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Construction and functional analysis of stable cell lines expressing nsP1 of alphaviruses

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Title

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ABSTRACT

Alphaviruses are positive-strand RNA viruses that are transmitted by mosquitoes and cause various (often serious) diseases in their vertebrate hosts, including humans. Similar to other positive-strand RNA viruses, alphaviruses encode for RNA replicase. RNA replicase of alphaviruses consists of four virus-encoded subunits, called non-structural proteins 1-4 (nsP1-4). nsP1 is a membrane anchor or the replicase complex. It forms a dodecameric ring that holds other components in their place, its guanine-7-methyltransferase (MTase) and guanylyltransferase (GTase) activities are essential for the capping of viral RNA genomes. In this study, tetracycline-inducible stable cell lines expressing nsP1 or its mutants of different alphaviruses were generated. Trans-complementation assay was performed by supplementing homologous or heterologous wild-type nsP1 or its mutant version to functionally defective replicase of Chikungunya virus (CHIKV) in which the defect was caused by mutations of nsP1. We found that expression of wild-type nsP1 of matching or closely related alphaviruses can significantly compensate for capping defects of CHIKV replicase. The compensation was less effective, or not observed at all, for replicase mutants where the defect was due to its compromised membrane attachments. Adding the defective nsP1 to the CHIKV replicase which harbours the same mutation worsens the activity of replicase; however, activities of mutated replicase were restored when it was supplemented with nsP1 harbouring different functional defects. Furthermore, expression of these findings expands our knowledge about alphavirus RNA replication and trans-complementation of replicase-associated defects. Besides, tetracycline-inducible stable cell lines can be used as tools to trans-complement alphaviruses with lethal defects in nsP1 allowing obtaining conditionally infectious alphaviruses virions which can be used at low biosafety level laboratories for high-throughput neutralization and antiviral testing.

Keywords: alphavirus, nsP1 stable cell lines, T-Rex system, trans-complementation. The CERCS code of this thesis is B230.

Pealkiri: Alfaviiruse nsP1 valku ekspresseerivate rakuliinide konstrueerimine ja kasutamine viiruse RNA replikatsiooni uurimiseks

Alfaviirused on lülijalgsete vektorite vahendusel levivad positiivse polaarsusega RNA genoomiga viirused. Oma selgroogsetes peremeestes, seal hulgas inimestes, tekitavad alfaviirused erinevaid, sagel raskeid, haiguseid. Sarnaselt teistele RNA viirustele kodeerivad alfaviirused RNA replikatsiooniks olulisi valke mida nimetatakse mittestruktuurseteks valkudeks 1-4 (nsP1-4). nsP1 on alfaviiruse replikaasi komplesi oluline komponent vahendades selle seondumist raku membraanidele. nsP1 moodustab dodekameerse rõnga-taolise struktuuri mis seondab ja hoiab koos replikaasi ülejäänud komponente. Lisaks on nsP1 valgul metüültransferaasne ja guanülüültransferaasne aktiivsus, need on vajalikud viiruse RNAdele cap-struktuuride lisamiseks.

Käesolevas töös konstrueeriti alfaviiruste metsikut tüüpi nsP1 ja selle mutatnsteid variante ekspresseerivad tetratsükliiniga indutseeritavad rakuliinid. Neid rakuliine kastutati uurimaks rakus toodetud nsP1 võimet komplementeerida Chikungunya viiruse (CHIKV) replikaaside, mis on mitteaktiivsed nendes sisalduvate nsP1 vahendatud cap-sünteesiks olulisi aktiivsuseid blokeerivate mutatsioonide tõttu, võimet sünteesida viiruse RNAsid. See analüüs näitas, et mutantse CHIKV replikaasi aktiivsust suurendab nii CHIKV enda kui ka CHIKV'le lähedaste alfaviiruste nsP1 ekspressioon. Samas ei olnud rakus ekspresseeritavad nsP1 valgud võimelised aktiveerima CHIKV replikaasi mille kooostisesse kuuluv nsP1 ei seondu korralikult raku membraanidele. Samuti leiti, et ka teatud defektsete nsP1 vormide ekspresseerimine võib taastada mutantse CHIKV replikaasi võime RNAsid sünteesida; selle tingimuseks on, et mutatsioonid replikaasis endas leiduvas nsP1 valgus ja raku poolt ekspresseeritavas nsP1 valgus on erinevad. Lisaks leiti, et indutseeritavad püsiliine saab kasutada nsP1 valgus letaalseid defekte omavate CHIKV mutantide vabastamiseks. See võimaldab saada ja paljundada viiruseid, mis on võimelised paljunema vaid selles kindlas rakuliinis ja mis on seetõttu ohutuks töövahendiks viirusvastaste neutraliseerivate antikehade ja viiruse RNA replikatsiooni inhibiitorite uurimisel madala bioohutustaseme labori tingimustes.

Märksõnad: alfaviirus, nsP1, stabiilsed rakuliinid, T-Rex süsteem, trans-komplementatsioon
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ABBREVIATIONS

3’R – 3’ region

5’R – 5’ region

BSL-2 – biosafety level 2

cDNA – complementary DNA

CHIKV – chikungunya virus

CMV – cytomegalovirus

ConA – ConcanavalinA

CSE - conserved sequence element

DMSO – dimethylsulfoxide

dNTP – deoxyribonucleoside triphosphate

DOX – doxycycline

DPBS – Dulbecco's phosphate-buffered saline

dsRNA - double-stranded RNA

EEEV – Eastern equine encephalitis virus

EGFP – enhanced green fluorescent protein

EILV – Eilat virus

FCS - fetal calf serum

Fluc – firefly luciferase

Gluc – *Gaussia* luciferase

GTase – guanyltransferase

IMDM – Iscove's Modified Dulbecco's Medium

m^7GMP – 7-methyl-GMP

mRNA – messenger RNA

MTase – methyltransferase

nsP1-4 – non-structural proteins 1 to 4

nt – nucleotide (residue)

ONNV – o’nyong’nyong virus

ORF – open reading frame

PBS – phosphate buffer saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

RC – replication complex

RdRp – RNA-dependent RNA polymerase

RRV – Ross River virus

RT-qPCR – reverse transcription quantitative PCR

SG – subgenomic

SFV – Semliki Forest virus

SINV – Sindbis virus

ssRNA – single-stranded RNA

T-Rex – tetracycline-regulated expression system

TetR – tet repressor protein

TetO2 – tet operator system

U2OS – human bone osteosarcoma epithelial cells

UTR – untranslated region

VEEV – Venezuelan equine encephalitis virus

WEEV - Western equine encephalitis virus

WGA – wheat germ agglutinin

INTRODUCTION

Alphaviruses are arthropod-borne positive-strand RNA viruses belonging to the *Togaviridae* family, that are distributed globally. The alphavirus genus includes over 30 known species. For example, chikungunya virus (CHIKV) and Ross River (RRV) viruses, which are highly pathogenic to humans, and Semliki Forest virus (SFV) and Sindbis virus (SINV), that act as the model viruses of the genus. Among alphaviruses, CHIKV has become a global threat due to the massive outbreaks in the Indian Ocean region in 2004 and the Caribbean region and the Americas since 2013. Symptoms of CHIKV infection include sudden onset of high fever, rash, and polyarthralgia. Currently, there are no antiviral drugs available against alphavirus infection.

Non-structural protein 1 (nsP1) of alphaviruses is an important component of viral RNA replicase. The localization of the replication complex (RC) to the cellular plasma membrane depends mostly on the events held by nsP1. nsP1 is the only membrane anchor for the RC as it locates in the neck of the spherule, the physical structure of RC. The N-terminal region of nsP1 possesses methyltransferase (MTase) and guanylyltransferase (GTase) activities involved in the capping of newly synthesized viral RNAs; capping is essential for viral mRNA translation and to avoid detection by host innate immune sensors. Studies reveal that nsP1 is a promising target for antiviral compounds. Thus, conducting research on nsP1 elucidates the importance of this protein in viral replication and in the capping of viral RNA and contributes to the development of antivirals.

This thesis focuses on the nsP1 of different alphaviruses, establishing tetracycline-inducible stable cell lines that can express alphaviruses nsP1 or their mutants forms. We also designed trans-complementation system by using defective RNA replicase of CHIKV and nsP1 individually expressed by the cell line to check whether the formation of active replicase can be achieved using trans-complementation. Furthermore, we used cell line expressing nsP1 of CHIKV for rescue of CHIKV harbouring lethal mutations in nsP1 encoding region. We observed that expression of wild-type nsP1 of viruses closely related to CHIKV can also significantly compensate for capping defective CHIKV replicases. However, adding the defective nsP1 to the CHIKV replicase which harbours the same mutation worsens the activity of replicases. We observed that the conduction of expression of CHIKV nsP1 can compensate for the infectivity of CHIKV infectious cDNA harbouring mutations in nsP1 that block synthesis of cap structures but cannot restore infectivity of cDNA containing mutations blocking the ability of nsP1 to bind cell membranes.

1. LITERATURE REVIEW

1.1.Overview of alphaviruses

Alphaviruses are small, enveloped, positive-strand RNA viruses belonging to the *Togaviridae* family (Powers et al. 2001). Most of the alphaviruses are arboviruses and spread through arthropods, using mainly *Aedes albopictus* and *Aedes aegypti* mosquitoes as vectors (Navarro et al. 2017). The majority of alphaviruses can efficiently replicate in vertebrates and arthropods. Their infection in vertebrate cells is highly cytopathic whereas that in arthropod cells is noncytotoxic (Raghow et al. 1973). The *Alphavirus* genus contains 32 recognized virus species (https://ictv.global/taxonomy). On the basis of nucleotide sequences encoding E1 protein alphaviruses are divided into eight antigenic complexes (nine, including the fish alphaviruses) visible in the phylogenetic tree (https://ictv.global/report_9th/RNApos/Togaviridae). The largest groups are the Semliki Forest complex, the Venezuelan equine encephalitis complex, and the Sindbis complex (Weaver et al. 1997) (Figure 1).

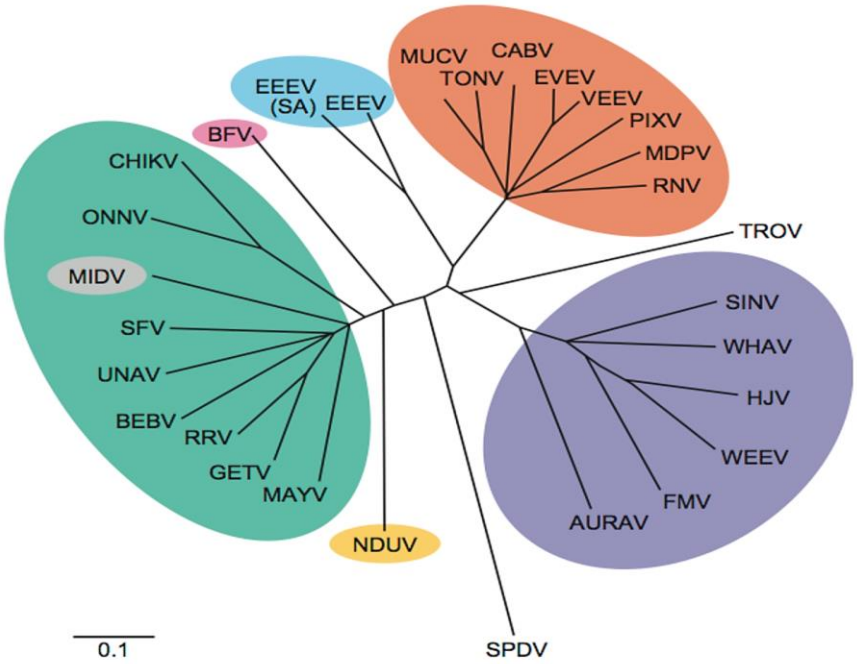


Figure 1. Unrooted phylogenetic tree of alphaviruses generated from nucleotide sequences of E1 encoding regions. The figure originates from (https://ictv.global/report_9th/RNApos/Togaviridae).

There are significant differences between alphaviruses belonging to different clades and sometimes even between members of the same clade. Semliki forest virus (SFV) and Sinbis virus (SINV) are known as model viruses to study alphavirus genome replication. SFV is not known to cause disease

in humans. In contrast, SINV, CHIKV, o'nyong-nyong virus (ONNV), Ross River virus (RRV) and Eastern quine encephalitis (EEEV) are alphaviruses associated with human diseases. Particularly, CHIKV has recieved a lot attention due to the major epidemics in tropical/subtropical regions since 2004 (Josseran et al. 2006; Weaver 2014). Based on geographical distribution, alphaviruses are divided into Old World alphaviruses and New World alphaviruses. Old World viruses, including CHIKV, ONNV, SINV, RRV, and SFV tend to cause a clinical syndrome characterized by fever, rash, and arthritis, while New World viruses, including EEEV, Venezuelan equine encephalitis virus (VEEV), and Western equine encephalitis virus (WEEV) cause encephalitis in humans or domestic animals (Strauss and Strauss 1994). In addition, there are fish-infecting alphaviruses and the recently discovered insect specific alphaviruses including Eilat virus (EILV), which infect only mosquitoes and cannot replicate in vertebrate cells (Nasar et al. 2012).

1.2. Alphavirus virion

Alphavirus virions are spherical, enveloped particles about 70 nm in diameter (Mancini et al. 2000) (Figure 2). The particle consists of a single copy of positive strand RNA genome in a nucleocapsid core surrounded by a lipid bilayer in which two glycoproteins E1 and E2 are embedded. These two glycoproteins are arranged as 80 trimers of heterodimers known as spikes (Fuller 1987). E3 glycoprotein can be found in virions of some, but not all, alphaviruses. It functions during synthesis and transports of envelope proteins to the plasma membrane. There is also a peptide called 6K protein that associates with the E2/E1 heterodimer and is transported to the site of viral assembly at the plasma membrane (Lusa et al, 1991). 6K itself is not be part of mature virions but its form is called TF (from trans-frame).

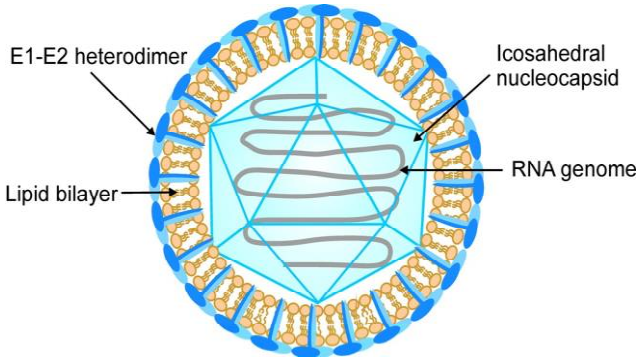


Figure 2. An overview of an alphavirus virion. The scheme is modified from PhD thesis of Age Utt.

1.3. Alphavirus genome

Alphaviruses RNA genome is approximately 12 kb in length. The RNA contains a 5’ type-0 N 7-methylguanosine cap structure and a 3’ poly(A) tail (Strauss and Strauss 1986). The naked RNA genome of alphaviruses is sufficient to initiate the complete replication cycle (Strauss and Strauss 1994). The alphavirus genome contains two open reading frames (ORF) (Figure 3). The first ORF is translated directly from genomic RNA and encodes for four non-structural proteins (nsP1-4) which are required for viral RNA replication. The second ORF is translated from subgenomic (SG) RNA that is synthesized during the infection and encodes the structural proteins that are crucial for the assembly of new virions (Rupp et al. 2015; Strauss and Strauss 1994).

The alphavirus genome contains three untranslated regions (UTRs), all of which play crucial roles in virus infection. The length of 5’ UTR varies between 26-85 nt while the 3’ UTR is up to 911 nt in length (Hyde et al. 2015). A third, short intragenic, non-coding region, typically of around 50 nt, located between the two ORFs. All these regions contain important cis-acting elements and are important for viral genome replication, protein expression and viral - host interactions.

The most important cis-acting elements are conserved between different alphaviruses and are therefore called conserved sequence elements (CSE). In total, there are four CSEs in the alphavirus genome (Strauss and Strauss 1994; Rupp et al. 2015). The first CSE is located at the 5’ end of the genome and the second is located in the nsP1 encoding region. The third CSE mostly overlaps with the region encoding the C-terminus of nsP4 while the fourth one immedialy preceeds the 3’poly(A) tail. The first and the last CSE respectively, are important for the synthesis of the negative and positive strands of the virus genomes (Kuhn et al; 1990).

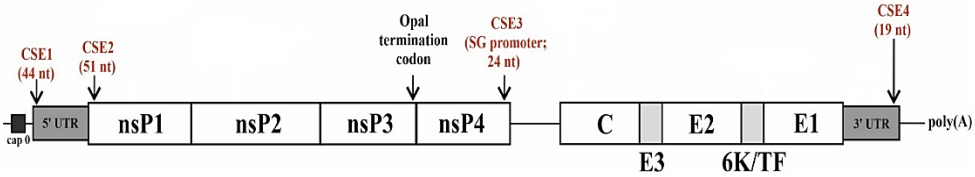


Figure 3. Organization of CHIKV genome. The genome contains Cap0 structure in the 5’ terminus and a poly-A tail in the 3’ terminus. The first ORF encodes for a nonstructural polyprotein, a precursor of the nsP1-nsP4. The second ORF encodes precursors of structural proteins (C, E3, E2, 6K, TF and E1). Localization of conserved *cis*-acting sequence elements (CSE1-4) is shown in red. Figure is modified from PhD thesis of Liubov Cherkashchenko.

1.4. Non-structural proteins (nsPs)

Alphaviruses encode four essential nsPs, all of which are neccessary for the replication of the viral genome and the transcription of SG RNA (McInerney and Merits 2021). Each of these proteins has important individual role in viral replication.

nsP1(~60 kDa) is a multifunctional protein engaged in all steps of alphavirus RNA replication. It possesses guanine-7-methyltransferase (MTase) and guanyltransferase (GTase) activities and is responsible for viral genomic and SG RNA 5′ cap-0 synthesis (Jose et al. 2009). Thus, during positive-strand RNA synthesis, 5' end of the genome becomes modified to m^7GpppA (the cap0 structure) which is important for protection of viral RNAs from degradation and its translation (Cross 1983). The capping of alphavirus RNAs takes place in RCs located in cytoplasm of infected cells and is performed in three steps. During the first step, 5′ γ-phosphate is removed from the RNA by the triphosphatase activity of nsP2. This is followed by reaction carried out using MTase activity of nsP1 which catalyzes the transfer of methyl group from S-adenosyl methionine (AdoMet) to GTP to yield m^7GTP. In the third step, m^7GTP forms covalent bond with nsP1 and is then transfered to the 5' end of RNA by the GTase activity of nsP1 (Rupp et al. 2015). The capping mechanism is as follows :

1) pppRNA → ppRNA + P_I

2) GTP + AdoMet → m^7GTP + AdoHcy

3) m^7GTP + nsP1 → m^7GMP-nsP1 + ppRNA → m^7GpppRNA

Among the four viral non-structural proteins, nsP1 is the only nonstructural protein that can directly binds to the plasma membrane while the other replication proteins have no independent membrane associations in cells. Thus, nsP1 is solely crucial for localization of RC to the membrane (Spuul et al. 2007; Gottipati et al, 2020). The strong membrane association is caused by covalent palmitoylation of cycteine residues in the nsP1 (Kiiver et al. 2008). Palmitoylation of nsP1 causes several changes in virus infected cells. For instance, it causes induction of filopodia-like structures, about 2-7 μm in length which are mainly composed of actin not tubulin (Martinez et al. 2014). When nsP1 is expressed alone in mammalian cells, both palmitoylated and nonpalmitoylated forms of nsP1 localize to the cytoplasmic surface of the plasma membrane (Kääriäinen et al. 2002). Analysis of palmitoylation-defective mutants has demonstrated that palmitoylation is not necessary

for the enzymatic activities of nsP1 wherase it is crucial for the formation of filopodia-like structures by nsP1 (Karo-Astover et al. 2010; Žusinaite et al. 2007). For example, SFV lacking palmitoylation results in the loss or fewer filopodia-like structures, however, mutant virus recover its fitness by adapting the compensatory mutation in nsP1 (Žusinaite et al. 2007). Despite nsP1 palmitoylation is universal for alphaviruses, however, the impact of palmitoylation mutation are different. For CHIKV nsP1, lack of palmitoylation is lethal as it completely abolishes activity of CHIKV RNA replicase; one the other hand palmitoylation of nsP1 is dispensable for SINV (Bakhache et al. 2020).

Alphaviruses nsP1 is an important component of RC. Alphavirus RCs are located at necks of membrane-bound structures called spherules. nsP1 is located in the neck region (Figure 4) forming a dodecameric ring structure (Jones et al. 2021; Tan et al. 2022; Zhang et al. 2021). 10 out of 12 copys of nsP1 included in the dodecameric ring form direct contact with nsP4; the remaining two form channels between nsP4 that are assumed to be used for transport of viral RNAs. Besides direct contact with nsP4, nsP1 may have indirect association with nsP2, as one molecule of nsP2 sits on top of nsP4.

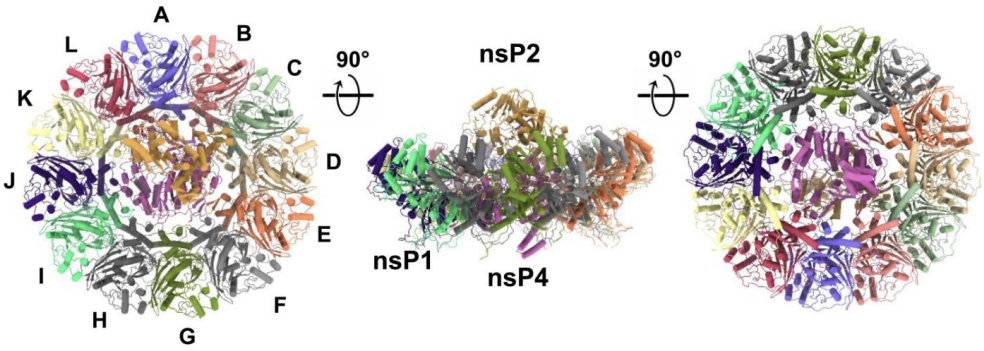


Figure 4. The graphical illustration of RC core (CHIKV nsP1+nsP2+ONNV nsP4). The left figure shows the top-view, the middle one shows side-view and the right one shows the bottom-view of architecture. The figure originates from (Tan et al. 2022).

nsP1 is a promising target for antiviral compounds. 3-deaza-adenosine inhibits CHIKV replication through nsP1 (Scholte et al. 2013). A novel class of small molecules are found to inhibit CHIKV specifically inhibiting guanylyation of nsP1 (Delang et al. 2016).

nsP2 (~90 kDa) is the largest nsP with multiple functions. It has several domains, a N-terminal region which possesses NTPase and RNA triphosphatase activities and a C-terminal protease domain. The N- and C-terminal regions of nsP2 are connected by a flexible linker (Law et al. 2021).

The presence of two regions are needed for the nsP2 helicase activity (Das et al. 2014). Enzymatic activities of nsP2 are essential for viral genome replication, including the protease function used for ns polyprotein processing (Hardy et al. 1989). Furthermore, nsP2 has functions related to shutoff of host cell transcription and antiviral responses (Fros et al. 2015).

nsP3 (~60 kDa) consists of three domains. A highly conserved macro domain is located on the N-terminus of the protein and can be found in other RNA viruses such as coronaviruses hepatitis E virus (Gorbalenya et al. 1991; Koonin et al. 1992). Recently the ADP-ribosylhydrolase activity of CHIKV macro domain was shown to be critical for CHIKV replication and virulence. In addition, macro domain is required to assist nsP2 for cleaving of the P23 polyprotein (Lulla et al. 2012; McPherson et al. 2017). An alphavirus unique domain (AUD), which is only found in alphaviruses, is important for the SG RNA synthesis (Gao et al. 2019). The C-terminal hypervariable domain of nsP3 has been shown to interact extensively with host proteins (Meshram et al. 2018; Mutso et al. 2018). Although nsP3's role alphavirus replication has been less understood than that of other nsPs, it is still absolutely essential for viral RNA synthesis (Tuittila et al. 2000).

nsP4 (~70 kDa) comprises the N-terminal domain (NTD) and C-terminal RNA-dependent RNA polymerase (RdRp) domain. The alphaviruses RdRp have a highly conserved catalytic motif GDD (Gly-Asp-Asp) which is essential for viral RNA synthesis (Utt et al. 2016). In addition to RdRp activity, nsP4 also has terminal adenylyl transferase (TATase) activity which is needed for the synthesis of poly (A) tail of positive strand RNAs (Chen et al. 2017). The quantity of nsP4 present in alphavirus-infected cells is lower than that of the other nsPs. One explanation for that is the opal codon and thus translation of two kinds of polyprotein from genomic RNA: the P123 and by a read-through event also the P1234 with a 10-20% efficacy. Secondly, the N-terminal residue of nsP4 is a conserved tyrosine which results in rapid degradation of nsP4 within the cell (Jose et al. 2009).

1.5. Alphavirus infection cycle

Most of alphaviruses replicate in both arthropod hosts and vertebrate hosts. In general, the infection of alphaviruses starts with a mosquito bite on a vertebrate host and quickly results in a high viral titer in the blood. The high-level of viremia can result in the spread of virus to other tissues such as the brain, joints, and muscle (Pingen et al. 2016). Studying a virus replication *in vivo* (i.e. in living host or vector) is very difficult. Therefore, most of knowledge about alphavirus infection has been obtaind using different cell-culture models (i.e. *in vitro*).

1.5.1. Binding and entry

Alphavirus virions bind to the cellular membrane receptors and/or attachment factors and enter the cytoplasm. Different alphaviruses use different receptors. For example, the main cellular receptor for CHIKV and number of related alphaviruses is Mxra8 (Vancini et al. 2015). The interaction between virion and cellular receptor (Figure 5) is mediated by the viral E2 glycoprotein (Schnierle et al. 2019). Entering the alphavirus virion into the host cells occurs through clathrin-mediated endocytosis followed by a drop of pH in endocytotic vesicle (Schnierle et al. 2019). Low pH triggers fusion of virion envelope and endosomal membrane and release of nucleocapsid. Subsequently, the nucleocapsid disintegrates, releases viral RNA genome into the cytoplasm where it is used as mRNA for translation of ns polyprotein (Singh et al. 1992).

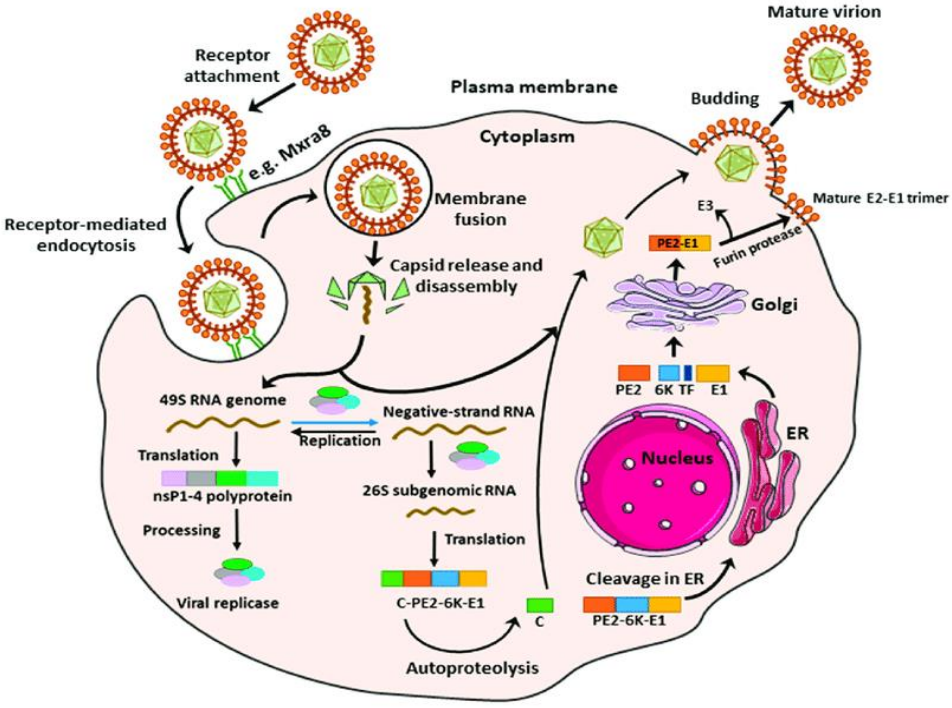


Figure 5. Infection cycle of alphaviruses. The infection cycle begins with the attachment of the virion to the host-cell membrane followed by the entry of the virion into the cytoplasm and releasing the viral nucleocapsid and then the RNA genome. The figure originates from (Abdelnabi and Delang 2020).

1.5.2. Alphavirus RNA replication

The replication of the alphavirus genome occurs in the membrane-derived RCs. Ultrastructure termed “spherule“ (Figure 6) contains the negative-strand copy of the RNA genome likely in form of dsRNA (Laurent et al. 2022) and replicase located at the neck (Figure 6).

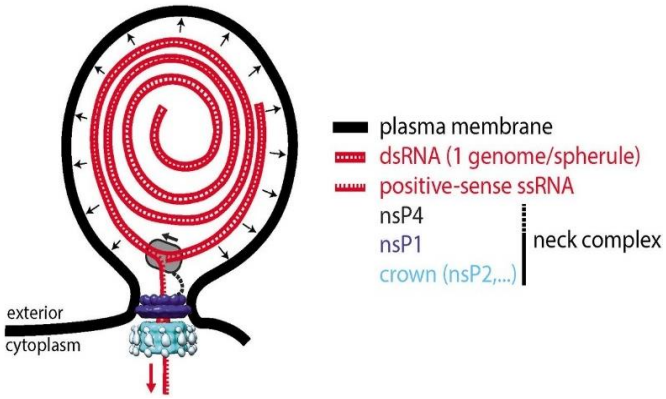


Figure 6. Schematic of alphavirus spherules. Each spherule contains a single viral dsRNA. The membrane shape is determined by the confined neck geometry, the pressure exerted by the confined genome, and the tension and stiffness of the membrane. nsP1 determines the neck of this structure. The figure originates from (Laurent et al. 2022).

Genomic RNA is translated to yield the P1234 (10-20%) and prevalent P123 polyprotein (90%) due to the opal codon at the end of nsP3 encoding region. However, the full-length P1234 polyprotein is unable to perform RNA replication and requires specific cleavage for activation of its activities (Figure 7) (Vasiljeva et al. 2003). At first, the protease activity of nsP2 cleaves P1234 into P123 and nsP4 which forms early replicase and function in the synthesis of negative-strand RNA using the genomic RNA as a template (Rupp et al. 2015). Next, cleavage site between nsP1 and nsP2 is processed and complex consisting form nsP1, short-lived P23 and nsP4 is formed. This is rapidly followed by trans cleavage in P23 and the formation of late replicase which is composed of the individual nsPs and is capable for positive-strand RNA synthesis (Jose et al. 2009).

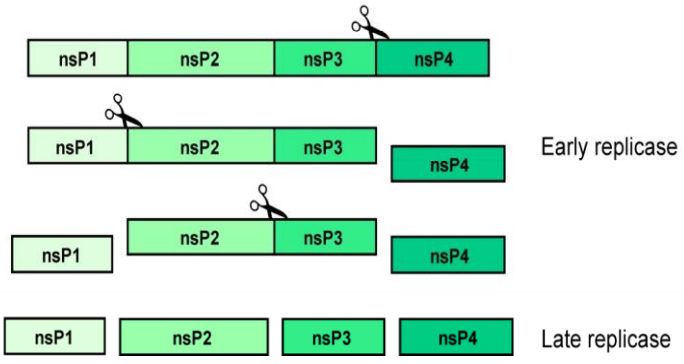


Figure 7. The order of nonstructural polyprotein processing. The figure is originated by PhD thesis of Age Utt.

1.5.3. Synthesis of structural proteins, virion assembly and budding

Precursors of structural proteins are translated from SG RNA using the free ribosomes. Due to the autocatalytic removal of capsid protein, the N-terminus of E3, responsible for translocation of the rest structural polyprotein to endoplasmic reticulum membranes, is released and synthesis continues using membrane-bound ribosomes. The synthesized polyprotein is processed by cellular enzymes into p62 precursor (E3+E2), 6K/TF, and E1; then in the trans-Golgi network p62 is cleavaged into E3 and E2 by cellular peptidase. Capsid protein together with genomic RNA forms nucleocapsids which are delivered to plasma membrane through interaction with E2. The new virion are formed and releases by budding on plasma membrane (Jose et al. 2009).

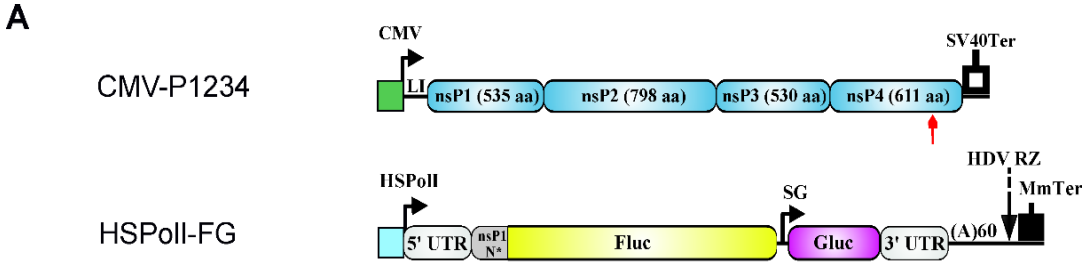
1.6. Trans-replication system

The first trans-replication assay was elaborated for SFV (Spuul et al. 2011). The theory behind the assay is to use an easily quantified method to check how much genomic and SG RNA being produced by viral RNA replicase. During natural alphavirus replication genomic RNA acts as an mRNA for translation of ns proteins. Thus, the expression of nsPs is tightly coupled with RNA replication (i.e., less replication produces fewer nsPs and *vice versa*). In the trans-replication systems, the expression of the replicase proteins is separated from the replication of their mRNA (Utt et al. 2019); this is achieved by cotransfecting replicase-coding and replication-competent RNA template coding plasmids. The replicase-coding plasmid encodes the same viral ns polyproteins as the genomic RNA, however, mRNAs synthesized from this plasmid lack cis-acting elements essential for RNA replication. Hence, the expressed replicase proteins need to interact with the separately transcribed RNA-template that contains all necessary cis-elements crucial for replication and transcription, but does not encode for ns-proteins. The interaction leaks to the synthesis of negative-strand RNA and spherule formation and is followed by the amplification of template RNA (replication) and the production of SG RNA (transcription). The plasmid expressing the template RNA in the trans-replicase system typically contains sequences encoding two reporters: firefly luciferase (Fluc) used as a a substitute for most of the ORF1 and *Gaussia* luciferase (Gluc) used as a substitute for the structural ORF (Lello et al. 2021; Utt et al. 2019) (Figure 8A). Theoretically, genomic and SG RNA synthesized by viral replicase can be detected and quantified directly using northern blotting or RT–qPCR. However, these methods are expensive and time consuming and are therefore substituted by more convenient detection of reporters which, as indicated above, typically are luciferases, but can also be fluorescent proteins

or other easily detectable markers (Figure 8A). Due to need to account for background activity of the reporters in the absence of RNA replication a negative control, typically plasmid expressing P1234 where the conserved GDD motif in the active center of nsP4 was replaced with GAA motif, is also necessary component of trans-replication assay.

One benefit of trans replicase system is that it allows use of "mix and match" approach: one can take replicase (or some ns-protein) for one virus and template RNA from another virus and test do they work together or not. In publications originating from our lab, combinations of plasmids expressing proteins and template RNAs from one and the same alphavirus are referred to as "matching combinations" whearas the combinations expressing components from different viruses are referred to as "heterologous combinations" (Lello et al. 2021) (Figure 8).

Trans-replication systems have been used to investigate RC formation (Frolova et al. 2010), for analysis of the effects of mutations introduced into replicase proteins on viral RNA synthesis in mammalian (Kallio et al. 2016) and mosquito cells (Bartholomeeusen et al. 2018), to study the impact of the length of RNA template on spherule size (Hellström et al. 2016) and also for tagging of different replicase proteins (Utt et al. 2016). This system also allows the study of compatibility of different alphaviruses RNA replicases (Lello et al. 2021) and simplifies the study of RNA replication of highly pathogenic alphaviruses. As all test systems trans-replicases have also contain certain technical limitations. For instance, the efficiency of this system is restricted to the properties of promoters used to generate replication competent template RNA. The alphavirus trans-replication systems have traditionally used several promoters: the T7 RNA promoter, which requires co-expression of T7 RNA polymerase; cellular RNA polymerase II promoters, such as human cytomegalovirus (CMV) promoter for mammalian cells or the *Aedes aegypti* polyubiquitin promoter (Ubi) for mosquito cells or cellular RNA polymerase I promoters. Each of these systems has its benefits and specific problems, so the question - what type of trans-replicase is most suitable? – has no universal answer. Instead, the answer depends on goal of the study: in one case one type of system is prefered while in another case different trans-replicase type will be optimal.



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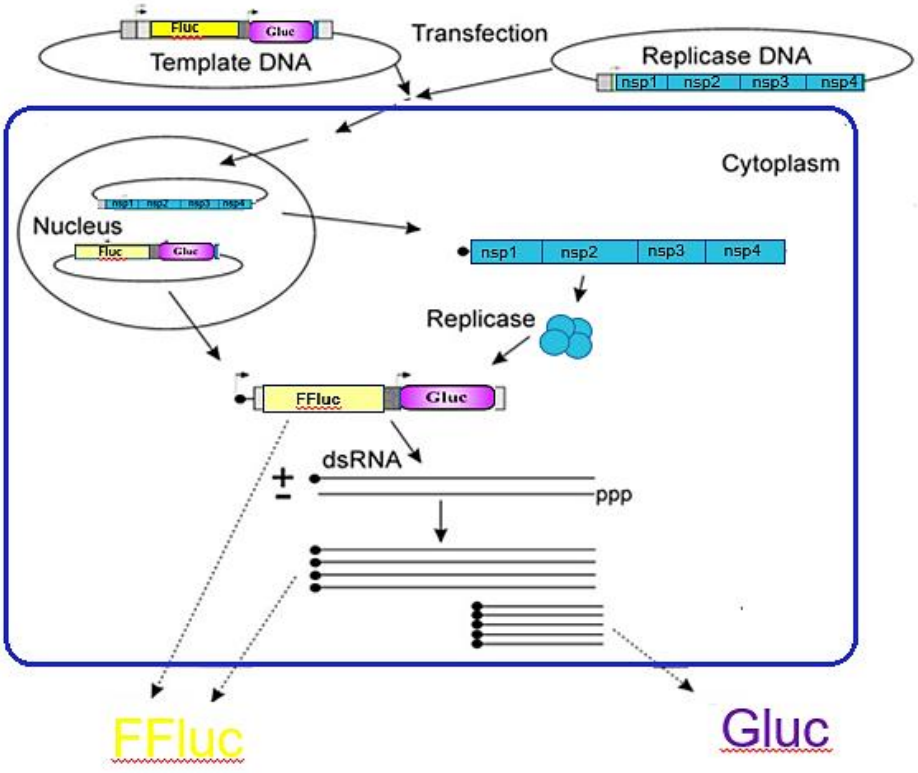

Figure 8. A) Template RNA and replicase proteins expression plasmids. B) Schematic representation of the trans-replication assay. Cells are co-transfected with plasmids expressing mRNA encoding P1234 and a plasmid expressing RNA template. The replicase and template plasmids will be transcribed to RNA. Replicase proteins cannot replicate their own mRNA and bind to the template RNA instead. This initiates synthesis of negative-strand RNA and following synthesis of genomic RNA and SG RNAs abundance of which can be estimated by measuring activities of reporters translated from these RNAs. The figure is modified from (Lello et al. 2020).

1.7. Alphaviruses included in this work

In this project, we established inducible cell lines expressing nsP1 proteins of different alphaviruses. A summary of viruses and used mutations is provided below:

CHIKV is an Old World alphavirus causing millions of infections worldwide (Bakhache et al. 2020) and is considered an emerging threat for the United States and Europe (Bartholomeeusen et al. 2018). CHIKV infection cause fever, rash and specifically severe joint pain and muscle pain as well as long-lasting chronic symptoms. It is transmitted to humans by *Aedes aegypti* and *Aedes*

albopictus mosquitos. We also investigated a number of nsP1 mutants of CHIKV, the effects of mutants are described in below:

CHIKV nsP1^{H37A}, the mutation abrogates GTase activity of nsP1. **CHIKV nsP1^{D63A}**, the mutation blocks methyltransferase activity of nsP1. Both H37A and D63A mutations abolish positive-strand RNA synthesis (Kallio et al. 2016).

CHIKV nsP1^{3C3A}, where palmitoylation site cysteine residues of nsP1 are substituted by alanine residues; this mutation completely abolish the replication of CHIKV (Bakhache et al. 2020; Utt et al. 2019).

CHIKV nsP1^{W258A}, the mutation lead to a temperature sensitive phenotype as it inhibits replication at 39 ^{o}C but has little effect at 28 ^{o}C (Bartholomeeusen et al. 2018).It was originally though to affect membrane attachment of nsP1 but the new data about structure of nsP1 does not support this idea as this residue has no clear contact with membranes.

CHIKV nsP1$^{1C\ GFP}$, is an insertion mutant harbouring GFP after residue 516 of nsP1. Accordingly, the expressed protein is nsP1-GFP fusion protein that can be easily detected in living cells. The insertion of GFP in this position is tolerated but it still reduces activities of trans-replicase (Utt et al. 2016).

SFV is a well characterised model of virus from Semliki Forest complex. In mice it is efficiently neuroinvasive (Fragkoudis et al. 2018). **SFV nsP1^{3C3A}** harbours the mutation ^{418}CCC^{420} to AAA in the nsP1 palmitoylation site. In contrast to similar mutation in nsP1 of CHIKV, the substitution of cysteines 418–420 in nsP1 of SFV is not lethal for the virus, instead it causes accumulation of compensatory mutations (Žusinaite et al. 2007).

RRV is another member of Semliki Forest complex. It is prevalent throughout Australia and is a major concern in Pacific Island countries. On average, about 5000 clinic cases of RRV infection are reported annually in Australia (Barber et al. 2009; Russell et al. 2002). *Aedes camptorhynchus* and *Aedes vigilax* are the most important mosquito vectors for this virus (Dhama et al. 2014).

SINV is widely found in insects and vertebrates in Eurasia, Africa, and Oceania and Northern Europe; it is also the only alphavirus occuring in Estonia. Mosquitoes of genera *Culex* and *Culiseta* are considered to be the main vectors of SINV transmition to humans. The symptoms include mild fever, and joint symptoms, particularly in wrists, hips, knees, and ankles.

EEEV is primarily found in North America and the Caribbean. EEEV is transmitted through the bite of *Culiseta melanura*. It is encephalitic alphavirus with high case-fatality rate in humans (Corrin et al. 2021).

2. EXPERIMENTAL WORK

2.1. Aims of the thesis

As mentioned before, nsP1 has multiple roles in viral replication, it has MTase and GTase activities which are crucial for viral RNA capping; it serves as the membrane anchor which forms dodecameric ring which is crucial for spherules formation; besides it is also a promising antiviral target. However, there are still unknown roles about nsP1 in alphaviruses replication, for instance if the homologous and heterologous nsP1 can be trans-complement functionally defective CHIKV replicases/viruses i.e., allow formation of functional RNA replicase. In addition, it was not known which type of defects (enzymatic and/or localization) in nsP1 of replicase or virus can be complemented and can this also be done using mutant forms of nsP1 or nsP1 from heterologous alphaviruses. In order to address these questions, we decided to establish stable cell lines using Tet-on system and express nsP1 in inducible manner.

The objectives of the thesis were as follows:

1. To establish T-REx inducible stable cell lines expressing nsP1 of different alphaviruses.
2. To check subcellular localization of nsP1 in obtained stable cell lines.
3. To perform trans-complementation assay using cell lines inducible for expression of nsP1 and study if nsP1 expressed by these cells can complement defective replicases that harbour mutations in their nsP1 region.
4. To check if the complementation also happens in the context of viruses i.e., to investigate whether CHIKV infection clones with lethal mutations in nsP1 can be rescued by induction of nsP1 expression in stable cell lines.

2.2. Materials and Methods

2.2.1. Construction of nsP1 expression plasmids

Sequence encoding for wt nsP1 of different alphaviruses and their mutants were amplified using PCR. PCR mix contained 1xPhusion HF buffer, 5% DMSO, 200 μM of dNTP, 200 μM of each forward and reverse primer, 10 ng of template DNA (selected according to the needs of experiment from existing collection of alphavirus expression plasmids), 0.02 U of Phusion high fidelity DNA polymerase (made in-house). The reactions were run using a three-step protocol based on guidelines taken from the manual of Phusion DNA polymerase; annealing temperature and

extension time were set according to the melting temperature of used primers and length of the amplicon. The PCR products were analysed using agarose gel electrophoresis and then purified with Zymoclean DNA Clean & Concentration Kit (Zymo Research) according to the manufacturer's instructions. In the next step, PCR products were cloned into pJET1.2/blunt vector using CloneJET PCR cloning kit (Thermo Fisher Scientific). The different constructs of pJET-alphavirus-nsP1 were used to transform *E. coli* (strain XL-10) competent cells using heat shock method. Following the transformation, the bacterial colonies grown on agar plates (SOY agar, ampicillin for selection) were picked and placed in 3 ml of SOY medium containing ampicillin. The cultures were grown shaking at 220 rpm and 37 ºC for 16 hours. In the next step, FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech Corp.) was used to purify the plasmid DNA, according to the manufacturer's instructions. To ensure that the obtained plasmids were correct, restriction analysis was performed. The Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific) was used to determine the DNA concentrations of the samples. Sequence of obtained plasmids were verified using Sanger sequencing.

The verified nsP1 genes were subsequently cloned into pcDNA4-TO vector using restriction enzyme-based cloning method. pcDNA4-TO is a vector designed for an inducible expression of proteins of interest. The induction is due to following property of the vector: it has two tetracycline operator 2 (TetO2) sites placed upstream of TATA box of the strong human cytomegalovirus immediate-earlyCMV promoter (https://www.thermofisher.com/order/catalog/product/V102020). To do the ligation, we first generated vector and insert fragments. The materials used for restriction were taken as follows:

Vector (pcDNA4-TO)	3 µg
Insert(pJET-aAlphavirus-nsP1)	2 µg
Cloning enzymes	0.5 µl of enzyme per 1 µg of DNA
Fast digest 10x buffer	2 µl (insert) / 5 µl (vector)
H_2O	Up to 20 µl (insert) / 50 µl (vector)

The vector fragments were also dephosphorylated by treatments for 1h on 37 ºC with FastAP (alkaline phosphatase, Thermo Fisher Scientific); this was done to reduce the self-ligation of the vector. 1 µl of FastAP was used to treat 1 µg of cut DNA. The FastAP treatment was followed by

purification of the vector fragments using Zymoclean DNA Clean & Concentration Kit (Zymo Research) according to the manufacturer's instructions. The insert fragment were separated using agaros gel electrophoresis, the correct fragments were cut out from the gel and purified using Zymoclean Gel DNA Recovery kit (Zymo Research) according to the manufacturer's instructions. The integrity of the purified vector and insert DNA fragments was checked via gel electrophoresis. Afterby, the produced vector and insert constructs were used in ligation reaction that was carried out using T4 DNA ligase (Thermo Fisher Scientific) at 12 ^{o}C for overnight. The ligation products were used to transform competent *E coli* cells; the process of picking colonies, DNA purification, and restriction analysis were performed as described above. Then, the bacterial colonies confirmed to contain correct plasmids were used to prepare endotoxin free DNAs. To do so, bacteria were cultured in 50 ml of SOY medium containing ampicillin at 220 rpm and 37 ^{o}C for 16h and plasmid DNA was purified with the NucleoBond® Xtra Midi EF kit (Machery-Nagel). The resulting clones were designated as follows: pcDNA4-TO-EEEV-nsP1, pcDNA4-TO-SINV-nsP1, pcDNA4-TO-RRV-nsP1, pcDNA4-TO-SFV-nsP1, pcDNA4-TO-SFV nsP1^{3C3A} , pcDNA4-TO-CHIKV-nsP1, pcDNA4-TO-CHIKV nsP1^{3C3A} , pcDNA4-TO-CHIKV nsP1^{H37A}, pcDNA4-TO-CHIKV nsP1^{D63A} , pcDNA4-TO-CHIKV nsP1^{W258A} , and pcDNA4-TO-CHIKV nsP1$^{1C\ GFP}$.

2.2.2. Cell lines and media

In this thesis, the T-Rex U2OS cell lines were used for conducting experimental work in cell culture. The T-Rex U2OS cells were cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Corning) supplied with 10% FBS (Pan Biotech) and 60 mg/ml hygromycin to maintain the tet-repressor expression; for constructed T-Rex U2OS nsP1 cell lines 200 mg/ml zeocin was added to the media. The cells were cultured at 37 °C in the presence of 5% CO_2.

2.2.3. Determining the minimum antibiotic sensitivity of untransfected host cell line

To successfully generate a stable cell line expressing our protein of interest, we determined the minimum concentration of selection antibiotic (Zeocin) required to kill untransfected cells. The reason that we used zeocin as selection agent is that pcDNA4-TO vector has zeocin resistance gene. To perfom the experiement, we seeded T-REx U2OS cells at 25% confluency in the 6 well cell culture plate. After 24h incubation at 37 ^{o}C, new medium containing varying concentrations of Zeocin™ (0, 10, 20, 40, 60, 80, 100, 150, 200 and 300 μg/mL) was added. The plates were incubated as described above for 3 weeks, with the medium changed every 3–4 days. Cell death

was monitored using light microscopy. It should be mention that the mode of cell death and presence of zeocin is quite different from that at the presence of other antibiotics. Upon exposure to zeocin cells do not round up or detach from the plate. Instead they exhibit vast increase in size, abnormal morphological shape and large empty vesicles are present in the cytoplasm of these cells (https://www.sciencedirect.com/topics/neuroscience/zeocin.com). The minimum concentration of zeocin which was capable to kill parential T-Rex U2OS cells after three weeks selection was 60 μg/mL.

2.2.4. Generation of inducible stable cell lines

In the T-REx™ System, expression of the gene of interest is repressed in the absence of tetracycline or its derivative (doxycycline; DOX). This is due to the expression of TetR repressor that in the absence of DOX binds tet-operator sequences in the CMV promoter present in pcDNA4-TO expression plasmid. In the presence of DOX, the TetR protein is released from the promoter resulting in activation of transcription of the desired gene (Yao et al. 1998). As it is effective and easy to use the T-REx system has been used broadly in many different studies including analysis of the properties of individual virus proteins, studies of the effects of these on cellular metabolism, viability and virus replication. Inducible expression of recombinant protein from stable cell lines has several advantages over systems based on transient transfection. The induction with DOX is easy, causes no damage to the cells and have high efficiency. Furthermore, it is precise, reversible, allows efficient spatiotemporal control of gene expression, and saves money and effort by avoiding repeated (or large-scale) transient transfection.

To establish T-REx U2OS stable cell lines expressing nsP1 of different alphaviruses, each constructed plasmid was linearized by PvuI restriction enzyme. Afterwards, we intoduced linearized nsP1 constructs into T-REx U2OS cells by electroporation. 48 h post electroporation, we added the zeocin at optimal concentration (60 μg/mL) to select cells where the used DNA has been successfully integrated to the cell genome. Approximately after one month selection cell colonies appeared; from these we picked three to six single colonies for each cell line and transfered them to 24 well plates. After the colonies have expanded to cell lines, the cells were split and part of them were treated with DOX to induce transcription of our gene of interest.

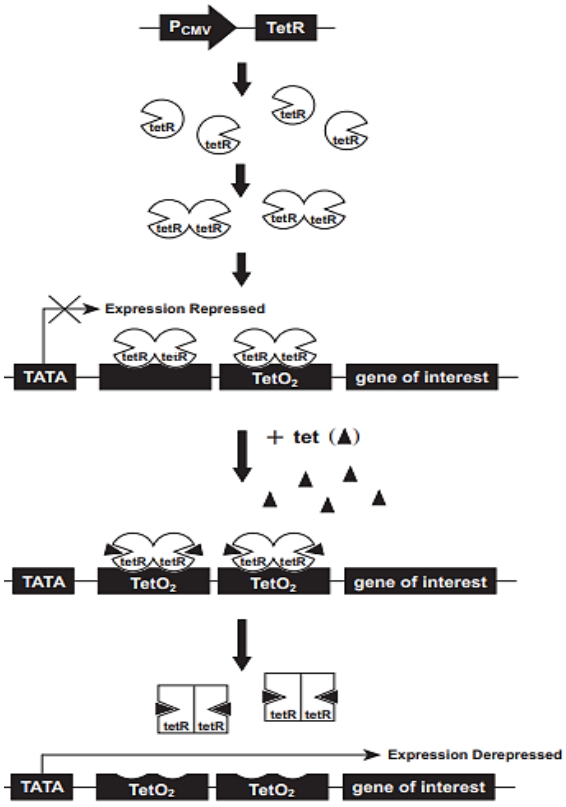

Figure 9. Principle of T-REx system. The Tet repressor protein (tetR) is expressed from the gene present in T-REx U2OS cells. In the absence of tetracycline, the TetR forms a homodimers that binds to two Tet operator (TetO2) sequences in the CMV promoter in pcDNA4-To vector repressing transcription of the gene of interest. Upon addition of tetracycline (tet) or its derivative DOX, the antibiotic binds to tetR homodimers, causes a conformational change and release of repressor from the Tet=2 sequence activating transcription from the gene of interest (Hillen and Berens 1994).

2.2.5. Verification of nsP1 expression

2.2.5.1 Western blotting and Flow cytometry assays

In order to find the optimal concentration of DOX, timepoint for the highest expression of nsP1 and select cell clonies with highest level of nsP1 expression, western blot was performed for two randomely picked T-REx U2OS-SFV nsP1, T-REx U2OS-SFV nsP1^{3C3A} and T-Rex U2OS-CHIKV-nsP1 (wt or mutant versions) cell lines as follows. Cells were induced with various concentrations of DOX and harvested at different timepoints, uninduced cells were used as control. For these expreiments cells were grown in 6-well plates and after incubation with DOX for 24 or 48 h after which cells were lysed with 1× Laemmli buffer and boiled for 5 min. Proteins were separated by SDS–PAGE in 12% gel and transferred to polyvinylidene difluoride membranes

followed by staining with primary antibody. Anti-CHIKV-nsP1 or anti-SFV-nsP1(1:10000 dilution, all in house, polyclonal rabbit antisera) were used to detect nsP1 of CHIKV and SFV respectively, anti-beta-actin antibody (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) in 1:5000 dilution was used to detect the loading control. The dilutions of primary antibodies were prepared in solution containing Odyssey Blocking Buffer (TBS, ref: 927-50000) supplemented with 0.2% Tween20; filters were incubated on the shaker overnight at 4 ^{o}C. The next day, the membrane was washed three times in 1x TBS/0.1%Tween20 then incubated at room temperature for 1h with IRDye 680 RD conjugated goat anti-rabbit (925-68071) secondary antibody (1:20,000 dilution) and the IRDye 800CW conjugated goat anti-mouse (925-32210) secondary antibody (1:20,000 dilution). Washing was performed as described above and Li-COR Odyssey Fc machine was used to acquire the images.

Flow cytometry assay was used to quantify the nsP1 expression level in T-Rex U2OS-CHIKV nsP1$^{1C\ GFP}$ cells. For this assay cells were seeded in 6-well plate. 24h post induction, cells were collected in 500 µl Phosphate Buffered Saline (PBS, 2.7 mM KCl, 137 mM NaCl, 1.8 mM KH_2PO4, 10 mM Na_2HPO4, pH 7.4) and analyzed with a Attune NxT Acoustic Focusing Cytometer. The obtained data were analyzed using Attune NxT software to determine the percentage of EGFP positive cells. The sample which contained the highest percentage of EGFP was considered as the best colony.

2.2.5.2. RT-qPCR

Antibodies against nsP1 of RRV, nsP1 of EEEV or nsP1 of SINV are not available in our lab nor can these be purchased from commercial companies. Therefore the induction of synthesis of corresponding RNAs at presence of DOX was monitored using RT-qPCR. Selected stable cell lines (T-Rex U2OS-EEEV nsP1, T-Rex U2OS-RRV nsP1 and T-Rex U2OS-SINV nsP1) were seeded in 6-well plate. Cells were harvested 24h post-induction. Using 1ml of TRIzol RNA isolation reagent the total RNAs were extracted by TRIzol method following the manufacturer's instructions. The isolated total RNAs were dissloved in 20 µL of nuclease-free water. Quantitative RT-qPCR was performed using Luna® Universal One-Step RT-qPCR Kit. The reaction setup was as follows and each sample was running 4 times :

COMPONENT	10 µl REACTION	FINAL CONCENTRATION
Luna® Universal One-Step Reaction Mix(2X)	5 µl	1X
Luna WarmStart RT Enzyme Mix (20X)	0.5 µl	1X
Forward primer (µM)	0.4 µl	0.4 µM
Reverse primer (1µM)	0.4 µl	0.4 µM
Template RNA		500 ng (total RNA)
Nuclease-free water	Up to 10 µl	

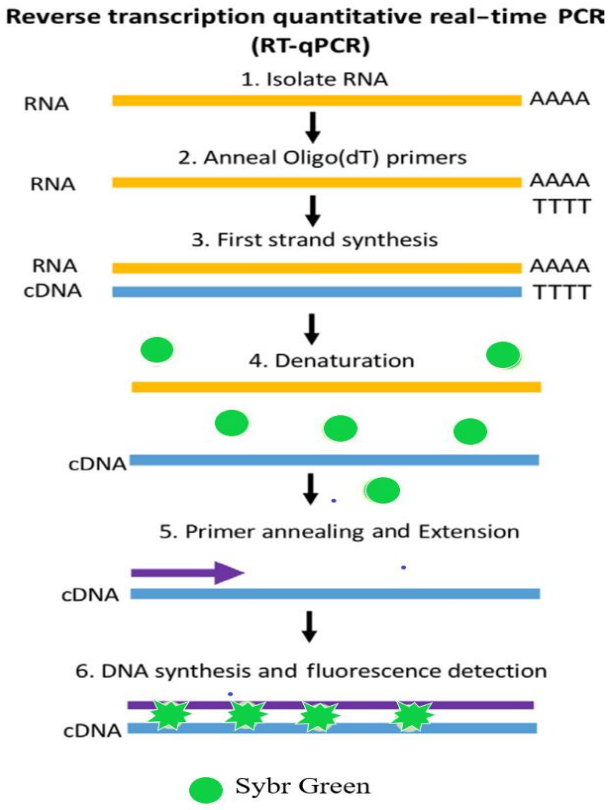

Figure 10. Schematic of SYBR green-based RT-qPCR steps. The cDNA obtained by reverse transcription is amplified in a PCR, which involves 40-45 rounds of denaturation, primer annealing, and extension by *Taq* DNA polymerase. The PCR product is detected by binding of fluorecent dye. For simplicity the second primer and subseqent PCR cycles are not shown. The figure is modified from (Graham et al. 2021).

RT-qPCR conditions was as follows:

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55	10 minutes	1
Initial Dentaturation	95	3 minutes	1
Denaturation Annealing Extension	95 60 72	30 seconds 30 seconds 30 seconds	40-45
Melt Curve	60-95	various	1

The relative expression level of nsP1 was calculated by the comparative C_T Method ($2^{-\Delta C_T}$), using GAPDH as an endogenous control. C_T (threshold cycle), is the cycle number at which flouroscence intensity crosses the background signal.

The ΔC_T value was calculated by formula:

$$\Delta C_T = C_{T\ nsP1} - C_{T\ GAPDH}$$

The standard deviation of the ΔC_T was calculated from the standard deviations of the nsP1 and GAPDH mRNA values using the formula:

$$S = (S_1^{\,2} + S_2^{\,2})^{1/2}$$

Thus, determining the best colony with highest expression of nsP1 was performed for each of these stable cell lines according to the number of $2^{-\Delta C_T}$. The higher value corresponds to the higher level of nsP1 mRNAs and presumably the highest levels of the protein.

2.2.6. Immunofluorescence assay (IFA)

To study the intracellular localization of nsP1, each of selected stable cell lines was examined by immunofluorescence assay (IFA); parental U2OS-T-Rex cells were used as controls. Cells were plated in 6 well cell culture plates (wells were containing glass cover slips) at 25% confluency and incubated overnight at 37°C. Next, cells were induced with DOX. 24h post-induction, cells on cover slips were washed three times with PBS and fixed for 20 minutes with 4 % paraformaldehyde (PFA) in PBS. After removing of fixative, cells were washed again three times with PBS and were permeabilized with 0.5% Triton-X100 in PBS for 5 minutes at room temperature; permeabilization

was followed by a washing step. In the next step, non-specific binding sites were blocked by incubation in 5% foetal calf serum overnight at 4 ^{o}C. When blocking was completed, cells were washed again. Immunostaining step was performed by adding the desired concentrations of primary antibody in the blocking solution. Anti-CHIKV-nsP1 antibody (1:1000 dilution) and anti-SFV-nsP1 antibody (1:500 dilution) was used as a primary antibody in the case of CHIKV-nsP1 and its mutations and SFV nsP1 and its derivative, respectively. In the case of RRV nsP1, SFV-nsP1 antibody (1:500 dilution) was used as a primary antibody; it is possible because of RRV nsP1 protein is similar enough to nsP1 of SFV to allow detection with corresponding antibody. After 24 hours incubation with primary antibody at 4 ^{o}C, cells were washed three times with PBS and treated with the goat anti-rabbit antibody conjugated to Alexa Fluor™ 568 (1:500) and with rhodamine-conjugated concanavalin A (ConA) (1:20) to stain plasma membrane. In the case of CHIKV-nsP1$^{1C\text{-}GFP}$, wheat germ agglutinin (WGA) conjugated with Alexa Fluor™ 555 (1:20 dilution) was used to stain cytoplasm while nsP1-EGFP fusion protein was detected using its autofuoresence at 488 nm. All the antibody dilutions were made in the same blocking solution. Following 1 hour incubation with secondary antibodies, cells were washed three times with PBS solution; 4′,6′-diamidino-2-phenylindole (DAPI) (Life Technologies) was added to counterstain nuclei. Slides were mounted using SlowFade Gold reagent. Cells were imaged using EVOS M5000 Imaging System.

2.2.7. Trans-replicase assay

The plasmids which were transfected to our selected cell line were previously constructed in our laboratory and named as follows. The CHIKV replicase expression plasmids that include variants harbouring different mutations in nsP1 were CHIKV P1234, CHIKV P1^{H37A}234, CHIKV P1^{D63A}234, CHIKV P1^{R252E} 234, CHIKVP1^{3C3A}234, CHIKV P1^{Y258A}234, and CHIKV P1234^{GAA}. The template RNA expression plasmid was based on use of human RNA polymerase I promoter and named HSPolI-FG-CHIKV (Figure 8).

All selected cell lines (20,000 cells/well) were seeded onto 48-well plate one day before transfection. For the trans-replicase assays in 48-well plate, 250 ng of plasmids encoding for the replicases and 250 ng of plasmids encoding for the CHIKV template RNA were used. Transfection was performed using Lipofectamine LTX® with PLUS^{TM} reagent (Invitrogen^{TM}) according to manufactures instructions. 5 hours post-transfection, the media was replaced with growth media with or without 1µg/ml of DOX. At 24 hours post transfection, cells were lysed in Passive Lysis

Buffer and the activitis of Fluc and Gluc were measured using the Dual Luciferase® Reporter Assay System Kit (Promega) by the GloMax® 20/20 luminometer (Promega). Each transfection was performed in triplate and experiments were repeated at least twice, Fluc and Gluc activities in experiemental cells were normalized to these from control cells transfected using CHIKV P1234^{GAA}. Statistical analysis was performed using GraphPad Prism 8.2.0 software. Data was analyzed using Student's unpaired one-tailed *t* test. P values of <0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****) were considered staistically significant.

2.2.8. Rescue of virus with lethal defects in CHIKV nsP1 cell lines

The cells which can stably express CHIKV nsP1 or parental T-Rex U2OS cells were seeded in 6-well plates. At next day cells were transfected with 5 μg of endotoxin-free plasmid CMV-ICRES1, CMV-ICRES1^{D63A}, CMV-ICRES1^{3C3A}, CMV-ICRES1^{R252E}, CMV-ICRES1^{Y148A} (these plasmids containing infectious cDNA of CHIKV under the control of CMV promoter were previously constructed in our lab). 5h post-transfection, media was replaced with media containing 2% FBS plus 1 μg/ml DOX. 48h post-transfection, primary virus stocks (P_0) were collected. The transfected cells were collected at the same time as the corresponding stocks. Cells were lysed by boiling in 1xLaemmli buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreitol, 0.2% bromophenol blue). The primary virus stock was used to infect parental cells and wt nsP1 expressing cells using 0.5 ml viral P_0 stock and 1,5 ml viral growth media containing 0.2% BSA. 4 h post infection media was replaced with growth media containing 2% FBS containing and DOX 1 μg/ml. 48 h later, supernatants (P_1 stocks) were collected; at the same time cells were harvested and lysed as described above. The presence of virus infection in transfected and P_0 stock infected cells was checked by detecting capsid protein using western blot. Lysate cells were loaded on a 12% polyacrylamide gel. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected using primary antibodies against CHIKV capsid protein (in-house), loading control was detected using anti-β-actin antibody (catalog number sc-47778; Santa Cruz Biotechnology). The membranes were then incubated with appropriate secondary antibodies conjugated to fluorescent labels, and proteins were visualized using a LI-COR Odyssey Fc imaging system. All these experiments were repeated three times. Due to classification of CHIKV as biosafety level 3 pathogen, the work with infectious viruses was performed in biosafety level 3 (BSL3) laboratory. Due to the restriction of working in BSL3, viral rescue was completely performed by my supervisor, Sainan Wang.

2.3. Results and discussion

2.3.1 Selection of optimal conditions of induction of nsP1 expression.

To reveal optimal conditions of nsP1 expression we used SFV nsP1^{3C3A} and CHIKV nsP1 cell lines as examples. In these experiments we induced expression with DOX taken at 0.5, 1 or 2 μg/mL and induced cells for 24 or 48 h. As shown on Figure 11, DOX taken at 1 μg/mL was sufficient to induce nsP1 expression at maximal (or close to maximal) level; as for optimal time points the levels of nsP1 at 24 h post induction were higher than 48 h post induction. Since similar results were obtained for two different nsP1 proteins and because performing such an optimization for all cell lines would have been time-consuming and impractical, the selected conditions (DOX taken at 1 μg/mL, induction for 24 h) were used to evaluate all other selected cell lines.

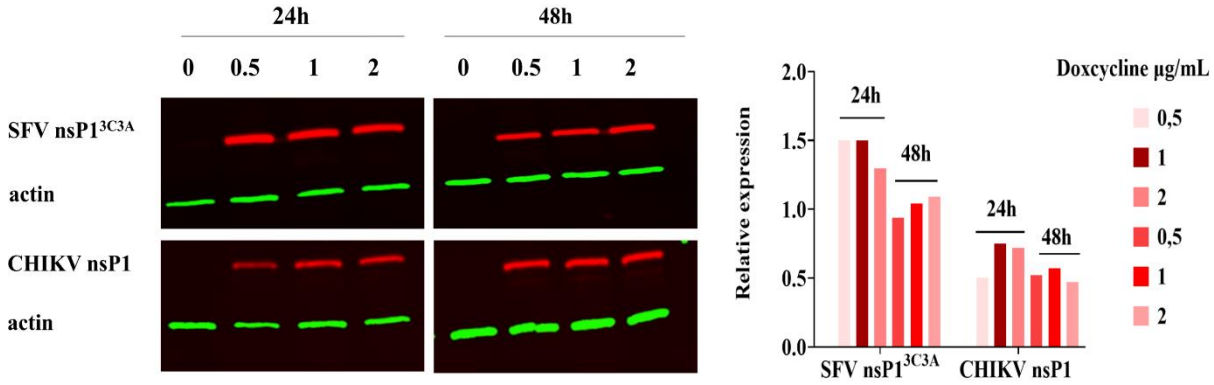

Figure 11. Determination of optimal DOX concentration and induction time for cell lines expressing SFV nsP1^{3C-3A} and CHIKV nsP1. Induced protein were detected using corresponding rabbit polyclonal antibodies, anti-beta-actin antibody was used to detect loading control. The results of western blot were quantified using ImageJ software and levels of nsP1 were normalized to these of actin (loading control). The graphs were obtained by graphpad using data of ImageJ.

2.3.2. Selection of best colony for CHIKV and SFV nsP1 and its mutant cell lines using western blot

Using conditions established in previous experiments, we performed comparison of CHIKV nsP1 (and its mutants) and SFV nsP1 (and its mutant) expression in individual cell lines derived from zeocin resistant different colonies. It was found that for cell lines expressing some nsP1 proteins, for example CHIKV nsP1^{3C3A}, relative expression levels in all analyzed cell lines were similar. For other nsP1 proteins, such as SFV nsP1 and CHIKV nsP1^{W258A}, large variation of relative expression in different cell lines was detected (Figure 12). No obvious correlation between the origin (SFV or CHIKV) or functionality (wt or mutant) and variation of expression levels between different cell

lines was observed. Thus, most likely the variation was caused by random events such as copy number of integrated expression cassets and/or sites of their integration. Based on this analysis cell lines with the highest relative expression levels were selected for further analysis as follows: CHIKV nsP1 – colony 3; CHIKV nsP1^{H37A} – colony 3; CHIKV nsP1^{D63A} – colony 2; CHIKV nsP1^{3C3A}- colony 1, CHIKV nsP1^{W258A} – colony 3; SFV nsP1 - colony 1 and SFV nsP1^{3C3A} – colony 3.

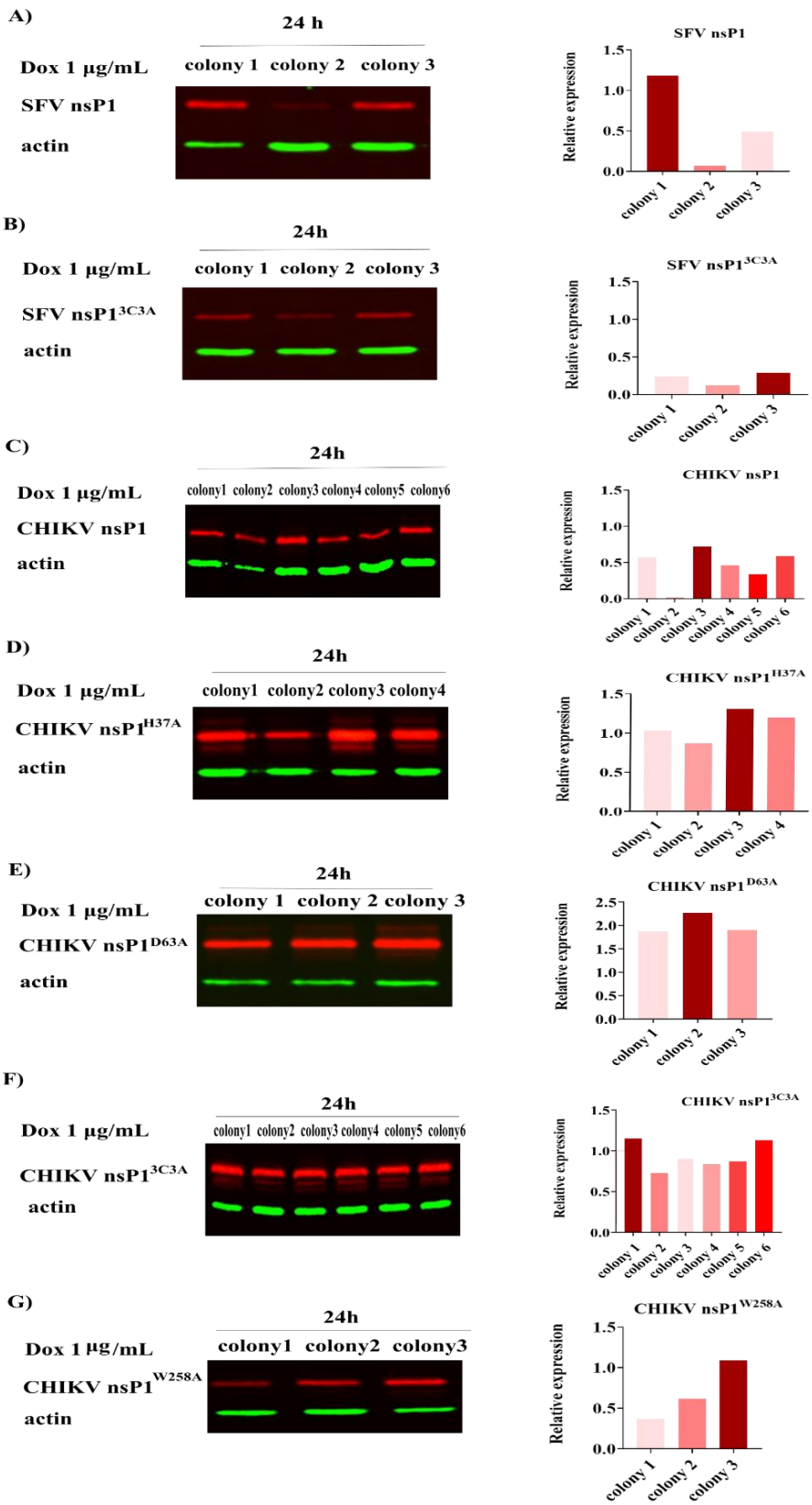

Figure 12. Relative expression of SFV and CHIKV nsP1 proteins in induced cell lines. Analysis was performed as described in legend of Figure 11 except that only one concentration of DOX and one induction time were used.

CHIKV nsP1$^{1C\ GFP}$ cell line expresses nsP1-EGFP fused protein that is detectable by its auto-fuorescence. Therefore, we decided to use flow cytometry assay, a method of cell seperation that can target the proteins of interest (in this case nsP1-EGFP) based on the specific light scattering and fluorescent characteristics of each cell (Cerda et al. 2021), and find out not only relative expression level of the fusion protein but also the proportion of EGFP-positive cells for each single colony derived cell line. As shown on the Figure 13A, the percentage of EGFP-positive cells was the highest in case of cell line derived from colony 4. However, suprisingly, it was still way below expected 100% (that would be the case for single cell derived cell line) indicating that either colonies were not made by a single progenitor, or some cells lost the gene of interest during of colony expansion or, that assay conditions were not ideal and lot of cells were counted as false negative. Subsequent analysis performed using fluorecent microscopy are coherent with the third possibility (see Figure 17). In addition, we also did western blot to detect CHIKV nsP1-EGFP expression (Figure 13B). Thus, we can conclude that this colony can express the highest amount of nsP1 and that the protein expressed is indeed nsP1-EGFP fusion protein.

A)

Comparsion the GFP ratio for CHIKV nsP1$^{1C\ GFP}$

nsP1	GFP %
Colony 1	6.27
Colony 2	8.81
Colony 3	1.96
Colony 4	24.92

B)

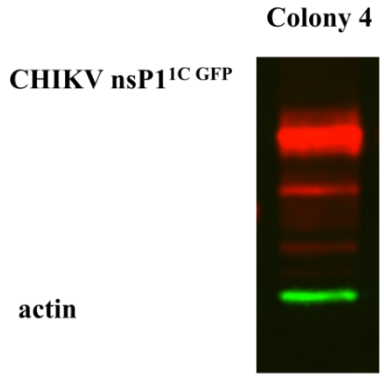

Figure 13. A) Comparison of the percentage of EGFP positive cells in different induced CHIKV nsP1$^{1C\ GFP}$cell lines. Cells were seeded in 6-well plates and induced with 1 µg/mL of DOX. At 24 h post-induction, cells were collected, and analyzed with an Attune NxT Acoustic Focusing Cytometer**. B) Detection of expression of nsP1-EGFP in CHIKV nsP1$^{1C\ GFP}$ selected cell line.** nsP1-EGFP fusion protein was detected using a rabbit polyclonal antibody against CHIKV nsP1. A mouse monoclonal antibody against actin was used to detect loading control.

2.3.3. Selection of EEEV, SINV, and RRV cell lines

As we didn't have primary antibodies against nsP1 proteins of EEEV, SINV and RRV, we decided to set up and used RT-qPCR for quantification of mRNAs of nsP1 of EEEV, SINV and RRV in corresponding induced cell lines. Among the available alternatives, one-Step procedures are preferred as they are fast and remarkably diminish preanalytical errors (Cerda et al. 2021). As shown before, we used comparative C_T method to analyze the results. The colony which has higher $2^{-\Delta C_T}$ was considered as the one with the highest inducible expression level of nsP1 for each selected cell line: for nsP1 of EEEV it was colony 3, for SINV nsP1 it was colony 1 and for RRV nsP1 it was colony 1. Cell-lines derived from these colonies were used for subsequent expreiments.

Cell line	nsP1 (Average C_T)	GAPDH (Average C_T)	ΔC_T (nsP1 – GAPDH)	$2\text{-}\Delta C_T$
EEEV colony 1	**15.04**	**11.82**	**3.22**	**0.10732068**
EEEV colony 2	**15.25**	**11.55**	**3.69**	**0.07707998**
EEEV colony 3	**13.80**	**11.75**	**2.05**	**0.24148408**
SINV colony 1	**11.46**	**11.47**	**-0.01**	**1.00695555**
SINV colony 2	**11.71**	**11.50**	**0.20**	**0.870550563**
RRV colony 1	**16.43**	**12.15**	**4.28**	**0.05147444**
RRV colony 2	**17.94**	**12.45**	**5.49**	**0.02225078**
RRV colony 3	**17.15**	**11.11**	**6.03**	**0.01530344**

Figure 14. Comparison of relative levels of nsP1 mRNAs in different DOX induced EEEV, SINV and RRV cell lines by RT-qPCR. Data were analyzed using relative quantitation of gene expression.

2.3.4. Subcellular localization of wild type and mutant nsP1 proteins

To study the subcellular localization of different nsP1 proteins the T-Rex U2OS cell lines expressing proteins of CHIKV and SFV were seeded in 6-well plates and induced with 1 µg/mL of DOX. 24h post-induction, cells were fixed, permeabilized, and treated with corresponding primary and secondary antibodies. Results from immunofluorescence microscopy are shown in Figures 15, 16 and 17.

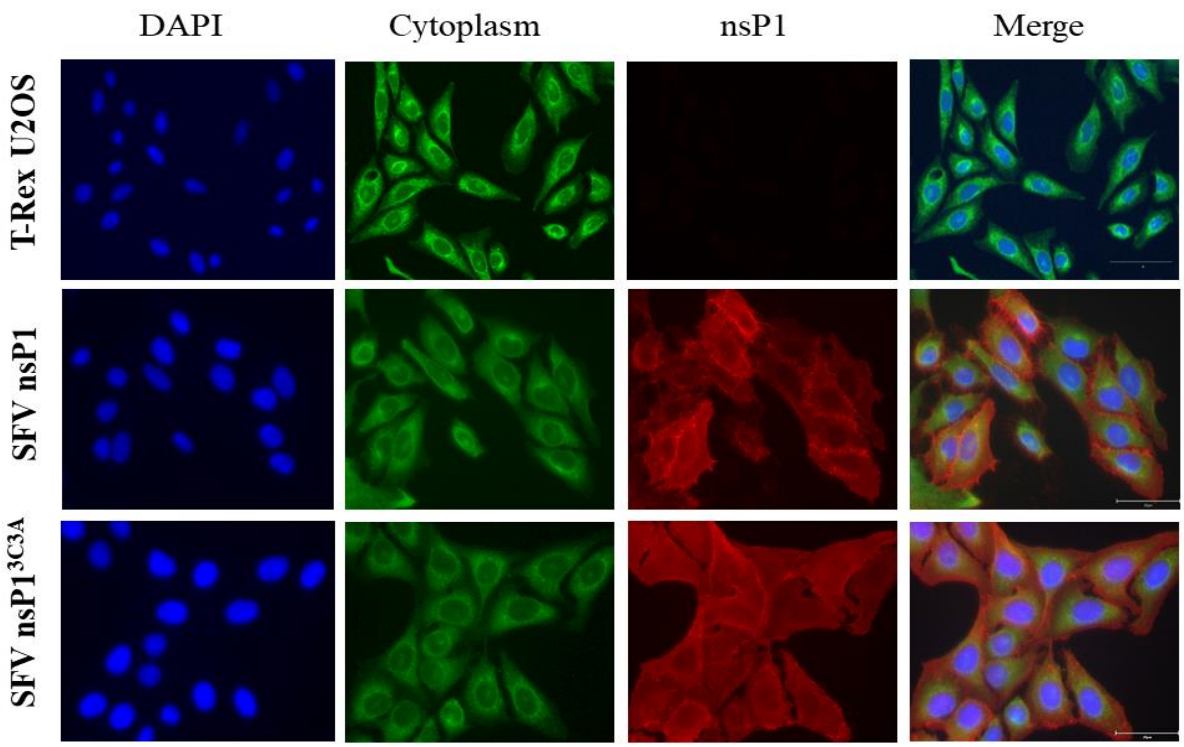

Figure 15. Subcellular localization of SFV nsP1 and SFV nsP1^{3C3A} in induced in T-Rex U2OS based cell lines. nsP1 staining is shown with red, plasma membrane was stained with rhodamine conjugated concanavalin A (green). Upper row – parential cells not expressing nsP1 (negative control).

Expression of nsP1 is known to cause significant morphological changes in induced cells, especially in the plasma membrane. In induced T-REx-nsP1 cells, wt nsP1 indeed localizes mostly to the plasma membrane, resulting in the extensive formation of filopodia-like structures. This was the same in our results - we did observe both localization of SFV nsP1 in plasma membrane and formation of filopodia-like structures. SFV nsP1^{3C3A} (defective in palmitoylation site) induced fewer filopodia-like structures but has still dominant plasma membrane localization; this is consistent with previous studies (Laakkonen et al. 1998; Spuul et al. 2007). Formation of fewer filopodia-like structures is also characteristic for this mutant protein (Žusinaite et al. 2007).

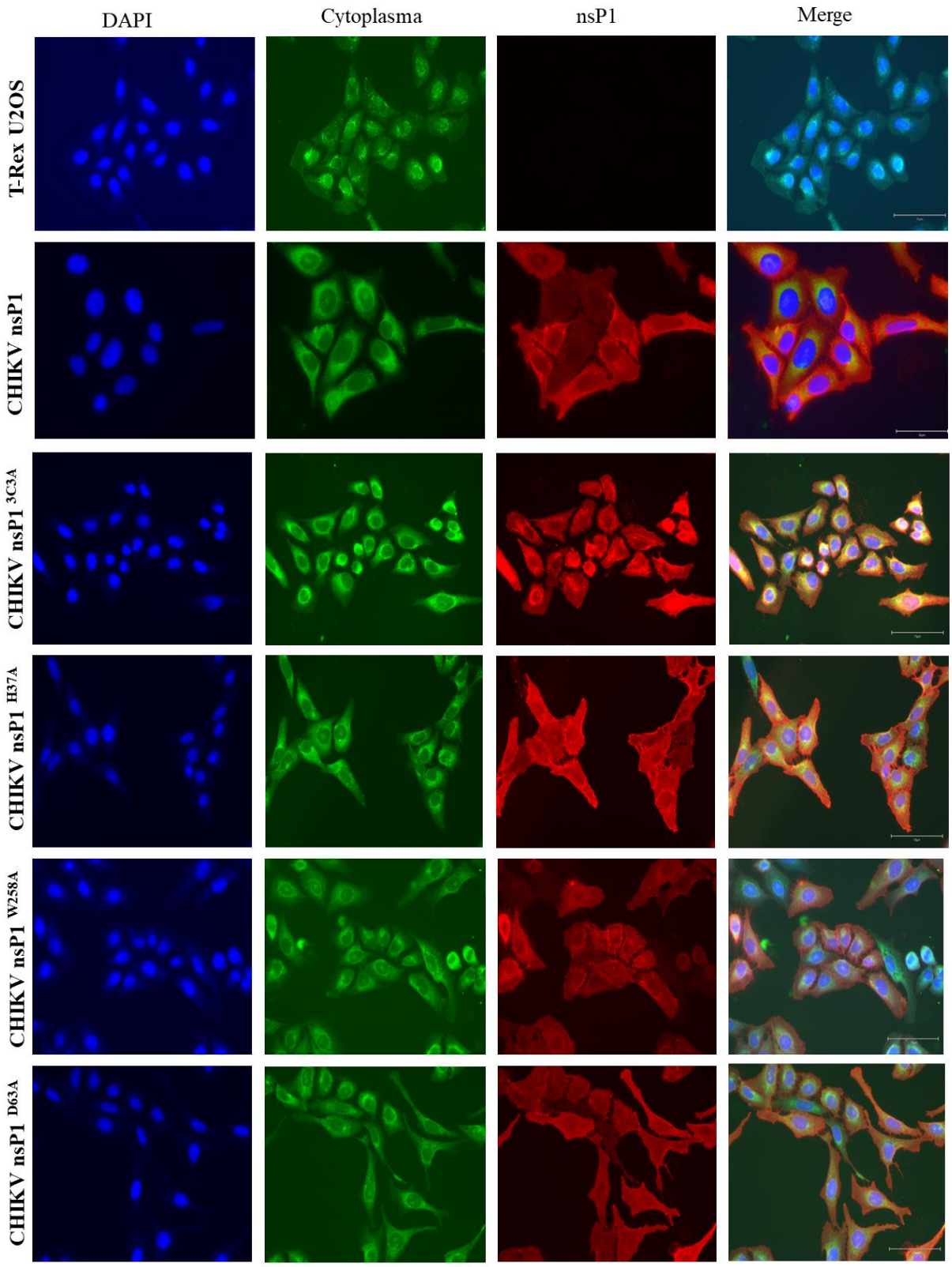

Figure 16. Subcellular localization of CHIKV nsP1 and its mutant variants in induced in T-Rex U2OS based cell lines. nsP1 staining is shown with red, plasma membrane was stained with rhodamine conjugated concanavalin A (green). Upper row – parential cells not expressing nsP1 (negative control).

The results obtained for cell lines expression CHIKV nsP1 were similar to those obtained for nsP1 of SFV. nsP1 and its mutants localized to plasma membrane. Expression of wt nsP1 and that of nsP1 harbouring W258A substitution resulted in formation of filopodia-like structures. Proteins harbouring H37A or D63A mutations behaved very similarly (possibly had minor reduction of the formation of filopodia). Expression of nsP1 harbouring mutation in palmitoylation site resulted in diminished formation of filopodia-like structures; however, even this mutation didn't change the localization of nsP1 to plasma membrane.

Several studies have demonstrated that the formation of the RCs of alphaviruses is associated with the binding of nsP1 to the plasma membrane (Zhang et al. 2021; Tan et al. 2022). Our results clearly demonstrate that, indeed, CHIKV nsP1 and its mutants did localize to plasma membrane where, with exception of protein harbouring mutation in palmitoylation site, caused formation of filopodia-like structures. Thus, the lethal defect associated with these mutations is not caused by wrong subcellular localization of mutant proteins. For H37A and D63A the nature of the defect is obvious (lack of capping of positive strand RNAs). The fact that W258A mutation did not alter localization of nsP1 of CHIKV also suggest that the mutation somehow interferes with enzymatic activities of nsP1 but not with its localization. Only nsP1^{3C3A}, a mutation that is completely lethal for CHIKV (Zhang 2019; Utt et al. 2019), caused loss of filopodia in the structure.

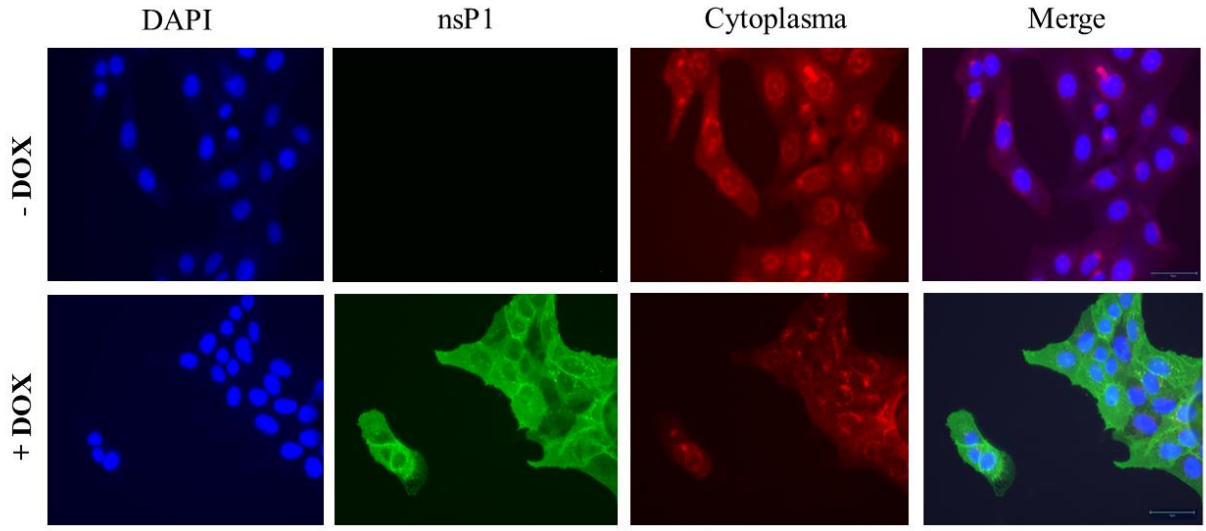

Figure 17. Subcellular localization of CHIKV nsP1-EGFP fusion protein. Induced (DOX+) and non-induced (DOX-) T-Rex U2OS cells encoding CHIKV nsP1^{1CGFP} were fixed, treated with WGA Alexa 555 to stain the cytoplasm (red) and DAPI to stain nuclei. nsP1-EGFP expression (green) was detected using its autofluorescence.

Since our CHIKV nsP1$^{1C\ GFP}$ cell lines express nsP1-EGFP fusion, it was detected using autofluoresence. As expected, the nsP1-EGFP is diffused in the cytoplasm with some localization on plasma membrane, these observations are consistent with previous reports (Utt et al. 2016; Bakhache et al. 2020). We could also detect induction of filopodia-like structures covering the entire cell surface. Thus, GFP-nsP1 behaves rather similar to native nsP1. This analysis also revealed expression of nsP1-EGFP in all induced cells which contrast with findings made using flow cytometry (Figure 13A). As at the case of all proteins, analyzed using immune florescence, the expression was always detected in all cells and the levels of nsP1 in different cells showed little to know variation it is almost certain that data obtained using flow cytometry was flawed, most likely because use of sub-optimal parameters. It is safe to conclude that each cell line for which the immunofluorescence analysis was performed indeed represents progeny of a single originally transfected cell.

2.3.5. The CHIKV nsP1 expressed in inducible cell lines can complement activity of CHIKV trans CHIKV replicase inactivated due to mutations in its nsP1 region.

We generated cell lines expressing nsP1 (or its mutants) of SFV, RRV, SINV and CHIKV from Old World alphaviruses and EEEV from New World alphaviruses. The expression and correct subcellular localization of nsP1, mean that it is a functional protein. To confirm this, it is needed to demonstrate that nsP1 expressed in these cells can act as a component of functional RC. Studies in our lab have revealed that transient expression of nsP1 together with P23 and nsP4 results in formation of RCs but the efficiency of such a system is rather low, possibly because of different subcellular localization of individually expressed nsP1 and P23. Therefore, we decided to use an alternative approach based on the use of highly sensitive *trans*-replication system. Previously our lab has generated panel of mutations in nsP1 region of P1234 expression plasmid and revealed that many of these mutant replicases have no ability to synthesize viral RNAs (Utt et al. 2019). Here we decided to analyze whether the activites of these replicases can be restored by nsP1 expressed by our cell lines.

Firstly, we assessed the replication and transcription activities of defective CHIKV replicases in the cell line expressing wt CHIKV nsP1. It is observed that induction of CHIKV nsP1 expression significantly increases Fluc and Gluc boosts (hereafter replication and transcription activities) of all mutant replicases except CHIKV P1^{R252E}234. At the same time induction of nsP1 expression had no impact on activities of CHIKV wild type replicase (Figure 18A). So, this data clearly

demonstrated that the functional nsP1 expressed by cell can be used in formation of active replicases by CHIKV P1234 with most of nsP1 mutations. It is not clear how this occurs but most likely defective (from mutant P1234) and functional (from cell) nsP1 subunits form „mixed" dodecamerics and if substantial number of its subunits are functional, a replicase using this ring is functional as well. It also come apparent that the extent to which replication and transcription are restored depends on the mutations in the nsP1 part of P1234 of CHIKV. As the results suggested, nsP1 mutations of H37A, D63A, Y248A, blocking the RNA capping, were efficiently trans complemented. Most likely this indicates either that only few enzymatically active subunits are needed to make the nsP1 ring-structure functional. No trans-complemetation whatsoever was observed for P1234 with R252E mutation. Most likely this means that the defect caused by this mutation is hard/impossible to compensate for. Very likely this means that wild type (cell expressed) and mutant nsP1 fail to form active ring structures. This may be beacuse mutation has dominant negative effect (i.e. one defective subunit makes all structure non-functional) or because the wt nsP1 and the one with R252E mutation (and P23 part attached to it) simply localize differently and cannot therefore interact with each other. Interestingly, the activity of CHIKV $P1^{3C3A}234$ could be restored by wt nsP1. This was interesting, as it has been shown that 3C3A mutation determining CHIKV nsP1 sensitivity to cholesterol distribution does change localization of the proteins in different subdomains of plasma membrane (Bakhache et al. 2020). Still, it looks likely that overall the localization of mutant protein is similar enough to the wild type one (indeed, our immunofluoresence analysis agrees with this) to allow interaction between mutant and wt nsP1 proteins.

Unlike the CHIKV nsP1, the expression of nsP1-EGFP fusion protein has no positive effect on activity of any mutant replicase except that formed by CHIKV $P1^{3C3A}234$ (Figure 18B); instead the expression even reduced activites of wild type CHIKV replicase. The reduced ability of fusion protein to support replication is consistent with previous finding (Utt et al. 2016), however how exactly the protein intereres with activity of wt replicase remains unclear. It is also not obvious why this protein enhanced activity of CHIKV $P1^{3C3A}234$-based replicase. This replicase apparently does not suffer from defects in enzymatic activities of nsP1 (see below) but does not locate correctly in plasma membrane (Bakhache et al. 2020). So, it is possible that nsP1-EGFP is able to compensate for this defect but not any other and, if so, this represents an interesting topic for futher studies. However, it may also be that the stronger attenuation of CHIKV $P1^{3C3A}234$ made the boosting effect from expression of nsP1-EGFP easier to observe.

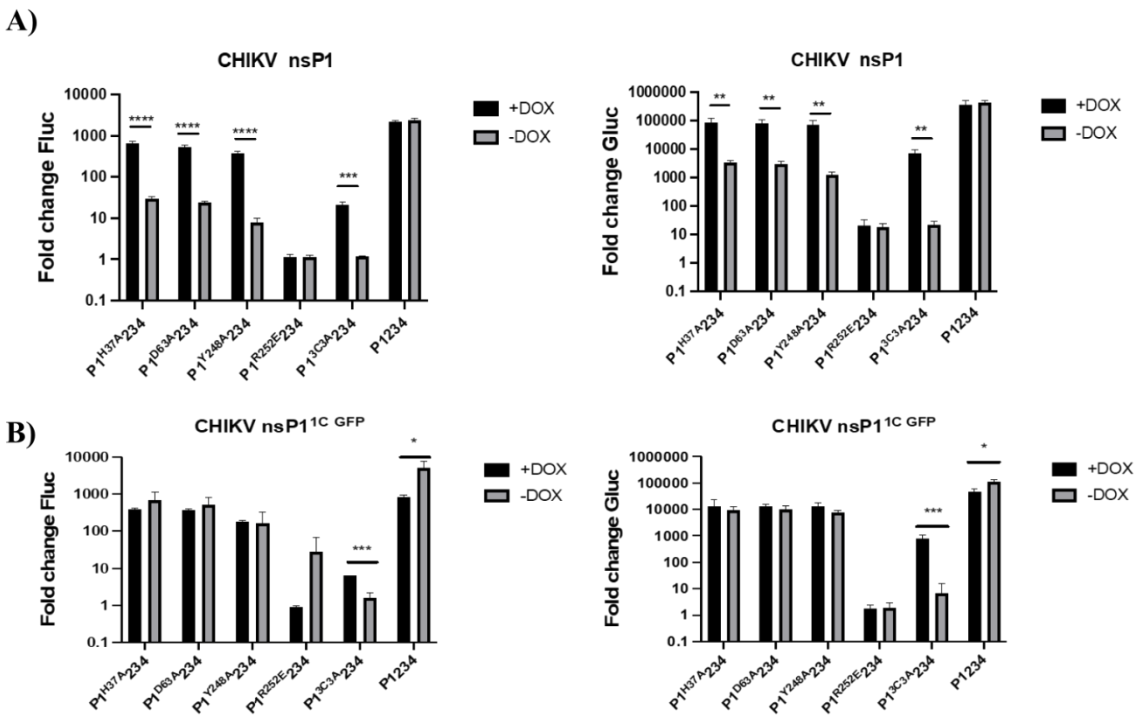

Figure 18. CHIKV trans replicases with inactivating mutations in nsP1 regions are differently activated upon induction of expression of CHIKV nsP1. T-Rex U2OS cells expressing CHIKV nsP1 were transfected with the HSPoll-FG-CHIKV template expression construct and plasmids for expression of indicated P1234 polyproteins. All activities measured in the experiment are presented as normalized to the control (cells transfected with polymerase inactive P1234^{GAA}).

2.3.6. Mutations affecting capping activities of CHIKV replicases can be compensated by expression of nsP1 of closely related alphaviruses.

As we have seen the restoration of activities of replicase mutants P1^{H37A}234, P1^{D63A}234 and P1^{Y248A}234 in the presence of CHIKV nsP1. Interestingly, very similar result was obtained using cell line for expression of nsP1 of RRV nsP1 (Figure 19A), clearly indicating that the nsP1 of RRV can be incorporated into CHIKV replicase and works together with CHIKV nsP2, nsP3 and nsP4. In contrast, expression of nsP1 of SINV or EEEV had much smaller impact; however it can be seen that these nsP1 proteins also restored, albeit not always significantly, replicase activity of P1^{H37A}234, P1^{D63A}234 and P1^{Y248A}234 polyproteins (Figure 19B, C). Besides, the expression of SINV and EEEV nsP1 also had negative effects on activities of CHIKV wild type replicase. Most likely all these effects originate from poor compatibility of nsP1 of CHIKV and that of more distantly related alphaviruses. For mutant replicases it was not enough to result in prominent increase of activity, for wt replicases it is possible that heterologous subunits interfered with

formation of correct replicase core structures. It may be that interactions between nsP1 subunits were altered or, even more likely, that interaction of nsP1 and nsP4 was disturbed.

Taken together, our results clearly demonstrated that the functional replication complexes could be formed when RRV nsP1 (and, to an extent, SINV or EEEV nsP1) were provided to the CHIKV replicases inactivated due to mutations (H37A, D63A and Y248A) blocking its own capping activity.

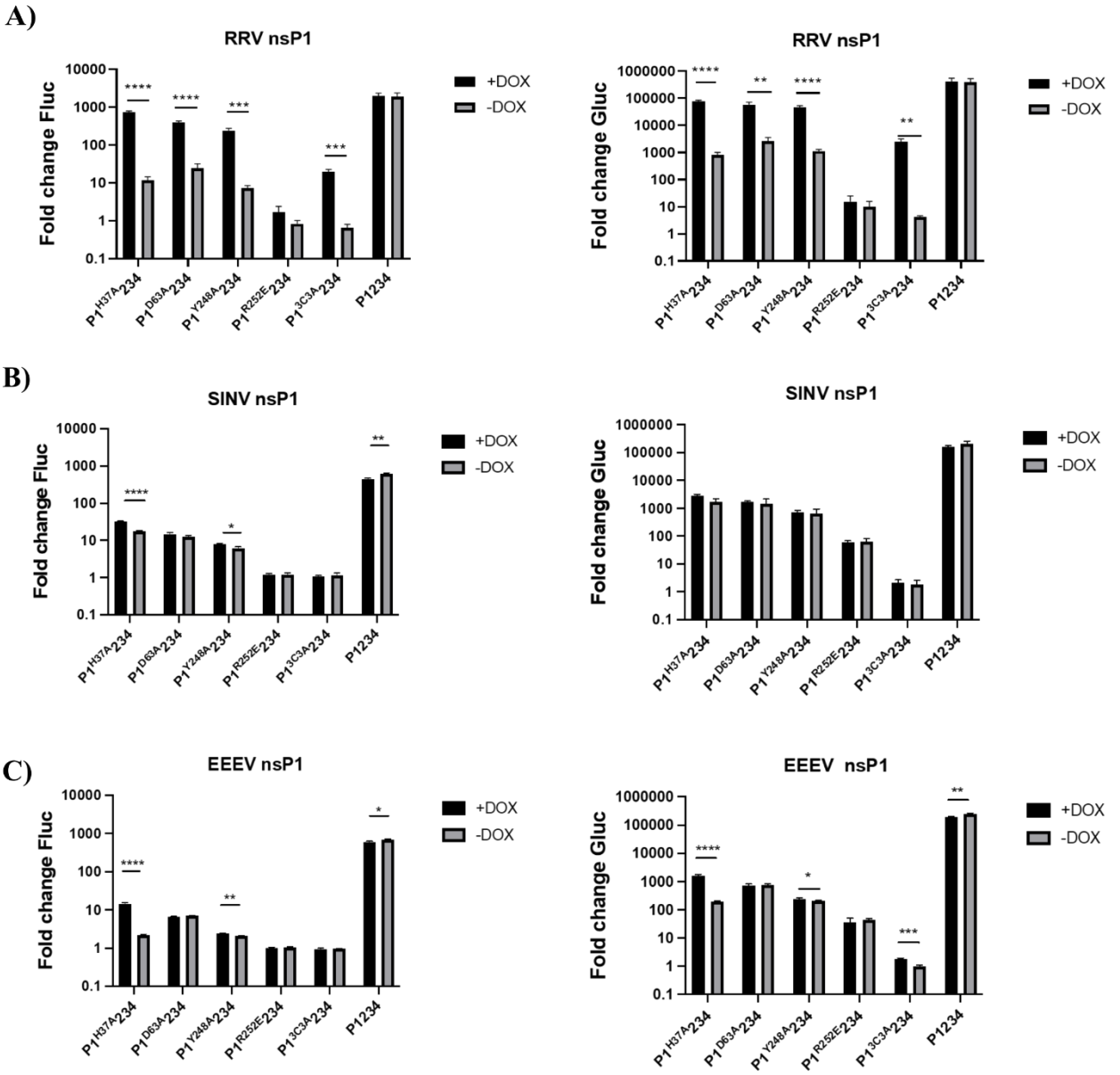

Figure 19. CHIKV trans replicases with inactivating mutations in nsP1 regions are activated upon induction of expression of nsP1 of RRV, SINV or EEEV. T-Rex U2OS cells expressing nsP1 of RRRV (A), SINV (B) or EEEV (C) were transfected with the HSPoll-FG-CHIKV template expression construct

and plasmids for expression of indicated P1234 polyproteins. All activities measured in the experiment are presented as normalized to the control (cells transfected with polymerase inactive $P1234^{GAA}$).

2.3.7. Activity of CHIKV replicase harbouring mutation in palmitoylation site can be restored by expression of enzymatically inactive variants of CHIKV nsP1.

General rule of trans complementation is that proteins with mutations affecting different functions can trans-complement each other while proteins harbouring same or similar defect cannot. This was also confirmed using cell lines expressing mutant nsP1 proteins of CHIKV transfected with panel of replicase mutations. Induction of expression of CHIKV $nsP1^{H37A}$ or CHIKV $nsP1^{D63A}$ had no positive impact on activities of replicases formed by $P1^{H37A}234$, $P1^{D63A}23$ or CHIKV $P1^{Y248A}234$.In contrast, expression of both of these proteins activated replicase formed by $P1^{3C3A}234$ and, at the case of induction of $nsP1^{D63A}$ expression, the effect was highly significant (Figure 20A, B). At the same time induction of expression of CHIKV $nsP1^{3C3A}$ compensated the replicase activities of $P1^{H37A}234$, $P1^{D63A}234$ and $P1^{Y248A}234$ but not these of replicase formed by $P1^{3C3A}234$ (Figure 20C). Results obtained for expression of CHIKV $nsP1^{W258A}$ were less clear, possibly because the experiment was conducted at not completely restrictive temperature (37 ^{o}C instead of 39 ^{o}C). Nevertheles the trend was more similar to that seen for CHIKV $nsP1^{H37A}$ or CHIKV $nsP1^{D63A}$ than that for CHIKV $nsP1^{3C3A}$ expression (Figure 20D) confirming above presented hypothesis that W258A mutation more likely affects capping activities of CHIKV nsP1 than its binding to memberanes.

The clear conclusions based on this data are following – lethal mutations in nsP1 either affect capping activities (H37A, D63A, Y248A and possibly W258A) or localization of nsP1 in general (likely R252E) or inside the microdomains of plasma membrane (3C3A). It is clear that the nsP1 harbouring mutations blocking its enzymatic activity can form functional (albeit not very active) replicase with nsP1 harbouring mutation in palmitoylation site. This implies that the profound negative effect of 3C3A mutation on CHIKV trans-replicase activity and for infectivity of corresponding mutant virus is not caused by lack of enzymatic activities of $nsP1^{3C3A}$. The mechanism behind the effect of this mutation remains elusive, further experiments are needed to check what is going on.

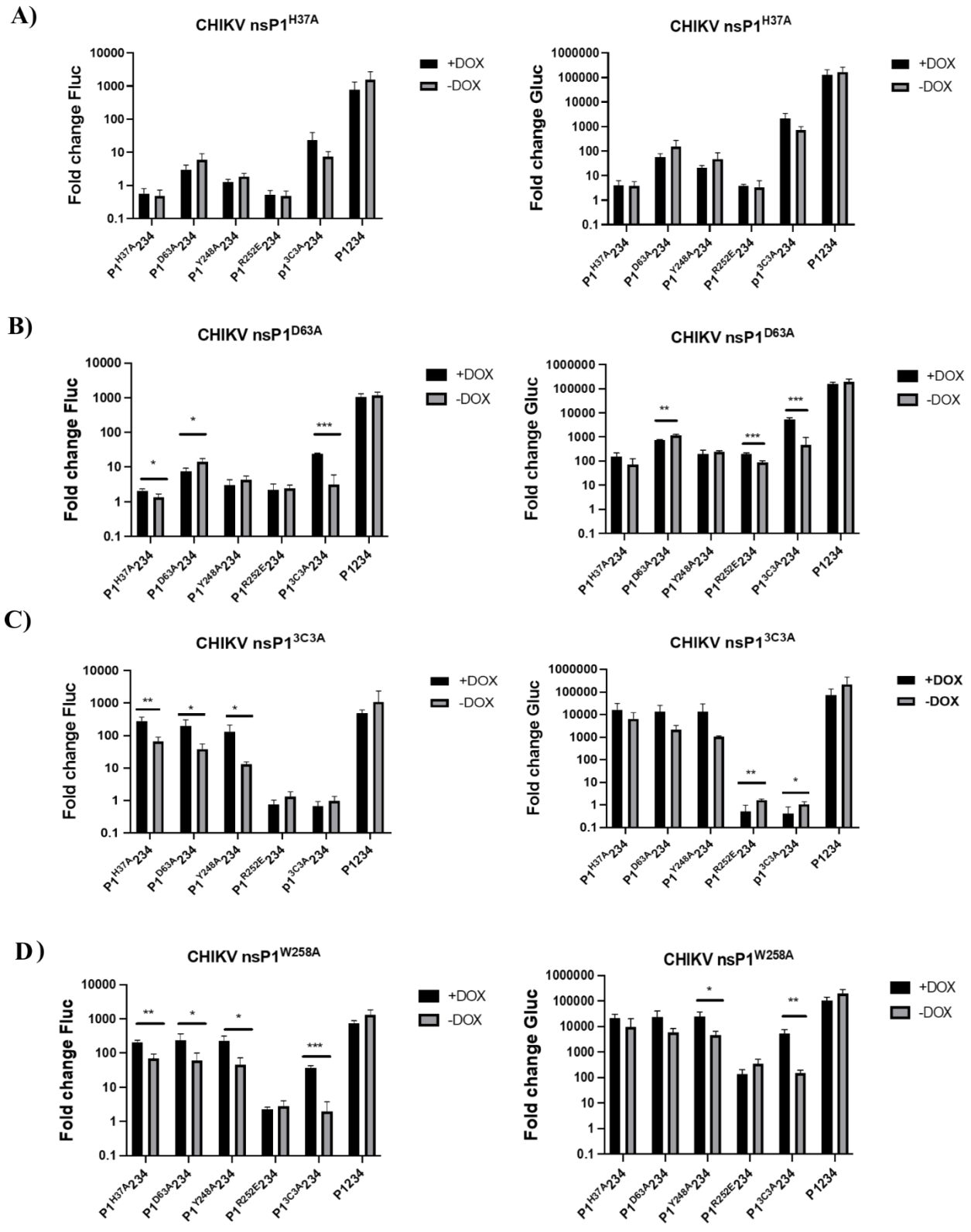

Figure 20. Lethal mutations in nsP1 of CHIKV can be devided into severla complementation groups. T-Rex U2OS cells expressing CHIKV nsP1 with H37A (A), D63A (B), 3C3A (C) or W258A (D) mutations were cotransfected with HSPolI-FG-CHIKV template expression construct and plasmids for expression of

indicated P1234 polyproteins plasmids. CMV-P1234^{GAA} was used instead of CMV-P1234 as a negative control. The expression of mutant nsP1 proteins was either induced with DOX or not induced. Data was collected, analyzed and presented as described for Figure 19.

2.3.8. The presence of CHIKV nsP1 could restore the infectivity of CHIKV infectious cDNA with D63A and Y248A mutation in nsP1.

Although we observed that expression of wt nsP1 can restore activity of mutant trans-replicases, it was important to analyse whether it also occurs in more relevant context – for mutant virus genomes. CMV promoter launched CHIKV infectious cDNA (icDNA) or its mutant variants (nsP1 with D63A, 3C3A, R252E or Y148A mutations) previously constructed in our lab were used to transfect T-Rex U2OS-CHIKV nsP1 induced for the expression of viral proteins and parential T-Rex U2OS cells (as a control). It was found that, as expected, wild type CHIKV can be rescued in both cell lines. At the presence of wt CHIKV nsP1 expression, CHIKV mutants harbouring Y248A and D63A substitutions in nsP1 were also rescued with the clear detection of huge amount of capsid protein in transfected cells. However, these mutants could not be rescued in parential T-Rex U2OS cells. The presence of viruses was also confirmed by propagation of P_0 stocks which was, again, possible in induced T-Rex U2OS-CHIKV nsP1 cells but not in parential T-Rex U2OS cells (Figure 21).

The regain infectivity of CHIKV D63A and Y248A in nsP1 are highly consistent with our trans-replicase data, as we see highest restoration effects on those two mutants. In this case the rescue was not due to reversions or pseudoreversions. The presence of D63A and Y248A mutation in genomes of rescued viruses was confirmed by Sanger sequencing of corresponding fragments generated using RT-PCR. Interestingly, in one (out of three) experiment performed using native T-Rex U2OS cells, virus with R252E mutation was also rescued. At this case sequencing revealed that rescue was due to pseudoreversion, the Glu252 was substituted to Lys252. The positive charged lysine most likely functioned similar to the original arginine restoring infectivity of the virus. The pseudorevesion (instead of true reversion) was probably due to the feasibility of nucleotide change – in alphaviruses (pseudo)reversions are almost exclusively due to single nucleotide changes. At this case single nucleotide substitution could change from glutamic acid (GAG) to lysine (AAG) while for true reversion to arginine two substitutions would be required.

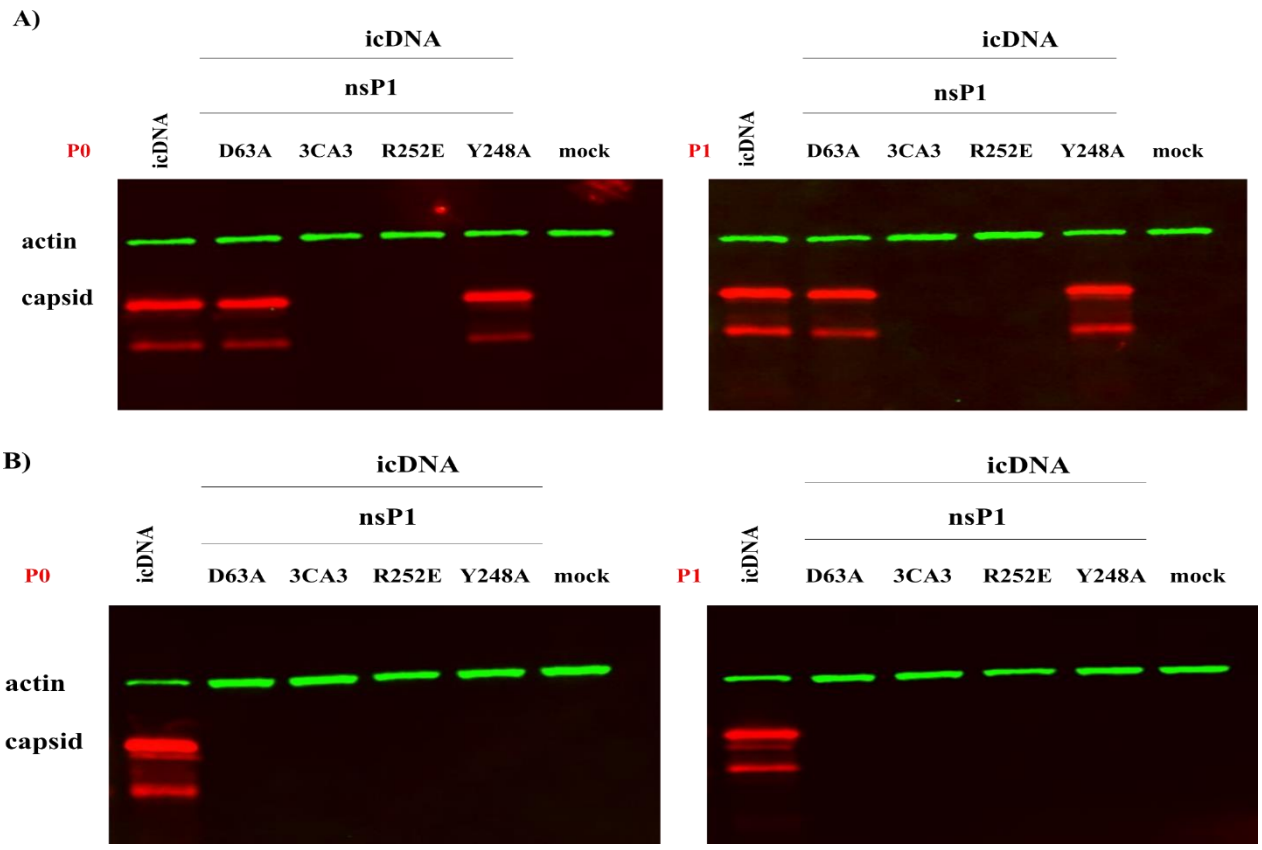

Figure 21. CHIKV with D63A and Y249A mutations in nsP1 can be rescued and propagated in cell line expressing wt nsP1 of CHIKV. Capsid proteins, that serve as marker for successful genome replication and transcription were detected using antibodies against CHIKV capsid protein; antibody against β-actin was used to detect loading control.

SUMMARY

nsP1 of alphaviruses is required for synthesis and capping of viral RNA. It is also an important target for antiviral compounds. Thus, conducting functional studies of nsP1 of alphaviruses can extend the knowledge we hold on viral RNA replication and may contribute to the develpement of antiviral strategies. In this work, we constructed and characterized a set of stable inducible cell lines expressing wt and mutant nsP1 of CHIKV or nsP1 of SFV, RRV, EEEV and SINV. We also compared subcellular localization of nsP1 of aforementioned alphaviruses. In the next step, we performed trans-complementation of defective RNA replicase of CHIKV by individually expressed nsP1 to check whether it can be functional as a part of replication complex. In the final step of this work, we used cell line expressing nsP1 of CHIKV for rescue of CHIKV harbouring lethal mutations in nsP1 encoding region.

We clearly observed that induction of expression of nsP1 causes the formation of filopodia-like structures in the plasma membrane of cells. However, nsP1 of different alphaviruses and mutants thereof caused formation of filopodia like structures of different appearence and localization. Trans-replicase experiments revealed that expression of nsP1 of CHIKV compensates for certain mutations in nsP1 region of replicase. The same was observed for nsP1 of related RRV but generally not for nsP1s from distanly related EEEV and SINV. Interestingly, it was revealed that defective nsP1 expressed by the cell can still work together with defective nsP1 from the replicase as long as these defects in these proteins were different. In contrast, complementation was not observed if used nsP1s harboured identical defects. Furthermore, expression of wt nsP1 could compensate for enzymatic defects in nsP1 of replicase but could not compensate for mutation causing wrong localization of nsP1. Presumably, enzymatic defects are easier to compensate as they do inhibit only positive strand synthesis and/or translation of these RNAs while defects from incorrect localization of nsP1 hampers all stages of replication making it harder to compensate for. In line with this, no form of nsP1 could compensate for defect caused by R252E mutation in nsP1 from CHIKV replicase.

Although we observed the restoration of activity of trans-replicases, we were curious to check whether the effect is big enough to allow rescue a mutant virus. We observed that the conduction of expression of of CHIKV nsP1 restores the infectivity of CHIKV infectious cDNAs harbouring D63A or Y248A mutations in nsP1 but not infectious cDNA with 3C3A or R252E mutations confirming biological relevance of data obtained using trans-replicas assay.

ACKNOWLEDGEMENT

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