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New World alphavirus protein interactomes from a therapeutic perspective

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ABSTRACT

The New World alphaviruses, Venezuelan, eastern and western equine encephalitis viruses (VEEV, EEEV, and WEEV), are important human pathogens due to their ability to cause varying levels of morbidity and mortality in humans. There is also concern about VEEV and EEEV being used as bioweapons. Currently, a FDA-approved antiviral is lacking for New World alphaviruses. In this review, the function of each viral protein is discussed with an emphasis on how these functions can be targeted by therapeutics. Both direct acting antivirals as well as inhibitors that impact host protein interactions with viral proteins are described. Non-structural protein 3 (nsP3), capsid, and E2 proteins have garnered attention in recent years, whereas little is known regarding host protein interactions of the other viral proteins and is an important avenue for future study.

1. Introduction

The New World (NW) alphaviruses (Venezuelan, eastern, and western equine encephalitis viruses-VEEV, EEEV, and WEEV) cause significant disease in humans and equines (Table 1). However, to date there are no FDA-approved therapeutics or vaccines available for human use for these pathogens. Traditional antivirals are typically developed to target key viral proteins such as viral enzymes (e.g. proteases, polymerases) in an effort to specifically prevent viral replication. However, the acceptance of host-based antiviral strategies has become increasingly popular during the past decade. As resistance to viral targeting drugs persists, host-based interactions are of great interest for antiviral development. Host targeting offers significant advantages aside from evading resistance such as providing broad spectrum effectiveness against various serotypes and/or viral groups and offering a larger range of potential targets. For instance, the iminosugar drug UV-4B inhibiting glycosylated mediated protein folding at the host's endoplasmic reticulum (ER) provides antiviral activity *in vivo* for dengue virus (DENV; family *Flaviviridae*), influenza A virus (IAV; family *Orthomyxoviridae*) and influenza B virus (IBV; family *Orthomyxoviridae*) in mice (Perry et al., 2013; Warfield et al., 2016). This mechanism can be considered a model for host-based targeting as it impedes a common functionality in the host to provide protection against different viral families.

Therapeutic development can be facilitated through a more detailed molecular understanding of viral replication and host processes that are utilized to produce a productive infection. Presented within is a review of NW alphavirus proteins including their role in the viral life cycle and known host protein interactions constituting the viral interactome to date. Post-translational modifications of viral proteins are also highlighted. The current state of antiviral strategies from both the direct acting and host-based antiviral perspective for each alphavirus protein is discussed. When information is lacking about specific NW alphavirus proteins, information about Old World (OW) alphaviruses [Sindbis virus (SINV), Semliki Forest virus (SFV) and chikungunya virus (CHIKV)] proteins is presented in an effort to highlight potential avenues for future exploration.

1.1. Molecular biology and life cycle

Alphaviruses are of the *Togaviridae* family characterized as enveloped, positive-sense single stranded RNA viruses with a genome size ranging from 11 to 12 kb. The genome is broken down into two reading frames (Fig. 1). The first reading frame encodes the non-structural polyprotein that is further processed into four non-structural proteins, nsP1-nsP4. The non-structural proteins form the replication complex and copy viral RNA first in the negative sense and then back to the positive sense RNA that is incorporated into the virion. The second reading frame is controlled by a 26S promoter on the negative strand RNA and encodes the structural polyprotein. The structural polyprotein is made up of the capsid protein and the envelope glycoproteins, E1 and E2.

There are several steps in the alphavirus life cycle (Fig. 2). Entry at the plasma membrane of host cells involves binding of host protein receptors varying between species of alphaviruses through fusion or receptor binding mediated by the viral E2 protein. Subsequent

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Table 1
NW alphaviruses: Geographic distribution, human disease, and route of transmission.

Virus	Endemic Regions	Disease in Humans	Transmission
VEEV	• United States, Central and South America o (Weaver and Barrett, 2004)	• Febrile illness, encephalitis • Mortality ~1% • Neurological sequelae in up to 14% of survivors o (Ronca et al., 2016)	• Mosquito • Aerosol route via laboratory accidents o (Lennette and Koprowski, 1943; Steele and Twenhafel, 2010)
EEEV	• North, Central and South America, Caribbean o (Kumar et al., 2018)	• Febrile illness, encephalitis • Mortality 50–78% • Neurological sequelae in up to 75% of survivors o (Ronca et al., 2016)	• Mosquito • Aerosol route via laboratory accidents o (Steele and Twenhafel, 2010)
WEEV	• North and South America • Has not been detected in mosquitoes since 2008 • Last human case was in 2009 in Uruguay o (Delfraro et al., 2011; Kumar et al., 2018)	• Febrile illness, encephalitis • Mortality 3–7% • Neurological sequelae in up to 90% of survivors o (Ronca et al., 2016)	• Mosquito • Aerosol route via laboratory accidents o (Steele and Twenhafel, 2010)

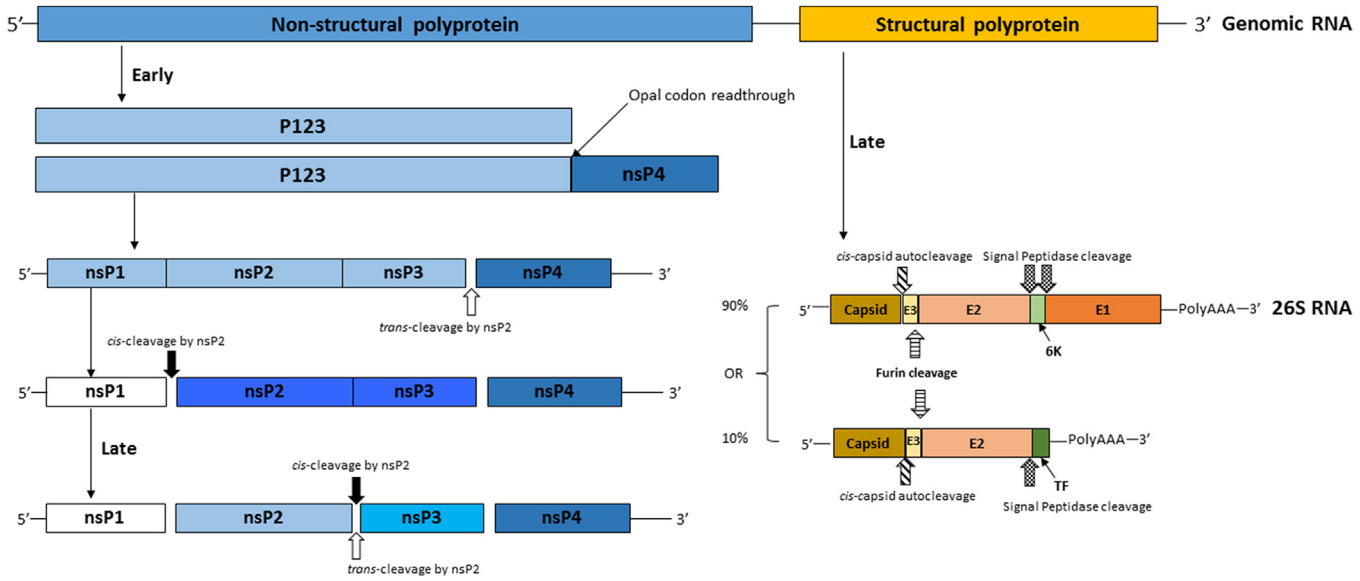

Fig. 1. Alphavirus genome and protein coding. The alphavirus genome encodes two distinct reading frames: genomic (blue) which encodes the non-structural polyprotein and subgenomic (yellow) which encodes the structural polyprotein. The genomic reading frame is translated first into either the polyprotein P123 or P1234. P123 contains a stop codon at the end of the nsP3 protein which has readthrough activity to allow the translation of nsP4 RdRp. After P1234 is translated, nsP4 (dark blue) is cleaved in *trans* by nsP2 resulting in P123 and nsP4 (if no readthrough occurs this step does not happen). Next, P123 is cleaved in *cis* by nsP2 resulting in nsP1 (white), P23 (royal blue), and nsP4. Finally, P23 is cleaved both in *cis* and *trans* by nsP2 to separate nsP2 (light blue) and nsP3 (teal). The nonstructural polyproteins form the replication complex that replicates the viral RNA. Once the negative strand RNA is produced, a 26S promoter controls the transcription of the subgenomic reading frame. During translation of the structural polyprotein, capsid (gold) is the first protein translated and is cleaved in *cis* through its own proteolytic activity. This autocleavage event frees an ER localization sequence on the N-terminus of the E3 protein (light yellow). Once the ER localization sequence is recognized, the remainder of the structural polyprotein is translated into the ER. E3 and E2 (salmon) remain as the p62 complex while signal peptidase cleaves the 6K (light green) or TF (dark green) protein from the polypeptide chain. This cleavage frees E1 (orange) to form a dimer with p62. Furin cleaves E3 from E2 to form the mature glycoprotein spike.

internalization is driven by clathrin-mediated endocytosis in which the endocytic vesicle increases in acidity during maturation and the low pH environment induces dissociation of the E1/E2 dimer to allow E1 insertion into the endosomal membrane and subsequent fusion of the viral and cell membrane for the release of the viral nucleocapsid into the host's cytosol (Brown et al., 2018). Genomic RNA (gRNA) is translated immediately into two non-structural precursors, P123 or P1234. P1234 is produced by a read-through of the opal termination codon (UGA) between nsP3 and nsP4 followed by cleavage of P123 and nsP4 in either cis or trans and cleavage of nsP1 and P23 in cis only (Strauss et al., 1983). P123-nsp4 and nsP1-P23-nsP4 utilize host proteins to synthesize negative strand viral RNA intermediates through formation of early replication complexes. A final cleavage event between P23 occurs to produce all four mature non-structural proteins and these late stage replication complexes are responsible for the synthesis of positive-sense genomic (49S) and subgenomic (26S) RNA (Abu Bakar and Ng, 2018; Shirako and Strauss, 1994).

A second open reading frame (ORF) constitutes roughly ~1/3 of the alphaviral genome and is controlled by its own subgenomic promoter on the negative-strand of the viral genome (Brown et al., 2018). This ORF encodes for capsid, E3, E2, 6K, and E1 proteins, which are primarily structural proteins. Like the non-structural proteins, the structural proteins are translated as a polyprotein precursor in which the capsid protein proteolytically cleaves itself and subsequently encapsidates gRNA into the nucleocapsid. Cleavage of capsid from the structural polyprotein exposes an ER localization signal on pE2 (precursor to E3 and E2), which directs the remaining polyprotein to the ER where the proteins enter the secretory pathway for export to the plasma membrane. Late in this pathway, furin-mediated cleavage of pE2 forms the E2 and E3 proteins. Trimers of E1 and E2 proteins are transported to the plasma membrane. Within the 6K coding region, ribosomal frameshifting causes a −1 reading of the ORF during translation resulting in the production of the transframe (TF) protein (Brown et al., 2018; Firth et al., 2008). Budding at the cell surface occurs as assembly of capsid

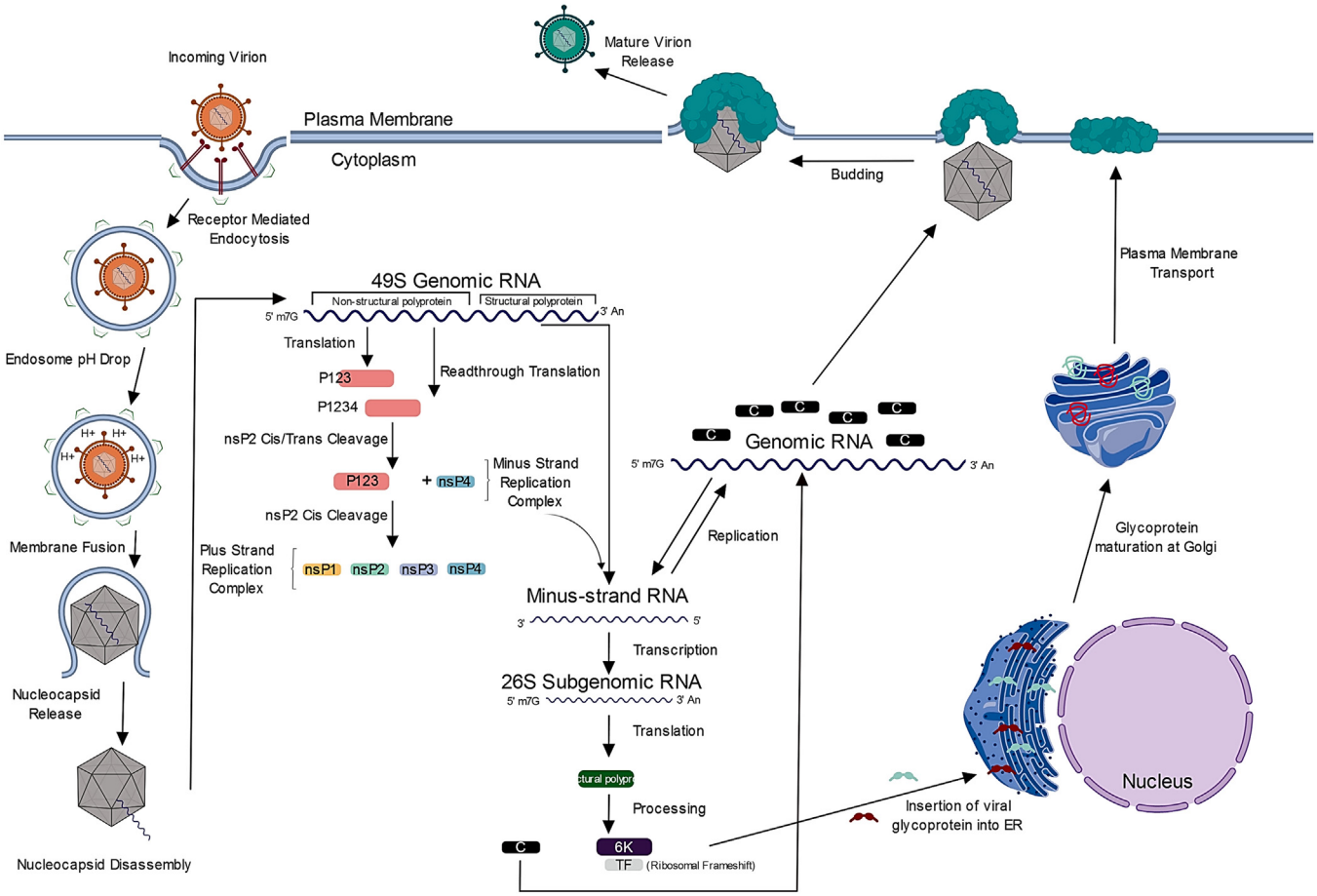

Fig. 2. Alphavirus replication cycle. Incoming virions are taken into the cell via clathrin mediated endocytosis. Once endocytosis is complete, the drop in pH within the endosome causes a conformational change in the E1 glycoprotein causing it to fuse with the endosomal membrane releasing the viral nucleocapsid. Nucleocapsid disassembly happens almost immediately and genomic viral RNA is translated producing the non-structural polyprotein. A series of cleavage events occur separating the non-structural polyprotein into the individual non-structural proteins nsP1-4. The non-structural proteins form the viral replication complex where viral RNA is transcribed into minus strand RNA. Minus strand RNA is used as a template for the full viral genome to be transcribed by the replication complex for incorporation into new viral particles. Simultaneously, minus strand RNA contains a 26S promoter for the subgenomic RNA that is transcribed into the 5′ direction to be translated. During translation of the 26S subgenomic RNA, capsid is the first protein to be produced in the structural polyprotein. Capsid possesses autoprotease activity and cleaves itself from the growing polypeptide chain. The cleavage of capsid exposes an ER localization sequence in the E3 protein that causes the translating ribosome to translocate to the ER membrane and continue to translate the remainder of the structural polyprotein. A series of cleavage events and post translational processing occur in the ER/golgi and the mature glycoproteins are transported to the cell membrane. Capsid binds with newly transcribed viral RNA and translocates to the membrane where it interacts with the E2/E1 glycoprotein heterodimers already incorporated in the membrane. The interaction of capsid with the glycoproteins provides the energy for the virion to bud from the cell.

dimers forming spherical particles acquire the lipid envelope with E1 and E2 glycoprotein dimers embedded within (Abu Bakar and Ng, 2018; Perera et al., 2003).

2. nsP1

Nonstructural protein 1 (nsP1) contains methyltransferase and guanylyltransferase activities that contribute to viral mRNA capping that is critical to the translation activities of alphaviruses (Li et al., 2015). Alphavirus mRNAs contain 5′ cap structures not unlike eukaryotic mRNA cap structures, but lack *2′O* methylation on the 5′ end of viral mRNA's. These mRNA cap-0 structures [m^7G(5)ppp(5′)N] aid in avoidance of host-defense mechanisms such as host-mediated 5′-exonuclease activity (Li et al., 2015). The currently proposed mechanism of alphavirus mediated RNA capping was characterized through enzymatic identification of both methyltransferase and guanylyltransferase activities of nsP1 of SINV, SFV and VEEV. Early studies of OW alphaviruses identified methyltransferase activity of SINV nsP1 in insect cells lacking methionine (Li et al., 2015; Mi et al., 1989). Additionally, *in vitro* assays confirmed the presence of SFV nsP1 methyltransferase activities in insect cells and *Escherichia coli* (*E.coli*) (Laakkonen et al., 1994; Li et al., 2015). Following OW alphavirus characterization, amino acid residues within nsP1 that correspond to methyltransferase activity were identified to be conserved in the Sindbis-like group of alphaviruses (Rozanov et al., 1992). More recent studies have revealed that not only are amino acid sequences conserved but also capping mechanisms elucidated in OW alphaviruses are conserved in NW alphaviruses such as VEEV. In studies utilizing purified VEEV nsP1 recombinants, it was confirmed that VEEV nsP1 contains methyltransferase activity that results in an S-Adenosyl methionine (AdoMet) dependent-methylation of a guanosine triphosphate (GTP) molecule. Following methylation, guanylyltransferase activity results in release of pyrophosphate and covalent link formation between m^7GTP and nsP1 resulting in m^7 GMP-nsP1. The last step of VEEV nsP1-mediated capping results in the transfer of the m^7Gp to the to the 5′ end of VEEV genome (Li et al., 2015). Following specific characterization through the use of VEEV recombinants, mutagenesis experiments, and enzyme assays it was confirmed that the alphavirus capping activities identified in SFV and SINV were conserved.

Antiviral targets of nsP1 has not been extensively researched but

Table 2
Known alphavirus antivirals and the viral component they target.

Inhibitor	Virus	Viral Target	EC_{50}	Cell Type	*In vivo* Model	Reference
Sinefungin	VEEV	nsP1	ND[a]	n/a[b]	ND	(Li et al., 2015; Scheidel and Stollar, 1991)
Aurintricarboxylic acid (ATA)	VEEV, SINV	nsP1	ND	n/a	ND	(Li et al., 2015; Scheidel and Stollar, 1991)
Ribavirin	VEEV	nsP1	ND	n/a	ND	(Li et al., 2015; Scheidel and Stollar, 1991)
ML-336	VEEV	nsP2	0.8 μM	Vero76	C3H/HeN mice	Schroeder et al. (2014)
CID15997213	VEEV	nsP2	0.84 μM	Vero76	C3H/HeN mice	Chung et al. (2014)
β-D-N^4-hydroxycytidine	VEEV	nsP4	0.426 μM	Vero	ND	Urakova et al. (2017)
Favipiravir	WEEV, CHIKV, VEEV, EEEV	nsP4	7.5 μM (WEEV), 25 μM (CHIKV), 11 μM (VEEV). 18 μM (EEEV)	Vero-E6	AG129 mice	Delang et al. (2014)
LBP	VEEV	E2	ND	n/a	ND	Bondarenko et al. (2004)

[a] ND = not determined.
[b] n/a = Not applicable.

several guanosine analogs and small molecule inhibitors have been assessed for inhibition of nsP1-related capping activities in alphaviruses (Table 2). Small molecule inhibitors targeting methyltransferase and guanylyltransferase activities of VEEV, SINV and CHIKV have been assessed through enzymatic assays, viral replication and viral growth assays (Bullard-Feibelman et al., 2016; Gigante et al., 2014, 2017; Li et al., 2015). In studies utilizing purified VEEV nsP1, Sinefungin, Aurintricarboxylic acid (ATA) and Ribavirin triphosphate were assessed for their ability to inhibit capping activities of VEEV (Li et al., 2015).

An AdoMet/AdoHcy analogue, Sinefungin, was found to inhibit VEEV nsP1 methyltransferase activities and formation of m^7GMP-nsP1 complex when applied in increasing concentrations to purified nsP1. In addition to inhibition of methyltransferase activities, Sinefungin also inhibits guanylyltransferase activities through inhibition of activation of AdoHcy guanylyltransferase reactions. Additionally, an inhibitor of flavivirus mRNA capping, ATA, was found to have antiviral activity towards alphaviruses (Li et al., 2015; Scheidel and Stollar, 1991). Through inhibition of methyltransferase and guanylyltransferase activities when applied to purified VEEV nsP1, similar to that of Sinefungin, the mechanism of inhibition of ATA is not fully elucidated due to non-specific inhibition of numerous other viral proteins such as Hepatitis C virus (HCV; *Flaviviridae*) NS3 and IAV neuraminidase. Ribavirin triphosphate, a broad spectrum antiviral was observed to inhibit VEEV nsP1 activities but to a lesser degree and is thought to function through inhibition of cellular IMP dehydrogenase which results in reduction in the GTP pool required for viral replication. Following treatment of VEEV nsP1 with ribavirin, subsequent compensatory mutations were observed in the N terminus of nsP1 in response to ribavirin triphosphate treatment and due to less potent inhibition of viral activities, this inhibitor is considered to likely have a non-specific effect on nsP1 capping activities (Li et al., 2015; Scheidel and Stollar, 1991).

Through screening for antiviral compounds for inhibition of CHIKV viral activities, [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one-, was identified as an inhibitor of CHIKV replication in Vero cells with a determined EC_{50} as low as 0.75 μM for the Congo 95 (2011) strain of CHIKV (13). Additionally, drug resistant strains of CHIKV were derived when treated with [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one, and mutations were observed in the form of substitutions within CHIKV nsP1. Under selective pressure induced by [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one, it is thought that this compound inhibits the guanylyltransferase activities of nsP1 of CHIKV. Additionally, [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one-, was found to inhibit VEEV guanylyltransferase activities of nsP1, but not impact the overall viral activity (Gigante et al., 2014, 2017).

Additionally, a pilot screening was conducted in order to identify natural, FDA approved molecules that may interfere with CHIKV nsP1 activities by GTP displacement or interference of guanylyltransferase activities. Following a screening of 3051 small molecules, lobaric acid, pyrantel pamoate and garcinolic acid acted as inhibitors of CHIKV nsP1 guanylyltransferase activities through use of fluorescently labeled GTP and subsequent nsP1 interaction studies. All three small molecules are capable of inhibiting formation of guanosine monophosphate (GMP)-protein intermediate produced by the guanylyltransferase activities of CHIKV nsP1 in a dose-dependent manner. Interestingly, lobaric acid was the only small molecule identified to attenuate viral growth of alphaviruses. Lobaric acid treatments attenuated viral growth of CHIKV and SINV in BHK-21 and Huh-7 cells at both 24 and 48 h post-infection (Feibelman et al., 2018).

In addition to viral RNA capping activities, nsP1 plays an essential role in directing the viral replication complex to membrane surfaces in order to aid in viral RNA replication. Alphavirus nsP1 membrane association is thought to be mediated through palmitoylation of specific residues within nsP1 that direct the viral replication complexes to membrane surfaces. Palmitoylation of alphavirus nsP1 was first identified in studies with SFV in which specific cysteine residues, 418–420 were identified to be palmitoylated. In these studies, removal of

palmitate groups resulted in reduced membrane associability and hydrophobicity of nsP1. Additionally, palmitoylated nsP1 was identified in filopodial extensions of the plasma membrane. When palmitate groups are removed from nsP1, subcellular localization of nsP1 is altered (Laakkonen et al., 1996). In later studies, it was confirmed that SINV nsP1 is also palmitoylated on cysteine residue 420, indicating conservation of palmitoylation of nsP1 in alphaviruses (Ahola et al., 2000). Palmitoylation of SFV and SINV nsP1 was also observed to contribute to the development of filopodial extensions at cellular plasma membranes in HeLa cells (Laakkonen et al., 1996, 1998). Interestingly, SINV or SFV-induced filopodia containing nsP1 did not contain F-actin in HeLa cells. Additionally, only palmitoylated forms of nsP1 were localized to induced filopodial extensions. These current studies implicate nsP1 in regulation of cellular cytoskeletal networks and specific palmitoylated forms of nsP1 are associated with altered cytoskeletal structures (Laakkonen et al., 1998).

Therapeutic targeting of nsP1 palmitoylation has not been extensively investigated. Studies have been conducted in which mutations in nsP1 have been introduced in an effort to disrupt palmitoylation activities in SFV and SINV nsP1 and subsequently disrupt viral replication. In SFV and SINV containing mutations which prevent palmitoylation of nsP1, normal viral replication was observed, but growth was delayed in BHK cells. Additionally, nsP1 of SINV and SFV did not localize to filopodial extensions in viral mutants lacking nsP1 palmitoylation. Interestingly, in mice infected with SFV nsP1 mutants lacking palmitoylation sites, SFV pathogenesis was altered. SFV mutant infected mice all survived in comparison to WT infected mice and infectious virus was not detected in the brain following infection (Ahola et al., 2000). Targeting nsP1 has great therapeutic potential; however there is little information known about specific host interacting partners and is an area that should be explored further.

3. nsP2

Three functions are characterized for nsP2–helicase, phosphatase, and protease activity. The N-terminal acts as an RNA helicase with nucleoside triphosphatase (NTPase) capabilities that associates with the polymerase nsP4 to exert its unwinding function efficiently (Gomez de Cedron et al., 1999; Rupp et al., 2015). In addition to this, the N-terminal domain contains RNA triphosphatase activity to initiate viral RNA capping reactions through removal of the γ-phosphate at the 5' end of nascent viral RNA that allows for proper capping by nsP1 (Vasiljeva et al., 2000). The papain-like C-terminal domain is the protease responsible for cleaving the non-structural polyprotein in which the mechanism of proteolysis is modulated by the other domains of nsP2 as well as the other viral proteins it is associated with during the course of infection (Lulla et al., 2006; Vasiljeva et al., 2000). Data has proven the significance of the protease domain as deletion of this domain renders the nsP2 helicase functionality inactive (Kim et al., 2013). Recent data has also suggested that nsP2 may play a role either directly or indirectly in the encapsidation and packaging process of viral RNA for the production of infectious virions (Kim et al., 2013).

Although NW alphaviruses utilize their capsid protein to induce shutoff of cellular transcription and will be discussed later in this review, it is worth noting the role of nsP2 in the context of OW alphaviruses. OW alphaviruses utilize nsP2 to suppress cellular transcription in host cells and this is the primary means for control of the host's innate antiviral defense (Frolova et al., 2002; Garmashova et al., 2006; Gorchakov et al., 2005). The mechanism of which OW alphaviruses control cellular transcription is through efficient degradation of the catalytic subunit of RNA polymerase II, Rpb1. This degradation is surprisingly caused by ubiquitination triggered by the transcription-coupled repair (TCR) pathway rather than the inherent protease activity of nsP2. Instead, the helicase and NTPase domains are responsible for nsP2-mediated Rpb1 ubiquitination as mutations in these eliminated degradation completely (Akhrymuk et al., 2012).

More recently, data has indicated that transcriptional cellular shutoff is delayed in comparison to translational shutoff within alphavirus infected cells and that these events are temporally independent. Mutagenesis data of VEEV nsP2 demonstrates that control of host translation is attributed to the C-terminal domain of the viral protein but not within the canonical "PGG" domain conserved between alphaviruses (Bhalla et al., 2016). Because EEEV nsP2 contains a "KGG" sequence in place of the canonical PGG domain, it is presumed that this sequence is not responsible for cellular translational shutoff as the mutation for VEEV nsP2 is similar to the inherent sequence of EEEV (Bhalla et al., 2016). Whereas, mutations in the PGG domain for SINV and CHIKV result in abrogation of transcription and translation shutoff by the viruses (Burke et al., 2009; Frolov et al., 2012). Additionally, data demonstrated VEEV nsP2 induced shutoff of host translation provides a condition suitable for viral propagation through the reduction of interferon-stimulated gene expression. Rendering interferon stimulation inadequate to control VEEV replication attests to its resistance phenotype and increased antiviral activity in comparison to other alphaviruses (Bhalla et al., 2016). The differences in host cell utilization for antiviral subversion validates the understanding that although alphaviruses are structured and classified similarly, there are strong differences between not only OW and NW but within these groupings that may provide challenges for developing broad-spectrum therapeutics.

Host-based therapeutics for inhibiting host interactions with alphaviral nsP2 are considerably lacking. This is in large part due to the instability of nsP2 as a protease and studying overexpression constructs *in vitro* has proven challenging (Atasheva et al., 2007). Research groups have previously been able to study nsP2 through replicon-encoding plasmids for the purposes of investigating transcriptional inhibition as a whole in host cells (Garmashova et al., 2007). For this review, we were unable to verify and/or identify literature in which host-based targeting was achieved for disruption of nsP2 activity upon alphaviral infection. However, we were able to identify direct inhibitors of viral nsP2 and will instead discuss these antivirals (Table 2).

Molecular Libraries Small Molecule Repository (MLSMR) screening and subsequent medicinal chemistry optimization led to the advancement of compound ML-336 that proved to be effective for control of VEEV infection for three VEEV strains, TC-83, TrD, and V3526. With the use of ML-336 viral titers were reduced by at least 7 $\log_{10}$ PFU/mL using low micromolar amounts of the compound. The pharmacokinetic profile and its moderate blood brain barrier permeability shows promise for *in vivo* studies. Preliminary data through *in silico* modeling suggests ML-336 targets the viral nsP2 protein but further mechanism of action studies are needed for this validation (Chung et al., 2010). Another quinazolinone compound, CID15997213, displayed antiviral effects for VEEV and WEEV but had little to no effect on EEEV. This inhibitor targets the N-terminal domain of the alphaviral nsP2 protein which was revealed through mutational analyses and serial passaging of VEEV TC-83 while increasing the inhibitor concentration (Chung et al., 2014). Reverse genetic screening indicated Y102 and D116 as key target residues that may attest to the difference in susceptibility of the drugs action among alphaviruses since OW alphaviruses have K102 present in their nsP2 sequence (Chung et al., 2014). Viral inhibition was successful up to 2 h post infection (hpi) and addition of the drug at 4 hpi allowed 90% of viral production suggesting the mechanism of action for CID15997213 is during minus strand synthesis (Chung et al., 2014). *In vitro* testing showed CID15997213 having acceptable BBB crossing potential and thus was tested in mice. *In vivo* studies demonstrated no toxicity and complete efficacy upon VEEV TC-83 infection (Schroeder et al., 2014). Overall, both inhibitors display promising data, but mutational and genetic fitness studies over time are needed for determining development of viral resistance.

The host interactome for nsP2 of NW alphaviruses has not been well defined in part due to the inherently complex properties of nsP2 rendering investigation of host interactions more challenging in overexpression constructs due to induction of host translational shutoff

Table 3
Known interactions with OW alphavirus nsP2.

General Function	Host Protein	Virus	Reference
Protein Degradation	UBQLN4 – Ubiquilin 4	CHIKV	(Bourai et al., 2012; Burnham et al., 2007)
	WWP1 – WW Domain containing E3 ubiquitin protein ligase 1		Bourai et al. (2012)
	RCHY1 – Ring finger and CHY zinc finger domain containing 1		
Apoptosis	PDK2 – Pyruvate dehydrogenase kinase 2	CHIKV	Bourai et al. (2012)
RNA Binding	HNRNPK – Heterogeneous nuclear ribonucleoprotein K	CHIKV, SINV	Bourai et al. (2012)
	HNRNPU – Heterogeneous nuclear ribonucleoprotein U	SINV	Atasheva et al. (2007)
	YBX1 – Y-Box binding protein 1		
	HNRNPC – Heterogeneous nuclear ribonucleoproteins C1/C2		
	RBM12B – RNA binding motif protein 12B	CHIKV	Bourai et al. (2012)
Cytoskeletal Organization	VIM – Vimentin	CHIKV, SINV	(Atasheva et al., 2007; Bourai et al., 2012)
	ACTB – Actin beta	SINV	Atasheva et al. (2007)
	KLC4 – Kinesin light chain 4	CHIKV	Bourai et al. (2012)
	GFAP – Glial fibrillary acidic protein/Intermediate filament protein		
RNA Processing	PABPC1 – Poly(A) binding protein cytoplasmic 1	SINV	Atasheva et al. (2007)
	HNRNPA3 – Heterogeneous nuclear ribonucleoprotein A3		
Protein Folding	HSPA8 – Heat shock protein family A member 8	SINV	Atasheva et al. (2007)
RNA Helicase	DDX5 – DEAD-box Helicase 5	SINV	Atasheva et al. (2007)
Stress Granule & Viral Replication Complex Formation	G3BP1 – Ras GTPase-activating protein-binding protein 1	SINV	Atasheva et al. (2007)
	G3BP2 – Ras GTPase-activating protein-binding protein 2		
Protein Synthesis	EEF1A1 – Eukaryotic translation elongation factor 1 alpha 1	SINV	Atasheva et al. (2007)
	RPL4 – Ribosomal protein L4		
RNA Transport	TPR – Translocated promoter region	CHIKV	Bourai et al. (2012)
Transcription Activation	ASCC2 – Activating signal cointegrator 1 complex subunit 2	CHIKV	Bourai et al. (2012)
	EWSR1 – EWS RNA binding protein 1		
Cytoskeletal Signaling	CEP55 – Centrosomal protein 55	CHIKV	Bourai et al. (2012)
Miscellaneous	MRFAP1L1 – Morf4 Family associated protein 1 like 1	CHIKV	Bourai et al. (2012)
	TTC7B – Tetratricopeptide repeat domain 7B		
Zinc Finger Motif	TRIM27 – Tripartite motif containing 27	CHIKV	Bourai et al. (2012)
	IKZF1 – IKAROS family zinc finger 1		
	ZBTB43 – Zinc finger and BTB domain containing 43		
Autophagy	CALCOCO2 – Calcium binding and coiled-coil domain 2	CHIKV	Bourai et al. (2012)

(Bhalla et al., 2016). However, studies of OW alphavirus nsP2 interactions by yeast two-hybrid screens (Bourai et al., 2012), comparative 2 dimensional electrophoresis (Burnham et al., 2007), and a transposon based approach (Atasheva et al., 2007) have been successful and are summarized in Table 3. The table highlights intriguing interactions revealed from nsP2 investigation for OW alphaviruses and these may be of relevance in the context of NW alphaviruses as well.

Although the mechanism of action and functionality for nsP2 has been established, the interactome for NW alphaviruses is deficient. Interactors for OW alphaviruses have been elucidated to some extent but in the context of therapeutic intervention, host-based strategies are lacking. Instead, antivirals targeting alphaviral nsP2 directly (Table 2) have historically been the primary focus for viral control. Viral targeting inhibition can become problematic if mutational resistance is achieved over time. This issue and the deficiency in host-based targeting further emphasizes the need for developing a full proteomic picture to better understand host processes utilized for successful viral replication.

4. nsP3

Unlike the other non-structural proteins, the exact functionality and mechanism of nsP3's contribution to viral RNA replication has yet to be elucidated but it is widely accepted that this viral protein drives the assembly of viral replication complexes (vRCs) within host cells. It is known that it is present in the RNA replicase complex and is critical for negative sense and subgenomic RNA synthesis but it's precise mechanism of action is currently unknown (Hahn et al., 1989; Lastarza et al., 1994a, 1994b; Wang et al., 1994). Mutations in the nsP3 coding region have demonstrated unfavorable effects on viral replication confirming its role in RNA synthesis (De et al., 2003).

Three domains have been characterized for nsP3—the N-terminal macrodomain, the alphavirus unique domain (AUD), and the hypervariable (HVD) C-terminal domain. The alphavirus macrodomain is considered conserved among OW and NW viruses where it displays characteristics comparable to human homologs of Adenosine diphosphate (ADP)-ribose 1′-phosphate phosphatase. Additionally, an aspartic acid site in the active pocket of this domain was found to be the binding site for nucleic acids, specifically adenines (Foy et al., 2013; Malet et al., 2009; Rungrotmongkol et al., 2010). The AUD domain contains a zinc coordination site from four cysteine residues where amino acid site mutations rendered each cysteine residue to be critical for early RNA replication (Shin et al., 2012). Regardless, the exact implication of this domain for successful viral propagation has yet to be clarified. The C-terminal portion of nsP3 contains the HVD and has historically been the most confounding portion of the protein. Large sequence variability between alphaviruses has rendered this region significant for interacting host proteins each alphaviral nsP3 utilizes. In the context of SFV, the HVD was found to be phosphorylated through mass spectrometric means. Alanine mutations made at threonine 344 and 345 to reduce phosphorylation levels inhibited early levels of viral RNA synthesis and resulted in low pathogenicity (Vihinen et al., 2001). Additionally, mutation of SINV nsP3 showed that a decrease in phosphorylation of the HVD resulted in a reduction of minus strand RNA synthesis in chicken embryo fibroblasts (LaStarza et al., 1994a, b). Conversely, phosphorylation of the HVD in VEEV was found to be dispensable for replication within vertebrate host cells but indispensable within mosquito cells. The HVD is also not required for successful viral replication in BHK-21 cells but a repeat sequence in the carboxy-terminal region of the HVD is indispensable for viral replication in cell lines apart from BHK-21 cells (Foy et al., 2013). The carboxy terminal peptide presence in the HVD is critical for VEEV but is lacking in other NW alphaviruses. This peptide drives the formation of nsP3-containing protein complexes and highlights the uniqueness of viral-

host interactions for each alphavirus. The HVD of OW alphaviruses were shown to rely on Ras-GTPase activating protein SH3 domain binding protein (G3BP) family members for formation of vRCs and NW alphavirus, VEEV, was demonstrated to utilize the fragile X related (FXR) family members (Kim et al., 2016) through means of Flag-green fluorescent protein (GFP) tagged chimeric replicons. A separate group also demonstrated FXR family host interaction using hemagglutinin (HA)-tagged nsP3 of the TC-83 VEEV strain through immunoprecipitation pull downs followed by mass spectrometric means (Amaya et al., 2014). Therefore, two independently and vastly differing experimental means confirm this host interaction. On the other hand, EEEV, another representative NW alphavirus, has acquired redundancy and was shown to be able to rely on interactions with FXR or G3BP family members (Frolov et al., 2017). Fluorescent tagged chimeric viruses expressing EEEV replication machinery were constructed and challenged in G3BP and FXR knockout cell lines independently (Frolov et al., 2017). Domains in the HVD of EEEV nsP3 found to interact with each of these families were mutated and challenged in the knockout cell lines (Frolov et al., 2017). When interaction of both FXR and G3BP family members is abrogated, replication of the chimeric virus is drastically reduced but when the virus is able to utilize FXR or G3BP proteins, viral replication is only moderately compromised (Frolov et al., 2017). The aggressive phenotype observed for EEEV could be explained by its ability to use the FXR and G3BP families in an either/or manner highlighting its ability to independently interact with these host factors. Because its utilization of the host falls somewhere between OW and NW alphaviruses for replication, the HVD of EEEV can be considered evolutionarily advantaged and emphasizes the diversity of host factors an alphavirus may exploit. These differences observed between alphaviruses further highlight the complexity of determining viral-host interactions for a group of related viruses.

Efforts in defining the alphaviral nsP3 host protein interactome have been successful and reveal relevant pathways including cytoskeletal reorganization, vesicle trafficking, stress granule and viral replication formation, RNA binding, and protein synthesis (Table 4). One particular pathway that is influenced after VEEV infection is the Nuclear Factor Kappa Beta (NF-κB) pathway and transcription factor p65, a subunit of the NF-κB protein complex known to regulate the expression of many genes upon cellular stress (Le Negrate, 2012; Perkins, 2007). Various viruses have proven to stimulate this signaling cascade for its own benefit in terms of pathogenesis and life cycle propagation (Gamble et al., 2012; Israel, 2010; Le Negrate, 2012; Liu et al., 2012). It was determined that VEEV infection also activates the NF-κB pathway as indicated by macromolecular reorganization of IκB kinase (IKKβ), phosphorylation of the Iκβα complex and p65 as well as nuclear localization of p65. Because of this utilization, it was speculated that upon viral infection, kinase complexes may alter their substrate specificities so as to exhibit their phosphorylation functionality preferentially on viral target proteins to enhance infection (Amaya et al., 2014). Treatment with IKKβ inhibitors, specifically BAY-11-7082, whether pre- or post-infection significantly reduced viral titers in the context of glial and neuronal cell lines in both the attenuated and virulent strains of VEEV. This inhibitor was also shown to reduce viral titers when model cell lines were challenged with WEEV and EEEV thus regarding this host-based inhibitor as a model for broad-spectrum applicability. BAY-11-7082 was also able to reduce viral titers by 1 log10 PFU/mL *in vivo* when mice were challenged with the attenuated VEEV TC-83 strain (Amaya et al., 2014). Furthermore, both VEEV and WEEV nsP3 were shown to interact with IKKβ. Confocal imaging additionally validated co-localization of this interaction in the context of both WEEV and VEEV. However, upon treatment with BAY-11-7082, co-localization with VEEV nsP3 was disrupted indicating that phosphorylation of IKKβ is crucial for this interaction between virus and host to occur and that alphaviral nsP3 proteins may need phosphorylation by the IKKβ enzyme for successful propagation (Amaya et al., 2014).

Another interesting interaction that has been defined is the interaction between VEEV nsP3 and DEAD-box RNA helicases, specifically DDX1 and DDX3. DDX1 and DDX3 were shown to have significant regulatory roles involving nsP3 in the context of infection for both the attenuated VEEV TC-83 strain and the fully virulent VEEV TrD (Amaya et al., 2016). These helicases are multifunctional for RNA processing employing aid for transcription, splicing, transport, translation initiation, decay and investigation with HIV and IAV demonstrates that these helicases are utilized during the lifecycle of RNA viruses (Edgcomb et al., 2012; Fang et al., 2004; Fuller-Pace, 2013; Xu et al., 2010). The extent to which these DDX helicases are relied upon during infection was explored in the context of VEEV infection despite its own nsP2 protein exhibiting helicase activity. Time dependent analyses determined through small interfering RNA (siRNA) knockdown of DDX1 and/or DDX3 resulted in diminished viral multiplication for both the TC-83 and TrD strains with the virulent TrD strain depending less on DDX3 than that of the attenuated TC-83 strain (Amaya et al., 2016). It is thought that the viral nsP3 protein co-opts DDX1/DDX3 early during infection to assist in the unwinding of incoming genomic RNA for proper initiation of translation and/or replication (Amaya et al., 2016). It is known that during cell stress upon viral infection, preinitiation complexes assemble into stress granules made of eukaryotic initiation factors (eIF's), poly(A)-binding protein (PABP) and more for viral propagation (Lloyd, 2012; Scholte et al., 2015). Immunoprecipitation of an HA tagged nsP3 expression construct in glioblastoma cell lines showed viral:host interactions with eIF4A, eIF4G, PABP and DDX3. Because of this, VEEV infection is thought to hijack pre-existing translational machinery for its own proliferation (Amaya et al., 2016). Small molecule RK-33, an inhibitor of DDX3 exerts its mechanism of action by halting the unwinding function of the helicase through binding in the ATP-binding cleft (Bol et al., 2015). Use of RK-33 demonstrated a reduced interaction of nsP3 with the host translational machinery attesting to the significance of DDX3 in facilitating the viral protein:host translational protein(s) interaction (Amaya et al., 2016). Because DDX3 is capable of nucleating the formation of stress granules, the addition of RK-33 further establishes the notion that these helicases are in fact critical for viral dependence on the host translational machinery. The small, but validated, interactome of vnsP3:DDX3:eIF4A:eIF4G:PABP offers more possibilities for the use of therapeutic intervention targeting the host translational system.

It can be confidently stated that a considerable amount of host protein interactors have been identified for NW alphaviral nsP3. However, the lack of host-based therapeutic implementation highlights not only the need, but the opportunity for furthering antivirals in this manner. Additionally, the actual defined functionality of viral nsP3 has yet to be determined. Understanding the mechanism of this viral protein may shed more light on how it utilizes its host for successful replication so that researchers are able to pinpoint categories of machinery as potential therapeutic targets.

5. nsP4

nsP4 is the RNA dependent RNA polymerase (RdRp) responsible for viral genome replication and RNA transcription. It is a 67 kDa protein consisting of 606 amino acids. VEEV nSP4 expression is regulated by an opal (UGA) stop codon that also exists in several other alphaviruses. The non-structural polyprotein is translated as normal but when a read-through of the opal codon occurs, P1234 is produced and nsP4 is quickly cleaved by nsP2 resulting in P123' and nsP4 (Firth et al., 2011; Strauss et al., 1983). The existence of nsP4 during infection is brief as degradation is regulated through the N-end rule pathway, however it is stable once sequestered into replication compartments. Mutagenesis of the N-terminal residue of nsP4 causes less degradation of the protein (de Groot et al., 1991).

CHIKV nsP4 has been shown to form an interaction with heat shock protein 90 (HSP90) during infection (Table 5). Disruption of HSP90 with either siRNA or the small molecule inhibitor geldanamycin causes

Table 4
Known host protein interactions and inhibitors for NW alphavirus nsP3.

General Function	Host Protein	Inhibitor	EC_{50}	Cell Type	*In vivo* Model	Virus	Reference
Transcription Regulation from Cellular Stress	IKKβ	BAY-11-7082	ND[a]	U-87MG, AP7	C3H/HeN Mice	VEEV, EEEV, WEEV	Amaya et al. (2014)
RNA Helicase	DDX3	RK-33	ND	U-87MG	ND	VEEV, EEEV, WEEV	Amaya et al. (2016)
Cytoskeletal Reorganization	CAPZA1	n/a	n/a[b]	n/a	n/a	VEEV, EEEV	(Frolov et al., 2017; Kim et al., 2016)
	CAPZA2	n/a	n/a	n/a	n/a	VEEV, EEEV	(Frolov et al., 2017; Kim et al., 2016)
	CAPZB	n/a	n/a	n/a	n/a	VEEV, EEEV	(Frolov et al., 2017; Kim et al., 2016)
	CD2AP	n/a	n/a	n/a	n/a	VEEV, EEEV	(Frolov et al., 2017; Kim et al., 2016)
	MAP1B	n/a	n/a	n/a	n/a	VEEV, EEEV	Amaya et al. (2016)
	PLEC	n/a	n/a	n/a	n/a	VEEV	Amaya et al. (2016)
	SH3KBP1/CIN85	n/a	n/a	n/a	n/a	VEEV, EEEV	Frolov et al. (2017)
Cytoskeletal Reorganization & Vesicle Trafficking	SNX33	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
	SNX9	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
Vesicle Trafficking	CLTC	n/a	n/a	n/a	n/a	EEEV	(Amaya et al., 2016; Fang et al., 2004; Frolov et al., 2017)
RNA Binding	FMR1	n/a	n/a	n/a	n/a	VEEV, EEEV	(Amaya et al., 2016; Fang et al., 2004; Frolov et al., 2017)
	FXR1	n/a	n/a	n/a	n/a	VEEV, EEEV	(Amaya et al., 2016; Fang et al., 2004; Frolov et al., 2017)
	FXR2	n/a	n/a	n/a	n/a	VEEV, EEEV	(Amaya et al., 2016; Fang et al., 2004; Frolov et al., 2017)
	HNRNPA1	n/a	n/a	n/a	n/a	VEEV	Frolov et al. (2017)
	IGF2BP1	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
	IGF2BP2	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
	IGF2BP3	n/a	n/a	n/a	n/a	VEEV, EEEV	Frolov et al. (2017)
	YBX1	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
Stress Granule & Viral Replication Complex	G3BP1	n/a	n/a	n/a	n/a	EEEV	Frolov et al. (2017)
	G3BP2	n/a	n/a	n/a	n/a	EEEV	(Amaya et al., 2016; Frolov et al., 2017)
Protein Synthesis	RPL10A	n/a	n/a	n/a	n/a	VEEV	Amaya et al. (2016)
	RPL6	n/a	n/a	n/a	n/a	VEEV	Amaya et al. (2016)
	RPLP0	n/a	n/a	n/a	n/a	VEEV	Amaya et al. (2016)
	RPS8	n/a	n/a	n/a	n/a	VEEV	Frolov et al. (2017)
Mitochondrial Solute Carriers & Phosphatases	SLC25A3	n/a	n/a	n/a	n/a	VEEV	Frolov et al. (2017)
	SLC25A5	n/a	n/a	n/a	n/a	VEEV	Frolov et al. (2017)
	PGAM5	n/a	n/a	n/a	n/a	VEEV, EEEV	Frolov et al. (2017)
Protein Folding	HSPA1B	n/a	n/a	n/a	n/a	VEEV	Frolov et al. (2017)
Miscellaneous	S100A4	n/a	n/a	n/a	n/a	VEEV, EEEV	Frolov et al. (2017)

[a] ND = not determined.
[b] n/a = Not applicable.

a significant reduction in viral titers. Furthermore treatment of CHIKV infected mice with HSP90 targeting drugs, SNX-2112 or HS-10, reduced the severity of infection and inflammation (Rathore et al., 2014). CHIKV nsP4 has also been implicated in suppressing the phosphorylation of eIF2α by activating the unfolded protein response (UPR) pathway (Rathore et al., 2013).

NW alphavirus nsP4 interactions with host factors have not been extensively studied, however, there have been a few promising antiviral treatments developed that target nsP4 (Table 2). The nucleoside analogue β-D-N^4-hydroxycytidine (NHC) has been identified as a potent inhibitor of VEEV. NHC induces mutations in viral RNA and affects the infectivity of the virus and the release of functional virions. Addition of NHC at early times post infection showed the most robust decrease in viral titer and viral RNA. Furthermore, the earlier NHC is added to infected cells, the fewer infectious viral particles are released. Resistance to NHC develops rather inefficiently, however, when resistant mutants do develop, they have the most mutations in nsP4 and those mutations are amino acids involved in coordination of NTP phosphates (Urakova et al., 2017). These data can be used to exploit VEEV for potential attenuation sites or drugs targeting nsP4.

nsP4 has also been implicated as a possible target for the antiviral, favipiravir. Similar to NHC, favipiravir is a nucleobase mimetic and has been shown to inhibit IAV, IAB, and influenza C virus (IAC, *Orthomyxoviridae*) (Furuta et al., 2002; Kiso et al., 2010), as well as yellow fever virus (YFV, *Flaviviridae*) (Julander et al., 2009a), West Nile virus (WNV, *Flaviviridae*) (Furuta et al., 2009), and norovirus (NOV, *Caliciviridae*) (Rocha-Pereira et al., 2012). Mutations in nsP4, specifically K291R in CHIKV nsP4 confers resistance to favipiravir (Delang et al., 2014). Favipiravir is also effective at treating WEEV in infected mice. Mice treated with 400 mg/kg/day favipiravir and infected with WEEV had a significant improvement in mean day to death and survival vs. mice treated with saline. However, there wasn't a significant decrease in viral titer in brain tissue. Several mice that showed signs of brain disease did recover from the disease suggesting that favipiravir can improve the chances of recovering (Julander et al., 2009b). nsP4 is a promising target for both viral and host based therapeutics.

Table 5
Known host protein interactions and inhibitors for nsP4.

General Function	Host Protein	Inhibitor	Virus	EC50	Cell Type	*In vivo* Model	Reference
Protein Chaperone	HSP-90	Geldanamycin	CHIKV	ND[a]	HEK293T	Sv/129 Mice	Rathore et al. (2014)
Protein Translation	eIF2α	n/a	CHIKV	n/a[b]	HEK293T	n/a	Rathore et al. (2013)

[a] ND = not determined.
[b] N/A = Not applicable.

Additional studies should be performed to further elucidate the effect of targeting nsP4 during infection.

6. Capsid

The capsid protein is divided into two separate domains—N-terminal and C-terminal. The C-terminal is highly conserved amongst all alphaviral capsid proteins however the N-terminal domain is extremely variable. Capsid has several functions during the lifecycle of the virus. Capsid's C-terminal domain has protease activity which is responsible for cleaving capsid from the translating polypeptide. This frees capsid from the polypeptide and exposes an ER localization sequence on the remaining polypeptide so that the glycoproteins can be further processed in the ER. The N-terminal domain is highly positively charged and is responsible for binding viral RNA during assembly. NW capsid proteins also play an important role in cytopathogenicity. Early studies have shown that VEEV and EEEV capsid is responsible for host transcriptional shutoff leading to cytopathic effects. Further studies showed that host transcriptional shutoff in VEEV infected cells is due to the existence of a nuclear localization signal (NLS) and a nuclear export sequence (NES) [reviewed in (Lundberg et al., 2017)].

VEEV capsid's role in host transcriptional shutoff is an important mechanism for evasion of the innate immune response (Garmashova et al., 2007). The ability to shutdown transcription is largely due to capsid's ability to inhibit nucleocytoplasmic trafficking (Atasheva et al., 2010a). The NES in the N-terminal domain of capsid interacts with Chromosomal Maintenance 1 (CRM1), also known as exportin 1, in the absence of RanGTP (Atasheva et al., 2010b). Interestingly, the presence of an NLS in capsid has been shown to allow capsid's association with host proteins importin α (Impα) and importin β (Impβ), which facilitate entry into the nucleus. Inhibition of this mechanism is an attractive target for potential therapeutics. Inhibitors of nuclear import such as mifepristone and ivermectin prevent VEEV capsid's import into the nucleus and decrease VEEV replication (Table 6) (Lundberg et al., 2013). Treatment with nuclear import inhibitors also reduce VEEV capsid induced cytopathic effects. Additional studies were performed to identify more selective inhibitors of VEEV capsid through blocking its ability to bind to Impα/β1. A high-throughput screening approach using AlphaScreen technology identified G281-1564 as an inhibitor of VEEV capsid binding to Impα/β1 (Thomas et al., 2018). G281-1564 inhibited VEEV replication at low μM concentrations, while showing minimal toxicity. An *in silico* structure-based-drug-design approach identified compound 1111684 as an additional inhibitor of capsid:Impα/β1 binding (Shechter et al., 2017). Compound 1111684 reduced VEEV replication at μM concentrations, concomitant with reduced capsid nuclear accumulation in infected cells. Blocking VEEV capsid nuclear export is also an effective means of blocking viral replication as inhibition of CRM1 via either the classical CRM1 inhibitor Leptomycin B or selective inhibitors of nuclear import (SINE) significantly decrease VEEV replication by trapping capsid in the nucleus (Lundberg et al., 2013, 2016). Inhibition of CRM1 mediated nuclear export suppressed EEEV, WEEV, CHIKV, but not SINV replication (Lundberg et al., 2016). Nuclear trafficking has also been targeted for potential vaccine strategies. Mutation of VEEV capsid in the NLS region in context of the TC-83 strain, has shown the ability to decrease cytopathic effects (Atasheva et al., 2010c). The mutant virus (VEEV_Cm) infects cells and allows the induction of the innate immune response. Further, VEEV_Cm is attenuated in mice and protects mice after challenge with fully virulent VEEV (Atasheva et al., 2015).

EEEV and WEEV capsid proteins have a conserved NLS and a NES in their N-terminal (Atasheva et al., 2010a); however neither have been shown to impair nucleocytoplasmic trafficking. EEEV capsid blocks interferon production, suppresses host mRNA accumulation and induces phosphorylation of eIF2α (Aguilar et al., 2007). The N-terminal of EEEV capsid is responsible for preventing RNA polymerase II dependent transcription and deletion of amino acids 55–75 results in viral

Table 6
Known host protein interactions and inhibitors for capsid.

General Function	Host Protein	Inhibitor	Virus	EC_{50}	Cell Type	*In Vivo* Model	Reference
Nuclear Import	Importin α/β	Mifepristone, Ivermectin, G281-1564, 1111684	VEEV	10.8 μM (G281-1564), 9.9 μM (1111684)	Vero, MEF	ND[a]	(Atasheva et al., 2010b; Lundberg et al., 2013; Shechter et al., 2017; Thomas et al., 2018)
Nuclear Export	CRM1	Leptomycin B, SINE compounds	VEEV, EEEV, WEEV, CHIKV	0.093–0.622 μM (SINE)	Vero, MEF	ND	(Atasheva et al., 2010b; Lundberg et al., 2013, 2016)
Phosphatases	PP1α	1E7-03	VEEV, EEEV, WEEV, CHIKV, SINV	0.6 μM	Vero	ND	Carey et al. (2018)
Kinase, Signal Transduction	PKCδ	Rottlerin	VEEV, EEEV, SINV, CHIKV	0.45 μM	Vero	ND	Unpublished
Ubiquitin proteasome system	undetermined	Bortezomib	VEEV, EEEV, and WEEV	ND	U87MG, Vero	ND	Amaya et al. (2015)

[a] ND = not determined.

Table 7
Known host protein interactions and inhibitors for E2.

General Function	Host Protein	Inhibitor	Virus Studied	EC_{50}	Cell Type	*In vivo* model	Reference
Viral Entry	DC-SIGN	n/a[b]	SINV	n/a	n/a	n/a	Klimstra et al. (2003)
	L-SIGN	n/a	SINV	n/a	n/a	n/a	Klimstra et al. (2003)
	Heparin sulfate	n/a	SINV	n/a	n/a	n/a	(Bernard et al., 2000; Klimstra et al., 1998)
G Protein Signaling	Rac1	EHT1864	VEEV	ND[a]	n/a	ND	Radoshitzky et al. (2016)
	Rac1	NSC23766	VEEV	ND	n/a	n/a	Radoshitzky et al. (2016)
Actin Organization	Arp3	CK548	VEEV	ND	n/a	n/a	Radoshitzky et al. (2016)
	Arp3	CK869	VEEV	ND	n/a	n/a	Radoshitzky et al. (2016)
Cytoskeleton	Actin	Cytochalasin D	VEEV	ND	n/a	n/a	Radoshitzky et al. (2016)
	Actin	Latrunculin A	VEEV	ND	n/a	n/a	Radoshitzky et al. (2016)
Endocytosis	Eps15	n/a	VEEV	n/a	n/a	n/a	Kolokoltsov et al. (2006)
	Rab5	n/a	VEEV, SFV	n/a	n/a	n/a	Kolokoltsov et al. (2006)
	Rab7	n/a	VEEV	ND	n/a	ND	Surviladze et al. (2010)
	Rab7	n/a	VEEV	ND	n/a	ND	Agola et al. (2012)

[a] ND = not determined.
[b] N/A = Not applicable.

attenuation as shown by lack of disease in inoculated mice (Aguilar et al., 2007, 2008). WEEV capsid also blocks antiviral signaling induced by poly I:C, but not interferon β, through inhibition of pattern recognition receptors downstream of interferon regulatory factor 3 (IFF-3) (Peltier et al., 2013). This activity of WEEV capsid is independent of global inhibition of host translation or transcriptional. Thus while the NW alphavirus capsid proteins block antiviral signaling, the mechanism by which this is accomplished is likely different amongst these viruses.

VEEV capsid has been shown to be phosphorylated and ubiquitinated and modulation of these post-translational modification alter viral replication (Amaya et al., 2015; Carey et al., 2018). Treatment of VEEV infected cells with the proteasome inhibitor, Bortezomib, suppresses VEEV replication. Further, VEEV capsid was found to be K48 ubiquitinated both in cells and within the virion. As K48 ubiquitin linkages are associated with proteasomal degradation, a model was proposed in which ubiquitination and subsequent proteasomal degradation of VEEV capsid is needed to enable viral uncoating (Amaya et al., 2015). VEEV capsid is phosphorylated on Thr 93, Thr 108, Thr 127, and Ser 124 (Carey et al., 2018). The host protein phosphatase, Protein Phosphatase 1α (PP1α), interacts with VEEV capsid and regulates phosphorylation. Infected cells treated with a PP1α small molecule inhibitor, 1E7-03, show an increased level of phosphorylation on capsid than cells treated with a solvent control. Further, inhibition with the PP1α inhibitor caused a decrease in viral titer and ablated the interaction between capsid and PP1α. siRNA knockdown of PP1α also caused a decrease in viral titer. Finally, in an effort to elucidate the importance of phosphorylation on capsid, it was found that less viral RNA is bound to capsid during infection when PP1α is inhibited (Carey et al., 2018). Furthermore, unpublished data from our lab suggests that the kinase responsible for phosphorylating capsid is Protein Kinase C type delta (PKCδ). PKCδ siRNA or treatment with the PKCδ inhibitor, Rottlerin, causes a significant decrease in VEEV titers (unpublished data). While kinase, phosphatase and proteasome inhibitors also reduce EEEV and WEEV replication (Amaya et al., 2015; Carey et al., 2018), the phosphorylation and ubiquitination status of EEEV and WEEV capsid proteins has not been assessed.

7. E2

The envelope glycoproteins E1 and E2 are processed in the ER after capsid autocleavage allows for recognition of the ER localization sequence in E3. E2 is translated as a polyprotein called pE2 including both E3 and E2. Processing pE2 is a crucial step during infection as uncleaved pE2 incorporation has been shown to effect viral entry (Carleton et al., 1997; Ryman et al., 2004). E1 and E2 interactions are extremely important for viral budding and cell entry. The proteins exist as heterodimers in the lipid envelope (Mukhopadhyay et al., 2006; Navaratnarajah and Kuhn, 2007; Sjoberg and Garoff, 2003). E2 is glycosylated and is responsible for receptor mediated endocytosis. Entry into the cell is mediated by the interaction of the spike E2 component with receptors on the surface of the cell. Experiments with SINV E2 suggest that the cell receptors utilized are Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Klimstra et al., 2003), liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin (L-SIGN) (Klimstra et al., 2003) and Heparin sulfate (Bernard et al., 2000; Klimstra et al., 1998). E2 isn't just involved in cell attachment; it is also necessary for viral budding. Assembled nucleocapsids migrate to the cell surface where they interact with the C-terminal cytoplasmic domain of E2. The interaction alone provides enough energy for the virus to bud from the cell (Strauss and Strauss, 1994). Host proteins are generally not found in viral particles suggesting that budding may take place at dedicated sites on the cell surface (Brown et al., 2018). E2 mutations have also been implicated in epizootic virulence determinants as well as neurotropism of the disease (Greene et al., 2005; Trabalza et al., 2013).

Numerous host components have been implicated to be in association with E2 during viral infection (Table 7). Recently, an actin-remodeling pathway has been identified as an essential component for E2 trafficking to the plasma membrane. A high-content imaging based siRNA screen showed that E2 associates with actin during infection and co-localizes with Ras-related C3 botulinum toxin substrate 1 (Rac1)-Phosphatidylinositol-4-phosphate 5-kinase type-1 (PIP5K1-α). PIP5K1-α activates the RhoGTPase Rac1 and induces stress fiber formation leading to increased myosin binding and the bundling of actin filaments (Martin, 1998). Chemical inhibition of Rac1 (EHT1864 and NSC23766) and actin related protein 3 (Arp3) (CK548 and CK869) disrupted E2 trafficking from the Golgi to the cell surface and decrease viral infection. Furthermore, siRNA knockdown of PIP5K1-α, Rac1, and Arp3 caused a decrease in viral infection. Finally, inhibition of actin polymerization with Cytochalasin D or Latrunculin A also caused a decrease in viral titer (Radoshitzky et al., 2016).

E2 is heavily involved in viral endocytosis as well as budding (Mukhopadhyay et al., 2006; Navaratnarajah and Kuhn, 2007; Sjoberg and Garoff, 2003). Double knockout mutants of endocytosis proteins, Epidermal growth factor receptor substrate 15 (Eps15), Rab5, and Rab7, were able to resist endocytosis of VEEV virus like particles (Kolokoltsov et al., 2006). Small molecule inhibitors against Rab7 such as ML141 or CID1067700 (Agola et al., 2012; Surviladze et al., 2010) should be tested to determine their efficacy against VEEV. No inhibitors targeting Rab5 or Eps15 could be found during a literature search, however, Rab5 is a GTPase and could be affected by other GTPase inhibitors. Induction of the host protein tetherin/BST-2 has been shown to inhibit CHIKV budding by trapping viral particles at the host cell plasma membrane. BST-2 is induced by interferon expression however

further work is needed to determine if increased expression can cause a decrease in viral titer amongst other alphaviruses (Jones et al., 2013). E2 has also been shown to be palmitoylated (Schmidt et al., 1979) however no studies could be found to elucidate the mechanism for palmitoylation. Further studies should be done to determine the importance of this post-translational modification.

8. 6K/TF

The 6 kDa (6K protein) is a structural protein derived from the alphavirus subgenomic promoter. However, under frameshifting conditions, transframe protein (TF) can be derived from alphavirus 6K gene (Kendra et al., 2017; Ramsey and Mukhopadhyay, 2017). This frameshifting is a result of a heptanucleotide slippery sequence in the 6K region of the viral RNA and the presence of an RNA structural element called a pseudoknot. These two features cause the ribosome to stall and result in a −1 programmed ribosomal frameshift (−1 PRF) resulting in the production of the TF protein (Kendra et al., 2017). A 6K-like protein was first identified in early studies with SINV in which a 4.2 kD protein was identified to be produced during infection but not present in mature virion (Welch and Sefton, 1979). Similarly, a 6 kD protein was identified to be derived from SFV infection under subgenomic promoter and is not present in the mature virion (Welch and Sefton, 1980). Both the 4.2K SINV and 6K SFV proteins are not glycosylated (Ramsey and Mukhopadhyay, 2017; Welch and Sefton, 1979, 1980). Following identification of the presence of 6K-like proteins, sequencing approaches of the N-terminal regions of both SFV and SINV indicated conservation between the two viral N-termini (Ramsey and Mukhopadhyay, 2017; Welch et al., 1981). Additionally, it was determined that the 4.2K and 6K proteins fall in the alphavirus genome after E2 and prior to E1 glycoproteins (Ramsey and Mukhopadhyay, 2017; Welch et al., 1981).

Mutation of TF or ablation of TF protein expression has led to the characterization of the TF protein as an alphavirus virulence factor. Antiviral approaches targeting of 6K or TF activities of alphaviruses has consisted of viral attenuation strategies targeting the −1 PRF activity of alphaviruses such as VEEV and SINV (Kendra et al., 2017; Snyder et al., 2013). Through generation of silent protein coding mutants that attenuate VEEV -1 PRF activity, reduction in viral replication *in vitro* was observed. Interestingly, during aerosol exposure experiments, exposure of mice to a VEEV -1 PRF mutant virus resulted in reduced clinical signs and increased survival (Kendra et al., 2017).

6K is a transmembrane protein that contains two transmembrane helices and a cytoplasmic loop and resides within the ER membrane. It also contains a translocation signal for the E1 glycoprotein (Ramsey and Mukhopadhyay, 2017). In addition to transmembrane and signal sequence roles, the 6K protein acts as a viroporin, or ion channel protein. In overexpression studies in bacteria, overexpression of 6K or TF proteins of SINV resulted in bacterial cell lysis (Sanz and Carrasco, 2001). It is suggested that the role of viral viroporins and ion channel characteristics contribute to viral exit from the host-cell through alteration of membrane permeability (Melton et al., 2002; Ramsey and Mukhopadhyay, 2017). Additionally, approaches targeting 6K protein viroporin activities have not been thoroughly investigated but strategies targeting viroporin activities of other viruses have proved to be successful antiviral strategies. Specifically, inhibitors targeting a viroporin, p7, of HCV have been successful antiviral strategies and have moved into human clinical trials (Madan and Bartenschlager, 2015).

9. E1

The E1 glycoprotein is responsible for viral fusion in the endosome and conformational changes caused by a drop in endosomal pH causes the release of nucleocapsid into the cytoplasm. After entering the cell by clathrin mediated endocytosis, E1 is still bound to E2 and is inactive. Endosome acidification disrupts association with E2 and allows for fusion with the endosomal membrane. Fusion also requires the presence of sphingolipids in the endosomal membrane (Wahlberg et al., 1992). Other alphaviruses also require the presence of cholesterol in the host endosomal membrane but a P226S mutation ablates that requirement and VEEV naturally has this mutation (Chatterjee et al., 2000; Kolokoltsov et al., 2006; Vashishtha et al., 1998). During assembly, E2 is bound to a small protein called E3 in a complex called p62. E1 binds p62 which protects E1 from exposure to low pH during trafficking. Late in the secretory pathway, proteolytic maturation frees E2 from E3 forming the E1/E2 dimer that is incorporated into the membrane. The E1/E2 dimer will trimerize with other E1/E2 dimers and form the glycoprotein spikes in the cell membrane (Sanchez-San Martin et al., 2009; Venien-Bryan and Fuller, 1994).

No small molecule inhibitors could be found that inhibit the function of E1; however several studies have shown antibodies directed against E1 can confer protection. In 1951, mice were protected after vaccination with hyper immune serum from rabbits and then infected with WEEV (Sabin, 1951). Mice vaccinated with lipid-antigen-nucleic acid complexes (LANACs) containing VEEV E1 and WEEV E1 were completely protected against lethal EEEV, VEEV, and WEEV infection. LANAC vaccination is sterilizing against VEEV and WEEV and not sterilizing in EEEV infection (Rico et al., 2016). Furthermore, mice were also protected when vaccinated with a mutated strain of VEEV TrD. Mutations to the furin cleavage site in pE2 and a single amino acid in E1 significantly attenuated the virus; it was less neurovirulent than TC-83 and protected mice and non-human primates from infection with fully virulent VEEV (Davis et al., 1995; Fine et al., 2007; Ludwig et al., 2001; Pratt et al., 2003; Reed et al., 2005). Research into E1 is severely lacking in regards to host protein interactions as well as anti-viral development. E1 is a good target for vaccines but more research needs to be done to determine if targeting E1 can be beneficial for therapeutics.

10. Additional host protein inhibitors

VEEV replication is influenced by numerous other host proteins whose purpose within the viral life cycle have yet to be elucidated (Table 8). Glycogen Synthase Kinase-3β (GKS3β) inhibition has been shown to be important during VEEV infection. GSK3β is a host protein that mediates pro-inflammatory gene expression (Stambolic and Woodgett, 1994). Inhibition of GSK3β with either siRNA or small molecules caused a decrease in viral titer. GSK3β inhibition caused an increase in the anti-apoptotic gene, survivin, and a decrease in the pro-

Table 8
Known host protein interactions and inhibitors for an unknown VEEV component.

Host Protein	Inhibitor	Virus Studied	EC_{50}	Cell Type	*In vivo* model	Reference
GSK3β	BIOder	VEEV	ND[a]	U87MG	C3H/HeN mice	Kehn-Hall et al. (2012)
LIMK	R10015	VEEV	ND	Vero	C3H/HeN mice	Yi et al. (2017)
Ago2	Acriflavine	VEEV, EEEV, WEEV	0.2 μM	U87MG	C3H/HeN mice	Madsen et al. (2014)
ERK	Ag-126	VEEV, EEEV, WEEV	ND	Vero	ND	Voss et al. (2014)
IKKβ	IKK2 Compound IV	VEEV	ND	U87MG, Vero	C3H/HeN	Amaya et al. (2014)
Unknown	Sorafenib	VEEV, EEEV, CHIKV, SINV	4.2 μM	Vero	ND	Lundberg et al. (2018)

[a] ND = not determined.

apoptotic gene, BH3 interacting-domain death agonist (BID). The GSK3β small molecule inhibitor, BIOder, was also shown to partially protect mice from VEEV induced mortality (Kehn-Hall et al., 2012). LIM domain kinase (LIMK), which modulates actin cytoskeletal rearrangements, has also been implicated in VEEV infection. The LIMK inhibitor, R10015, was developed as an HIV-1 inhibitor but has also shown efficacy against numerous other viruses including VEEV (Yi et al., 2017). Argonaute 2 (Ago2) is an important component of the RNA-induced silencing complex and loss of Ago2 or inhibition with the small molecule inhibitor acriflavine (ACF) causes a decrease in viral titer and capsid expression. Furthermore, treatment with ACF promotes increased survival of neuronal cells over infected cells left untreated and was able to also prevent replication of EEEV and WEEV (Madsen et al., 2014). Another host factor that has been implicated in VEEV replication is extracellular signal-regulated kinase (ERK). VEEV infection causes an activation of the ERK-signaling cascade and inhibition of ERK1/2 by the small molecule inhibitor Ag-126 causes a decrease in VEEV, EEEV, and WEEV viral titers. Viral protein expression was also down regulated in cells treated with Ag-126 and the inhibition is due to an effect on both the early and late stages in the viral life cycle (Voss et al., 2014). Finally, the FDA approved drug, sorafenib, can reduce replication of multiple alphaviruses *in vitro* with studies suggesting that viral translation is affected. Currently, the mechanism is unknown however data suggest that the dephosphorylation of eIF4E and p70 S6 kinase (p70 S6K) could be involved (Lundberg et al., 2018). Further research into what viral component is involved in each of these important proteins could lead to better therapeutics and viral attenuation strategies.

11. Future directions

The roles of host proteins in mediating multiple aspects of the alphavirus replication process cannot be overstated and hence, the multitude of host components that can be targeted for therapeutic development becomes apparent. However, a strong pipeline for therapeutic countermeasure development for alphaviruses is still a challenge and one potential reason is the lack of a fairly complete mechanistic picture of how the viral components interact with the host components during various aspects of the replication process. A critical requirement in this area is the need for a large picture interactome map of how multiple viral proteins utilize host proteins to complete different stages of infection. For example, a useful strategy would be to understand how the early translation of nonstructural proteins is effected and how the translation is dependent on interactions of nsP1 with the host translational machinery, particularly in cap-dependent translation methods. The initiation of replication of viral RNA can be seen as a subsequent step in which multiple viral proteins, especially nsP3 and nsP4 are involved. We propose that the interactomes of proteins like nsP3 are likely to be different during the early translation steps versus the replication processes and it is important to know how those protein complexes differ before they can be effectively targeted for therapeutic intervention.

In addition to understanding how the interactomes of the various viral proteins are modulated and evolve during the infection, the role of post translational modification of these proteins (both viral and host components) should not be ignored. In fact, this area provides maximum room for drug repurposing as several kinase, ubiquitin ligase, phosphatase and methyl transferase inhibitors are likely candidates. As indicated in the protein interaction sections, several alphavirus proteins are phosphorylated and ubiquitinated during infection (Amaya et al., 2015; Carey et al., 2018). Inhibitors that can impact kinases or components of the ubiquitin proteasome machinery have been demonstrated to have broad spectrum impact on VEEV, EEEV and WEEV. Mutational analyses of such modified residues have emphasized their relevance for the establishment of a productive infection. Targeting the effectors of such modification events will be effective as broad-spectrum intervention strategies. While identification of such approaches can be translationally useful, mechanistic studies that can address the impact of inhibition of post translational modification on viral protein function is essential, not only from a basic research perspective, but also to develop novel therapeutic approaches from a structure-function perspective.

Several host-based inhibitors of alphavirus infection have been identified for which an understanding of how it affects viral multiplication is lacking. For example, inhibitors of chaperones and the cytoskeleton have shown efficacy; however, it remains to be determined if the impact transitions a more general requirement of a chaperon to assist protein folding and function of large proteins like the nsP4 polymerase. In a similar manner, it is important to know how cytoskeletal components can impact a productive infection beyond a simplistic influence on intracellular trafficking of viral components, their packaging and viral egress. Several viral proteins such as nsP2, capsid, nsP1 appear to have extensive intracellular transport involved as part of their functionality and are bound to be dependent on the cytoskeletal machinery for critical steps beyond viral packaging such as transcription and translation inhibition of host cell function and hence, control of innate immunity.

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