2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
*master in de biomedische wetenschappen*

# Masterproef

## Identification of the virus-host protein interaction landscape of the Chikungunya virus


Promotor :
Prof. dr. Niels HELLINGS

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Terence Agombin Aka


*Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen*


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## ABSTRACT

### Identification of the virus-host protein interaction landscape of Chikungunya virus

**Introduction:** Chikungunya virus (CHIKV) is a disease-causing arthropod-borne virus (arbovirus) which is transmitted to humans by *Aedes* mosquitos. CHIKV is a prototype alphavirus belonging to the family of the *Togaviridae*. CHIKV causes a number of symptoms including fever, rash and most prominently polyarthralgia. The positive stranded RNA genome of the virus allows for the direct translation of the non-structural polyprotein which undergoes self-cleavage into 4 non-structural proteins (nsP1-4) and which form the replicase complex required to synthesize negative sense, genomic and sub-genomic RNA. The mechanisms of nsP interactions with host cell factors to support viral replication within human and mosquito cells still remain elusive. The objective of this study is to identify host factors that associate with CHIKV nsPs to gain insight into the replication process within targeted host cells. The specific aim is to optimize the affinity purification of nsPs to allow optimal yield and subsequent identification of the interacting host proteins.

**Materials and Methods:** HEK293T and C6/36 cells were transfected with plasmid constructs expressing CHIKV nsPs with a Strep-tag inserted at the C-terminus (nsP-CT) or N-terminus (nsP-NT). The proteins were purified by affinity purification (AP), using beads coated with streptavidin to capture tagged proteins. Proteins were subsequently eluted with biotin and analysed by SDS-PAGE, immunoblotting, and Coomassie and silver staining. Proteins will be identified by Mass Spectrometry (MS).

**Results:** Elution with high biotin concentration showed that nsPs were expressed but remained stuck to the beads. NsP3 solubilisation was impaired. Modified elution conditions with RNase and high salt concentration disrupt RNA-dependent interactions and potentially improved the specificity of interactions of nsP1-CT. Optimization of detergent and sonication parameters allowed for the solubilization and AP of nsP3-CT.

**Conclusion:** Identifying host factors interacting with CHIKV nsPs allows insight into the mechanism of replication of the virus in human and mosquito cell lines. We optimized the conditions that allows for optimal yield and downstream identification of the CHIKV-host protein complexes.

## SUMMARY

Chikungunya virus (CHIKV), a prototypic alphavirus, utilizes its replication complex to synthesize genomic strand (49S), minus strand and sub genomic strand (26S) RNAs. CHIKV infects both human and mosquito cells and replicates within them. However, the infection of humans by CHIKV causes Chikungunya fever and most prominently, painful polyarthralgia that are mostly self-resolving within weeks to months after the infection but can also result in chronic joint pains in a significant number of CHIKV infected individuals. No approved treatment or vaccine exists for its management but outbreaks continue to surface in recent times. The molecular mechanisms involved in the virus replication strategy is still obscure. Although the role of the viral proteins in the replication process have been described, the involvement of host cell factors is unclear. The goal of this study is to elucidate host cell factors that support CHIKV replication in human and mosquito cells through interaction with the viral non-structural proteins. We have optimized affinity purification schemes for the identification of these host factors by mass spectrometry (AP-MS).

# CHAPTER ONE
# INTRODUCTION

## 1.1 – General Background

Chikungunya virus (CHIKV) is a re-emerging (1), arthropod-borne virus (arbovirus) (2) that causes chikungunya disease, sometimes referred to as chikungunya fever (CHIKF). First isolated from patients with high fever in the former Tanganyika (now Tanzania) in 1952, CHIKV was named using the native language Makonde with literal meaning "to bend up". This is apparently because patients infected with the virus exhibited characteristic symptoms including high biphasic fever, rash and persistent joint pain. The arthritic joint pain caused a stooped walk, thus the name "Chikungunya".

CHIKV is an alphavirus belonging to the family of *Togaviridae*. It contains a positive sense (+) ribonucleic acid (RNA) genome confined within an assembly of capsid (CP) structural proteins, encoded by the subgenomic strand (29S) alongside the envelope glycoproteins (E3-E2-6K-E1). As an arbovirus, it is sustained in nature by replication in both insect and vertebrate organisms. CHIKV is transmitted by two kinds of mosquitos of the genus *Aedes (Ae.)* (2)(3)(4) (Fig.1.1). *Ae. aegypti* was initially implicated in the transmission of CHIKV (2)(3), however in recent outbreaks, CHIKV adapted to replication in *Ae. albopictus* with a significant increase in transmission of the virus by this widespread mosquito vector (2)(5). The mutation caused a one amino acid substitution (A226V) in the E1 glycoprotein, which was identified at least in four different strains and occasions (6,7). Recent unprecedented outbreaks in the Americas (8), have allowed for the vector competence to be analysed and defined. Results showed that *Ae. aegypti* and *Ae. albopictus* are competent enough to establish spread of CHIKV infection beyond the borderline of rural regions of Africa, with a tropical climate, to more temperate urban regions (2).

CHIKV was initially an enzootic virus, maintained in a sylvatic cycle, and circulating in and around rural areas in Africa with little spillover infections in humans (9). Due to increased travel and trade, genetic adaption of CHIKV to widespread vectors (i.e. *Ae. albopictus*), immunological naïve human populations in some geographical regions (like the Americas), there is an emergence of an urban transmission cycle with *Ae. albopictus* being the main biological vector. Both the virus and its vector have now moved from their initial site of discovery where they were endemic; Africa, South-East Asia, and Indian Ocean regions, to colonise new areas in more temperate regions of Europe (10) and North-America, as well as in Central- and South-America (8)(11).

Phylogenetic studies have shown three strains distributed over different parts of the world; the Indian Ocean Lineage (IOL), the Asian lineage and the Eastern, Central and Southern African lineage (ECSA). The ECSA lineage was the main lineage identified in 1952 in Tanzania. Over the years, the IOL and Asian lineages were identified in the Indian Ocean basin, the Indian subcontinent and Southeast Asia (Fig. 1.2) (12). Recently, the Asian lineage was shown to cause the epidemic in the Americas (13).

Over the years, research has mainly focused on identifying and describing the role of the viral proteins involved in alphavirus replication and infectivity. Interestingly, the alphavirus replication complex, composed of non-structural proteins (nsP1 – nsP4), is fairly well described. However, the interactions with host factors and how these factors support alphavirus replication in time and space is not clear. The alphavirus genome encodes non-structural and structural proteins involved in viral genome replication, packaging of viral particles and entry, respectively (14)(15). We also know that it is highly probable that host factors play a role in alphavirus replication and these viruses cannot gain entry into the host cell without utilising the host cell machinery (15)(14). Therefore, understanding the landscape of the virus-host protein interactome provides an insight into the viral replication cycle within infected mosquito and vertebrate cells.

Currently, there are no approved vaccines or specific therapeutic options for CHIKV disease. There are candidate vaccines that have shown efficacy in animal models of CHIKV infection but have to be validated in human subjects before commercialization (16)(17). One of the earliest CHIKV vaccines developed at the clinical stage after showing efficacy in mice was the formalin-inactivated vaccine from the African 167 CHIKV strain and produced from green monkey cells (17). This vaccine was reported to elicit neutralizing antibody response with no adverse effects detected. Later, a live attenuated vaccine was purified from strain 15561, an isolate from the 1962 Thai outbreak. Another vaccine at clinical stage of development is based on virus-like particles (VLPs), which are produced by introducing CHIKV glycoprotein in HEK293T cells using a lentivirus. The VLP-based vaccine is capable of eliciting neutralizing antibody response. Other CHIKV vaccines still at the preclinical stage include CHIKV/IRES (Chikungunya virus-internal ribosomal entry site) vaccine and a DNA-based vaccine designed using CHIKV envelop proteins (E1, E2, E3), both being tested in animal models for the moment.

In the absence of a preventive vaccine and therapy, the US Centres for Disease Control and Prevention (CDC) as well as many local health authorities, advise on preventive

measures to protect against mosquito bites, such as use of air conditioning, window or door screens, emptying of standing water reservoirs, wearing of long sleeved shirts, use of mosquito repellents. The use of larvicides such as juvenile hormone analogs (methoprene) and chitin synthesis inhibitors (Novaluron) prevent the development of the larvae stage of the mosquitos. However, a limitation to this is the lack of correlation between the larva index and the adult mosquito abundance.

Unfortunately, even a combined approach of preventive measures is not able to eliminate CHIKV spread. Therefore, good diagnostic strategies and the development of therapeutics is required.

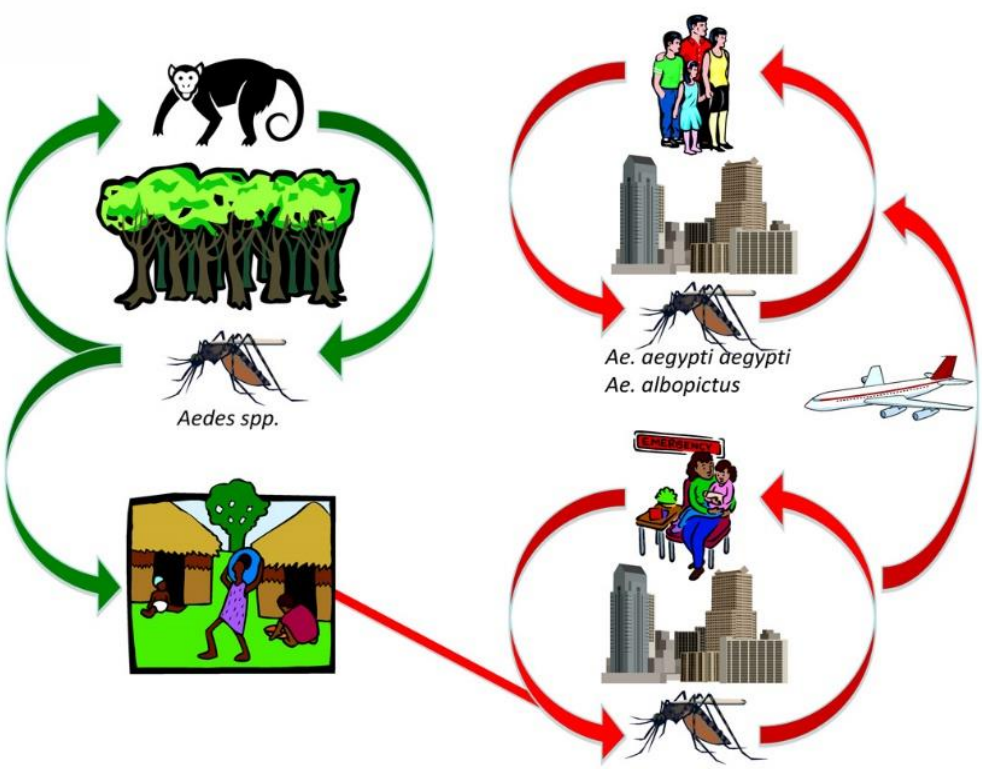


**Fig. 1.1- Sylvatic transmission (Green arrows) and urban transmission (Red arrows) cycles of chikungunya virus**. Non-human primates initially harboured the virus and *Aedes* mosquito transmitted to humans in rural setting. As migration and travelling increased over the years, the virus is transported to urban setting with vector mosquitos that mediate transmission.

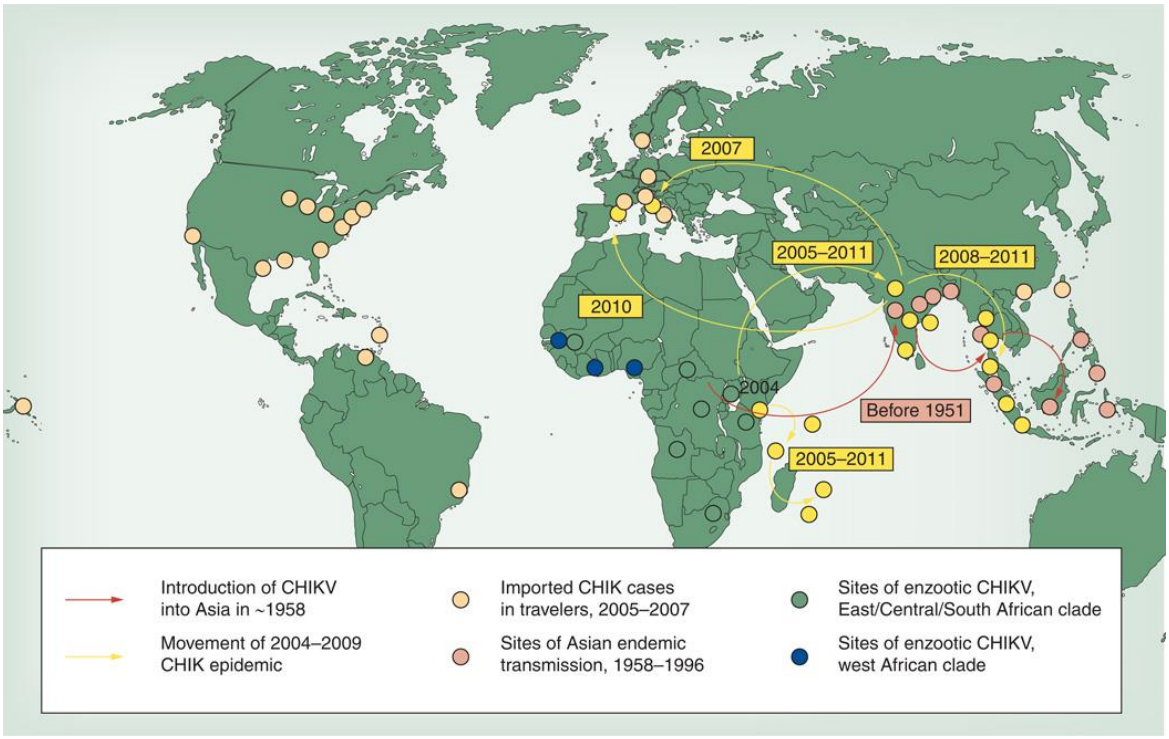


**Fig. 1.2- Chikungunya virus epidemiology.** Reproduced from (56).

## 1.2 – Viral structure

CHIKV is a spherical alphavirus with a diameter of approx. 65-70 nm (Fig.1.3B) and contains an 11.8 kb positive sense single stranded, capped and polyadenylated RNA genome (Fig.1.3A) (18) surrounded by a capsid (CP). The CP is embedded inside a host-derived membranous envelope sandwiched by glycoproteins translated during the later phase in the viral infection process (Fig.1.3C). The viral genome constitutes of two open reading frames (ORF); one encoding the non-structural proteins (nsP1-4) from the genomic RNA and the other encoding the structural proteins (CP, E1-3, 6k) from the subgenomic 26S RNA (Fig.1.3A) (26)(19). The glycoproteins E1 and E2 form heterodimers that reunite during posttranslational modification in infected cells to form a trimeric spike (Fig.1.3C) (20), which assembles within the host-derived membrane of the virus and is required for virus entry (21,22).

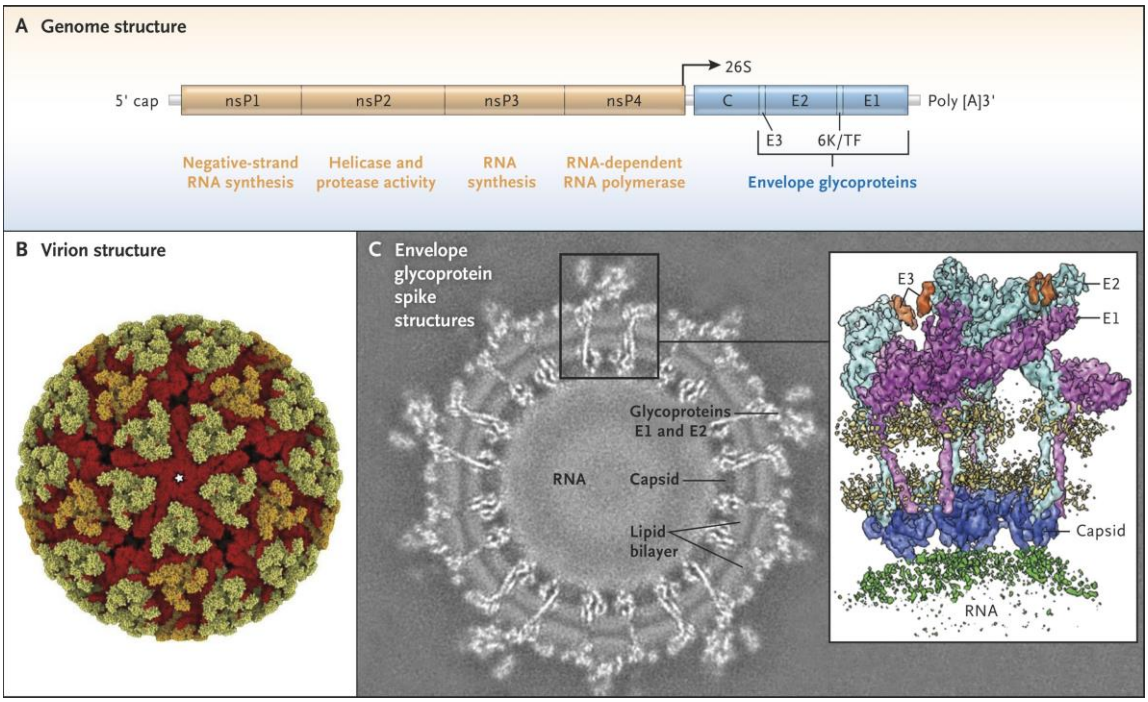


**Fig. 1.3- CHIKV structure**. Panel A shows the genome organization of CHIKV with two ORFs; first encoding non-structural polyprotein, P1234; second encoding structural proteins, C-E3-E2-6K-E1. 5´ methyl cap provides initiation signal for translation, a poly (A) 3´tail is added to provide stability. Each ORF is separated by a UTR segment with a potential role in translation initiation. Panel B shows the icosahedral structure of CHIKV. Panel C shows the envelop glycoproteins E1 and E2 forming a trimeric spike of heterodimers on the virus surface. Image reproduced from (23).

The pathogenesis of CHIKV infection is still poorly understood. Fibroblasts are the main targets of CHIKV, which upon infection release interferon (IFN-α and IFN-β) and other inflammatory cytokines that elicit an immune response and account for a high fever (24). Several of the CHIKV nsPs function in a counter response to bypass the innate immune response, as described below. Local tissue destruction in the joints and ensuing autoimmune responses as well as virus persistence and lack of clearance have been suggested to contribute to the prevalent chronic joint pain (23).

### 1.3 – Actors of viral replication

The current understanding on alphavirus replication is mainly derived from studies with other, but similar, alphaviruses such as Semliki Forest virus (SFV) and Sindbis virus (SINV). Some proteins are translated early and others late during the course of viral infection. Those proteins translated early in replication help the virus during the entire cycle of infection including replication, maturation, development and spread of the virus within the infected cells. These nsPs are essential components of the replication complex, which assembles

at endosomal vesicles and the plasma membrane and give rise to spherules (25). However, a significant fraction of these replicase proteins exist as solitary proteins in infected cells (30).

### 1.3.1 – Functions of non-structural proteins

NsP1 is a central component of the alphaviral replication complex and locates the entire complex towards the plasma membrane and in filopodium-like membrane extensions (25). With an approximate weight of 60kDa and about 500 amino acid residues in length, nsP1 has been shown to have two domains that characterise its functions in CHIKV replication. These include the N-terminal methyltransferase (MTase) and Guanyltransferase (GTase) domains (26) which are involved in the capping reaction during the processing of newly synthesised viral genomes (26). When the strand is capped with a 7' methylguanine triphosphate head, it becomes mature and is packaged into the viral particle through the assembly of other structural proteins. Another important function of nsP1 is its membrane binding affinity (27) that steers localisation of the replicase complex and enables the production of new minus, genomic and subgenomic strands (28). This membrane affinity is partly contributed by the palmitoylation of cysteine residues on nsP1, allowing anchorage on membranes (29,30). Studies have shown that although palmitoylation is an important process directing nsP1 binding to membranes, to effectively generate replicative strands, palmitoylation is dispensable in the enzymatic activity of nsP1 (29,30). This function depends on the three-dimensional structure of nsP1 and its capacity to interact with other proteins of the replication complex (30). Nevertheless, Ahola *et al.*, showed that depalmitoylation mutants exhibited reduced pathogenesis of Semliki forest virus (SFV) (29). During alphaviral replication, there is development of *de novo* type I cytoplasmic vacuoles, which are modified lysosomes and endosomes containing membranous structures, called spherules (25). NsP1 together with other nsPs form an associations with these components and are bound to the membranes of cytoplasmic structures such as the nucleus. Co–immunoprecipitation of nsP1, as well as yeast-two-hybrid experiments, showed that this viral protein interacts with nsP3 and nsP4 and potentially also with nsP2 (31). Outside of the replicase complex, nsP1 was shown to counteract the antiviral effects of tetherin. This interferon response factor is a broad-spectrum antiviral protein which is targeted for degradation through interaction with nsP1 (32).

NsP2 (90kDa MW) is a highly multifunctional protein. The two main functions of nsP2, based on the structural domains identified by bioinformatics analysis, are the nucleoside triphosphatase (NTPase) (33) and protease (34) activities. The domains responsible for these activities are N-terminal NTPase and C-terminal protease domains, respectively. The

NTPase catalyses the resolution of complex secondary structures of the viral genome and render them linear for transcription and packaging. The protease domain processes the viral nsP polyprotein leading to the generation of mature nsPs with functional activity (34). It is speculated based on mutational analysis, that the N-terminal domain of nsP2 could contain extensions with possible function useful to the virus (35). The activities of nsP2 are likely modulated by these domains yet to be characterised (36). In addition to its role in maturing the replicase complex and viral genome replication, the presence of a nuclear localisation signal allows substantial amounts of nsP2 to translocate to the nucleus. Here, the CHIKV nsP2 induces degradation of Rpb1, the main component of the cellular RNA polymerase II, to prevent transcriptional activation of interferon induced genes (37).

NsP3 (60kDa MW) has three domains but the functions are still obscure (36). However, mutation of nsP3 have shown a reduction in the generation of negative sense and subgenomic strands by preventing both formation and function of replicase complex. This indicates nsP3 is involved in both negative sense and subgenomic strand synthesis (38). The N-terminal macrodomain (approximately 150 amino acids long), which is conserved amongst alphaviruses, is found to bind nucleic acids, i.e to the ribose fragment of polyadenosinediphosphate (39). The central alphavirus unique domain (AUD) also shows a strong homology amongst alphaviruses. About 200 amino acids long, AUD contains a zinc-binding site and through genetic manipulations of the AUD region, the development of minus strand RNA was affected indicating a possible role in viral replication (40). The hypervariable domain exhibits strong variation both in terms of length and amino acid sequence amongst the alphaviruses. Shown to play a role in the formation of complexes within infected cells (41), this C-terminal domain of nsP3 could be the main determinant of pathogenesis in infected cells. Some studies have indicated interactions between nsP3 hypervariable domain and the mammalian host cell stress-granule proteins G3BP1 and G3BP2 (42,43). Accordingly, an association of nsP3 with the mosquito homologue of these G3BPs, rasputin, has been identified in mosquito cells. The interaction of nsP3 with these factors prevents the formation of stress-granules upon viral infection and alleviates the translational shut-down of the viral RNA in both human and mosquito cells (44).

### 1.3.2 – Functional properties of the viral structural proteins

Structural proteins are encoded and translated by the subgenomic vRNA strand that constitutes the second ORF. This occurs during the late stage of the CHIKV replication cycle, with the replicase complex playing an important enzymatic role in the synthesis of minus strand from which the subgenomic strand is transcribed. The glycoproteins E1 and E2 form a trimeric spike on the surface of the virus during their release from the infected cell (20). The glycoproteins are modified in the endoplasmic reticulum immediately after synthesis and proteolytic cleavage from the capsid protein. The capsid encapsulating the CHIKV genome. E1 and E2 are key players during CHIKV entry into the target cells. E2 recognises and attaches to an unknown receptor on the target cell, which activates the entry process (Fig.1.4).

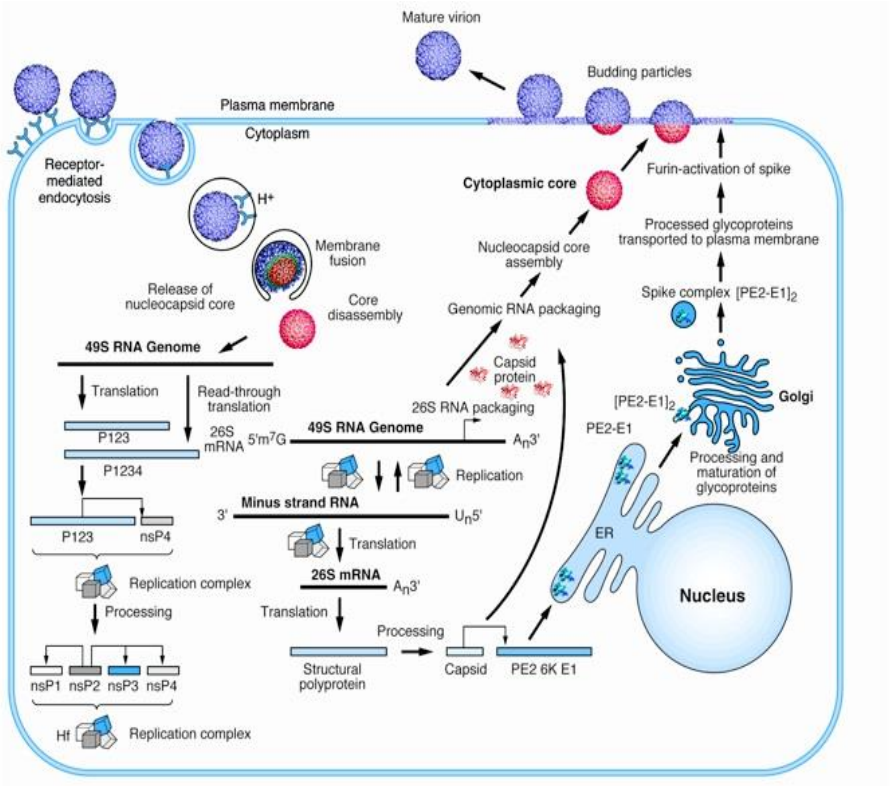


**Fig. 1.4 – Replication cycle of the CHIKV**. Virus glycoprotein E2 recognises an unknown receptor on the surface of the cell. The release of the viral genome into the cell allows for immediate translation into non-structural proteins (nsP1234), which form the replication complex, important for downstream activities involved in viral replication. The structural proteins are translated through a subgenomic promoter followed by a posttranslational modification and are used to package the genomic strand before the new CHIKV particles bud from the cell.

## 1.4 - Objectives of the thesis

The general objective is to identify host factors associated with nsPs to gain a detailed molecular insight in the CHIKV replication process. More specifically, this study will optimize the purification and elution methods for nsPs to allow reliable downstream identification of host factors using AP-MS and the study will define the expression levels of CHIKV nsPs in cell types relevant for CHIKV infection and pathogenesis.

# CHAPTER TWO
# MATERIALS AND METHODS

## 2.1 – Virus, Cells, Culture and Cell Count

Human embryonic kidney cells expressing SV40 large T antigen (HEK293T) and mosquito cells (*Ae. albopictus* C6/36) were used. HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Lonza), supplemented with 10% Fetal Calf Serum (FCS) and L-Glutamine and incubated at 37ºC with 7% $CO_2$. C6/36 cells were maintained in Minimum Essential Medium (MEM) (Gibco) and incubated at 28ºC with 7% $CO_2$. Culture medium was replaced every 2 days to maintain the cells at high quality growth rate. After every passage, a record of cell growth rate was noted. At every cell count, cell suspension and trypan blue were mixed in equal proportion. Cell viability was recorded.

## 2.2 – Plasmid Lipofection and cell lysis

The FuGENE 6 transfection kit (Promega) was used to introduce PCDNA4TO plasmid DNA designed for mammalian cell expression or pIEX for insect cell expression of CHIKV nsP1, nsP2 and nsP3, with either N-terminal or C-terminal Strep-tags. Transient transfection was done as described. Cells were seeded 24h before to about 70% confluency and incubated as described before. In two separate tubes, the plasmid and the FuGENE 6 were mixed with Opti-medium (medium without serum); 60µg plasmid in 450µl Opti-medium; 120 µl FuGENE in 390 µl. After mixing both constitutions, the mixture was incubated at room temperature and a cloudy appearance indicated the formation DNA- FuGENE complexes. The cells were incubated at 37ºC with 7% $CO_2$ for 48h.

## 2.3 – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS – PAGE)

The PAGE gels were hand-casted. The 12 mini-format linear gradient gels, which required the Model 485 Gradient Former and Mini-PROTEAN 3 Multi-Casting chamber, was made for the electrophoresis analysis. The gel was prepared according to protocol described (Bio-Rad). Samples including eluates, beads, inputs and pellets, were prepared as follows; 16µl sample and 4µl loading buffer (in 5X concentration: 250mM Tris-XHCl pH6.8, 10% SDS, 40% glycerol, 0.01% bromophenol blue, and stored at r.t.p). DDT was added and the set up was allowed to boil for 5 min at 95ºC. The running buffer (250 mM Tris, 1.92 M glycine, 1% SDS, pH 8.3) was poured into electrophoresis cell containing the casted gel. 20 µl of protein samples was loaded into each well and the ladder added in descending volumes; 7 µl, 2.5 µl. the samples were run at 144 volts for 50 mins.

**2.4 – 2x STREP Affinity Purification**

Each step of the process was completed with tubes placed on ice to ensure the structural integrity of the protein and their interactions are maintained. The principle is based on the fact that beads, which are coated with streptavidin bind with high affinity to the Strep tag fixed to the CHIKV nsPs. After purifying the CHIKV protein complexes from the cells using beads, biotin molecules are used to elute the proteins with their interactors from the beads. The steps (lysing – beading – washing – eluting) were done according to a standard protocol. Briefly, after washing the cells twice with PBS (Lonza), the cells were collected in a 10 ml tube and the lysis buffer (2 tablets of protease inhibitor, 500µl of 10% NP40, 12 ml PBS) added to lyse the cells. Cell lysate was either or not sonicated (Amplitude: 90, Timer: off, Pulser: 1, 5 pulses, three times, cool in between) (Sonics and Materials Inc. Danbury, Connecticut, U.S.A). The lysed solution was then transferred into Eppendorf tubes and centrifuged at 3500 rpm in 4$^{o}$C precooled centrifuge for 4 min. In the meantime, the beads were washed twice with 900 µl washing buffer (1 ml of 10% NP40 in 20ml of PBS) and centrifuged at 1500 rpm for 3 min. The lysate was removed and added to the tubes containing washed beads. The tubes were placed on a rotator for 1h at 4$^{o}$C. The proteins were eluted from the beads using the different elution buffers (supplementary table). The tubes containing eluates and beads were stored overnight at -20$^{o}$C.

**2.5 – Western Blotting**

After electrophoresis, the gel was placed on the nitrocellulose membrane (Bio-Rad) interspersed within two blotting papers (Whatman) and placed in the cassette of the Trans-Blot machine (Bio-Rad). The transfer process was run using the standard protocol (25V, 0.3A, and 83.3Ω) for 30 min. The gel was then removed and discarded and the nitrocellulose membrane was covered with blocking buffer (5 g dry milk in 50 ml PBS-Tween 20) in a petri dish and placed on a shaker for 1h. 6µl anti-strep antibody (1:5000 dilution) was added and incubated for 1h at room temperature and overnight at 4$^{o}$C. The membrane was washed using TBST buffer (0.05% Tween-20 in TBS buffer) and then incubated with secondary antibody conjugated with peroxidase. After incubation, the membrane was washed again with TBST. The signals were developed using the Stain-Free ChemiDoc$^{TM}$ MP imager (Bio-Rad).

**2.6 – Coomassie and silver stains**

The gels were stained following the protocol as described (ThermoFisher Scientific, Catalog number LC6065). For a single gel, the composition of the stain constituted; Stainer A, 10ml; Stainer B, 2.5ml; Methanol, 10ml; deionized water, 27.5ml. After electrophoresis, gels were placed in a petri dish containing the Coomassie Blue stain and then placed on a gentle shaker. The gels were incubated overnight at room temperature. After overnight incubation on gentle shaker, the gels were destained for about 7 hours to have a clear background to view protein bands. The silver stain was done as described in the manufacturer's protocol (Novex, Invitrogen).

**2.7 – MALDI-TOF Mass Spectrometry**

Spectrometric analysis was done at the Centre for Proteomics (CFP), University of Antwerp. Samples were prepared as described (45). After analyzing the eluate on SDS-PAGE, the gel was washed in water for 2 hours. The bands were cut into small cubes ($1mm^3$) and transferred into microcentrifuge tubes. To avoid contamination, this was done under a laminar hood and glass plates used were cleaned with soap and water. 100µl of 100mM ammonium bicarbonate/acetonitrile (1:1, volume/volume) was added and incubated in a vortex for 30 min. 500µl of acetonitrile was added and incubated at room temperature in a vortex until the gel piece shrank and turned white. The gel piece was then saturated with 50µl of Trypsin for 2 hours. To complete enzymatic cleavage, 10-20µl of ammonium bicarbonate was added on the gel piece for 90 min. The samples were then incubated overnight at $37^o$ C. Following this, the extraction of the peptides was done. 200µl of extraction buffer (1:2, vol/vol, 5% formic acid/acetonitrile) was added to each tube and incubated for 15 min at $37^o$ C in a shaker. The peptides were resuspended with 10-20µl of 0.1% trifluoroacetic acid (TFA), vortexed for 2 min in a sonication bath and centrifuged for 15 min at 10,000 rpm.

# CHAPTER THREE
# RESULTS

## 3.1 – Transient expression and purification of CHIKV nsPs with C- or N- terminal Strep tags

Expression of individual CHIKV nsPs allows for the identification of host cell associated factors that support their function or direct subcellular localization of the replication complex. Plasmid (pcDNATO) of CHIKV nsP1 or nsP3 with either C- or N- terminally tagged Strep, was used to transiently transfect cell lines to study their interactions with cellular factors. After cell lysing, the lysate was pulled down using streptavidin-coated beads followed by elution with standard elution buffer (0.5% NP40 in PBS, 10 mM biotin). The eluate was analyzed by SDS-PAGE and gels were stained with Coomassie and silver stain (Fig. 3.1). N-terminally and C-terminally Strep tagged nsP1 was expressed and pulled down as indicated by their approximate size of 60kDa whilst nsP2 did not express (data not shown). However, nsP1 (and its associated host factors) were stuck to the beads (Fig. 3.1, lane B). Therefore, the elution was optimized in order to release nsP1 from the beads.

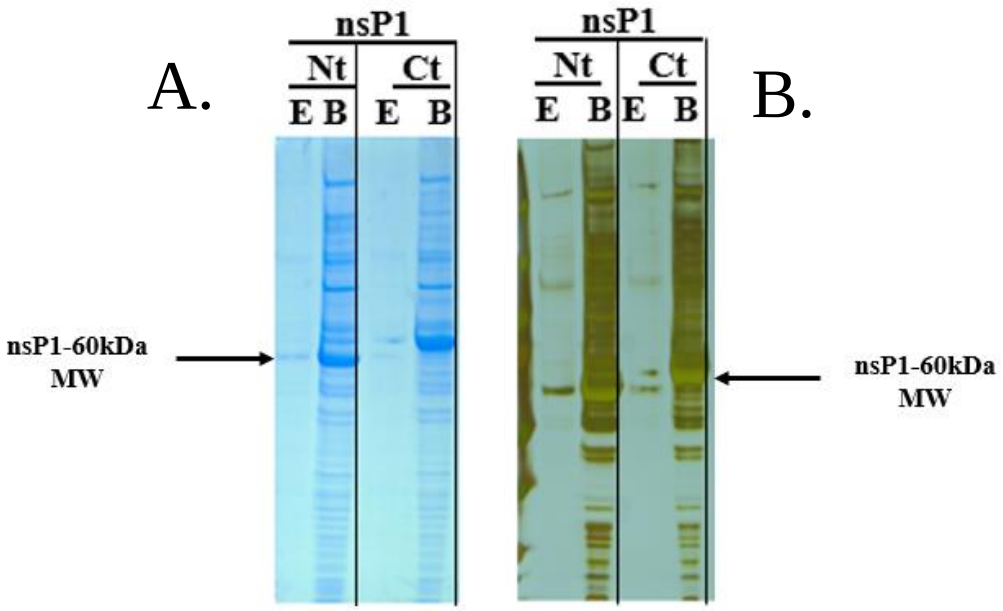


**Figure 3.1- Expression and AP of nsPs with C- or N- terminal Strep tag**. Analysis by Coomassie stain **(A**) and silver stain **(B)**. After 48 hr of transfection of pcDNATO containing constructs of nsP1 with Strep tag fixed at C-terminus (Ct) or N-terminus (Nt), HEK293T cells were lysed and proteins pulled down using beads (B stands for Beads fraction) and eluted with biotin (E stands for Eluate fraction).

### 3.2 – Optimization of elution conditions for optimal yield and detection of CHIKV nsP1-host factor interactions.

In order to achieve optimal yield and specificity for purification of CHIKV nsPs and interacting host factors, we modified the elution buffer to allow specific release of nsP1 and its interaction partners from the beads. In addition to standard biotin which was used in previous experiments (Fig. 3.1), we modified the elution buffer by adding higher salt concentration and/or addition of RNase in non-denaturing conditions. Denaturing SDS conditions were used as positive control. The use of high salt (650 mM) (Fig. 3.2, upper panel, condition 2) and RNase (10 ng/µl) (Fig. 3.2, upper panel, condition 3) showed an improved elution of nsP1 and its interactors. When both high salt and RNase were used, there was no discernible improvement in the release of nsP1 and its interactors from the beads as compared to high salt alone (Fig. 3.2, upper panel, compare conditions 2 and 4). SDS showed an effective disruption of proteins from the beads, as previously reported (Fig. 3.2, upper panel, condition 5). The background bands could possibly be streptavidin and other independent proteins efficiently released by SDS treatment (Fig. 3.2, lower panel, compare conditions 1-4 to condition 5) as evidenced by the non-transfected control. High salt conditions thus allows improved and specific elution of nsP1 and its interactors. However, improved elution of nsP1 and interactors following RNase treatment suggests the presence of nsP1 in a ribonucleoprotein complex (RNP) where several nsP1 interacting proteins are potentially associated through secondary RNA-mediated interactions which do most likely not represent direct protein interactions.

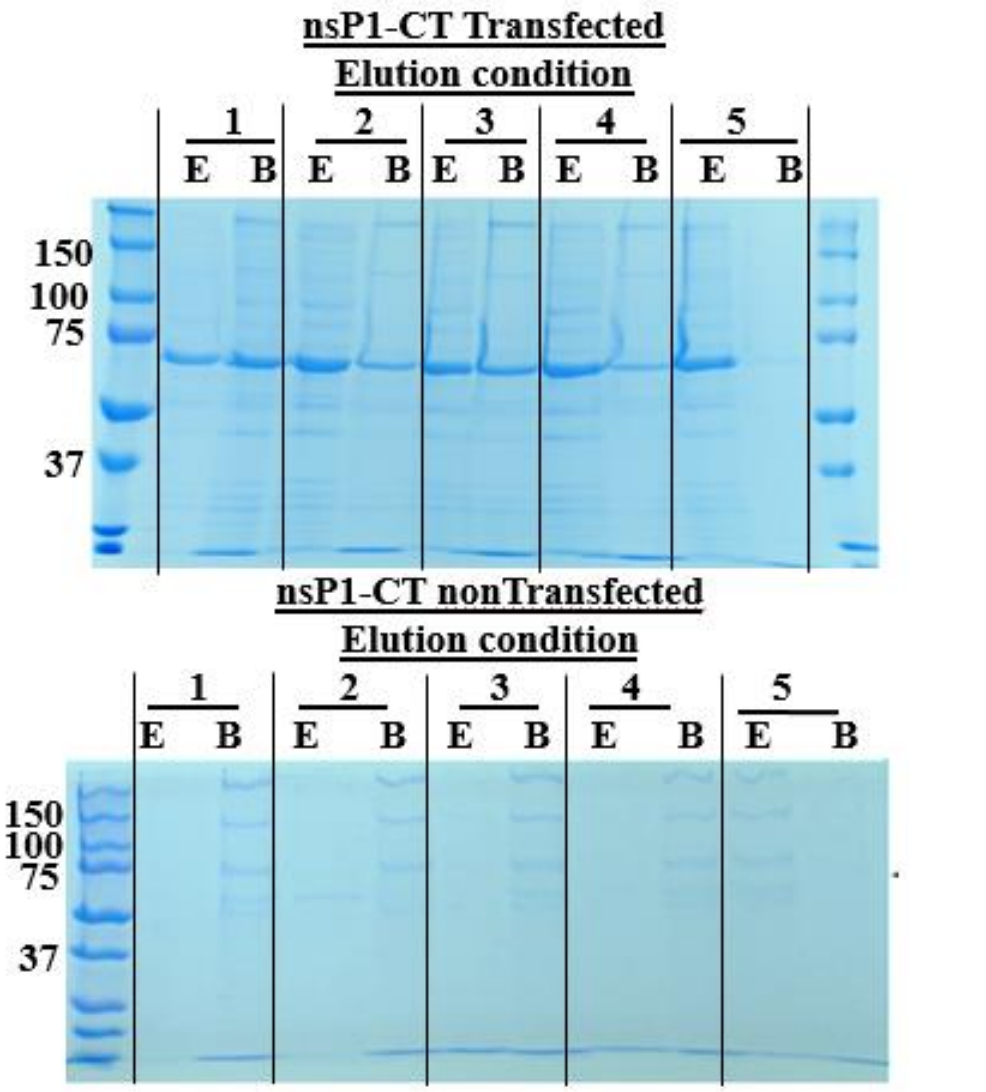


**Figure 3.2-Optimization of elution condition for nsP1-CT.** HEK293T cells were transfected with pcDNATO construct for nsP1 with a C-terminal strep tag. After 48 hrs, cells were lysed and proteins captured with beads (B stands for bead fraction). Standard elution conditions with Biotin and 0.5% NP40, i.e. condition 1 were modified: condition 2 (650 mM NaCl), condition 3 (10 ng/µl RNase), condition 4 (650 mM NaCl and 10 ng/µl RNase), condition 5 (1% SDS). E-eluate, B-beads. Upper panel, transfected. Lower panel, untransfected control.

### 3.3 – Disruption of RNA-dependent interactors to nsP1 by prior treatment with RNase

Here, we tested the potential of RNase treatment to allow separation of RNA-dependent and RNA-independent interactors of nsP1. We performed a two-step elution after pulling down nsP1 with streptavidin beads. Cells were lysed and nsP1 captured with its potential interactors via the strep tag linked to its C-terminus. Prior to elution of nsP1 using biotin, the beads were treated with RNase (Fig. 3.3A) and the supernatant containing RNA-dependent interactors removed from the beads (further referred to as elution fraction 1 (E1)), was analysed. Subsequently, nsP1 and associated factors were eluted using high salt elution buffer (further referred to as elution fraction 2 (E2)) (Fig. 3.3A). Indeed, pre-treatment of the nsP1 affinity purifications with RNase allowed removal of a number of proteins (Fig. 3.3A).

To confirm this activity for RNase and identify optimal conditions for separation of RNA dependent interactors, we titrated increasing amounts of RNase (Fig 3.3B) and found that higher RNase concentration did not produce a more potent activity than the RNase concentration used before (Fig. 3.3B).

In order to prepare eluate for MS analysis, we applied every step of the optimized scheme. We transfected both HEK293T and C6/36 cells with nsP1-CT plasmid, lysed the cells after 48h and performed the two-step elution as described above. As compared to the untransfected control (UTC), primary elution with RNase disrupted RNA-dependent binders to nsP1-CT (Fig. 3.3C) whilst secondary elution with standard biotin and high salt allowed for specific release of nsP1-CT interactors (Fig.3.3C).

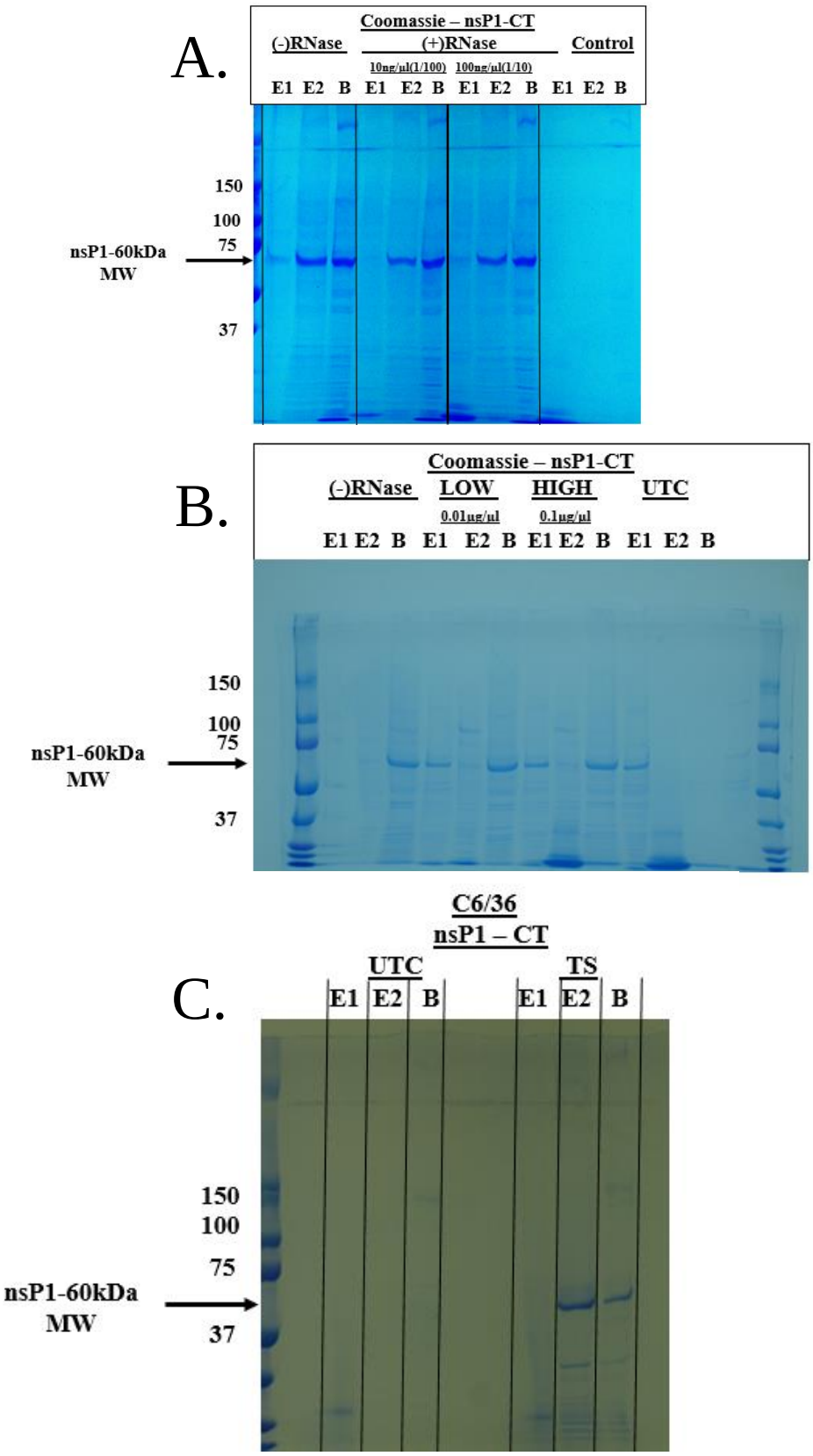


**Figure 3.3- Disruption of RNA dependent interactors to nsP1-CT.** (A) and (B) show analysis by Coomassie stain for the effect of using RNase to remove RNA-dependent interactors of nsP1-CT. E1-eluate fraction with unspecific binders to nsP1-CT, E2-eluate fraction with specific binders to nsP1-CT, B-bead fraction. (C) Applying the use of RNase and standard elution condition in C6/36 cells.

### 3.4 – Optimization of nsP3 solubilisation

NsP3 was shown by immunoblot assay to be highly expressed in both HEK293T and C6/36 cells but could not be solubilized from the pellet fraction (Fig. 3.4A). Here, we optimized the lysing method to allow for nsP3 release and solubilization. We transfected nsP3-CT and 48h later cells were lysed using different concentrations of non-denaturing detergent (0,5% NP40 (Low) or 0,5% NP40 + 0,5% Tween-20 (High)). In addition, mild sonication was evaluated for its potential to release nsP3 from the aggregates. The pellet, input, eluate and bead fractions were analysed by SDS-PAGE and Coomassie staining and Western blot using anti-Strep antibody (Fig. 3.4B). While the use of detergent alone did not release nsP3 from the pellet fraction (Fig. 3.4B), additional sonication of the lysate allowed efficient release of nsP3 into the soluble fraction (Fig. 3.4B). Analysis of the eluate and bead fraction of the same experiment by Coomassie staining verified the increased presence of nsP3 and its interaction partners in the soluble fraction when the lysate was sonicated (Fig. 3.4C and 3.4E). While similar amounts of nsP3 could be purified in low and high detergent conditions, co-purification of interacting proteins was diminished in the presence of higher amounts of detergent (Fig. 3.4C and 3.4E).

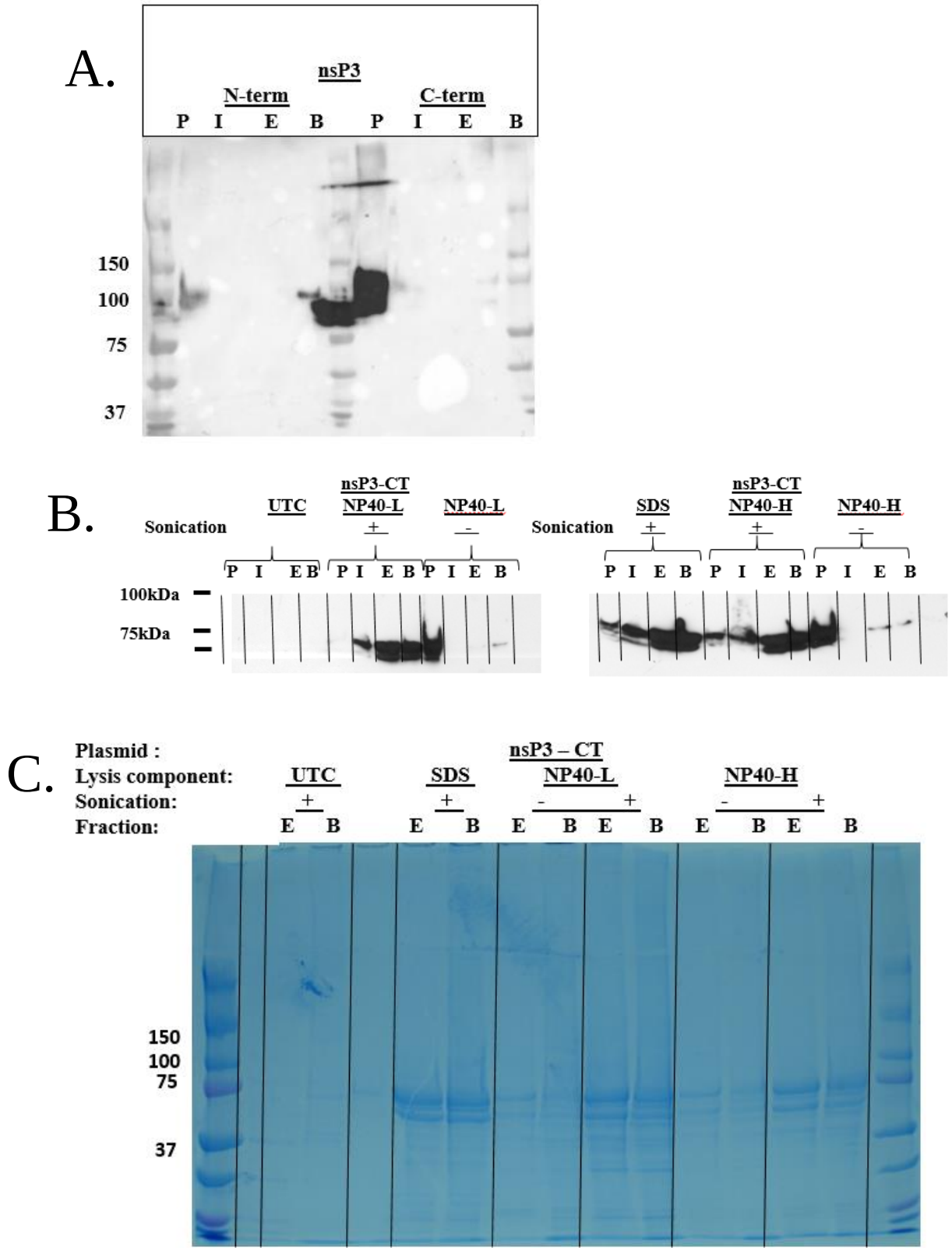


**Figure 3.4-Optimisation of nsP3 solubilisation.** (A) and (B) Western blot analysis, nsP3 probed with anti-strep antibodies, P-pellet, I-input, E-eluate, B-bead. (B) shows the use of NP40 with or without sonication to solubilize nsP3 from pellet shown in (A). (C) Coomassie stain of eluate (E) and beads (B) fractions.

# CHAPTER FOUR
# DISCUSSION AND CONCLUSION

The organization of the viral replication complex is a critical step that determines the outcome of CHIKV infection of both human and mosquito cells. The CHIKV nsPs interact with each other to organize the replication of the viral genome. However, significant amounts of the individual nsPs are found outside of the replication complex where they interfere with different cellular (antiviral) mechanisms. The main objective of this study was to identify host cellular factors that interact with CHIKV nsPs. In order to achieve this, it was important to optimize affinity purification schemes which allow for optimal yield and specificity of the viral proteins and their cellular interactors. Identifying these interactions is essential for an in-depth understanding of the mechanisms involved in CHIKV replication in both vertebrate and non-vertebrate organisms. In addition, elucidating the virus-host protein network allows for the exploration of selected cellular interactors as potential targets for therapeutic intervention. The identification of cellular interaction partners of the viral proteins in both human and mosquito cells will also allow for comparison of the interactome in these different organisms. While we expect to retrieve different protein interaction partners in the separate organisms, we also expect to retrieve homologous interaction partners, be it at structural or functional level. The overlap in protein interaction partners in human and mosquito cells will allow to define a core cellular machinery that CHIKV engages to allow viral replication in cells of very distinct organisms (Fig.4.1). Recently, new geographical locations are experiencing unprecedented outbreaks of CHIKV and other arbovirus infections partly attributed to adaptation of the virus, increased prevalence of mosquito vectors that transmit the virus and large immunologically naïve populations (9). It was shown that the replicase complex is dynamic and co-existing in different locations with respect to time and function of the replicase proteins within the host cell (46). Replication of alphaviruses occurs at the intracellular membranous structures such as endosomes and the plasma membrane. NsP1, which interacts with all other nsPs, is considered the main organiser of the replicase complex (47). The formation of spherules at membrane structures reveals the prolific synthesis of viral genomes and the production of new virion particles, which infect new cells (Fig.4.2).

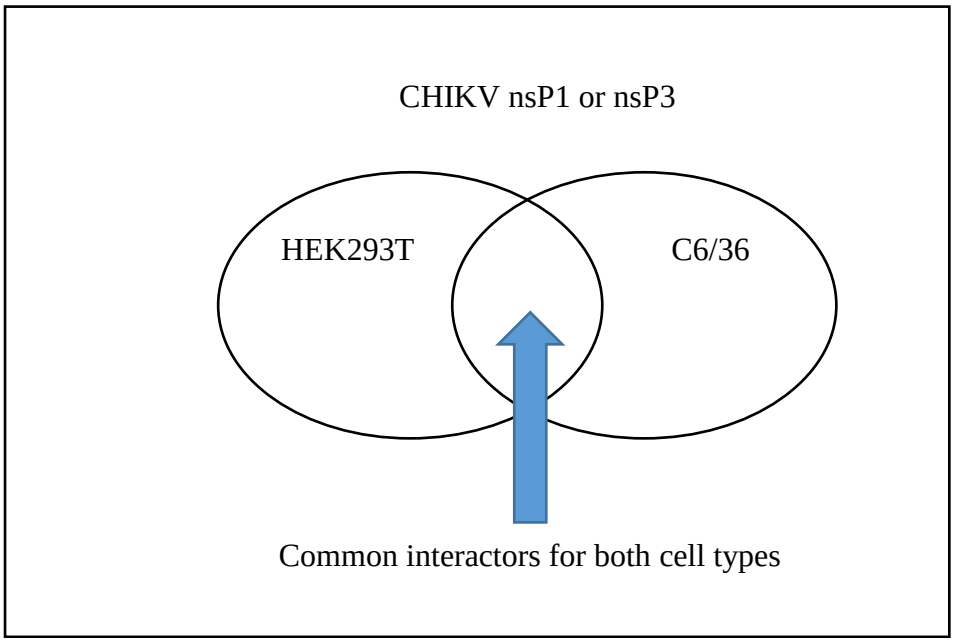


**Fig. 4.1 – Sketch of possible interactors for both human (HEK293T) and mosquito (C6/36) cells**. Each circle represents the interactors elucidated for each cell. When these interactors are compared, those which are found in both cell types are validated as potential interactors to CHIKV nsP1 and nsP3. The arrow indicates the common interactors for both cells.

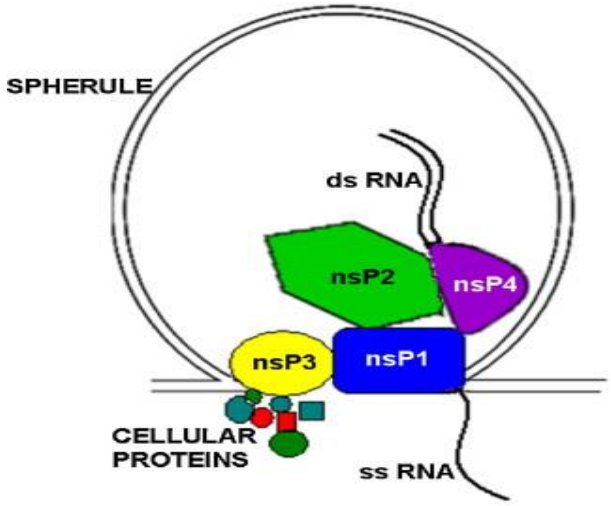


**Fig. 4.2 – Representation of Spherule formed during CHIKV infection process. Reproduced from** (47)**.**

Protein-protein interaction studies are very important to elucidate protein function. Yeast-two hybrid (Y2H) screens and pull-down experiments have elucidated interactions between nsP1 and all other nsPs defining its role as the main organiser of the replicase complex (47). Y2H screens are often used to study protein interactions (48) but

however possess some limitations; the yeast model is physiologically different from human and mosquito cells, specificity of interactions is low and interaction complexes are limited to one-on-one interactions. Another important tool is RNA interference (RNAi), which involves the introduction of RNAi reagents into cell to specifically knock-out a gene sequence in order to understand the gene function (49,50). It is widely applicable in the study of infection pathways in many diseases since it provides large-scale information about gene functions in the infection process. However, the main challenge are off-target effects (OTEs) and how to control for them (49,50). We have used affinity purification coupled to mass spectrometry (AP-MS) to isolate and identify stable host cellular interactors of nsP1 and nsP3.

We have expressed individual replicase proteins with Strep tags at either C-terminus or N-terminus of the protein in both human and mosquito cells. Although this does not represent the physiological replicase complex, expression and purification of separate nsPs is expected to allow for the identification of stable host cell interactors specific for a given nsP. While we expect to identify cofactors that support the role of the nsP in the replicase complex, we also expect to identify cofactors that support the role of the viral proteins outside of the replicase complex by counteracting antiviral activities (32) and in the inhibition of the cellular machinery (42,43). The sequence encoding for a specific nsP was cloned into plasmid vectors (pcDNATO for HEK293T, pCoofy for C6/36), with the gene encoding for the Strep tag affixed at either the C-terminus or N-terminus.

There are many intermediary steps in the affinity purification of proteins expressed in a cell type. These steps are important for the preparation of eluate fractions used to identify proteins in mass spectrometry analysis. In order to identify real and specific interactors to nsP1 and nsP3, we have optimised the affinity purification scheme (AP-scheme). The steps in AP include cell lysing and solubilisation, pull down and elution. All these steps need to be optimised in order to achieve a successful identification without interfering background.

The purification strategy for nsP1 was slightly different from nsP3 since both proteins end up in separate fractions after cell lysis. Whilst nsP1 is in the soluble fraction, nsP3 is retained in the insoluble pellet. During the affinity purification of nsP1, HEK293T cells were lysed and spun down. NsP1 was directly soluble, therefore, was present in the soluble fraction of the lysate after centrifugation. The AP proceeded with the soluble fraction, which was incubated with sepharose beads coated with streptavidin. These beads were used to pull down nsP1 through its strep tag. The principle lies in streptavidin specific affinity for strep tag. Upon

treatment with elution buffer, which constitutes of biotin, the streptavidin is displaced preferentially and the proteins are eluted. A significant problem is that nsP1, after elution with standard biotin, remained stuck to the beads (Fig.3.1, lane B). This means that the elution buffer was not strong enough to release nsP1 and its co-interactors. In the purification and characterization of HIV-human protein complexes, the method of washing the beads included 150mM NaCl before elution with biotin (51,52). We have included high salt in the modified elution condition and found that high salt (Fig. 3.2, lane 2) in addition to standard biotin was effective in disrupting non-specific interactions between nsP1 (and/or its interacting proteins) with the beads, leading to the release of nsP1 and its co-interactors into the eluate fractions. Likely because the non-specific interactions were ionic in nature and effectively disrupted by the higher ionic strength buffer. Looking at the replicase complex, it is possible to have non-specific interactors from cellular compartment that bind to RNA but not directly to nsP1. These could have a false positive effect. In this regard, we modified the standard elution buffer to include RNase in order to reduce non-specific association of RNA-dependent proteins (51)(Fig. 3.2). We observed an effective release of nsP1 and some of its co-interactors. This is probably logical, since the disruption of RNA in the replicase could disentangle the complex into separate units. But to what extent is RNase capable of releasing non-specific interactors to nsP1? We investigated this using high concentrations of RNase in a two-step elution approach (Fig. 3.3); (1) the first elution using elution buffer containing RNase but without biotin, was aimed at releasing non-specific interactors that depend on RNA to interact with nsP1, (2) the second elution using standard biotin in addition with high salt, which was found to be potent in releasing interactors of nsP1, was aimed at releasing specific binders to nsP1. We found that the RNase was effective and potent enough to release RNA dependent binders to nsP1 into E1 fraction (Fig. 3.3A, compare –RNase E1 to +RNase E1). However, many possible specific interactors to nsP1 were retained and upon elution with biotin and high salt, we found that these proteins were eluted into the E2 fraction (Fig. 3.3A). The application of the optimized AP-scheme in C6/36 cells showed equal effect as trace of non-specific binders to nsP1 were seen in the E1 fraction and specific interactors in the E2 fraction (Fig. 3.3C). Although likely not representing direct protein interaction partners of nsP1, the RNA dependent interactors, could potentially function in supporting RNA processing capacities during viral genomic replication. Indeed, these RNA dependent interactors, released in the E1 eluate fraction, will also be identified through MS and functionally studied for their potential role in CHIKV replication.

NsP3 is the replicase protein with the least elucidated function. In fact, it is speculated that it plays a role in the RNA synthesis pathway and also interacts with host cell factors (53). Recently, Varjak *et al.* showed a novel function of nsP3 in alphavirus replication by truncation of the C-terminus region of the protein. This had a significant effect on the multiplication of Semliki Forest virus (SFV) (54). NsP3 tagged with enhanced green fluorescent protein (EGFP) were found to form clusters in the cytoplasm (55). It also has strong binding affinity for nucleic acids, mainly through the nsP3 macrodomain (39). Using our AP-MS strategy, we found that solubilisation of nsP3 is problematic, hence its isolation and purification is suboptimal. When the plasmid containing nsP3-CT was expressed in HEK293T cells, the pellet, input, eluate and bead fractions were analyzed by Western blot and found that the entire protein was retained in the pellet fraction (Fig.3.4A). We optimized the process of solubilisation in order to bring nsP3 into solution. We used NP40, non-denaturing detergent, to lyse the cells with or without mild sonication (Fig.3.4B). Sonication uses sound energy to agitate particles in a sample. In our analysis after transfection and lysing with low NP40 detergent without sonication, we found that the entire nsP3 was stuck to the pellet fraction (Fig.3.4B, left panel, lane P, NP40L minus sonication), a similar observation as in previous experiments (Fig.3.4A). Mild sonication allowed nsP3 to be released into the input, eluate and bead fractions (Fig.3.4B). As a proof of effectivity, we compared effects with high NP40 detergent with or without agitation. The same effect was observed for high detergent with sonication (Fig.3.4B). However, when we considered analyzing the eluate and bead fractions using Coomassie stain, we found a higher retention of nsP3 interactors in the NP40 low condition with sonication (Fig.3.4C). It is worth noting here that low detergent (NP40 low) showed a better performance in retaining interactions between nsP3 and cellular factors than the high detergent (NP40 high and 0.2% Tween-20).

We showed for the first time that low detergent with mild sonication could be used in the affinity purification scheme for the purification of nsP3 in both human and mosquito cells. With this purification scheme, we are able to prepare large batches of eluates, which are used for mass spectrometry analysis for the identification of nsP3 host cellular interactors.

We analyzed pellet and input fractions for the expression of nsP2 by western blot and found that nsP2 was not expressed (data not shown). Also, we found in a Coomassie and silver staining that the eluate and bead fractions did not contain nsP2 (data not shown). NsP2 is a multifunctional replicase protein with variable activities by its different domains. The genome sequence encoding for nsP2 has a nuclear localization sequence (NLS) with motif of lysine and

arginine residues. This motif provides a signal that allows for the import of nsP2 into the nucleus where it degrades RNA pol II and blunts cellular transcription. This could have a downregulating effect on the activity of the RNA PolII dependent promoter present in pcDNATO to generate mRNA for the synthesis of nsP2. However, recently, a trans-replication system has been developed to uncouple the expression of replicase proteins and RNA synthesis. These systems; phage T7 polymerase and CMV cellular polymerase II, are shown to be sensitive in the functional analysis and tagging of CHIKV replicase protein (55). The trans-replication system can be used to express nsP2 and elucidate its interactions. Also, it is possible to transfect cells with full-length CHIKV RNA to allow expression of internally tagged nsP2 and purification of host interactors.

Our optimization process is a novel scheme in affinity purification, which allows for eluates to be prepared for the study and identification of protein-protein interactions using AP-MS. We have shown that; (1) the use of high salt in addition to standard biotin and NP40, can induce effective release of host cellular factors associated with CHIKV nsPs, (2) the use of RNase can improve the specificity of these interactions and (3) low detergent with mild sonication can induce solubilisation of nsP3.

In conclusion, optimization of the affinity purification scheme allows for optimal yield and specificity of CHIKV-host protein interactions. This helps in the identification of these host cell factors, which thus provide a molecular insight of their role in CHIKV infection, replication and pathogenesis in human and mosquito cells.

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## LIST OF ABBREVIATIONS

1. AP – MS: Affinity purification and mass spectrometry
2. AUD: Alphavirus Unique domain
3. CDC: Centre for Disease Control
4. CHIKF: Chikungunya fever
5. CHIKV: Chikungunya virus
6. CHIKV/IRES: Chikungunya virus-internal ribosomal entry site
7. CPE: Cytopathic effect
8. CT: Carboxyl terminus end of protein
9. C6/36: Mosquito cell line
10. DMEM: Dulbecco´s modified eagle medium
11. ECDC: European Centre for Disease Control and Prevention
12. FCS: Fetal Calf Serum
13. HEK293T: Human Embryonic Kidney cell expressing large SV40 antigen
14. MEM: Minimum Essential medium
15. nsP: Non-structural proteins (1,2,3,4)
16. nsP1-CT: Non-structural protein 1 with C-terminus strep tag
17. nsP3-CT: Non-structural protein 3 with C-terminus strep tag
18. NT: Amino terminus end of protein
19. ORF: Open reading frame
20. OTE: Off target effects
21. SDS-PAGE: Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis
22. SFV: Semliki forest virus
23. SINV: Sindbis virus
24. siRNA: short interference RNA
25. UTR: Untranslated region
26. +RNA: positive sense RNA
27. vRNA: viral RNA
28. 2X STREP: Strep tag protein affixed at either C-terminus or N-terminus of nsPs.

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