throughput Screening Assay for Evaluation of Inhibitors Against Emerging Chikungunya Virus," *Scientific Reports* 8, no. 1 (2018): 10831, https://doi.org/10.1038/s41598-018-29024-2.

148. H. Singh, R. Mudgal, M. Narwal, et al., "Chikungunya Virus Inhibition by Peptidomimetic Inhibitors Targeting Virus-Specific Cysteine Protease," *Biochimie* 149 (2018): 51–61, https://doi.org/10.1016/j.biochi.2018.04.004.

149. B. G. de Souza, S. Choudhary, G. G. Vilela, et al., "Design, Synthesis, Antiviral Evaluation, and In Silico Studies of Acrylamides Targeting nsP2 From Chikungunya Virus," *European Journal of Medicinal Chemistry* 258 (2023): 115572, https://doi.org/10.1016/j.ejmech.2023.115572.

150. G. Giancotti, M. Cancellieri, A. Balboni, et al., "Rational Modifications on a Benzylidene-Acrylohydrazide Antiviral Scaffold, Synthesis and Evaluation of Bioactivity Against Chikungunya Virus," *European Journal of Medicinal Chemistry* 149 (2018): 56–68, https://doi.org/10.1016/j.ejmech.2018.02.054.

151. R. Tardugno, G. Giancotti, T. De Burghgraeve, et al., "Design, Synthesis and Evaluation Against Chikungunya Virus of Novel Small-Molecule Antiviral Agents," *Bioorganic & Medicinal Chemistry* 26, no. 4 (2018): 869–874, https://doi.org/10.1016/j.bmc.2018.01.002.

152. M. Lucas-Hourani, A. Lupan, P. Desprès, et al., "A Phenotypic Assay to Identify Chikungunya Virus Inhibitors Targeting the Nonstructural Protein nsP2," *Journal of Biomolecular Screening* 18, no. 2 (2012): 172–179, https://doi.org/10.1177/1087057112460091.

153. P. K. Tripathi, A. Soni, S. P. Singh Yadav, et al., "Evaluation of Novobiocin and Telmisartan for Anti-CHIKV Activity," *Virology* 548 (2020): 250–260, https://doi.org/10.1016/j.virol.2020.05.010.

154. P. Mishra, A. Kumar, P. Mamidi, et al., "Inhibition of Chikungunya Virus Replication by 1-[(2-Methylbenzimidazol-1-Yl) Methyl]-2-Oxo-Indolin-3-Ylidene] Amino] Thiourea(MBZM-N-IBT)," *Scientific Reports* 6, no. 1 (2016): 20122, https://doi.org/10.1038/srep20122.

155. S. S. Jadav, B. N. Sinha, R. Hilgenfeld, B. Pastorino, X. de Lamballerie, and V. Jayaprakash, "Thiazolidone Derivatives as Inhibitors of Chikungunya Virus," *European Journal of Medicinal Chemistry* 89 (2015): 172–178, https://doi.org/10.1016/j.ejmech.2014.10.042.

156. E. M. El-labbad, M. A. H. Ismail, E. D. A. Abou Ei, et al., "Discovery of Novel Peptidomimetics as Irreversible CHIKV NsP2 Protease Inhibitors Using Quantum Mechanical-Based Ligand Descriptors," *Chemical Biology & Drug Design* 86, no. 6 (2015): 1518–1527, https://doi.org/10.1111/cbdd.12621.

157. H. Zhang, M. Harmon, S. R. Radoshitzky, et al., "Vinyl Sulfone-Based Inhibitors of Nonstructural Protein 2 Block the Replication of Venezuelan Equine Encephalitis Virus," *ACS Medicinal Chemistry Letters* 11, no. 11 (2020): 2139–2145, https://doi.org/10.1021/acsmedchemlett.0c00215.

158. J. Campos-Gomez, F. Ahmad, E. Rodriguez, and M. F. Saeed, "A Novel Cell-Based Assay to Measure Activity of Venezuelan Equine Encephalitis Virus nsP2 Protease," *Virology* 496 (2016): 77–89, https://doi.org/10.1016/j.virol.2016.05.012.

159. F. I. L. Hucke and J. J. Bugert, "Current and Promising Antivirals Against Chikungunya Virus," *Frontiers in Public Health* 8 (2020): 618624, https://doi.org/10.3389/fpubh.2020.618624.

160. T. Laurent, P. Kumar, S. Liese, et al., "Architecture of the Chikungunya Virus Replication Organelle," in *eLife*, eds. G. Zanetti and V. Malhotra, Vol. 11, (2022): e83042, https://doi.org/10.7554/eLife.83042.

161. M. Ehteshami, S. Tao, K. Zandi, et al., "Characterization of β-d-N4-Hydroxycytidine as a Novel Inhibitor of Chikungunya Virus," *Antimicrobial Agents and Chemotherapy* 61, no. 4 (2017), https://doi.org/10.1128/aac.02395-16.

162. L. Pohjala, A. Utt, M. Varjak, et al., "Inhibitors of Alphavirus Entry and Replication Identified With a Stable Chikungunya Replicon Cell

Line and Virus-Based Assays," *PLoS One* 6, no. 12 (2011): e28923, https://doi.org/10.1371/journal.pone.0028923.

163. S. Nam, Y. J. Ga, J. Y. Lee, et al., "Radicicol Inhibits Chikungunya Virus Replication by Targeting Nonstructural Protein 2," *Antimicrobial Agents and Chemotherapy* 65, no. 7 (2021), https://doi.org/10.1128/aac.00135-21.

164. D. H. Chung, C. B. Jonsson, N. A. Tower, et al., "Discovery of a Novel Compound With Anti-Venezuelan Equine Encephalitis Virus Activity That Targets the Nonstructural Protein 2," *PLoS Pathogens* 10, no. 6 (2014): e1004213, https://doi.org/10.1371/journal.ppat.1004213.

165. J. Lee, J. Parvathareddy, D. Yang, et al., "Emergence and Magnitude of ML336 Resistance in Venezuelan Equine Encephalitis Virus Depend on the Microenvironment," *Journal of Virology* 94, no. 22 (2020), https://doi.org/10.1128/jvi.00317-20.

166. P. Yin, E. B. Sobolik, N. A. May, et al., "Mutations in Chikungunya Virus nsP4 Decrease Viral Fitness and Sensitivity to the Broad-Spectrum Antiviral 4′-Fluorouridine," *PLoS Pathogens* 21, no. 1 (2025): e1012859, https://doi.org/10.1371/journal.ppat.1012859.

167. R. Abdelnabi, K. Kovacikova, J. Moesslacher, et al., "Novel Class of Chikungunya Virus Small Molecule Inhibitors That Targets the Viral Capping Machinery," *Antimicrobial Agents and Chemotherapy* 64, no. 7 (2020), https://doi.org/10.1128/aac.00649-20.

168. I. Akhrymuk, T. Lukash, I. Frolov, and E. I. Frolova, "Novel Mutations in nsP2 Abolish Chikungunya Virus-Induced Transcriptional Shutoff and Make the Virus Less Cytopathic Without Affecting Its Replication Rates," *Journal of Virology* 93, no. 4 (2019): e020622–18, https://doi.org/10.1128/JVI.02062-18.

169. C. D. Meshram, T. Lukash, A. Phillips, I. Akhrymuk, E. I. Frolova, and I. Frolov, "Lack of nsP2-Specific Nuclear Functions Attenuates Chikungunya Virus Replication Both In Vitro and In Vivo," *Virology* 534 (2019): 14–24, https://doi.org/10.1016/j.virol.2019.05.016.

170. A. T. Russo, R. D. Malmstrom, M. A. White, and S. J. Watowich, "Structural Basis for Substrate Specificity of Alphavirus nsP2 Proteases," *Journal of Molecular Graphics and Modelling* 29, no. 1 (2010): 46–53, https://doi.org/10.1016/j.jmgm.2010.04.005.

171. Y. H. Chan, T. H. Teo, A. Utt, et al., "Mutating Chikungunya Virus Non-Structural Protein Produces Potent Live-Attenuated Vaccine Candidate," *EMBO Molecular Medicine* 11, no. 6 (2019): e10092, https://doi.org/10.15252/emmm.201810092.

172. X. Liu, M. Mutso, A. Utt, et al., "Decreased Virulence of Ross River Virus Harboring a Mutation in the First Cleavage Site of Nonstructural Polyprotein Is Caused by a Novel Mechanism Leading to Increased Production of Interferon-Inducing RNAs," *mBio* 9, no. 4 (2018): e000444–18, https://doi.org/10.1128/mBio.00044-18.

173. S. Y. Folimonova, "Superinfection Exclusion Is an Active Virus-Controlled Function That Requires a Specific Viral Protein," *Journal of Virology* 86, no. 10 (2012): 5554–5561, https://doi.org/10.1128/jvi.00310-12.

174. R. H. Adams and D. T. Brown, "BHK Cells Expressing Sindbis Virus-Induced Homologous Interference Allow the Translation of Nonstructural Genes of Superinfecting Virus," *Journal of Virology* 54, no. 2 (1985): 351–357, https://doi.org/10.1128/jvi.54.2.351-357.1985.

175. B. G. Bolling, F. J. Olea-Popelka, L. Eisen, C. G. Moore, and C. D. Blair, "Transmission Dynamics of an Insect-Specific Flavivirus in a Naturally Infected Culex pipiens Laboratory Colony and Effects of Co-Infection on Vector Competence for West Nile Virus," *Virology* 427, no. 2 (2012): 90–97, https://doi.org/10.1016/j.virol.2012.02.016.

176. B. T. Eaton, "Heterologous Interference in *Aedes albopictus* Cells Infected With Alphaviruses," *Journal of Virology* 30, no. 1 (1979): 45–55, https://doi.org/10.1128/jvi.30.1.45-55.1979.

177. J. Hobson-Peters, A. W. Y. Yam, J. W. F. Lu, et al., "A New Insect-specific Flavivirus From Northern Australia Suppresses Replication of West Nile Virus and Murray Valley Encephalitis Virus in Co-Infected Mosquito Cells," *PLoS One* 8, no. 2 (2013): e56534, https://doi.org/10.1371/journal.pone.0056534.

178. A. R. Karpf, E. Lenches, E. G. Strauss, J. H. Strauss, and D. T. Brown, "Superinfection Exclusion of Alphaviruses in Three Mosquito Cell Lines Persistently Infected With Sindbis Virus," *Journal of Virology* 71, no. 9 (1997): 7119–7123, https://doi.org/10.1128/JVI.71.9.7119-7123.1997.

179. M. Laureti, P. N. Paradkar, J. K. Fazakerley, and J. Rodriguez-Andres, "Superinfection Exclusion in Mosquitoes and Its Potential as an Arbovirus Control Strategy," *Viruses* 12, no. 11 (2020): 1259, https://doi.org/10.3390/v12111259.

180. D. M. Tscherne, M. J. Evans, T. von Hahn, et al., "Superinfection Exclusion in Cells Infected With Hepatitis C Virus," *Journal of Virology* 81, no. 8 (2007): 3693–3703, https://doi.org/10.1128/jvi.01748-06.

181. F. Nasar, J. H. Erasmus, A. D. Haddow, R. B. Tesh, and S. C. Weaver, "Eilat Virus Induces Both Homologous and Heterologous Interference," *Virology* 484 (2015): 51–58, https://doi.org/10.1016/j.virol.2015.05.009.

182. L. Cherkashchenko, K. Rausalu, S. Basu, L. Alphey, and A. Merits, "Expression of Alphavirus Nonstructural Protein 2 (nsP2) in Mosquito Cells Inhibits Viral RNA Replication in Both a Protease Activity-Dependent and -Independent Manner," *Viruses* 14, no. 6 (2022): 1327, https://doi.org/10.3390/v14061327.

183. M. U. Ehrengruber and A. L. Goldin, "Semliki Forest Virus Vectors With Mutations in the Nonstructural Protein 2 Gene Permit Extended Superinfection of Neuronal and Non-neuronal Cells," *Journal of Neuro-Virology* 13, no. 4 (2007): 353–363, https://doi.org/10.1080/13550280701393204.

184. C. M. Reitmayer, E. Levitt, S. Basu, et al., "Mimicking Superinfection Exclusion Disrupts Alphavirus Infection and Transmission in the Yellow Fever Mosquito Aedes aegypti," *Proceedings of the National Academy of Sciences of the United States of America* 120, no. 37 (2023): e2303080120, https://doi.org/10.1073/pnas.2303080120.

185. J. Bettadapura, L. J. Herrero, A. Taylor, and S. Mahalingam, "Approaches to the Treatment of Disease Induced by Chikungunya Virus," *Indian Journal of Medical Research* 138, no. 5 (2013): 762–765.

186. A. Taylor, J. Melton, L. Herrero, et al., "Effects of an In-Frame Deletion of the 6k Gene Locus From the Genome of Ross River Virus," *Journal of Virology* 90, no. 8 (2016): 4150–4159, https://doi.org/10.1128/JVI.03192-15.

187. J. M. Carr, S. Mahalingam, C. S. Bonder, and S. M. Pitson, "Sphingosine Kinase 1 in Viral Infections," *Reviews in Medical Virology* 23, no. 2 (2013): 73–84, https://doi.org/10.1002/rmv.1718.

188. S. S. Foo, P. C. Reading, S. Jaillon, A. Mantovani, and S. Mahalingam, "Pentraxins and Collectins: Friend or Foe During Pathogen Invasion?," *Trends in Microbiology* 23, no. 12 (2015): 799–811, https://doi.org/10.1016/j.tim.2015.09.006.