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The Life Cycle of the Alphaviruses: From an Antiviral Perspective

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Abstract

The alphaviruses are a widely distributed group of positive-sense, single stranded, RNA viruses. These viruses are largely arthropod-borne and can be found on all populated continents. These viruses cause significant human disease, and recently have begun to spread into new populations, such as the expansion of Chikungunya virus into southern Europe and the Caribbean, where it has established itself as endemic. The study of alphaviruses is an active and expanding field, due to their impacts on human health, their effects on agriculture, and the threat that some pose as potential agents of biological warfare and terrorism. In this systematic review we will summarize both historic knowledge in the field as well as recently published data that has potential to shift current theories in how alphaviruses are able to function. This review is comprehensive, covering all parts of the alphaviral life cycle as well as a brief overview of their pathology and the current state of research in regards to vaccines and therapeutics for alphaviral disease.

Keywords

alphaviruses; viral replication; positive-sense RNA viruses; viral life cycle

1. Introduction

Alphaviruses are positive sense, single stranded, RNA viruses in the family *Togaviridae*, which are classified as members of the domain *Riboviria*[1]. The alphaviruses currently encompass more than thirty members that infect a wide range of host and vector species, both terrestrial and aquatic. These viruses are widely dispersed geographically as well, with at least one alphavirus being present on every populated continent[2–6]. Alphaviruses are

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Declaration of interests

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continually emerging into naïve populations, and there are currently no licensed treatments or vaccines for alphaviral disease, making these viruses important research targets.

The alphaviruses have historically been divided into two clades based upon the location of their isolation. The Old-World viruses, which were first isolated in the eastern hemisphere, and the New-World viruses which were first discovered in the Americas. The Old-World viruses generally cause arthralgia and fever, with some also causing a rash[7]. There is some recent evidence that Old-World members may be able to cause encephalitis as well, with the adaptation of neurologically invasive Sindbis virus (SINV) for use in mice, as well as its association with rare cases of viral encephalitis in Europe[8–10]. The New-World viruses are known to cause encephalitis; the three most prominent members of this clade, Eastern (EEEV), Western (WEEV), and Venezuelan Equine Encephalitis viruses (VEEV) demonstrate high levels of neurological pathogenicity[7]. Recently it has been proposed that certain South American clades of EEEV be split into the new species Madariaga virus, as they are genetically distinct and are less pathogenic[11]

Continuing efforts of alphavirus discovery, characterization, and sequencing have now indicated that the division between the Old and New-World viruses may be losing usefulness, as there are now several known New-World alphaviruses that don’t cause any disease[12], as well as the recent discovery of alphaviruses that are native to the New-World but have disease phenotypes that are similar to the Old-World clade, such as Mayaro virus[2,13].

2. Alphaviral Disease

The Old-World virus of most concern is Chikungunya virus (CHIKV), which has recently expanded into naive populations across Asia, southern Europe, and most dramatically, in the Caribbean[3–6]. This has resulted in CHIKV becoming endemic in several of these regions. The primary risk of CHIKV is a sustained arthralgia that can last for months, with one study in Mexico indicating that over a third of confirmed CHIKV cases have arthralgia twelve months after acute disease[14]. Similar pathologies have also been reported after infection with Ross River virus[15].

The New-World alphaviruses generally cause more severe disease than the Old-World viruses; however, the three most common (EEEV, WEEV, VEEV) are noted for a high rate of asymptomatic infection[12]. This asymptomatic infection rate varies between the three viruses and in the two primary populations of interest, equids and humans, with equids having significantly higher rates of symptomatic disease[12]. Disease is also generally more severe in equid hosts than in humans, with most equid cases being lethal[7]. EEEV has the highest reported rate of neurological involvement and lethality, VEEV has the least, and WEEV falls between the two[12]. However, VEEV is the New-World virus of most concern as it has historically caused the largest and most frequent outbreaks affecting both human and equid populations, and resulting in many thousands of human cases and equid deaths[12,16]. EEEV remains rare in the human population, but there was a significant increase in the number of cases reported in 2019. This increase retreated in 2020, the most recent year with data available from the CDC, with that year having an average number of reports[17]. WEEV

has virtually disappeared from the human population, and also become much rarer in its enzootic hosts in North America[18,19]

VEEV is also a high risk for accidental release and exposure due to it propensity to infect via aerosols[20]. This led to VEEV being developed as a bioweapon by both the former USSR and the USA, making VEEV a select agent, a classification it shares with EEEV[21]. Select agents are those toxins and organisms that "could threaten public health and safety"[22] and have additional security regulations in addition to those controls indicated by biosafety. VEEV generally causes a mild febrile illness that occasionally results in encephalitic infection, resulting in death in approximately 10% of cases when encephalitis is present[7], however the total case fatality rate is only around 1%[23]. Those patients that do survive neurological symptoms are likely to suffer from long term sequelae[24].

3. Natural Transmission of the Alphaviruses

Alphaviruses are vector-borne viruses that generally require the use of an intermediate species to transmit to a naïve vertebrate host[7] (Figure 1). Due to this cycle, the viruses must efficiently infect and replicate in multiple species. Alphaviruses infect a wide variety of both vector and host species. Single species of alphavirus can often infect multiple different species of vector, and different vector species are typically responsible for endemic maintenance and epidemic/epizootic outbreaks of disease[23].

The virus first enters the mosquito or other vector through a blood meal that is taken from an infected host. The virus then encounters the cells of the mosquito midgut, before passing into the haemocoel, the circulatory system of the mosquito. Eventually virus arrives in the salivary glands where it replicates to high levels and is transmitted to the next vertebrate host during a blood meal[29,30]. Not only does vector transmission complicate control of these viruses, but infection of the mosquito is an important selection process, and different strains of these viruses can behave differently in the vector. In particular, epidemic VEEV strains behave very differently in the mosquito than those are isolated from enzootic infection[29–31].

Upon blood meal from an infected mosquito, the alphavirus is injected into the skin of a naïve host. As these viruses have various cellular tropisms that will result in differing pathologies; we will here outline the general series of events that occur during infection of a susceptible and permissive host cell.

4. Alphaviral replication

The steps outlined here are common to all alphaviruses unless otherwise indicated. After inoculation into the vertebrate host alphaviruses enter permissive and susceptible host cells to manufacture new virions. The alphaviruses are noted to have highly efficient infection. While in infection there is an excess of genomes produced compared to plaque forming units, as time goes on these numbers reach near parity[32]. The alphaviral replication process is here described in detail, and a summary can be found in Figure 2.

4.1 Receptor-mediated endocytosis

The primary mechanism by which alphaviruses enter naïve host cells is via receptor mediated endocytosis, and the viruses are highly promiscuous[33]. Of particular interest are DC-SIGN and L-SIGN, which may influence myeloid cell infection[34]. Additional receptors continue to be discovered[35,36]. Heparan sulfate is an important binding partner in cell culture, and this appears to be a specific adaptation that occurs in response to passaging virus repeatedly in cells[33,37–39]. The exception to this is EEEV, which has been found to have affinity for heparan sulfate in naturally circulating strains[40]

After receptor binding, the alphaviruses are then transported into the cell via clathrin mediated endocytosis[41,42], resulting in a virus-containing endosome passing through the stages of acidification and maturation. The New-World viruses remain in the vacuoles until they reach the endosome stage, whereas the Old-World viruses escape from the early endosomal compartment[43].

There is also now evidence that some alphaviruses may be able to utilize alternative entry strategies, such as direct entry at the host cell plasma membrane[44,45]. This has been well characterized in CHIKV, with research indicating that even though an acidification step is required, it can occur in a manner that is independent of the activity of clathrin[46]. However, the importance of this entry method remains unclear, but it shows one way these viruses may be able to display such wide cell tropisms. This does indicate that these viruses may have the potential to develop resistance to entry inhibitors rapidly.

4.2 Viral entry as a target for antiviral drug development.

Receptor attachment and entry are common drug targets, and an example of a drug that targets this activity in the alphaviruses is suramin. Suramin was initially discovered as a treatment for trypanosomal diseases in Africa, and it is also known by the names naganol, suramine, forneau, and germanin[47,48]. Suramin has a long history of being tested for potential therapeutic effects in many different diseases.

Before it was tested in the treatment of alphaviral disease, suramin was already known to function to inhibit trypanosomal disease, to potentially act as an inhibitor of the HIV reverse transcriptase, and to have anticancer effects[47,49,50]. Suramin has recently been studied in the treatment of CHIKV infection and was found to inhibit multiple stages of the replication cycle, including viral entry. Suramin was also mildly efficacious in the treatment of SINV and SFV. Treatment with suramin was also found to reduce viral load in a mouse model of infection, and reduced infection relation swelling in the foot[51,52]. As it has a known safety profile, suramin is a promising lead compound for further refinement via modeling and medicinal chemistry. The structure of suramin can be found in table 2 below.

4.3 Fusion/Uncoating and RNA release

Fusion of the viral and host cell membranes is achieved by the activity of the E1 protein, and expression of E1 without the other glycoproteins is enough to mediate viral membrane fusion[63,64]. This fusogenic activity is initially prevented by the interaction of E1 with E2, but this interaction is disrupted at low pH[42,65].

After escape from the endosome, the nucleocapsid interacts with ribosomes, which disassemble the capsid in a non-catalytic manner, which is dependent on conserved capsid sequences[66,67]. The disassembly of the nucleocapsid is enhanced by low pH, and the pore forming activities of the E proteins are implicated to induce these pH changes[68–70].

4.4 Translation and processing of the nsPs

Upon release the alphavirus genome is available as an mRNA for cellular ribosomes and recruits all the factors required for protein synthesis in a similar manner as a cellular RNA[7]. First to be translated is the nonstructural polyprotein, which contains the proteins that are responsible for the replication of the viral RNA. The viral nsPs are numbered in the order that they occur in the genome from 5' to 3', 1-4. The genomic organization of alphaviruses can be found in Figure 3 A. The initial polyprotein is translated as either nsP123 or nsP1234, depending on read through of a stop codon that may or may not be present in the genome depending on the alphavirus in question[71–73,].

After the initial translation of these proteins, they undergo tightly controlled autologous cleavage events which are independent from cellular processes and result in the formation of multiple intermediates as well as the final mature replicase complex nsP1/2/3/4[74,75]. This fully cleaved, mature complex is highly stable. Control of this cleavage process is important as it controls the levels of viral RNA species that are present at different times during infection[76,77]; this regulation is discussed in more detail in the following section. This cleavage process appears to have unique regulatory features such as having morphological cleavage recognition instead of sequence specificity[78]. This regulatory process is highly important to viral biology as altering it leads to attenuation[79]. Proper cleavage is also important to immune evasion, as viruses with incomplete cleavage result in alterations of the viral RNA species prevalence, increasing type I interferon induction as well as the sensitivity of the viruses to interferon[80].

4.5 Viral RNA Replication

The process of viral RNA synthesis is outlined in Figure 3 B. To make additional molecules of RNA genome, the virus is required to first transcribe the positive-sense genome into negative-sense template strand. This activity is performed by the partially cleaved polyprotein nsp123/4[77]. However, the protein cleavage activity of nsP2 rapidly degrades the polyprotein into its constitutive parts. This initially produces intermediate forms of the replicase complex that are short lived and produce both negative and positive-sense RNA[76,77]. The final cleavage between nsP2 and nsP3 leads to the formation of the mature replicase complex nsP1/2/3/4 which produces only positive-sense RNA[74,75,81]. This self-proteolytic behavior creates a distinct expression profile of the viral RNA. Initially the immature forms of the complex produce higher levels of negative-sense RNA. As the complex is processed the synthesis of negative-sense RNA is reduced and eventually eliminated. This causes most negative-sense RNA to be produced early in infection, as well as less negative-sense RNA being produced overall[62]. Following cleavage and assembly of the mature replicase complex, RNA synthesis converts to the synthesis of positive-sense genomic and subgenomic RNA[82].

The positive-sense genomic RNA functions primarily as the genetic material of the next generation of virus, as well as being translationally active in the cell to produce additional nonstructural proteins. However, recent work has indicated that the genomic RNA may have biological functions that are not dependent on its function as a viral mRNA, as increasing the amount of capped RNA decreased viral fitness, indicating that there is some important role played by the noncapped RNA which isn't replicatively competent[83].

Late in infection an additional positive sense RNA is produced, the small subgenomic RNA, which is used to produce the structural proteins[71]. Additionally, when viral RNA is examined on agarose gels, there is a third RNA species that appears, the so called replication intermediate RNA [62,84]. This an RNA species that runs at very large size, however its significance is unknown, and there is no research indicating its role in the viral life cycle.

4.6 Viral RNA replication as a target of antiviral drug development.

One of the most common targets of antiviral drug development has been reducing replication of the viral genetic material, as this limits viral spread in the host and thus disease. A common drug class that has been found to have these effects is the nucleoside analogs. This class includes the drug ribavirin, used in the treatment of many viruses, but which is largely ineffective as an anti-alphaviral therapy[85]. Multiple nucleoside analogues have been tested as treatments for the alphaviruses and two will be described here, favipiravir, and β-D-N4-hydroxycytidine.

Favipiravir is a nucleoside analogue that is approved for use by the Pharmaceuticals and Medical Devices Agency, the Japanese drug regulatory body, for the treatment of influenza under the tradename Avigan[54]. Favipiravir has also been tested against a wide variety of other viruses, including the alphaviruses, both *in vitro* and *in vivo*. Favipiravir inhibits the activity of viral polymerases by competing with purines for incorporation in to the viral RNA. This activity locks the strand and prevents its use in further viral replication[55,86].

When tested in models of alphaviral infection, favipiravir has been found to be mildly efficacious against WEEV, and treatment with this drug resulted in the clearance of CHIKV from infected mice when given in the acute phase of infection, but had no effect in the chronic phase[86–88]. However, favipiravir treatment for alphaviruses has also only been tested via intraperitoneal injection, which is not a preferred method of delivery in humans[87,89]. There have been no tests with the oral formulation currently approved for use in humans for influenza. Sensitivity to favipiravir in the alphaviruses also appears to be strain dependent. With the strains of CHIKV that are of most concern being less sensitive to treatment than strains that are less involved in human outbreaks[90]. While favipiravir is currently not clinically approved for use in the alphaviruses, the knowledge about its efficacy could lead to future compounds with enhanced anti-alphaviral properties.

Not all nucleoside analogues function by the same mechanism to inhibit viral replication. Another nucleoside analogue that has been examined in alphaviral infection is β-D-N4-hydroxycytidine or NHC[60]. NHC has been tested for therapeutic efficacy against many viruses including influenza and respiratory syncytial virus, and is structurally highly similar to molnupiravir, one of the currently approved antiviral treatments for SARS-CoV-2[91,92].

NHC functions by multiple mechanisms, one of which is the inducement of hypermutation during replication of the viral genome[60]. This mutagenesis as well as the secondary effect of reduced viral infectivity results in inducing minimal resistance to treatment in the viral population[60] which indicates that NHC and its derivatives/related compounds show promise as potential treatments of the alphaviruses. The structures of both favipiravir and NHC can be found above in table 2.

4.7 Importance of the untranslated regions of alphaviruses

The genomes of alphaviruses have large 5'and 3' untranslated regions (UTRs) which are biologically active. The 3' UTR is important in avoiding the immune bottlenecks that exist in the arthropod phase of the life cycle[93], and the sequence variability that occurs in this region can have significant effects on transmission in mosquito vectors and on vector specificity[93]. The main variation in this region is due to size, which directly relates to the number of repeated regions that occur in the sequence[94,95]. This variation in the 3' UTR does not only occur between different viruses but can also vary significantly within viral species and has been well documented to differ in the differently pathogenic strains of CHIKV[96]. The 3' UTR is also involved in pathogenicity of alphaviruses, such as in EEEV where it binds to micro RNA and promotes neurological disease[97], and is involved in the synthesis of the negative sense RNA[98].

The 5' UTR functions in many ways to promote translation of the viral RNA both via its structure and sequence[99,100]. These structures can vary significantly between species and aren't necessarily interchangeable[101]. The structure of the 5' UTR is also important to evasion of the interferon response in alphaviruses, with the stability of the structure playing an important role in preventing recognition of the cap structure of the viral RNA[100,102]. The importance of this region to virulence is seen in the attenuation of VEEV strain TC-83, a mutation in this region alters the ratios of viral RNA types and results in a significant increase in sensitivity to interferon compared to wild-type viruses[103,104]

4.8 Localization of genome replication

Alphaviruses demonstrate a sequestration of their replication to intracellular membranes, which is similar to other RNA viruses which also largely replicate in and on membranous structures[105–107]. The alphaviruses utilize microinvaginations called spherules[105]. These are sites where the viral RNA has been found to localize in infected cells[108,109]. It has been confirmed *in vitro* that these structures contain viral RNA synthetic activity through the use of purified spherules to produce viral RNA[110]. It has been recently determined that the initial formation of the spherules is dependent solely on the activity of the nsPs with no requirement for viral RNA being present[111]. However, the size of the individual spherules is dependent on the length of the RNA that is transcribed within, which appears to be a feature unique to alphaviruses[112]. These spherules have been suggested to play a role in viral immune evasion by isolating viral double-stranded RNA away from cytoplasmic pattern recognition receptors[113,114]. There is also evidence from the flaviviruses that in general, membranous association of viral RNA replication can protect RNA from enzymatic digestion[115]. Many of these phenotypes have also now been confirmed in CHIKV using cutting edge microscopy and structural biology methods[116]

Spherules were initially identified on large, endosomal-like compartments in infected cells. In several of the alphaviruses these spherules form at the plasma membrane and later traffic to intracellular compartments[117]. In vertebrate cells, recent work has indicated the movement of the spherules away from the plasma membrane is dependent on the activation of PI3K-Akt-mTOR, and reduction of this activation is associated with an increased proportion of the spherules remaining at the cellular membrane[118]. However, inhibition of this activity has no effect on viral titer, indicating that localization of replication may not be important to replicative success[119]

4.9 Translation of the structural genes

The structural genes of the alphaviruses are produced via translation of the subgenomic RNA. The synthesis of this RNA initiates independently from the full-length genomic RNA, but how this secondary initiation happens remains unknown. The initial gene product is a polyprotein that contains the capsid, E proteins, 6K, and TF proteins[7,71]. This translation is carried out in the cytosol and the structural proteins are synthesized at very high levels and are found throughout the cell[120,121]. Production of the structural genes occurs primarily later in infection due to the increased expression of the subgenomic RNA[7]. The capsid protein contains a serine protease domain and uses this to rapidly cleave itself from the other structural genes after it is translated[71].

After cleavage of the capsid protein, the glycoproteins pass into the endoplasmic reticulum, and move through the Golgi apparatus before being embedded into the plasma membrane of the cell[71]. The glycoproteins are also highly post-translationally modified via glycosylation and palmitoyaltion[71].

4.10 Packaging of the viral RNA and release of the virion

Unlike some other viruses, the alphaviruses don’t readily produce empty particles[122]. However, they are known to produce defective interfering (DI) particles when passaged at high concentrations repeatedly, although this occurs less frequently than in other viruses such as vesicular stomatitis virus and influenza[123]. These particles have a range of sizes and are often smaller than normal virions, and they usually incorporate deletions in the viral RNA. The deletions in these DI particles get larger with increased passages[123]. The alphaviruses are noted for generally producing highly uniform, icosahedral particles[124] though this can be altered by mutating the structural proteins[125]. Current data indicate that while multicore, and other irregular virion shapes do occur with these viruses, these are highly selected against, and particles largely only contain a single unit of genomic RNA[7] and capsid cores select for carrying only single cargo units[122].

After translation of the structural proteins, the viral RNA and capsid undergo interactions based on molecule size and charge, resulting in nucleocapsid like structures occurring in the cytoplasm[71,126,127]. Alphaviruses bud directly from the plasma membrane of the infected cell[7]. However, it is unclear how this budding process is initiated[127]. It has been found that both the preformed nucleocapsid like structures and the glycoproteins are able to drive budding[127,128]. However, when either of these functions occurs independently of the

other, there is a marked reduction in efficiency, indicating that it is likely that these two mechanisms interact to allow for the maximal budding of virions[128].

Transport of the structural proteins to the plasma membrane requires the host secretory system[128]. However the exact proteins that are used remain unknown[128]. Release of virions can also be inhibited by host proteins. In particular tetherin has been shown to prevent the release of virions from infected cells[128]. The general replication scheme of alphaviruses is outlined in Figure 2.

Reducing viral release and decreasing the infectivity of viral particles is an activity of some anti-alphaviral compounds, in particular ß-D-N4-hydroxycytidine, a compound discussed earlier in section 4.6.

5. Functions of the alphaviral nonstructural proteins

The alphaviruses make four nonstructural proteins. These proteins are responsible for viral RNA replication as well as many other enzymatic functions. The nonstructural proteins are also intimately involved in the pathogenesis of the alphaviruses. The functions of these proteins will now be described in greater detail. While the functions of the nsPs are highly conserved, differences between the Old and New-World viruses will be indicated when necessary.

5.1 Nonstructural protein 1

NsP1 is the capping enzyme for the viral genomic RNA, and this activity occurs independent of the activities of the other nsPs[129]. The activity of this protein has recently been examined in VEEV, having previously been studied only in Old-World viruses. This was also the first time that each individual step, including the final guanyl transfer, has been described[130]. The steps occur as follows. 1) The transfer of a methyl group from *S-adenosylmethionine* to position N7 of a molecule of GTP is catalyzed; 2) nsP1 receives the methyl-GTP becoming guanylated, releasing pyrophosphate in the process; 3) the 7 methyl-GMP is transferred to the 5’ end of the target RNA[130,131]. For this reaction to occur properly, the RNA being capped must have had its 5’ terminal phosphate removed by nsP2[132].

NsP1 is also responsible for the anchoring of the viral replicase complex to cellular membranes which are the site of RNA replication, and this activity is required for capping to be carried out as well[133–135]. Very recently a cryo-em structure was published that showed how nsP1 influences the structure of the membrane spherules and potentially controls entry and exit of materials[136]. NsP1 was found to form a ring-like structure that appears to act as a gate and controls movement to and from the compartment[136]. This detailed structure has now also been used to examine the capping activity of CHIVK in depth as well, as can be seen in recent publication from the Law lab[137]

5.2 Nonstructural protein 2

NsP2 is a multifunctional protein with several distinct domains with discreet enzymatic activities. First, nsP2 is responsible for host cell transcriptional shutoff in the Old-World viruses, and loss of this phenotype reduces viral cytotoxicity[138,139]. In the New-World

viruses this activity is instead carried out by the capsid protein, and nsP2 is responsible for shutoff of host cell protein synthesis and may have a role in packaging of viral RNA[138,140–142]. In VEEV this translational shutdown has been shown to mediate resistance to a pre-existing antiviral state[141]. Interestingly, a recent publication has indicated that both expression of a heterologous nsP2 as well overexpression of an nsP2 in the presence of infection is inhibitory for the alphaviruses in mosquito cells, and is likely one way that the infected cells resist superinfection[143]

There are three recognized domains in nsP2. The N-terminal region contains a helicase domain and NTPase activity that serves to provide energy for the helicase[144,145]. This same region also has RNA 5'-triphosphatase activity which prepares RNA for capping, allowing for translation and packaging in virions[132]. The previous 2 years have seen significant advances in the understanding of the structure of alphaviral nsP2 protein. First, the N terminal region of nsP2 from CHIKV, including the helicase domain, has recently been crystalized[146], and this portion of the protein was used to characterize RNA binding activity[147]. Following this, there is now a full length crystallization of nsP2 available for CHIKV released by the same research group[148]. Here it was revealed that the N and C termini of this protein are connected by a flexible linker, and that this linker is highly important for normal protein function and viral pathogenicity[148]. Structures from this group have already proven useful for prediction of the structures of the nsP2 proteins of other alphaviruses[62]. These structures are also already being used to further research into potential therapeutics, in particular using computer modeling for drug selection and binding prediction[149,150].

The most interesting feature of the nsP2 N-terminal crystal structure was the large number of accessory domains that were present, as these domains had not previously been predicted by structural modeling. Of particular interest is the so called stalk domain, which based upon recent research appears to have an important function in viral RNA synthesis as shown by the activity of the recently characterized antiviral compound ML336[62]. This compound is discussed later in section 5.5

Large portions of the N-terminal region of nsP2 remain poorly characterized. Studies have implicated that in VEEV this region may be important to packaging of the viral genome[142]. However, in SINV a transposon insertion approach using the sequence for GFP found that this region was involved in the cleavage between nsP2 and nsP3, controlling the ratio of genomic and subgenomic RNA, and regulation of RNA synthesis[151]. This range of phenotypes indicates that this region is highly important to these viruses, but further characterization and research are needed.

NsP2 also contains a cysteine protease domain that is responsible for the cleavage of the nsPs from the polyprotein into its constitutive members[152–154]. As described earlier, this cleavage is responsible for the transition from the synthesis of negative-sense viral RNA to positive-sense viral RNA[77,155]. The protease has also been shown to target cellular proteins, a common feature of viral proteases, and this is related to resistance to innate immune responses[156].

Lastly, nsP2 contains a putative methyltransferase domain that was predicted using homology modeling[154]. However, it is currently thought to be inactive as it lacks a key active site residue[129]. Recently there has been work that has shown a potential alternative activity for this region. There is data that indicates this domain may play a role in interferon shutoff due to its interaction with signal transducer and activator of transcription proteins 1 (STAT1)[157]. This activity appears to be mediated by enhancing the nuclear export of STAT1, which prevents the magnification of downstream immune signaling, including the interferon response[157]. Work with CHIKV nsP2 has also continued with groups now using the complete crystal structure to predict potential interactions with innate immune proteins[158], and this structure can now be utilized for studies altering the structures and components of the nsP2 protein to further elucidate its functions[159]

5.3 Nonstructural protein 3

NsP3 is poorly understood, but mutations within this protein have resulted in defects in both negative-sense and subgenomic RNA synthesis[160].

NsP3 contains a macrodomain with both adenosine diphosphate ribose (ADPr) binding and hydrolase activity[129,161,162], and these activities have begun to be characterized in the context of infection models. The ADP ribosylase activity is necessary for infection of neural cells and the hydrolase activity results in an increase in replicase complexes[163]. In a SINV model, reductions in hydrolase activity led to reduced neurovirulence while increases in ADP ribosylase activity increased neurovirulence[164].

NsP3 also contains the highly conserved alphavirus unique domain, or AUD[129]. This domain is maintained across all alphaviruses[129]. Recent work using CHIKV has indicated that it potentially has many functions, particularly in subgenomic RNA replication[165]. Disruption of the AUD resulted in decreased infectivity, potentially due to decreased interaction with the viral RNA and the subgenomic promoter[165].

The last feature of note in nsP3 is the hypervariable domain or HVD. This domain is so varied that it can be distinct between strains of a single viral species, such as in VEEV[166]. This region is tolerant of significant mutation and even deletion, which is unique compared to the rest of the nsPs and their domains[167]. Natural duplications and insertions in this region can even have positive effects on viral fitness[168]. The HVD is also involved in interaction with host cell proteins, resulting in the formation of distinct protein complexes in Old and New-World viruses[169]. These interactions include the cellular machinery responsible for the formation of stress granules, which alphaviruses utilize to their own replicative benefit[170–173]. These interactions are highly specific to viral species as well, and may partially drive the differences in pathogenesis seen between species[173–175].

5.4 Nonstructural protein 4

NsP4 is produced in small amounts by most alphaviruses due to the inclusion of a stop codon between nsP3 and nsP4[71,176]. Altering expression levels of nsP4 decreases viral fitness, indicating that tight control of expression is highly important[177]. The tight limit on expression of nsP4 is also promoted by it being targeted by N-end rule degradation[178]. Recent work performed using trans replication systems has elegantly shown how nsP4 is

involved in RNA selectivity, yet this occurs in tandem with the other nsPs[179]. Interestingly these authors have shown that in trans and in a transfection system, increasing nsP4 levels does not have these same detrimental effects[179]

NsP4 is the RNA dependent RNA polymerase (RDRP) of the alphaviruses and is active in both positive and negative-sense RNA synthesis, with the specificity being determined by the cleavage state of the other nsPs[77,129,180]. NsP4 can display RNA synthesis activity alone, but its activity is enhanced by the presence of the other nsPs[180–182]. There has been recent publication of crystal structures of the RDRP domains of both RRV and SINV[183]. This work indicated that the RDRP pocket of these viruses was highly dynamic and flexible, as well as being well conserved between the two viruses[183].

NsP4 also has a large N terminal region that lacks predicted structure or function. Recent work has predicted that this region is somehow involved in viral RNA synthesis, as mutations in this region result in resistance to the effects of a drug that inhibits the production of new viral RNA[62,184]. However, the function of this region remains unclear. Work by others has also shown that mutations in this region has a broad range of effects on viral RNA synthesis[185]. This work, as well as the antiviral resistant VEEV isolates that have been recovered[184], indicate that this region plays an important role in RNA synthesis of these viruses, potentially in tandem with nsP2. This indicates that these proteins have additional, complex interactions and roles in viral biology that remain to be understood. The functions of the nsPs are summarized in Table 3.

5.5 The nsPs as potential antiviral drug targets

In recent years there has been increasing interest in viral proteins as targets for antiviral drug development. This is primarily due to their being largely distinct from cellular proteins, as well as being required for viral replication. Additionally, many nucleoside and non-nucleoside analogs broadly target replication of RNA and DNA resulting in significant side effects and toxicity, making novel targets attractive. Targeting viral proteins directly theoretically allows for increased specificity and reduced side effect potential. The approval of sofosbuvir/ledipasvir combination treatment for hepatitis C infection and its incredible success in the clinic has increased interest in these targets, as it has proved that viral protein targets are therapeutically viable. Of particular note is that sofosbuvir/ledipasvir combination treatment has fewer side effects and higher cure rates than the previous standard of care using ribavirin/interferon combination treatment[186].

With regards to the alphaviruses, researchers have so far reported a novel class of anti-VEEV drugs based around a highly aromatized core[184]. These compounds were then further developed into the benzamidine drug ML336, which is highly effective at inhibiting RNA synthesis, likely due to interactions with nsP2 and nsP4[62]. This compound is highly specific as well, having only minimal effects on cellular RNA synthesis[62], and this combined with its low EC_{50} value indicate that it is unlikely to have significant side effects. While ML336 is highly specific to VEEV, further manipulation of these drugs has potential to lead to treatments for additional alpahviruses[62,184]. The structure of ML336 can be found in table 2 above.

There is also work looking at the other nsPs for potential antiviral targets. These include nsP3 in CHIKV, which has been used for modeling studies examining small molecule libraries[187]. The other nsPs of CHIKV have been examined as well, and a review summarizing these investigations has been published by Sundar, Piramnanyagam, and Natarajan, and it also compares these efforts to dengue and zika[188]. Further review of CHIKV drug discovery can be found in Kovacikova and Hemert[189]. A review of promising pre-clinical antivirals against the encephalitic alphaviruses can be found in Kehn-Hall and Bradfute[190].

6. Functions of the alphavirus structural proteins

As outlined above, the alphaviruses manufacture their structural proteins through a second open reading frame derived from the genomic RNA termed the subgenomic RNA[7]. The subgenome encodes six proteins: capsid, E3/2, 6K/TF, and E1. These proteins are produced as a polyprotein similar to the nonstructural proteins, and the capsid has self-cleavage functionality to release itself from the polyprotein[71]. The function and processing of these proteins will be further described below.

6.1 Capsid protein

The capsid is the first structural protein that is translated after subgenomic RNA production. This protein is responsible for forming the primary structure of the virion and selects for packaging of the viral genomic RNA by recognition of a conserved packaging signal[191], but the formation of nucleocapsid cores is not dependent on this signal[192–194]. Encapsidation selects specifically for only single units of cargo to be packaged[122], and in infected cells, this packaging activity is highly specific for viral RNA. After packaging the RNA, the nucleocapsid cores translocate to the plasma membrane where they will bud into the extracellular environment while collecting a membrane as well as the glycoprotein spikes[7]. The initiation of budding remains poorly defined and there are contributions from both the nucleocapsid core and the glycoproteins. While either can initiate budding independently, they likely function in a synergistic manner[127,128].

The capsid protein is one of the most produced proteins during alphaviral infection, and it has several pathogenic roles in addition to its structural function. In the New-World alphaviruses the capsid protein is able to block the nuclear pores and thus prevent the translation of new cellular proteins, enhancing viral pathogenesis, cytopathic effect, and assisting in immune evasion[138,140,195]. This protein synthesis inhibition functions in tandem with the nsPs which actively inhibit the synthesis of cellular proteins[141,196,197]. While several mechanisms have been proposed for this activity there is currently no definitive evidence to support one hypothesis over another and it is likely multifactorial. The capsid of the New-World viruses also contains sequences which result in its importation to the nucleus itself, which inhibits the transcription of cellular RNA[195]. Exactly how this activity is caried out remains unknown.

While the capsid of the Old-World viruses contains a nuclear transport signal and is able to enter the nucleus, why it does this remains unclear as it is not involved in transcriptional shutoff in infection[198]. This transcriptional shutoff in the Old-World viruses is carried out

by nsP2, which ubiquinates subunits of the cellular RNA polymerase, targeting them for degradation[195,199].

These non-packaging activities of the capsid protein have been targeted by certain preclinical anti-alphaviral compounds, such as mifepristone and compounds derived from it, by inhibiting the normal trafficking of the capsid protein[200].

6.2 The glycoproteins

The alphaviruses manufacture three glycoproteins during infection, E1, E2, and E3. E3 and 2 are made as a single fused peptide that undergoes later processing after translation in the endoplasmic reticulum. E1 is encoded singly[7]. These proteins are translated in the ER and then traffic through the Golgi apparatus and undergo various glycosylation and palmytoilation events[201–207]. E2 is cleaved from E3 although the two remain in close association. E2 and E3 also may not always fully separate and this appears to be pH dependent[124,208]. The proteins are then arrayed at the plasma membrane and form heterotrimers of E1 and E2[209] where they are picked up by budding nucleocapsid cores and integrated into the mature virions[7].

As mentioned above, the glycoproteins are involved in the fusion of the viral and host cell membranes in the endosomal pathway, which mediates release of the viral genome into the cytoplasm. E1 alone is sufficient to induce this fusogenic activity[63,64]. E1 is also able to induce membrane pores, and this pore forming activity is likely to contribute to pH dependent viral particle disassembly as well as induce various physiological changes in the cells due to these membrane disruptions[68–70,210].

E3 is known to function as a signal sequence that guides the structural polyprotein into the endoplasmic reticulum after capsid cleavage[211]. E3 remains associated to E2 until furin cleavage separates the two proteins in the Golgi apparatus[211,212]. However, E3 must have additional functions as replacing it with an endoplasmic reticulum signal sequence results in the other glycoproteins being trapped in the ER[213]. Swapping the E3 sequences between alphaviral clades also results in attenuation while swapping within a clade does not have the same effect, and this phenotype is dependent on interactions that occur between E2 and E3[214]. Finally E3 has an important role in protecting E1 from the acidic pH of the secretory pathway and allowing for its secretion to the cell surface[215].

6.3 6K/TF protein

The 6K gene splits the E2 and E1 genes and forms two different protein products due to a frameshift that occurs in response to read through of a so called slippery sequence in the middle of the protein[7,216]. This frameshift is actively enhanced by the folding of the polyprotein that occurs simultaneously with translation of the viral RNA[217].

The more common form of the protein is simply termed 6K as it has a size of roughly six kilodaltons[216]. 6K is known to be important to budding of the mature virions and is hypothesized to act as a spacer for the glycoproteins[216,218]. There is also strong evidence that the 6K protein directly associates with the E2 glycoprotein, and a loss of this association

reduced budding efficiency[216,219–222]. However, this relationship and the resulting reduction in budding has yet to be fully described.

The second protein is called TF for “trans frame,” as it is generated from a one nucleotide frame shift[216,223]. This protein was relatively recently identified as a separate entity from 6K[216]. TF is found incorporated into the virion structure after release from the cell[216,224]. Recent work has also indicated that there is a role for TF protein in the evasion of the interferon response and that this is due to specific palmitoylation patterns[225], with mutant viruses causing no disease in interferon intact animals, but disease pathology being restored in ifnar knockout mice[225]. Additionally palmitoylation of this protein is self-regulated, with specific domains of TF protein affecting its own palmitoylation levels[226]

One or both of these proteins also function as a viroporin ,and when either or both of these proteins is over expressed in bacteria they are cytotoxic to the cultured cells[216].

It is currently understood that many of the phenotypes attributed to 6K are likely mediated instead by the TF protein; however, the exact differences in their activities remain poorly defined. Both are important for efficient release of progeny virus, both are known to be highly cytotoxic when expressed ectopically, and both are important to viral virulence, with viruses that lack these proteins causing less severe disease phenotypes[216].

Perhaps the most interesting finding about these two proteins is that they are the only alphaviral proteins that are not necessary to produce viable viruses in cell culture[216]. A deletion of both proteins does result in reduced viral titers in the supernatant of infected cells, but virus is produced[216,227]. If any of the other proteins coded for by the alphaviruses are deleted, however, it results in nonviability. The functions of the structural proteins are summarized in table 4.

7. Screening assays for antiviral drug development

As highlighted in the above sections, there is extensive ongoing work on discovery and testing of anti-alphaviral therapeutics. As with all antiviral development, this work has seen an explosion in productivity thanks to the development of high-throughput drug discovery assays and technology. The introduction of these techniques has allowed for an exponential increase in the number of compounds that can be screened, and has played roles in most of the already mentioned antiviral compounds. A summary of selected assays can be found in table 5 below. For a thorough review of the state of antiviral development studies more information can be found in Andersen et al.[230].

8. The study of alphaviruses in animal models

A critical part of studying infectious agents and the development of drug treatments is the availability of animal models that recapitulate disease as seen in human patients. The alphaviruses have been extensively studied since their initial discovery in mid 20th century. Due to this there have been a large number of animal models investigated for potential study of these viruses. VEEV in particular was widely studied in a variety of animal models in the mid-20th century in an attempt to determine a model that would closely mimic human

infection[244]. Currently mice and non-human primates are the most common alphavirus models. A summary of commonly used and well described animal models for alphaviral infection can be found in table 6 below.

9. The Development of alphaviral vaccines

While there are no approved vaccines against alphaviruses for human use, there are veterinary vaccines available for livestock. These vaccines are available as a trivalent dose for the three Equine Encephalitis viruses and are widely used in North, Central, and South America[302]. While this vaccine is highly effective at disease prevention it does require annual boosting[302]. The livestock vaccine is based on inactivated virus and is not replicatively competent[303];this is due to previous vaccination with the live TC-83 vaccine strain of VEEV resulting in detectable virus occurring in mosquitos in the area of testing, indicating that use of live attenuated virus could potentially result in outbreaks of disease if there were reversion events[304].

There have been many attempts at creating vaccines for the alphaviruses for human use. The most advanced vaccine for VEEV is the strain TC-83, a cell culture attenuated VEEV that was developed by the United States Army[305]. However, this vaccine is poorly immunogenic and has less than an 85% seroconversion rate when given as a single dose[306]. It also has a very high side effect rate with more than 20%, and in some studies more than 80%, of treated individuals reporting a side effect upon use[306,307]. TC-83 has also been noted to have the potential to revert to wild-type, epidemic strain VEEV[308,309]. There is also a specific booster for T-83, C-84, a formalin inactivated vaccine for those who have received TC-83 but did not seroconvert[306].

Despite its shortcomings, TC-83 remains in use for certain high-risk individuals such as those who frequently work with wild-type VEEV in high-risk research applications[310]. A rationally designed attenuated strain of VEEV termed V3526 was also tested as a vaccine, but it was found to have some remaining neurovirulence in a non-human primate model and was later abandoned early in clinical trials due to a high rate of side effects[311,312]. There are also candidate vaccines available for human use under special circumstances for both EEEV and WEEV, inactivated PE-6 and inactivated CM-4884, respectively. Though like TC-83 these vaccines have low response rates and these two inactivated vaccines often fail to produce durable immunity[313].

There has also been recent work examining the safety profile and immunogenicity of a trivalent virus like particle vaccine against VEEV, WEEV, and EEEV[314]. While this work is in the early stages of clinical development, the vaccine was extremely safe with only minimal and primarily localized side effects, and also demonstrated the production of a durable immune response as measure by neutralizing antibody titers against the three viruses. One caveat is that as time went on subjects became less likely to respond to all three of the viruses and instead responded preferentially to only one or two of the viruses[314]. A more detailed review of the state of vaccine development for the encephalitic alphaviruses can be found in Stromberg et al.[315].

With the spread of CHIKV across the tropics, it has also become a target of intense vaccine development research. The first candidate was the 181/25 strain of CHIKV which was abandoned due to a high rate of side effects[316,317]. Recently there has been significant promise shown by a variety of CHIKV vaccines based on live attenuated, vector launched, and subunit platforms[318–321]. Some of these have shown great promise for clinical approval[321].

10. Remaining questions.

While the alphaviruses have been well studied for many years, they remain significant public health threats due to challenges in the development of either vaccines or antiviral treatments. While vaccines are available for veterinary use[322,323], none of the available vaccine candidates have been found to meet the more stringent standards for humans due to significant side effects and relatively poor immunogenicity[324–326]. This lack of treatment means that these viruses require significant ongoing study.

A major challenge that remains in the field is the characterization of the remaining regions of the nsPs that have no predicted function. These proteins remain challenging to study, however recent advances in protein expression have ameliorated this somewhat[146,154,327]. There is evidence that these uncharacterized regions are important to viral replication[62,231] , which makes them promising targets for antiviral drug development.

In addition to being potential drug targets, further characterization of these uncharacterized protein regions has the potential to further our understanding of positive-sense RNA viruses generally. While these viruses are highly variable in their biology and pathogenesis, they retain highly similar replication strategies, as exemplified by the similarities of viral RNA dependent RNA polymerase proteins[328] and the consistent use of host cell membranes as scaffolding to develop their replication centers[329,330].

Lastly, there has been some recent work to show that the alphaviral RNAs themselves are likely to have biological activities in addition to their use for translation of the viral proteins. This can be seen in the necessarily tight control of capping[83], as well as in the highly complex structures that form in the RNA and affect viral replication[331,332]. This all indicates that there remain many outstanding questions about these viruses, and that further research will be needed as they remain significant threats to public health and for emergence into naïve populations.

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References

1. International Committee on Taxonomy of Viruses. Taxonomy. (2020).
2. Powers AM et al. Evolutionary Relationships and Systematics of the Alphaviruses. J. Virol 75, 10118–10131 (2001). [PubMed: 11581380]

3. Crosby L. et al. Severe manifestations of chikungunya virus in critically ill patients during the 2013–2014 Caribbean outbreak. Int. J. Infect. Dis 48, 78–80 (2016). [PubMed: 27208636]
4. Rezza G. et al. Infection with chikungunya virus in Italy: an outbreak in a temperate region. The Lancet 370, 1840–1846 (2007).
5. Grandadam M. et al. Chikungunya Virus, Southeastern France. Emerg. Infect. Dis 17, 910–913 (2011). [PubMed: 21529410]
6. Staples JE & Fischer M Chikungunya Virus in the Americas — What a Vectorborne Pathogen Can Do. N. Engl. J. Med 371, 887–889 (2014). [PubMed: 25184860]
7. Johnston Robert E., C. J. P Fields Virology, vol. 1 (Lippincott-Raven, 2018).
8. Griffin DE & Johnson RT Role of the Immune Response in Recovery from Sindbis Virus Encephalitis in Mice. 7 (1977).
9. Laine M. et al. Prolonged arthritis associated with Sindbis-related (Pogosta) virus infection. Rheumatology 39, 1272–1274 (2000). [PubMed: 11085809]
10. Adouchief S, Smura T, Sane J, Vapalahti O & Kurkela S Sindbis virus as a human pathogen—epidemiology, clinical picture and pathogenesis. Rev. Med. Virol 26, 221–241 (2016). [PubMed: 26990827]
11. Arrigo NC, Adams AP & Weaver SC Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision. J. Virol 84, 1014–1025 (2010). [PubMed: 19889755]
12. Go YY, Balasuriya UBR & Lee C Zoonotic encephalitides caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. Clin. Exp. Vaccine Res 3, 58–77 (2014). [PubMed: 24427764]
13. Acosta-Ampudia Y. et al. Mayaro: an emerging viral threat? Emerg. Microbes Infect. 7, (2018). [PubMed: 29362446]
14. Murillo-Zamora E. et al. Persistent Arthralgia and Related Risks Factors: A Cohort Study at 12 Months from Laboratory-Confirmed Chikungunya Infection. Arch. Med. Res 49, 65–73 (2018). [PubMed: 29703609]
15. Barber B, Denholm JT & Spelman D RACGP - Ross River virus. (2009).
16. Aguilar PV et al. Endemic Venezuelan equine encephalitis in the Americas: hidden under the dengue umbrella. Future Virol. 6, 721–740 (2011). [PubMed: 21765860]
17. CDC. Statistics & Maps | Eastern Equine Encephalitis | CDC. https://www.cdc.gov/easternequineencephalitis/statistics-maps/index.html (2021).
18. Bergren NA et al. “Submergence” of Western equine encephalitis virus: Evidence of positive selection argues against genetic drift and fitness reductions. PLOS Pathog. 16, e1008102 (2020). [PubMed: 32027727]
19. Brault AC, Fang Y & Reisen WK Multiplex qRT-PCR for the Detection of Western Equine Encephalomyelitis, St. Louis Encephalitis, and West Nile Viral RNA in Mosquito Pools (Diptera: Culicidae). J. Med. Entomol 52, 491–499 (2015). [PubMed: 26334826]
20. Rusnak JM, Dupuy LC, Niemuth NA, Glenn AM & Ward LA Comparison of Aerosol- and Percutaneous-acquired Venezuelan Equine Encephalitis in Humans and Nonhuman Primates for Suitability in Predicting Clinical Efficacy under the Animal Rule. Comp. Med 68, 380–395 (2018). [PubMed: 30282570]
21. CDC & USDA. Federal Select Agent Program - Select Agents and Toxins List. https://www.selectagents.gov/SelectAgentsandToxinsList.html (2020).
22. CDC & USDA. About Us | Federal Select Agent Program. https://www.selectagents.gov/overview/index.htm (2022).
23. Zacks MA & Paessler S ENCEPHALITIC ALPHAVIRUSES. Vet. Microbiol 140, 281 (2010). [PubMed: 19775836]
24. Steele KE & Twenhafel NA REVIEW PAPER: Pathology of Animal Models of Alphavirus Encephalitis. Vet. Pathol 47, 790–805 (2010). [PubMed: 20551475]
25. Lima Neto AS, Sousa GS, Nascimento OJ & Castro MC Chikungunya-attributable deaths: A neglected outcome of a neglected disease. PLoS Negl. Trop. Dis 13, e0007575 (2019). [PubMed: 31513593]

26. Willems WR et al. Semliki Forest Virus: Cause of a Fatal Case of Human Encephalitis. Science 203, 1127–1129 (1979). [PubMed: 424742]
27. Harley D, Sleigh A & Ritchie S Ross River Virus Transmission, Infection, and Disease: a Cross-Disciplinary Review. Clin. Microbiol. Rev 14, 909–932 (2001). [PubMed: 11585790]
28. Corbet PS, Williams MC & Gillett JD O'nyong-nyong Fever: An Epidemic Virus Disease in East Africa. Trans. R. Soc. Trop. Med. Hyg 55, 463–480 (1961). [PubMed: 13881254]
29. Smith DR, Adams AP, Kenney JL, Wang E & Weaver SC Venezuelan equine encephalitis virus in the mosquito vector Aedes taeniorhynchus: Infection initiated by a small number of susceptible epithelial cells and a population bottleneck. Virology 372, 176–186 (2008). [PubMed: 18023837]
30. Forrester NL, Coffey LL & Weaver SC Arboviral Bottlenecks and Challenges to Maintaining Diversity and Fitness during Mosquito Transmission. Viruses 6, 3991–4004 (2014). [PubMed: 25341663]
31. Arrigo NC, Smith DR, Weaver SC, Muehlberger LE & Leal G Infection and Dissemination of Venezuelan Equine Encephalitis Virus in the Epidemic Mosquito Vector, Aedes taeniorhynchus. Am. J. Trop. Med. Hyg 77, 176–187 (2007). [PubMed: 17620651]
32. Jose J, Taylor AB & Kuhn RJ Spatial and Temporal Analysis of Alphavirus Replication and Assembly in Mammalian and Mosquito Cells. mBio 8, e02294–16 (2021).
33. Leung JY-S, Ng MM-L & Chu JJH Replication of Alphaviruses: A Review on the Entry Process of Alphaviruses into Cells. Adv. Virol 2011, 1–9 (2011).
34. MacDonald GH & Johnston RE Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J. Virol 74, 914–922 (2000). [PubMed: 10623754]
35. Zhang R. et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. Nature 557, 570–574 (2018). [PubMed: 29769725]
36. Ma H. et al. LDLRAD3 is a receptor for Venezuelan equine encephalitis virus. Nature 588, 308–314(2020). [PubMed: 33208938]
37. Bernard KA, Klimstra WB & Johnston RE Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology 276, 93–103 (2000). [PubMed: 11021998]
38. Heil ML, Albee A, Strauss JH & Kuhn RJ An Amino Acid Substitution in the Coding Region of the E2 Glycoprotein Adapts Ross River Virus To Utilize Heparan Sulfate as an Attachment Moiety. J. Virol 75, 6303–6309 (2001). [PubMed: 11413296]
39. Klimstra WB, Ryman KD & Johnston RE Adaptation of Sindbis Virus to BHK Cells Selects for Use of Heparan Sulfate as an Attachment Receptor. J. Virol 72, 7357–7366 (1998). [PubMed: 9696832]
40. Gardner CL, Ebel GD, Ryman KD & Klimstra WB Heparan sulfate binding by natural eastern equine encephalitis viruses promotes neurovirulence. Proc. Natl. Acad. Sci 108, 16026–16031 (2011). [PubMed: 21896745]
41. Helenius A, Kartenbeck J, Simons K & Fries E On the entry of semliki forest virus into BHK-21 cells. J. Cell Biol 84, 404–420 (1980). [PubMed: 6991511]
42. Leung JY-S, Ng MM-L & Chu JJH Replication of Alphaviruses: A Review on the Entry Process of Alphaviruses into Cells. Adv. Virol 2011, 1–9 (2011).
43. Kolokoltsov AA, Fleming EH & Davey RA Venezuelan equine encephalitis virus entry mechanism requires late endosome formation and resists cell membrane cholesterol depletion. Virology 347, 333–342 (2006). [PubMed: 16427678]
44. Hernandez R, Luo T & Brown DT Exposure to low pH is not required for penetration of mosquito cells by Sindbis virus. J. Virol 75, 2010–2013 (2001). [PubMed: 11160702]
45. Paredes AM et al. Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. Virology 324, 373–386 (2004). [PubMed: 15207623]
46. Bernard E. et al. Endocytosis of Chikungunya Virus into Mammalian Cells: Role of Clathrin and Early Endosomal Compartments. PLOS ONE 5, e11479 (2010). [PubMed: 20628602]
47. Nok AJ Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. Parasitol. Res 90, 71–79 (2003). [PubMed: 12743807]

48. PubChem. Suramin. https://pubchem.ncbi.nlm.nih.gov/compound/5361.
49. De Clercq E Suramin: A potent inhibitor of the reverse transcriptase of RNA tumor viruses. Cancer Lett. 8, 9–22 (1979). [PubMed: 92362]
50. Stein CA, LaRocca RV, Thomas R, McAtee N & Myers CE Suramin: and anticancer drug with a unique mechanism of action. J. Clin. Oncol 7, 499–508 (1989). [PubMed: 2926472]
51. Albulescu IC et al. Suramin inhibits chikungunya virus replication through multiple mechanisms. Antiviral Res. 121, 39–46 (2015). [PubMed: 26112648]
52. Kuo S-C et al. Suramin treatment reduces chikungunya pathogenesis in mice. Antiviral Res. 134, 89–96 (2016). [PubMed: 27577529]
53. PubChem. Favipiravir. https://pubchem.ncbi.nlm.nih.gov/compound/492405.
54. PMDA. AVIGAN Tablets 200mg. 1–6 https://www.sukl.cz/file/92989_1_1/download/ (2019).
55. FURUTA Y, KOMENO T & NAKAMURA T Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci 93, 449–463 (2017).
56. CDC - African Trypanosomiasis - Resources for Health Professionals. https://www.cdc.gov/parasites/sleepingsickness/health_professionals/index.html (2022).
57. Büscher P, Cecchi G, Jamonneau V & Priotto G Human African trypanosomiasis. The Lancet 390, 2397–2409 (2017).
58. Kovacikova K & van Hemert MJ Small-Molecule Inhibitors of Chikungunya Virus: Mechanisms of Action and Antiviral Drug Resistance. Antimicrob. Agents Chemother 64, e01788–20 (2020). [PubMed: 32928738]
59. PubChem. N(4)-Hydroxycytidine. https://pubchem.ncbi.nlm.nih.gov/compound/197020.
60. Urakova N. et al. β-d-N4-Hydroxycytidine Is a Potent Anti-alphavirus Compound That Induces a High Level of Mutations in the Viral Genome. J. Virol 92, (2018).
61. PubChem. (E)-2-((1,4-dimethylpiperazin-2-ylidene)-amino)-5-nitro-n-phenylbenzamide. https://pubchem.ncbi.nlm.nih.gov/compound/71301451.
62. Skidmore AM, Adcock RS, Jonsson CB, Golden JE & Chung D-H Benzamidine ML336 inhibits plus and minus strand RNA synthesis of Venezuelan equine encephalitis virus without affecting host RNA production. Antiviral Res. 174, 104674 (2020). [PubMed: 31816348]
63. Omar A & Koblet H Semliki Forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. Virology 166, 17–23 (1988). [PubMed: 3413984]
64. Sanz MA, Rejas MT & Carrasco L Individual Expression of Sindbis Virus Glycoproteins. E1 Alone Promotes Cell Fusion. Virology 305, 463–472 (2003). [PubMed: 12573591]
65. Wahlberg JM, Boere WA & Garoff H The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. J. Virol 63, 4991–4997 (1989). [PubMed: 2479769]
66. Singh I & Helenius A Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. J. Virol 66, 7049–7058 (1992). [PubMed: 1433506]
67. Wengler G, Würkner D & Wengler G Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. Virology 191, 880–888 (1992). [PubMed: 1333127]
68. Wengler G & Wengler G In vitro analysis of factors involved in the disassembly of Sindbis virus cores by 60S ribosomal subunits identifies a possible role of low pH. J. Gen. Virol 83, 2417–2426 (2002). [PubMed: 12237423]
69. Schlegel A, Omar A, Jentsch P, Morell A & Kempf C Semliki Forest virus envelope proteins function as proton channels. Biosci. Rep 11, 243–255 (1991). [PubMed: 1724188]
70. Spyr CA, Käsermann F & Kempf C Identification of the pore forming element of Semliki Forest virus spikes. FEES Lett. 375, 134–136 (1995).
71. Strauss JH & Strauss EG The Alphaviruses: Gene Expression, Replication, and Evolution. MICROBIOL REV 72 (1994).
72. Strauss EG, Rice CM & Strauss JH Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. Proc. Natl. Acad. Sci 80, 5271–5275 (1983). [PubMed: 6577423]

73. Myles KM, Kelly CLH, Ledermann JP & Powers AM Effects of an Opal Termination Codon Preceding the nsP4 Gene Sequence in the O'Nyong-Nyong Virus Genome on Anopheles gambiae Infectivity. J. Virol 80, 4992–4997 (2006). [PubMed: 16641290]
74. Vasiljeva L, Valmu L, Kääriäinen L & Merits A Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. J. Biol. Chem 276, 30786–30793 (2001). [PubMed: 11410598]
75. Vasiljeva L. et al. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. J. Biol. Chem 278, 41636–41645 (2003). [PubMed: 12917405]
76. Lemm JA & Rice CM Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. J. Virol 67, 1916–1926 (1993). [PubMed: 8445717]
77. Shirako Y & Strauss JH Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. J. Virol 68, 1874–1885 (1994). [PubMed: 8107248]
78. Lulla A, Lulla V & Merits A Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein. J. Virol 86, 553–565 (2012). [PubMed: 22031949]
79. Lulla V. et al. Timeliness of Proteolytic Events Is Prerequisite for Efficient Functioning of the Alphaviral Replicase. J. Virol 92, (2018).
80. Liu X. 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, (2018).
81. Shin G. et al. Structural and functional insights into alphavirus polyprotein processing and pathogenesis. Proc. Natl. Acad. Sci 109, 16534–16539 (2012). [PubMed: 23010928]
82. Pietilä MK, Hellström K & Ahola T Alphavirus polymerase and RNA replication. Virus Res. 234, 44–57 (2017). [PubMed: 28104453]
83. LaPointe AT, Moreno-Contreras J & Sokoloski KJ Increasing the Capping Efficiency of the Sindbis Virus nsP1 Protein Negatively Affects Viral Infection. mBio 9, (2018).
84. Barton DJ, Sawicki SG & Sawicki DL Solubilization and immunoprecipitation of alphavirus replication complexes. J. Virol 65, 1496–1506(1991). [PubMed: 1847467]
85. Markland W, McQuaid TJ, Jain J & Kwong AD Broad-Spectrum Antiviral Activity of the IMP Dehydrogenase Inhibitor VX-497: a Comparison with Ribavirin and Demonstration of Antiviral Additivity with Alpha Interferon. Antimicrob. Agents Chemother 44, 859–866 (2000). [PubMed: 10722482]
86. Furuta Y. et al. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res. 100, (2013).
87. Julander JG, Smee DF, Morrey JD & Furuta Y Effect of T-705 treatment on western equine encephalitis in a mouse model. Antiviral Res. 82, 169–171 (2009). [PubMed: 19428608]
88. Abdelnabi R, Jochmans D, Verbeken E, Neyts J & Delang L Antiviral treatment efficiently inhibits chikungunya virus infection in the joints of mice during the acute but not during the chronic phase of the infection. Antiviral Res. 149, 113–117 (2018). [PubMed: 28958920]
89. Delang L, Abdelnabi R & Neyts J Favipiravir as a potential countermeasure against neglected and emerging RNA viruses. Antivir. Res 153, 85–94 (2018). [PubMed: 29524445]
90. Julander JG et al. Strain-dependent disease and response to favipiravir treatment in mice infected with Chikungunya virus. Antiviral Res. 182, 104904 (2020). [PubMed: 32791074]
91. Cavazzoni P. Emergency Use Authorization 108.
92. Yoon J-J et al. Orally Efficacious Broad-Spectrum Ribonucleoside Analog Inhibitor of Influenza and Respiratory Syncytial Viruses. Antimicrob. Agents Chemother 62, e00766–18 (2018). [PubMed: 29891600]
93. Filomatori CV, Merwaiss F, Bardossy ES & Alvarez DE Impact of alphavirus 3'UTR plasticity on mosquito transmission. Semin. Cell Dev. Biol 111, 148–155 (2021). [PubMed: 32665176]
94. Ou J-H, Trent DW & Strauss JH The 3'-non-coding regions of alpharivus RNAs contain repeating sequences. J. Mol. Biol 156, 719–730 (1982). [PubMed: 6288962]

95. Pfeffer M, Kinney RM & Kaaden O-R The Alphavirus 3'-Nontranslated Region: Size Heterogeneity and Arrangement of Repeated Sequence Elements. Virology 240, 100–108 (1998). [PubMed: 9448694]
96. Chen R, Wang E, Tsetsarkin KA & Weaver SC Chikungunya Virus 3' Untranslated Region: Adaptation to Mosquitoes and a Population Bottleneck as Major Evolutionary Forces. PLOS Pathog. 9, e1003591 (2013). [PubMed: 24009512]
97. Trobaugh DW et al. RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature 506, 245–248 (2014). [PubMed: 24352241]
98. Hardy RW & Rice CM Requirements at the 3' End of the Sindbis Virus Genome for Efficient Synthesis of Minus-Strand RNA. J. Virol 79,4630–4639(2005). [PubMed: 15795249]
99. Frolov I, Hardy R & Rice CM Cis-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis. RNA 7, 1638–1651 (2001). [PubMed: 11720292]
100. Hyde JL et al. The 5' and 3' ends of alphavirus RNAs – Non-coding is not non-functional. Virus Res. 206, 99–107 (2015). [PubMed: 25630058]
101. Gorchakov R, Hardy R, Rice CM & Frolov I Selection of Functional 5′ cis-Acting Elements Promoting Efficient Sindbis Virus Genome Replication. J. Virol 78, 61–75 (2004). [PubMed: 14671088]
102. Hyde JL et al. A viral RNA stmctural element alters host recognition of non-self RNA. Science 343, 783–787 (2014). [PubMed: 24482115]
103. Kulasegaran-Shylini R, Thiviyanathan V, Gorenstein DG & Frolov I The 5′UTR-specific mutation in VEEV TC-83 genome has a strong effect on RNA replication and subgenomic RNA synthesis, but not on translation of the encoded proteins. Virology 387, 211–221 (2009). [PubMed: 19278709]
104. White LJ, Wang J-G, Davis NL & Johnston RE Role of Alpha/Beta Interferon in Venezuelan Equine Encephalitis Virus Pathogenesis: Effect of an Attenuating Mutation in the 5′ Untranslated Region. J. Virol (2001) doi:10.1128/JVI.75.8.3706-3718.2001.
105. Paul D & Bartenschlager R Architecture and biogenesis of plus-strand RNA virus replication factories. World J. Virol 2, 32–48 (2013). [PubMed: 24175228]
106. Salonen A, Ahola T & Kääriäinen L Viral RNA Replication in Association with Cellular Membranes. Membr. Traffick. Viral Replication 285, 139–173 (2005).
107. Johan A den Boon & Ahlquist P Organelle-Like Membrane Compartmentalization of Positive-Strand RNA Virus Replication Factories. Annu. Rev. Microbiol 64, 241–256 (2010). [PubMed: 20825348]
108. Kopek BG, Perkins G, Miller DJ, Ellisman MH & Ahlquist P Three-Dimensional Analysis of a Viral RNA Replication Complex Reveals a Virus-Induced Mini-Organelle. PLoS Biol. 5, (2007).
109. Ertel KJ et al. Cryo-electron tomography reveals novel features of a viral RNA replication compartment. eLife 6, (2020).
110. Pietilä MK, van Hemert MJ & Ahola T Purification of Highly Active Alphavirus Replication Complexes Demonstrates Altered Fractionation of Multiple Cellular Membranes. J. Virol 92, (2018).
111. Hellström K et al. Partially Uncleaved Alphavirus Replicase Forms Spherule Structures in the Presence and Absence of RNA Template. J. Virol 91, (2017).
112. Kallio K et al. Template RNA Length Determines the Size of Replication Complex Spherules for Semliki Forest Virus. J. Virol 87, 9125–9134 (2013). [PubMed: 23760239]
113. Deshmukh D et al. New insights into culture negative endophthalmitis by unbiased next generation sequencing. Sci Rep 9, 844 (2019). [PubMed: 30696908]
114. Scutigliani EM & Kikkert M Interaction of the innate immune system with positive-strand RNA virus replication organelles. Cytokine Growth Factor Rev. 37, 17–27 (2017). [PubMed: 28709747]
115. Uchil PD & Satchidanandam V Architecture of the Flaviviral Replication Complex: PROTEASE, NUCLEASE, AND DETERGENTS REVEAL ENCASEMENT WITHIN DOUBLE-LAYERED MEMBRANE COMPARTMENTS *. J. Biol. Chem 278, 24388–24398 (2003). [PubMed: 12700232]

116. Laurent T et al. Architecture of the chikungunya virus replication organelle. eLife 11, e83042 (2022). [PubMed: 36259931]
117. Frolova EI, Gorchakov R, Pereboeva L, Atasheva S & Frolov I Functional Sindbis Virus Replicative Complexes Are Formed at the Plasma Membrane. J. Virol 84, 11679–11695 (2010). [PubMed: 20826696]
118. Thaa B 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. J. Virol 89, 11420–11437 (2015). [PubMed: 26339054]
119. Spuul P, Balistreri G, Kääriäinen L & Ahola T Phosphatidylinositol 3-Kinase-, Actin-, and Microtubule-Dependent Transport of Semliki Forest Virus Replication Complexes from the Plasma Membrane to Modified Lysosomes. J. Virol 84, 7543–7557 (2010). [PubMed: 20484502]
120. Martinez MG & Kielian M Intercellular Extensions Are Induced by the Alphavirus Structural Proteins and Mediate Virus Transmission. PLOS Pathog. 12, e1006061 (2016). [PubMed: 27977778]
121. Zheng Y & Kielian M Imaging of the Alphavirus Capsid Protein during Virus Replication. J. Virol 87, 9579–9589 (2013). [PubMed: 23785213]
122. Button JM & Mukhopadhyay S Removing the Polyanionic Cargo Requirement for Assembly of Alphavirus Core-Like Particles to Make an Empty Alphavirus Core. Viruses 12, 846 (2020). [PubMed: 32756493]
123. Stollar V Defective Interfering Alphaviruses. in The Togaviruses Biology, Structure, Replication 427–457 (Academic Press, 1980).
124. Zhang R et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. EMBO J 30, 3854–3863 (2011). [PubMed: 21829169]
125. Snyder AJ, Sokoloski KJ & Mukhopadhyay S Mutating Conserved Cysteines in the Alphavirus E2 Glycoprotein Causes Virus-Specific Assembly Defects. J. Virol 86, 3100–3111 (2012). [PubMed: 22238319]
126. Cheng F et al. The Packaging of Different Cargo into Enveloped Viral Nanoparticles. Mol. Pharm 10, 51–58 (2013). [PubMed: 22876758]
127. Garoff H, Sjöberg M & Cheng RH Budding of alphaviruses. Virus Res. 106, 103–116 (2004). [PubMed: 15567491]
128. Brown RS, Wan JJ & Kielian M The Alphavirus Exit Pathway: What We Know and What We Wish We Knew. Viruses 10, 89 (2018). [PubMed: 29470397]
129. Rupp JC, Sokoloski KJ, Gebhart NN & Hardy RW Alphavirus RNA synthesis and non-structural protein functions. J. Gen. Virol 96, 2483–2500 (2015). [PubMed: 26219641]
130. Li C et al. mRNA Capping by Venezuelan Equine Encephalitis Virus nsP1: Functional Characterization and Implications for Antiviral Research. J. Virol 89, 8292–8303 (2015). [PubMed: 26041283]
131. Decroly E, Ferron F, Lescar J & Canard B Conventional and unconventional mechanisms for capping viral mRNA. Nat. Rev. Microbiol 10, 51–65 (2012).
132. Vasiljeva L, Merits A, Auvinen P & Kääriäinen L Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. J. Biol. Chem 275, 17281–17287 (2000). [PubMed: 10748213]
133. Ahola T, Lampio A, Auvinen P & Kääriäinen L Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J. 18, 3164–3172 (1999). [PubMed: 10357827]
134. Spuul P et al. Role of the Amphipathic Peptide of Semliki Forest Virus Replicase Protein nsP1 in Membrane Association and Virus Replication. J. Virol 81, 872–883 (2007). [PubMed: 17093195]
135. Kujala P et al. Biogenesis of the Semliki Forest Virus RNA Replication Complex. J. Virol 75, 3873–3884 (2001). [PubMed: 11264376]
136. Jones R, Bragagnolo G, Arranz R & Reguera J Capping pores of alphavirus nsP1 gate membranous viral replication factories. Nature 1–5 (2020) doi: 10.1038/s41586-020-3036-8.
137. Zhang K et al. Molecular basis of specific viral RNA recognition and 5′-end capping by the Chikungunya virus nsP1. Cell Rep. 40, 111133 (2022). [PubMed: 35905713]

138. Garmashova N et al. Analysis of Venezuelan Equine Encephalitis Virus Capsid Protein Function in the Inhibition of Cellular Transcription. J. Virol 81, 13552–13565 (2007). [PubMed: 17913819]
139. Akhrymuk I, Lukash T, Frolov I & Frolova EI Novel Mutations in nsP2 Abolish Chikungunya Virus-Induced Transcriptional Shutoff and Make the Virus Fess Cytopathic without Affecting Its Replication Rates. J. Virol 93, e02062–18, /jvi/93/4/JVI.02062-18.atom (2018).
140. Atasheva S, Fish A, Fornerod M & Frolova EI Venezuelan Equine Encephalitis Virus Capsid Protein Forms a Tetrameric Complex with CRM1 and Importin α/β That Obstmcts Nuclear Pore Complex Function. J. Virol 84,4158–4171 (2010). [PubMed: 20147401]
141. Bhalla N et al. Host translation shutoff mediated by non-structural protein 2 is a critical factor in the antiviral state resistance of Venezuelan equine encephalitis virus. Virology 496, 147–165 (2016). [PubMed: 27318152]
142. Kim DY, Atasheva S, Frolova EI & Frolov I Venezuelan Equine Encephalitis Virus nsP2 Protein Regulates Packaging of the Viral Genome into Infectious Virions. J. Virol 87, 4202–4213 (2013). [PubMed: 23365438]
143. Cherkashchenko L, Rausalu K, Basu S, Alphey L & Merits A 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, 1327 (2022). [PubMed: 35746799]
144. Gomez de Cedrón M, Ehsani N, Mikkola ML, García JA & Kääriäinen L RNA helicase activity of Semliki Forest virus replicase protein NSP2. FEBS Lett. 448, 19–22 (1999). [PubMed: 10217401]
145. Das PK, Merits A & Lulla A Functional cross-talk between distant domains of chikungunya virus non-structural protein 2 is decisive for its RNA-modulating activity. J. Biol. Chem 289, 5635–5653 (2014). [PubMed: 24407286]
146. Law Y-S et al. Structural insights into RNA recognition by the Chikungunya virus nsP2 helicase. Proc. Natl. Acad. Sci 116, 9558–9567 (2019). [PubMed: 31000599]
147. Law Y-S et al. Structural insights into RNA recognition by the Chikungunya virus nsP2 helicase. Proc. Natl. Acad. Sci 116, 9558–9567 (2019). [PubMed: 31000599]
148. Law Y-S et al. Interdomain Flexibility of Chikungunya Virus nsP2 Helicase-Protease Differentially Influences Viral RNA Replication and Infectivity. J. Virol 95, e01470–20 (2021). [PubMed: 33328310]
149. Ivanova L et al. Novel Analogues of the Chikungunya Virus Protease Inhibitor: Molecular Design, Synthesis, and Biological Evaluation. ACS Omega 6, 10884–10896 (2021). [PubMed: 34056242]
150. Silva Muniz L & Pita S. S. da R. In silico studies revealed interaction mechanisms of benzylidene-acrylohydrazide derivatives and nsP2 CHIKV. New J. Chem 46, 6414–6423 (2022).
151. Atasheva S, Gorchakov R, English R, Frolov I & Frolova E Development of Sindbis Viruses Encoding nsP2/GFP Chimeric Proteins and Their Application for Studying nsP2 Functioning. J. Virol 81, 5046–5057 (2007). [PubMed: 17329335]
152. Hahn YS, Strauss EG & Strauss JH Mapping of RNA- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J. Virol 63, 3142–3150 (1989). [PubMed: 2724421]
153. Hardy WR & Strauss JH 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. J. Virol 63, 4653–4664 (1989). [PubMed: 2529379]
154. Russo AT, White MA & Watowich SJ The Crystal Structure of the Venezuelan Equine Encephalitis Alphavirus nsP2 Protease. Structure 14, 1449–1458 (2006). [PubMed: 16962975]
155. Kim KH, Rümenapf T, Strauss EG & Strauss JH Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. Virology 323, 153–163 (2004). [PubMed: 15165827]
156. Morazzani EM et al. Proteolytic cleavage of host proteins by the Group IV viral proteases of Venezuelan equine encephalitis virus and Zika virus. Antiviral Res. 164, 106–122 (2019). [PubMed: 30742841]

157. Göertz GP et al. The Methyltransferase-Like Domain of Chikungunya Virus nsP2 Inhibits the Interferon Response by Promoting the Nuclear Export of STAT1. J. Virol 92, e01008–18, /jvi/92/17/e01008-18.atom (2018). [PubMed: 29925658]

158. Modeling interaction between non-structural protein 2 of Chikungunya Virus and various protein factors of innate pathway. Biomed. Lett 8, 162–169 (2022).

159. Wang S & Merits A G3BP/Rin-Binding Motifs Inserted into Flexible Regions of nsP2 Support RNA Replication of Chikungunya Virus. J. Virol 96, e01278–22 (2022). [PubMed: 36226983]

160. LaStarza MW, Lemm JA & Rice CM Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. J. Virol 68, 5781–5791 (1994). [PubMed: 8057460]

161. Malet H et al. The Crystal Structures of Chikungunya and Venezuelan Equine Encephalitis Virus nsP3 Macro Domains Define a Conserved Adenosine Binding Pocket. J. Virol 83, 6534–6545 (2009). [PubMed: 19386706]

162. Eckei L et al. The conserved macrodomains of the non-structural proteins of Chikungunya virus and other pathogenic positive strand RNA viruses function as mono-ADP-ribosylhydrolases. Sci. Rep 7, 1–18 (2017). [PubMed: 28127051]

163. Abraham R et al. ADP-ribosyl–binding and hydrolase activities of the alphavirus nsP3 macrodomain are critical for initiation of virus replication. Proc. Natl. Acad. Sci 115, E10457–E10466 (2018). [PubMed: 30322911]

164. Abraham R et al. Both ADP-Ribosyl-Binding and Hydrolase Activities of the Alphavirus nsP3 Macrodomain Affect Neurovirulence in Mice. mBio 11, e03253–19, /mbio/11/1/mBio.03253-19.atom (2020). [PubMed: 32047134]

165. Gao Y, Goonawardane N, Ward J, Tuplin A & Harris M Multiple roles of the non-structural protein 3 (nsP3) alphavirus unique domain (AUD) during Chikungunya virus genome replication and transcription. PLOS Pathog 15, e1007239 (2019). [PubMed: 30668592]

166. Oberste MS, Parker MD & Smith JF Complete Sequence of Venezuelan Equine Encephalitis Virus Subtype IE Reveals Conserved and Hypervariable Domains within the C Terminus of nsP3. Virology 219, 314–320 (1996). [PubMed: 8623548]

167. Foy NJ, Akhrymuk M, Shustov AV, Frolova EI & Frolov I Hypervariable domain of nonstructural protein nsP3 of Venezuelan equine encephalitis virus determines cell-specific mode of virus replication. J. Virol 87, 7569–7584 (2013). [PubMed: 23637407]

168. Aaskov J, Jones A, Choi W, Lowry K & Stewart E Lineage replacement accompanying duplication and rapid fixation of an RNA element in the nsP3 gene in a species of alphavirus. Virology 410, 353–359 (2011). [PubMed: 21185049]

169. Foy NJ et al. Hypervariable domains of nsP3 proteins of New World and Old World alphaviruses mediate formation of distinct, virus-specific protein complexes. J. Virol 87, 1997–2010 (2013). [PubMed: 23221551]

170. Kim DY et al. New World and Old World Alphaviruses Have Evolved to Exploit Different Components of Stress Granules, FXR and G3BP Proteins, for Assembly of Viral Replication Complexes. PLoS Pathog. 12, (2016).

171. Montero H & Trujillo-Alonso V Stress Granules in the Viral Replication Cycle. Viruses 3, 2328–2338 (2011). [PubMed: 22163347]

172. Valiente-Echeverria F, Melnychuk L & Mouland AJ Viral modulation of stress granules. Virus Res. 169, 430–437 (2012). [PubMed: 22705970]

173. Frolov I, Kim DY, Akhrymuk M, Mobley JA & Frolova EI Hypervariable Domain of Eastern Equine Encephalitis Virus nsP3 Redundantly Utilizes Multiple Cellular Proteins for Replication Complex Assembly. J. Virol 91, (2017).

174. Götte B, Utt A, Fragkoudis R, Merits A & McInerney GM Sensitivity of Alphaviruses to G3BP Deletion Correlates with Efficiency of Replicase Polyprotein Processing. J. Virol 94, (2020).

175. Meshram CD et al. Mutations in Hypervariable Domain of Venezuelan Equine Encephalitis Virus nsP3 Protein Differentially Affect Viral Replication. J. Virol 94, (2020).

176. Myles KM, Kelly CLH, Ledermann JP & Powers AM Effects of an Opal Termination Codon Preceding the nsP4 Gene Sequence in the O'Nyong-Nyong Virus Genome on Anopheles gambiae Infectivity. J. Virol 80, 4992–4997 (2006). [PubMed: 16641290]

177. Li GP & Rice CM Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. J. Virol 63, 1326–1337 (1989). [PubMed: 2521676]

178. de Groot RJ, Rümenapf T, Kuhn RJ, Strauss EG & Strauss JH Sindbis virus RNA polymerase is degraded by the N-end rule pathway. Proc. Natl. Acad. Sci. U. S. A 88, 8967–8971 (1991). [PubMed: 1924357]

179. Lello LS et al. nsP4 Is a Major Determinant of Alphavirus Replicase Activity and Template Selectivity. J. Virol 95,e00355–21 (2021). [PubMed: 34319783]

180. Rubach JK et al. Characterization of purified Sindbis Virus nsP4 RNA-dependent RNA Polymerase activity in vitro. Virology 384, 201–208 (2009). [PubMed: 19036396]

181. Tomar S, Hardy RW, Smith JL & Kuhn RJ Catalytic Core of Alphavirus Nonstructural Protein nsP4 Possesses Terminal Adenylyltransferase Activity. J. Virol 80, 9962–9969 (2006). [PubMed: 17005674]

182. Sreejith R et al. Mapping interactions of Chikungunya virus nonstructural proteins. Virus Res. 169, 231–236 (2012). [PubMed: 22951312]

183. Tan YB et al. Crystal structures of alphavirus nonstructural protein 4 (nsP4) reveal an intrinsically dynamic RNA-dependent RNA polymerase fold. Nucleic Acids Res. 50, 1000–1016 (2022). [PubMed: 35037043]

184. Chung D-H et al. Discovery of a Novel Compound with Anti-Venezuelan Equine Encephalitis Virus Activity That Targets the Nonstmctural Protein 2. PLOS Pathog. 10, e1004213 (2014). [PubMed: 24967809]

185. Rupp JC, Jundt N & Hardy RW Requirement for the amino-terminal domain of sindbis virus nsP4 during virus infection. J Virol 85, 3449–60 (2011). [PubMed: 21248049]

186. Gritsenko D & Hughes G Ledipasvir/Sofosbuvir (Harvoni): Improving Options for Hepatitis C Virus Infection. Pharm. Ther 40, 256–276 (2015).

187. Zhang S et al. Pyrimidone inhibitors targeting Chikungunya Virus nsP3 macrodomain by fragment-based drug design. PLOS ONE 16, e0245013 (2021). [PubMed: 33482665]

188. Sundar S, Piramanayagam S & Natarajan J A review on stmctural genomics approach applied for drug discovery against three vector-borne viral diseases: Dengue, Chikungunya and Zika. Virus Genes 58, 151–171 (2022). [PubMed: 35394596]

189. Kovacikova K & van Hemert MJ Small-Molecule Inhibitors of Chikungunya Virus: Mechanisms of Action and Antiviral Drug Resistance. Antimicrob. Agents Chemother 64, e01788–20 (2020). [PubMed: 32928738]

190. Kehn-Hall K & Bradfute SB Understanding host responses to equine encephalitis virus infection: implications for therapeutic development. Expert Rev. Anti Infect. Ther 0, 1–16 (2022).

191. Kim DY, Firth AE, Atasheva S, Frolova EI & Frolov I Conservation of a Packaging Signal and the Viral Genome RNA Packaging Mechanism in Alphavirus Evolution. J. Virol 85, 8022–8036 (2011). [PubMed: 21680508]

192. Frolova E, Frolov I & Schlesinger S Packaging signals in alphaviruses. J. Virol 71, 248–258 (1997). [PubMed: 8985344]

193. Volkova E, Gorchakov R & Frolov I The efficient packaging of Venezuelan equine encephalitis virus-specific RNAs into viral particles is determined by nsP1–3 synthesis. Virology 344, 315–327 (2006). [PubMed: 16239019]

194. Mendes A & Kuhn RJ Alphavirus Nucleocapsid Packaging and Assembly. Viruses 10, 138 (2018). [PubMed: 29558394]

195. Garmashova N et al. The Old World and New World Alphaviruses Use Different Virus-Specific Proteins for Induction of Transcriptional Shutoff. J. Virol 81, 2472–2484 (2007). [PubMed: 17108023]

196. Carrasco L, Sanz MA & González-Almela E The Regulation of Translation in Alphavirus-Infected Cells. Viruses 10, 70 (2018). [PubMed: 29419763]

197. Akhrymuk I, Frolov I & Frolova EI Sindbis Virus Infection Causes Cell Death by nsP2-Induced Transcriptional Shutoff or by nsP3-Dependent Translational Shutoff. J. Virol 92, e01388–18 (2021).

198. Rao S & Taylor A Arthritogenic Alphavirus Capsid Protein. Life 11, 230 (2021). [PubMed: 33799673]

199. Akhrymuk I, Kulemzin SV & Frolova EI Evasion of the Innate Immune Response: the Old World Alphavirus nsP2 Protein Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II. J. Virol 86, 7180–7191 (2012). [PubMed: 22514352]

200. DeBono A et al. Novel RU486 (mifepristone) analogues with increased activity against Venezuelan Equine Encephalitis Virus but reduced progesterone receptor antagonistic activity. Sci. Rep 9, 1–19 (2019). [PubMed: 30626917]

201. Curtis I. de & Simons K Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. Proc. Natl. Acad. Sci 85, 8052–8056 (1988). [PubMed: 3186706]

202. Ivanova L & Schlesinger MJ Site-directed mutations in the Sindbis virus E2 glycoprotein identify palmitoylation sites and affect virus budding. J. Virol (1993) doi:10.1128/jvi.67.5.2546-2551.1993.

203. Mulvey M & Brown DT Formation and rearrangement of disulfide bonds during maturation of the Sindbis virus E1 glycoprotein. J. Virol (1994) doi:10.1128/jvi.68.2.805-812.1994.

204. Pletnev SV et al. Locations of Carbohydrate Sites on Alphavirus Glycoproteins Show that E1 Forms an Icosahedral Scaffold. Cell 105, 127–136 (2001). [PubMed: 11301008]

205. Zhang X, Fugère M, Day R & Kielian M Furin Processing and Proteolytic Activation of Semliki Forest Virus. J. Virol (2003) doi:10.1128/JVI.77.5.2981-2989.2003.

206. Zhang X & Kielian M Mutations that promote furin-independent growth of Semliki Forest virus affect p62–E1 interactions and membrane fusion. Virology 327, 287–296 (2004). [PubMed: 15351216]

207. Soonsawad P et al. Stmctural Evidence of Glycoprotein Assembly in Cellular Membrane Compartments prior to Alphavirus Budding. J. Virol 84, 11145–11151 (2010). [PubMed: 20739526]

208. Holmes AC, Basore K, Fremont DH & Diamond MS A molecular understanding of alphavirus entry. PLOS Pathog. 16, e1008876 (2020). [PubMed: 33091085]

209. Mukhopadhyay S et al. Mapping the Structure and Function of the E1 and E2 Glycoproteins in Alphaviruses. Structure 14, 63–73 (2006). [PubMed: 16407066]

210. Wengler G, Koschinski A, Wengler G & Dreyer F Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. J. Gen. Virol 84, 173–181 (2003). [PubMed: 12533714]

211. Bonatti S & Blobel G Absence of a cleavable signal sequence in Sindbis virus glycoprotein PE2. J. Biol. Chem 254, 12261–12264 (1979). [PubMed: 500711]

212. Jain SK, DeCandido S & Kielian M Processing of the p62 envelope precursor protein of Semliki Forest virus. J. Biol. Chem 266, 5756–5761 (1991). [PubMed: 2005112]

213. Lobigs M, Zhao HX & Garoff H Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. J. Virol 64, 4346–4355 (1990). [PubMed: 2200886]

214. Snyder AJ & Mukhopadhyay S The Alphavirus E3 Glycoprotein Functions in a Clade-Specific Manner. J. Virol (2012).

215. Uchime O, Fields W & Kielian M The Role of E3 in pH Protection during Alphavirus Assembly and Exit. J. Virol (2013) doi:10.1128/JVI.01507-13.

216. Ramsey J & Mukhopadhyay S Disentangling the Frames, the State of Research on the Alphavirus 6K and TF Proteins. Viruses 9, 228 (2017). [PubMed: 28820485]

217. Harrington HR et al. Cotranslational folding stimulates programmed ribosomal frameshifting in the alphavirus structural polyprotein. J. Biol. Chem 295, 6798–6808 (2020). [PubMed: 32169904]

218. Gaedigk-Nitschko K & Schlesinger MJ Site-directed mutations in sindbis virus E2 glycoprotein's cytoplasmic domain and the 6K protein lead to similar defects in virus assembly and budding. Virology 183, 206–214 (1991). [PubMed: 1647069]

219. Ivanova L, Le L & Schlesinger MJ Characterization of revertants of a Sindbis virus 6K gene mutant that affects proteolytic processing and virus assembly. Virus Res. 39, 165–179 (1995). [PubMed: 8837882]
220. Ivanova L, Lustig S & Schlesinger MJ A Pseudo-Revertant of a Sindbis Virus 6K Protein Mutant, Which Corrects for Aberrant Particle Formation, Contains Two New Mutations That Map to the Ectodomain of the E2 Glycoprotein. Virology 206, 1027–1034 (1995). [PubMed: 7856077]
221. Schlesinger MJ, London SD & Ryan C An In-Frame Insertion into the Sindbis Virus 6K Gene Leads to Defective Proteolytic Processing of the Virus Glycoproteins, a Trans-Dominant Negative Inhibition of Normal Virus Formation, and Interference in Virus Shut off of Host-Cell Protein Synthesis. Virology 193, 424–432 (1993). [PubMed: 8094927]
222. Yao JS, Strauss EG & Strauss JH Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. J. Virol (1996) doi:10.1128/jvi.70.11.7910-7920.1996.
223. Firth AE, Chung BY, Fleeton MN & Atkins JF Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. Virol. J 5, 108 (2008). [PubMed: 18822126]
224. Ramsey J, Renzi EC, Arnold RJ, Trinidad JC & Mukhopadhyay S Palmitoylation of Sindbis Virus TF Protein Regulates Its Plasma Membrane Localization and Subsequent Incorporation into Virions. J. Virol 91, e02000–16 (2017). [PubMed: 27852864]
225. Rogers KJ, Jones-Burrage S, Maury W & Mukhopadhyay S TF protein of Sindbis virus antagonizes host type I interferon responses in a palmitoylation-dependent manner. Virology 542, 63–70 (2020). [PubMed: 32056669]
226. Ramsey J, Chavez M & Mukhopadhyay S Domains of the TF protein important in regulating its own palmitoylation. Virology 531, 31–39 (2019). [PubMed: 30852269]
227. Liljeström P, Lusa S, Huylebroeck D & Garoff H In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J. Virol (1991) doi: 10.1128/jvi.65.8.4107-4113.1991.
228. Garmashova N et al. Analysis of Venezuelan Equine Encephalitis Virus Capsid Protein Function in the Inhibition of Cellular Transcription. J. Virol 81, 13552–13565 (2007). [PubMed: 17913819]
229. Jose J, Taylor AB & Kuhn RJ Spatial and Temporal Analysis of Alphavirus Replication and Assembly in Mammalian and Mosquito Cells. mBio 8, e02294–16.
230. Andersen PI et al. Discovery and development of safe-in-man broad-spectrum antiviral agents.. Int. J. Infect. Dis 93, 268–276 (2020). [PubMed: 32081774]
231. Chung D-H et al. Discovery of a Novel Compound with Anti-Venezuelan Equine Encephalitis Virus Activity That Targets the Nonstructural Protein 2. PLOS Pathog. 10, e1004213 (2014). [PubMed: 24967809]
232. Promega. CellTiter-Glo® 2.0 Cell Viability Assay | ATP Assay | Promega. https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-2_0-assay/ (2022).
233. Andersen PI et al. Discovery and development of safe-in-man broad-spectrum antiviral agents. Int. J. Infect. Dis 93, 268–276 (2020). [PubMed: 32081774]
234. Bernatchez JA et al. Development and Validation of a Phenotypic High-Content Imaging Assay for Assessing the Antiviral Activity of Small-Molecule Inhibitors Targeting Zika Virus. Antimicrob. Agents Chemother 62, e00725–18 (2018). [PubMed: 30061280]
235. Bulanova D et al. Antiviral Properties of Chemical Inhibitors of Cellular Anti-Apoptotic Bcl-2 Proteins. Viruses 9, 271 (2017). [PubMed: 28946654]
236. Aggarwal M et al. Evaluation of antiviral activity of piperazine against Chikungunya virus targeting hydrophobic pocket of alphavirus capsid protein. Antiviral Res. 146, 102–111 (2017). [PubMed: 28842264]
237. Shen L et al. High-Throughput Screening and Identification of Potent Broad-Spectrum Inhibitors of Coronaviruses. J. Virol 93, e00023–19 (2019). [PubMed: 30918074]
238. Saotome K, Morita H & Umeda M Cytotoxicity test with simplified crystal violet staining method using microtitre plates and its application to injection drugs. Toxicol. In Vitro 3, 317–321 (1989). [PubMed: 20702298]

239. Li J-Q et al. Development of a replicon cell line-based high throughput antiviral assay for screening inhibitors of Zika virus. Antiviral Res. 150, 148–154 (2018). [PubMed: 29288699]

240. Cruz DJM et al. Identification of Novel Compounds Inhibiting Chikungunya Virus-Induced Cell Death by High Throughput Screening of a Kinase Inhibitor Library. PLoS Negl. Trop. Dis 7, e2471 (2013). [PubMed: 24205414]

241. Delekta PC et al. The Combined Use of Alphavirus Replicons and Psuedoinfectious Particles for the Discovery of Antivirals Derived from Natural Products. J. Biomol. Screen. 20, 673–680 (2015). [PubMed: 25550354]

242. Pohjala L et al. Inhibitors of Alphavirus Entry and Replication Identified with a Stable Chikungunya Replicon Cell Line and Virus-Based Assays. PLOS ONE 6, e28923 (2011). [PubMed: 22205980]

243. Varghese FS et al. The Antiviral Alkaloid Berberine Reduces Chikungunya Virus-Induced Mitogen-Activated Protein Kinase Signaling. J. Virol 90, 9743–9757 (2016). [PubMed: 27535052]

244. Gleiser CA, Gouchenour WS Jr, Berge TO & Tigertt WD The Comparative Pathology of Experimental Venezuelan Equine Encephalomyelitis Infection in Different Animal Hosts. J. Infect. Dis 110, 80–97 (1962). [PubMed: 13899188]

245. Jackson AC, Sengupta SK & Smith JF Pathogenesis of Venezulean Equine Encephalitis Virus Infection in Mice and Hamsters. Vet. Pathol 410–418 (1991). [PubMed: 1750167]

246. Victor J, Smith DG & Pollack AD The Comparative Pathology of Venezuelan Equine Encephalomyelitis. J. Infect. Dis 98, 55–66 (1956). [PubMed: 13295626]

247. Biodefense: Research Methodology and Animal Models. (CRC Press, 2005). doi:10.1201/9781420038118.

248. Vogel P et al. Venezuelan equine encephalitis in BALB/c mice: kinetic analysis of central nervous system infection following aerosol or subcutaneous inoculation. Arch. Pathol. Lab. Med 120, 164–172 (1996). [PubMed: 8712896]

249. Charles PC, Walters E, Margolis F & Johnston RE Mechanism of Neuroinvasion of Venezuelan Equine Encephalitis Virus in the Mouse. Virology 208, 662–671 (1995). [PubMed: 7747437]

250. Davis NL et al. A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis, in Positive-Strand RNA Viruses (eds. Brinton MA, Calisher CH & Rueckert R) 99–109 (Springer, 1994). doi: 10.1007/978-3-7091-9326-6_l1.

251. Grieder FB et al. Specific Restrictions in the Progression of Venezuelan Equine Encephalitis Virus-Induced Disease Resulting from Single Amino Acid Changes in the Glycoproteins. Virology 206, 994–1006 (1995). [PubMed: 7856110]

252. MacDonald GH & Johnston RE Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J. Virol 74, 914–922 (2000). [PubMed: 10623754]

253. Steele KE et al. Comparative Neurovirulence and Tissue Tropism of Wild-type and Attenuated Strains of Venezuelan Equine Encephalitis Virus Administered by Aerosol in C3H/HeN and BALB/c Mice. Vet. Pathol 35, 386–397 (1998). [PubMed: 9754544]

254. Steele KE & Twenhafel NA REVIEW PAPER: Pathology of Animal Models of Alphavirus Encephalitis. Vet. Pathol 47, 790–805 (2010). [PubMed: 20551475]

255. Ryzhikov AB, Tkacheva NV, Sergeev AN & Ryabchikova EI Venezuelan equine encephalitis virus propagation in the olfactory tract of normal and immunized mice. Biomed. Sci 2, 607–614 (1991). [PubMed: 1841630]

256. Danes L, Rychterová V, Kufner J & Hrusková V The role of the olfactory route on infection of the respiratory tract with Venezuelan equine encephalomyelitis virus in normal and operated Macaca rhesus monkeys. II. Results of histological examination. Acta Virol. 17, 57–60 (1973). [PubMed: 4405397]

257. Smith DR et al. Comparative pathology study of Venezuelan, eastern, and western equine encephalitis viruses in non-human primates. Antiviral Res. 182, 104875 (2020). [PubMed: 32755661]

258. Liu C, Voth DW, Rodina P, Shauf LR & Gonzalez G A Comparative Study of the Pathogenesis of Western Equine and Eastern Equine Encephalomyelitis Viral Infections in Mice by Intracerebral and Subcutaneous Inoculations. J. Infect. Dis 122, 53–63 (1970). [PubMed: 4914943]

259. Gardner CL et al. Eastern and Venezuelan Equine Encephalitis Viruses Differ in Their Ability To Infect Dendritic Cells and Macrophages: Impact of Altered Cell Tropism on Pathogenesis. J. Virol 82, 10634–10646 (2008). [PubMed: 18768986]
260. Reed DS et al. Severe Encephalitis in Cynomolgus Macaques Exposed to Aerosolized Eastern Equine Encephalitis Virus. J. Infect. Dis 196, 441–450 (2007). [PubMed: 17597459]
261. Adams AP et al. Common Marmosets (Callithrix jacchus) as a Nonhuman Primate Model To Assess the Virulence of Eastern Equine Encephalitis Virus Strains. J. Virol 82, 9035–9042 (2008). [PubMed: 18614636]
262. Espinosa BJ et al. Susceptibility of the Aotus nancymaae owl monkey to eastern equine encephalitis. Vaccine 27, 1729–1734 (2009). [PubMed: 19186197]
263. Nathanson N, Stolley PD & Boolukos PJ Eastern equine encephalitis: Distribution of central nervous system lesions in man and rhesus monkey. J. Comp. Pathol 79, 109–115 (1969). [PubMed: 4975613]
264. Trefry JC et al. The utilization of advance telemetry to investigate critical physiological parameters including electroencephalography in cynomolgus macaques following aerosol challenge with eastern equine encephalitis virus. PLoS Negl. Trop. Dis 15, e0009424 (2021). [PubMed: 34138849]
265. Aguilar MJ Pathological Changes in Brain and Other Target Organs of Infant and Weanling Mice After Infection with Non-Neuroadapted Western Equine Encephalitis Virus. Infect. Immun 2, 533–542 (1970). [PubMed: 16557874]
266. Logue CH et al. Virulence variation among isolates of western equine encephalitis virus in an outbred mouse model. J. Gen. Virol 90, 1848–1858 (2009). [PubMed: 19403754]
267. Monath TP & Kemp GE Necrotizing Myocarditis in Mice Infected with Western Equine Encephalitis Virus: Clinical, Electrocardiographic, and Histopathologic Correlations. J. Infect. Dis 158, 59–66 (1978).
268. Nagata LP et al. Infectivity variation and genetic diversity among strains of Western equine encephalitis virus. J. Gen. Virol 87, 2353–2361 (2022).
269. Hardy JL, Presser SB, Chiles RE & Reeves WC Mouse and Baby Chicken Virulence of Enzootic Strains of Western Equine Encephalomyelitis Virus from California. Am. J. Trop. Med. Hyg 57, 240–244 (1997). [PubMed: 9288823]
270. Julander JG et al. Effect of exogenous interferon and an interferon inducer on western equine encephalitis virus disease in a hamster model. Virology 360, 454–460 (2007). [PubMed: 17118420]
271. Zlotnik I, Peacock S, Grant DP & Batter-Hatton D The Pathogenesis of Westem Equine Encephalitis Virus (W.E.E.) in Adult Hamsters with Special Reference to the Long and Short Term Effects on the C.N.S. of the Attenuated Clone 15 Variant. Br. J. Exp. Pathol 53, 59–77 (1972). [PubMed: 5014245]
272. Reed DS et al. Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques. J. Infect. Dis 192, 1173–1182 (2005). [PubMed: 16136459]
273. Wyckoff RWG & Tesar WC Equine Encephalomyelitis in Monkeys. J. Immunol 37, 329–343 (1939).
274. Couderc T et al. A Mouse Model for Chikungunya: Young Age and Inefficient Type-I Interferon Signaling Are Risk Factors for Severe Disease. PLOS Pathog. 4, e29 (2008). [PubMed: 18282093]
275. Haese NN et al. Animal Models of Chikungunya Virus Infection and Disease. J. Infect. Dis 214, S482–S487 (2016). [PubMed: 27920178]
276. Levitt NH et al. Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine 4, 157–162 (1986). [PubMed: 3020820]
277. Werneke SW et al. ISG15 Is Critical in the Control of Chikungunya Virus Infection Independent of UbE1L Mediated Conjugation. PLOS Pathog. 7, e1002322 (2011). [PubMed: 22028657]
278. Gardner J et al. Chikungunya Virus Arthritis in Adult Wild-Type Mice. J. Virol 84, 8021–8032 (2010). [PubMed: 20519386]

279. Hallengärd D et al. Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice. J. Virol 88, 2858–2866 (2014). [PubMed: 24371047]

280. Morrison TE et al. A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease: Evidence of Arthritis, Tenosynovitis, Myositis, and Persistence. Am. J. Pathol 178, 32–40 (2011). [PubMed: 21224040]

281. Hawman DW et al. Chronic Joint Disease Caused by Persistent Chikungunya Virus Infection Is Controlled by the Adaptive Immune Response. J. Virol 87, 13878–13888 (2013). [PubMed: 24131709]

282. Poo YS et al. Multiple Immune Factors Are Involved in Controlling Acute and Chronic Chikungunya Virus Infection. PLoS Negl. Trop. Dis 8, e3354 (2014). [PubMed: 25474568]

283. Seymour RL, Adams AP, Leal G, Alcorn MDH & Weaver SC A Rodent Model of Chikungunya Virus Infection in RAG1 −/− Mice, with Features of Persistence, for Vaccine Safety Evaluation. PLoS Negl. Trop. Dis 9, e0003800 (2015). [PubMed: 26115459]

284. Binn LN, Harrison VR & Randall R Patterns of viremia and antibody observed in rhesus monkeys inoculated with Chikungunya and other serologically related group A arboviruses. Am. J. Trop. Med. Hyg 16, 782–5 (1967). [PubMed: 4965218]

285. Kam Y-W et al. Unique Epitopes Recognized by Antibodies Induced in Chikungunya Virus-Infected Non-Human Primates: Implications for the Study of Immunopathology and Vaccine Development. PLOS ONE 9, e95647 (2014). [PubMed: 24755730]

286. Labadie K et al. Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. https://www.jci.org/articles/view/40104/table/1 (2010) doi:10.1172/JCI40104.

287. Messaoudi I et al. Chikungunya Virus Infection Results in Higher and Persistent Viral Replication in Aged Rhesus Macaques Due to Defects in Anti-Viral Immunity. PLoS Negl. Trop. Dis 7, e2343 (2013). [PubMed: 23936572]

288. Paul SD & Singh KRP Experimental infection of Macaca radiala with chikungunya virus and transmission of virus by mosquitoes. Indian J. Med. Res 56, 802–11 (1968). [PubMed: 4971384]

289. Chen C-I et al. Comparative Pathogenesis of Epidemic and Enzootic Chikungunya Viruses in a Pregnant Rhesus Macaque Model. Am. J. Trop. Med. Hyg 83, 1249–1258 (2010). [PubMed: 21118930]

290. Heise MT, Simpson DA & Johnston RE Sindbis-Group Alphavirus Replication in Periosteum and Endosteum of Long Bones in Adult Mice. J. Virol 74, 9294–9299 (2000). [PubMed: 10982376]

291. Jackson AC, Morench TR, Griffen DE & Johnson RT Tha Pathogenesis of Spinal Cord Involvement in the Encephalomyelitis of Mice Caused by Neuroadapted Sindbis Virus Infection. Lab. Invest 56, 418–423 (1987). [PubMed: 3031369]

292. Klimstra WB et al. Infection of Neonatal Mice with Sindbis Virus Results in a Systemic Inflammatory Response Syndrome. J. Virol 73, 10387–10398 (1999). [PubMed: 10559357]

293. Orvedahl A et al. Autophagy Protects against Sindbis Virus Infection of the Central Nervous System. Cell Host Microbe 7, 115–127 (2010). [PubMed: 20159618]

294. Trgovcich J, Aronson JF & Johnston RE Fatal Sindbis Infection of Neonatal Mice in the Absence of Encephalitis. Virology 73–83 (1996).

295. Jupille HJ et al. Mutations in nsP1 and PE2 are critical determinants of Ross River virus-induced musculoskeletal inflammatory disease in a mouse model. Virology 410, 216–227 (2011). [PubMed: 21131014]

296. Morrison TE et al. Characterization of Ross River Virus Tropism and Virus-Induced Inflammation in a Mouse Model of Viral Arthritis and Myositis. J. Virol 80, 737–749 (2006). [PubMed: 16378976]

297. Morrison TE, Fraser RJ, Smith PN, Mahalingam S & Heise MT Complement Contributes to Inflammatory Tissue Destruction in a Mouse Model of Ross River Virus-Induced Disease. J. Virol 81, 5132–5143 (2007). [PubMed: 17314163]

298. Herrero LJ et al. Characterization of Barmah Forest virus pathogenesis in a mouse model. J. Gen. Virol 95, 2146–2154 (2022).

299. Seymour RL, Rossi SL, Bergren NA, Plante KS & Weaver SC The Role of Innate versus Adaptive Immune Responses in a Mouse Model of O'Nyong-Nyong Virus Infection. Am. J. Trop. Med. Hyg 88, 1170–1179 (2013). [PubMed: 23568285]

300. Santos FM et al. Animal model of arthritis and myositis induced by the Mayaro virus. PLoS Negl. Trop. Dis 13, e0007375 (2019). [PubMed: 31050676]
301. Fazakerley JK Semliki Forest virus infection of laboratory mice: a model to study the pathogenesis of viral encephalitis | SpringerLink. 10.1007/978-3-7091-0572-6_16 (2022).
302. USDA. USDA APHIS | Equine Encephalitis (EEE/WEE/VEE). https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/equine/eee-wee-vee/equine-encephalitis (2021).
303. MERCK. Compendium of Veterinary Products - Encevac® TC-4+VEE (MERCK ANIMAL HEALTH). https://merckusa.cvpservice.com/product/basic/view/1047320 (2021).
304. PEDERSEN CE JR, ROBINSON DM & COLE FE JR. ISOLATION OF THE VACCINE STRAIN OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS FROM MOSQUITOES IN LOUISIANA. Am. J. Epidemiol 95, 490–496 (1972). [PubMed: 4401801]
305. BERGE TO, BANKS IS & TIGERTT WD ATTENUATION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS BY IN VITRO CULTIVATION IN GUINEA-PIG HEART CELLS 1. Am. J. Epidemiol 73, 209–218 (1961).
306. Pittman PR et al. Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine. Vaccine 14, 337–343 (1996). [PubMed: 8744562]
307. Mckinney RW, Berge TO, Sawyer WD, Tigertt WD & Crozier D Use of an Attenuated Strain of Venezuelan Equine Encephalo-Myelitis Virus for Immunization in Man. Am. J. Trop. Med Hyg 12, 597–603 (1963). [PubMed: 14044773]
308. Kautz TF et al. Low-fidelity Venezuelan equine encephalitis virus polymerase mutants to improve live-attenuated vaccine safety and efficacy. Virus Evol. 4, vey004 (2018). [PubMed: 29593882]
309. Mckinney RW, Berge TO, Sawyer WD, Tigertt WD & Crazier D Use of an Attenuated Strain of Venezuelan Equine Encephalo-Myelitis Virus for Immunization in Man. Am. J. Trop. Med. Hyg 12, 597–603 (1963). [PubMed: 14044773]
310. Pittman PR & Plotkin SA Biodefense and Special Pathogen Vaccines. Plotkins Vaccines 149–160.e7 (2018) doi:10.1016/B978-0-323-35761-6.00012-2.
311. Fine DL et al. Neurovirulence evaluation of Venezuelan equine encephalitis (VEE) vaccine candidate V3526 in nonhuman primates. Vaccine 26, 3497–3506 (2008). [PubMed: 18508163]
312. Holley H Safety of an Attenuated Venezuelan Equine Encephalitis Virus (VEEV) Vaccine in Humans. in (Idsa, 2008).
313. Steele KE et al. Alphavirus Encephalitides. in Medical Aspects of Biological Warfare 241–270 (Office of the Surgeon General, 2007).
314. Coates EE et al. Safety and immunogenicity of a trivalent virus-like particle vaccine against western, eastern, and Venezuelan equine encephalitis viruses: a phase 1, open-label, dose-escalation, randomised clinical trial. Lancet Infect. Dis 22, 1210–1220 (2022). [PubMed: 35568049]
315. Stromberg ZR, Fischer W, Bradfute SB, Kubicek-Sutherland JZ & Hraber P Vaccine Advances against Venezuelan, Eastern, and Western Equine Encephalitis Viruses. Vaccines 8, 273 (2020). [PubMed: 32503232]
316. Perry JG et al. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. Am. J. Trop. Med Hyg 62, 681–685 (2000). [PubMed: 11304054]
317. Stromberg ZR, Fischer W, Bradfute SB, Kubicek-Sutherland JZ & Hraber P Vaccine Advances against Venezuelan, Eastern, and Western Equine Encephalitis Viruses. Vaccines 8, 273 (2020). [PubMed: 32503232]
318. Plante K et al. Novel Chikungunya Vaccine Candidate with an IRES-Based Attenuation and Host Range Alteration Mechanism. PLOS Pathog. 7, e1002142 (2011). [PubMed: 21829348]
319. Reyes-Sandoval A 51 years in of Chikungunya clinical vaccine development: A historical perspective. Hum. Vaccines Immunother 15, 2351–2358 (2019).
320. Hallengärd D et al. Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice. J. Virol 88, 2858–2866 (2014). [PubMed: 24371047]

321. Folegatti PM et al. A single dose of ChAdOxl Chik vaccine induces neutralizing antibodies against four chikungunya virus lineages in a phase 1 clinical trial. Nat. Commun 12, 4636 (2021). [PubMed: 34330906]
322. Barber TL, Walton TE & Lewis KJ Efficacy oftrivalent inactivated encephalomyelitis virus vaccine in horses. Am. J. Vet. Res 39, 621–625 (1978). [PubMed: 646197]
323. AAEP. Eastern & Western Equine Encephalomyelitis | AAEP. https://aaep.org/guidelines/vaccination-guidelines/core-vaccination-guidelines/easternwestern-equine-encephalomyelitis (2021).
324. Alevizatos AC, McKinney RW & Feigin RD Live, Attenuated Venezuelan Equine Encephalomyelitis Virus Vaccine: I. Clinical Effects in Man. Am. J. Trop. Med. Hyg 16, 762–768 (1967). [PubMed: 6066224]
325. Cole FE, May SW & Eddy GA Inactivated Venezuelan Equine Encephalomyelitis Vaccine Prepared from Attenuated (TC-83 Strain) Virus 27, 4 (1974).
326. Martin SS et al. Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB. Vaccine 28, 1031–1040 (2010). [PubMed: 19914193]
327. Law Y-S et al. Interdomain Flexibility of Chikungunya Virus nsP2 Helicase-Protease Differentially Influences Viral RNA Replication and Infectivity. J. Virol 95, e01470–20 (2021). [PubMed: 33328310]
328. Koonin EV, Dolja VV & Morris TJ Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences. Crit. Rev. Biochem. Mol. Biol 28, 375–430 (1993). [PubMed: 8269709]
329. Mackenzie JM, Jones MK & Westaway EG Markers for trans-Golgi Membranes and the Intermediate Compartment Localize to Induced Membranes with Distinct Replication Functions in Flavivirus-Infected Cells. J. Virol 73, 9555–9567 (1999). [PubMed: 10516064]
330. Lin W, Feng Z, Prasanth KR, Liu Y & Nagy PD Dynamic interplay between the co-opted Fis1 mitochondrial fission protein and membrane contact site proteins in supporting tombusvirus replication. PLOS Pathog 17, e1009423 (2021). [PubMed: 33725015]
331. Kutchko KM et al. Structural divergence creates new functional features in alphavirus genomes. Nucleic Acids Res 46, 3657–3670 (2018). [PubMed: 29361131]
332. Madden EA et al. Using SHAPE-MaP To Model RNA Secondary Structure and Identify 3′UTR Variation in Chikungunya Virus. J. Virol 94, e00701–20 (2020). [PubMed: 32999019]

Highlights:

- A broad review of all aspects of the alphaviral life cycle
- Particularly focused on the functions of the structural and nonstructural proteins
- Highlights recent discoveries in alphaviral replication
- Summarizes the current state of vaccine and antiviral development targeting the alphaviruses
- Points out current knowledge gaps in relation to alphaviral replication
- Poses additional areas of research focus to further the field

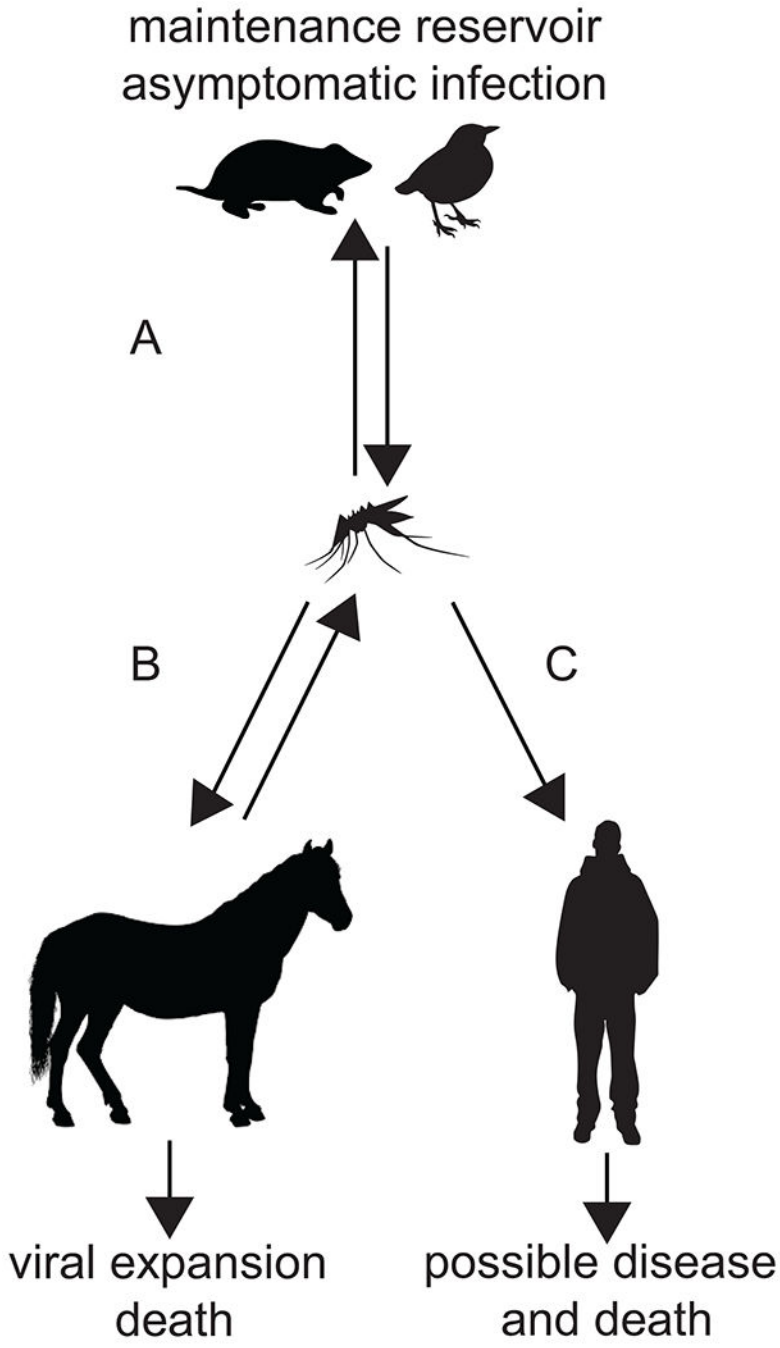


Figure 1.

The transmission cycle of encephalitic alphaviruses. A) Alphaviruses are maintained in nature by cycling between a host species, typically a bird or small mammal, and a mosquito vector species. B) Spillover events often occur into livestock, which reach high viral titers and readily transmit the virus to additional vectors. In the case of the New-World viruses this infection almost always leads to death[27]. C) Typically, after infection of livestock, humans that work in close association with these animals can also be infected by vector species.

Humans are regarded as dead-end hosts for most alphaviruses. In humans these infections may lead to disease, and, in severe cases, death.

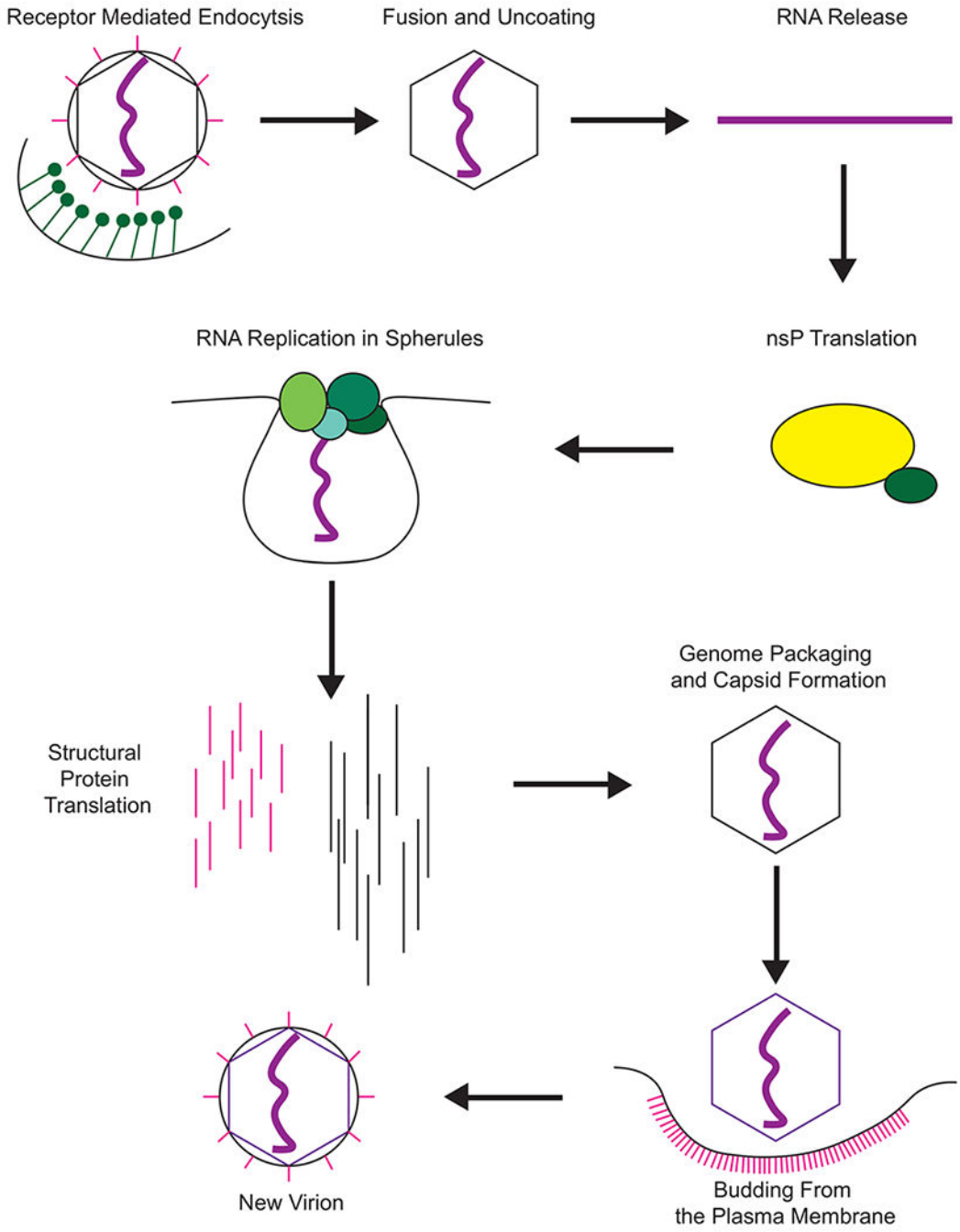


Figure 2.

The replication cycle of alphaviruses. The virion enters a susceptible cell via receptor mediated endocytosis, primarily mediated by clathrin (green) and due to pH changes of the endosome releases its RNA (purple) into the cytoplasm of the host cell. The positive sense genomic RNA is first used by ribosomes to translate the viral nsPs as a polyprotein (yellow = nsP123, green = nsP4). The polyprotein will undergo cleavage events that control the synthesis of the viral RNA species (individual nsPs represented as single green circles). This RNA synthesis occurs in membrane invaginations that are termed spherules. These spherules

protect the viral RNA and nsPs from detection by the host cell. Late in infection the structural genes are synthesized (pink = the E proteins, black = capsid). The capsid will form into nucleocapsid cores as it packages the viral RNA, and the glycoproteins are transported to the cell membrane. The nucleocapsid cores translocate to the cellular membrane where they bud off, collecting their envelope and glycoproteins and forming new infectious virions.

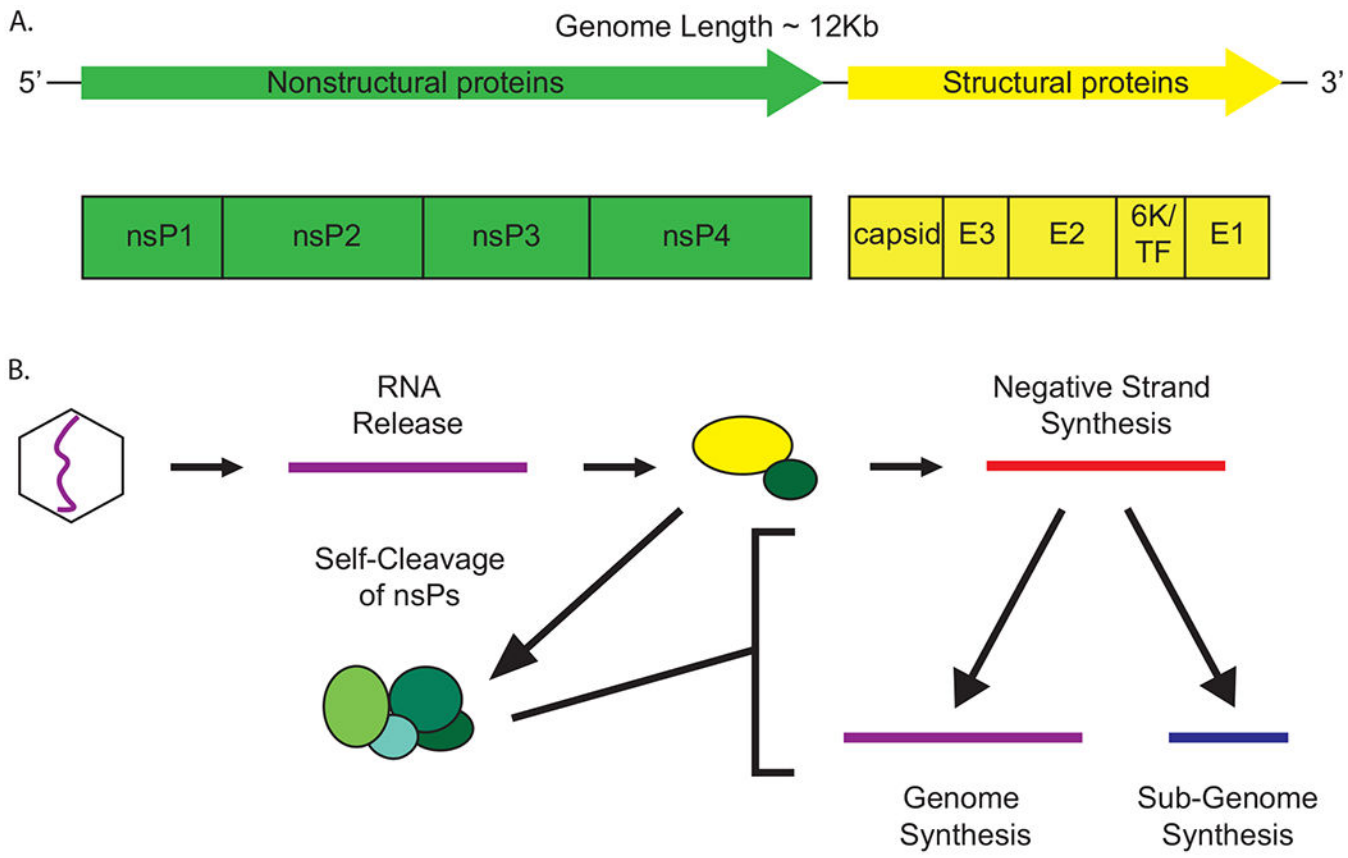


Figure 3.

A) The genetic structure of the alphaviruses. The alphaviruses have an approximately 12kb, linear, positive-sense genome. The genome has two open reading frames, the nonstructural and the structural. The nonstructural open reading frame is here displayed in green, and encodes the four nonstructural proteins, which are responsible for replication of the viral RNA. The structural open reading frame is displayed here in yellow and encodes for the E proteins and capsid as well as the 6K and TF proteins. The capsid and E proteins form the structure of the viral particle. **B)** RNA synthesis of alphaviruses. This RNA synthesis activity is carried out in spherules on the membranes of cellular organelles. After release into the cytoplasm the genomic RNA (purple) is used to synthesize the initial nonstructural polyprotein. nsP2 initially cleaves between nsP3 and 4 leading to nsP123/4 (nsP123 = yellow, nsP4 = green), which synthesizes primarily negative-sense template RNA (red). The protein undergoes rapid cleavage through intermediate states to reach the final replicase complex nsP1/2/3/4 (represented as individual green circles). This complex synthesizes new positive-sense genomic (purple) and subgenomic (blue) RNA and can no longer synthesize negative-sense RNA. The genomic RNA is used to synthesize additional genomes and is packaged into progeny virions. The subgenomic RNA is used to synthesize the structural genes that form the new virions.

Table 1:

A comparison of representative alphaviruses.

Virus	Old or New World	Arthritic	Neurologic	Mortality	Location
Chikungunya	OLD	+	−	Reports vary, as low as 0.03%. Some claim this is underreported.[25]	Tropics world wide
Semliki Forest	OLD	+	rarely	extremely rare with neurologic involvement[26]	Africa
Sindbis	OLD	+	rarely	extremely rare with neurological involvement[8–10]	Africa and Europe
Ross River	OLD	+	−	none[27]	Oceania
O'nyong-nyong	OLD	+	−	none[28]	Sub-Saharan Africa
Venezuelan Equine Encephalitis	NEW	−	+	≤1%[23]	South and Central America
Western Equine Encephalitis	NEW	−	+	3-7%[23]	Western North America and parts of South America
Eastern Equine Encephalitis	NEW	−	+	50-75%[23]	Eastern North and South America

Table 2:

A summary of selected anti-alphaviral compounds and their mechanisms of action. All structures are published in PubChem.

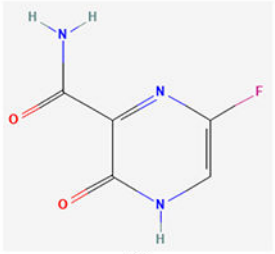
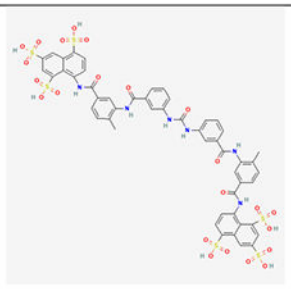
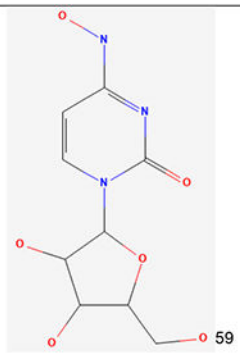
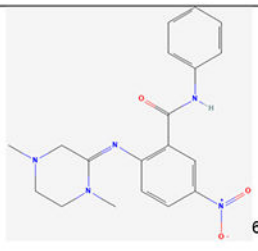
Compound Name	Structure	Licensed?	Mechanism of Action
favipiravir	 53	Yes, for use in influenza in Japan[54]	Competes with purines for incorporation into the RNA chain, arresting replication[55]
suramin	 48	Yes. Suramin is a treatment available from the WHO for African Trypansomiasis[56,57]	Inhibits viral replication as well as likely negatively effecting viral attachment[51,58]
ß-D-N4-hydroxycytidine	 59	No	Incorporates into the RNA chain, induces mutagenesis[60]
ML336	 61	No	Interferes with viral RNA synthesis, preventing replication[62]

Table 3:

A summary of the structures and functions of the alphaviral nsPs

Protein	Structures	Functions
nsP1	methyl transferase domain, guanyl transferase domain, membrane association domains	caps viral RNA making it usable by ribosomes, anchors the replication machinery to cellular membranes[111,129–131,133–136]
nsP2	helicase domain, ADP binding region, cysteine protease, methyl transferase like domain	unwinds viral RNA for replication, cleaves the polyprotein into its constitutive parts, digests host cell proteins[77,132,138–142,144–146,152–157]
nsP3	macrodomain, alphavirus unique domain, hypervariable domain	poorly described, necessary for replication, highly involved in host cell interactions[129,160–168,170–172,174,175]
nsP4	RNA dependent RNA polymerase domain	synthesizes new viral RNA[71,77,129,176–178,180–182,185]

Table 4:

Summary of the functions of the structural proteins of alphaviruses

Protein	Function
Capsid	Forms the virion structure, selects for packaging of viral RNA, involved in initiating budding from the cell, in the New-World viruses inhibits cellular translation and transcription[127,128,140,191–194,198,228]
E1	Forms heterotrimers with E2 to form the glycoprotein spikes, fuses the viral and host cell membranes, forms pores in the endosome to induce pH changes[63,68–70,210,229]
E2	Forms heterotrimers with E1 to from the glycoprotein spikes[209]
E3	Functions as the signal sequence for the structural polypeptide, protects E1 from acidic pH, additional functions involved in trafficking and virulence[213–215]
6K	Involved in budding, may help space the glycoproteins on the virion, may function as a viroporin[216,218]
TF	Involved in evading interferon due to specific palmitoylation, may be a viroporin, incorporated into the virion[216,224,225]

Table 5:

Selected antiviral screening assays

antiviral assay	readout type	Use	references
CelTiter-Glo	luminescence	cell viability	62,231,232
DNA staining and TUNEL	fluorescence	cell viability	233–235
MTT	colorimetric	cell viability	236,237
Crystal violet	cell staining	cell viability	238
viral reporter systems	fluorescence or luminescence	tracking viral protein production tracking viral replication determining stages of antiviral interference	233,237,239–243

Table 6:

Animal models of alphavirus infection

virus	model	Pathology	references
VEEV	guinea pig	high lethality, no encephalitis symptoms	244–246
	hamster	high lethality, no encephalitis symptoms	244–246
	mice (various immune competent strains)	similar to human infection, route dependent pathology	247–255
	cynomolgus macaques	Similar to human infection lymphatic pathology, myocarditis develop CNS lesions regardless of encephalitis symptoms	244,246,254,256,257
	various equids	high lethality but distinct from human disease	244
EEEV	mice (various immune competent strains)	develop seizures, no vasculitis	247,254,258,259
	hamsters	high lethality, no encephalitis symptoms	254
	guinea pigs	lethal with no obvious encephalitis	254
	macaques	similar to human infection	254,260–263
	owl monkeys	similar to human infection	262
	marmosets	similar to human infection	261
	NHP models	additional readouts such as brainwaves under development	264
WEEV	mice (Swiss, Balb/c, others)	Similar to human infection Also develop myocarditis	254,265–268
	hamsters	high mortality when infected in the periphery, rescuable with interferon	258,269–271
	macaques	Similar to human infection, Also develop myocarditis, lethality is strain and experiment dependent	254,257,272,273
CHIKV	neonatal mice	high lethality	274–277
	interferon deficient mice	high lethality	275
	mice (various immune competent strains)	similar to human arthralgia	275,278–280
	Rag knockout mice and similar	Similar to human arthralgia increased viral persistence	275,281–283
	adult macaques	similar to mild human disease, high dose mimics severe disease	275,284–288
	aged macaques	Similar to human disease, developed to study disease in specific human populations	275,289
	pregnant macaques	Similar to human disease, developed to study disease in specific human populations	287
SINV	mice (various immune competent strains)	specialized viral strains develop encephalitis can also be used for arthralgia, which is more similar to human pathology	8,290
	neonate and weanling mice	high lethality, some encephalitis	291–294
RRV	mice (various immune competent strains)	similar to human disease	295–297
BFV	mice (various immune competent strains)	similar to human disease	298
ONNV	mice (various immune competent strains)	similar to human disease	299
MAYV	mice (various immune competent strains)	similar to human disease	300
	cynomolgus macaques	similar to human disease	284

virus	model	Pathology	references
SFV	mice (various immune competent strains)	similar to human disease	[301]