COUPLING PROTEASE ACTIVITY AND ENDOPLASMIC RETICULUM ANCHORING PROPERTIES OF DENGUE VIRUS FOR MONITORING CLEAVAGE IN THE NATURAL CELLULAR COMPARTMENT

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Coupling Protease Activity and Endoplasmic Reticulum Anchoring Properties of
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ABSTRACT OF THE THESIS

Coupling Protease Activity and Endoplasmic Reticulum Anchoring Properties of Dengue Virus for Monitoring Cleavage in the Natural Cellular Compartment

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Dengue Virus (DenV), considered now an emerging viral pathogen in the US, is responsible for causing Dengue Fever, Dengue Hemorrhagic Fever, and Dengue Shock Syndrome, which are often fatal. Currently the only treatment for DenV infection is supportive care, which is non-specific and has no direct effect on the virus. Therefore, there is an increasing need for accurate assays to search for inhibitors that specifically target DenV.

DenV belongs to the Flaviviridae family. It is a positive sense, single-stranded RNA virus that is targeted to, translated, and anchored in the Endoplasmic Reticulum (ER) membrane of the infected cell. Once translated, proteolytic processing must occur by both cellular and virally encoded proteases. The N terminal one third of the Non-Structural (NS) Protein 3, and the central hydrophilic region of the NS2B cofactor comprise the domains for protease activity of the viral protease. Cleavage of viral targets by NS2B/NS3 occurs in the cytosolic side of the ER membrane and is essential for the maturation of new virions, making the viral protease an ideal target for DenV antivirals.

The goal of this project is to develop a cell-based assay that: (a) monitors protease activity in the natural cellular compartment: the cytosol, and (b) investigates the ER anchoring properties of viral proteins and their domains. The intricate topology of DenV and its complex protease activity will be exploited. The assay is based on the engineering of a fusion protein comprised of an anchoring domain of viral origin fused to the yeast transcription factor Gal4 through a sequence that serves as a putative protease substrate. Utilization of the DNA binding and transcription activation domains of Gal4 will be used to drive Gal4-dependent green fluorescent protein expression. The assay was developed to ensure that the viral protease, which is supplied in cis, acts both in the natural milieu of infection and cellular compartment. Flow cytometry and microscopy are used to show protein localization with or without cleavage. This assay will be further developed as a platform for high-throughput screening of novel protease substrates as well as for drug discovery of novel protease inhibitors.
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CHAPTER 1

INTRODUCTION

DENGUE VIRUS EPIDEMIOLOGY

Mosquito-borne diseases caused by viruses have plagued the animal kingdom throughout history, and, while programs have been implemented to monitor and abate mosquito populations, the growing number of emerging infectious diseases continues to threaten humanity. One such virus, transmitted by the bite of a virus-carrying mosquito, is Dengue Virus (DenV; see Appendix for abbreviations). First characterized in the 1950s, DenV has spread from the remote regions of the Philippines and Thailand, to spanning urban tropical and sub-tropical regions of the globe with the migration of mosquito populations [1, 2]. There are as many as 100 million DenV infections worldwide each year, with a devastating case-fatality rate of up to 10% [3]. This virus, like other viruses such as Yellow Fever Virus (YFV) and Chikungunya Virus, is transmitted preferably through the Aedes aegypti (primarily) and albopictus (secondarily) mosquito species [2, 4]. While the risk of contracting some viruses, such as Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV), weighs heavily on human behavior, including, but not limited to unprotected sexual intercourse and intravenous drug use, the at-risk population for DenV lies solely on the presence of infected mosquito populations in that region [5, 6]. Therefore, people living in or visiting sub-tropical regions (Figure 1 [7]) are at-risk and should take serious precautions [7]. According to the World Health Organization (WHO), 40% of the world’s population is at risk [5]. The continuous spread of mosquito populations makes DenV infection a global pandemic [4].

Diseases caused by DenV range from mild to severe, and an increased exposure to the virus will likely result in the onset of more severe symptoms [2, 8]. The most common and mild is Dengue Fever (DF). The sudden onset of flu-like symptoms, such as fever, fatigue, and muscle/joint aches, are characteristic of DF [3, 5]. Typically, the immune system fights
the virus with the production of serotype-specific neutralizing antibodies, which leads to the recovery of the infected individual within few weeks [9, 10]. The more severe disease caused by this virus is Dengue Hemorrhagic Fever (DHF), which can further lead to the even more dangerous Dengue Shock Syndrome (DSS) [2, 11]. These are often fatal, if diagnosed too late. The initial phase of the disease is similar to the symptoms of DF, during which the viral load is highest [12]. Once this subsides, plasma leakage and hemorrhaging will occur primarily into the abdominal and pleural cavities [1, 2]. Further progression of DHF leads to DSS. This occurs when the body goes into circulatory shock, due to the inability of oxygen to reach necessary tissues [2]. Many of the extreme cases of DHF and DSS occur in children under the age of ten years [5]. A successful diagnosis of DenV includes detection of viremia by real-time polymerase chain reaction (RT-PCR) and/or DenV-specific IgG and IgM enzyme-linked immunosorbent assay (ELISA); however, these tests do not predict the severity of the disease onset [2]. Importantly, there are no specific treatments to combat DenV; therefore, supportive care is the only option [13, 14]. Aggressive intravenous fluids must be administered immediately [2, 13, 14]. Due to the rapid onset of severe symptoms, it
is difficult to monitor specific treatments to compare survival rates, and is especially complex because there are no placebo controls, as it is unethical [2]. This highlights the desperate need for novel drugs that specifically target the viral replication cycle, and can be used in conjunction with supportive care.

The discovery of specific treatment is further hindered by the existence of diverse serotypes of the virus, and their complex interaction with the host immune system. DenV has four main serotypes, referred to as one through four [15]. Although the serotypes have similar clinical presentations, the human body develops serotype-specific neutralizing antibodies [15]. Accordingly, while infection with one serotype can usually be cleared by a healthy immune system, a secondary infection becomes more complicated. Upon infection of a distinct novel serotype, the previously developed antibodies will fail to fight the subsequent infection, because they will only recognize the virus and fail to neutralize the infection [2]. Superinfection with different serotypes is further complicated by the fact that the virus is known to bind non-neutralizing antibodies, which result in complexes that shuttle directly to macrophages [16, 17]. This allows for a more efficient infection of macrophages, through an increase in the number of virions endocytosing into cells, leading to the onset of more severe forms of the disease [9]. This phenomenon is known as antibody dependent enhancement, and is unique to DenV amongst the related Flaviviruses [9, 17].

**DENGUE VIRUS LIFE CYCLE**

DenV is a member of the *Flaviviridae* family in the genus of Flaviviruses. *Flaviviridae* are positive sense, single-stranded RNA (ss-RNA), icosahedral, enveloped viruses [18]. DenV, an 11Kb genome virus, has a tropism primarily for macrophages and dendritic cells, but has also been shown to spread to many other cell types including hepatocytes, endothelial cells, and myeloid cells of the bone marrow [19, 20]. The Envelope (E) protein is known to facilitate attachment and entry; however, the specific cellular receptors are not well elucidated, and remain controversial [21]. Entry into the host cell occurs via clathrin-mediated endocytosis [8]. In the endosome, a pH change encourages the fusion of the virion envelope with the endosome membrane, followed by uncoating and release of viral mRNA into the cytoplasm [22]. The RNA is then signaled to the membrane of the Endoplasmic Reticulum (ER), where it hijacks cellular ribosomes to begin cap-
mediated translation [23, 24]. The entire genome is then translated into one long polyprotein that weaves itself in and out of the ER membrane, and is ultimately anchored. Within the ER membrane, the proteome, containing four structural, and seven non-structural (NS) proteins, undergoes co and post-translational processing by both host and virally encoded proteases (Figure 2) [23, 24]. Once processed, the virus will begin to assemble new replicated genomes and capsids into virions that travel through the classical secretory pathway [23, 25]. The final modification needed for viral maturation is the cleavage event of the boundary between the peptide of the premature membrane (prM) protein ‘pr’ and membrane ‘M’ by a host furin-like peptidase, causing a conformational change leading to the maturation of M [26]. The virions exit the cell via exocytosis through the Golgi-trans Golgi network [22].

**NS2B/3 Protease Activity**

As mentioned, the viral polyprotein is embedded within the ER membrane and adapts a complex topology that is crucial for the following proteolytic events. All cytosolic facing protein boundaries are processed by the virally encoded protease NS2B/3 [22, 27]. The catalytic domain is located in the N-terminal one third of NS3. This domain contains the catalytic triad comprised of a Histidine, Aspartic acid, and Serine [28]. For maximal or full activity NS3 requires the interaction with its cofactor NS2B [27, 28]. Although NS2B is primarily hydrophobic, containing four transmembrane domains, the central 40 amino acid hydrophilic region is the primary necessary region responsible for the associated activity [28]. Further, studies have also shown that Flavivirus protease activity can be enhanced by the C-terminal two-thirds of NS3, which encodes for the viral helicase domain, but is not critical for enzymatic activity [29]. The NS2B/3 protease identifies very specific amino acid sequences for cleavage. This motif contains two basic amino acids at the P2 and P1 positions (either Glutamine, Arginine or Lysine), followed by a small amino acids at the P1’ position (either Glycine, Serine, or Arginine and sometimes Threonine) [28]. The NS2B/3 interaction leads to the exact cleavage at the boundaries of Capsid/pr, NS1/2A, NS2A/B, NS2B/3, within NS3, NS3/4A, and NS4B/5 [23, 28]. Cleavage of all these polyprotein boundaries by NS2B/3 protease is absolutely critical for the maturation and production of new infectious virions, making NS2B/3 a perfect target for antivirals. While protease inhibitors are already available
Figure 2. Dengue Virus proteome organization and topology in the ER membrane. The top panel shows a linear representation of the genome. The 5’ 1/3 region contains the four structural proteins necessary for virion assembly. The 3’ 2/3 of the genome contain the seven non-structural (NS) proteins, primarily for viral replication and processing. The bottom panel shows the DenV proteome as it is signaled to the ER, translated into one polyprotein that weaves itself in and out of the ER membrane. The arrows show the protein boundaries that are cleaved. The legend specifies which type of enzyme cleaves these positions, and also the nature of the transmembrane domains.

for many years against HIV, or recently against HCV, no such inhibitors exist against DenV [2, 30, 31].

**CELL-BASED ASSAYS**

The danger of antibody dependent enhancement has hindered the development of vaccine efforts against DenV. While mouse models exist for DenV these are controversial and no perfect animal model is available to mimic natural DenV infection [32, 33]. This setback further proves the immediate need of *ex vivo* assays to test for novel drugs. Cell-based assays can be considered a bridge between biochemical *in vitro* assays and animal model *in vivo* assays. Stable expression of an assay in an adherent mammalian cell line, such as hepatocytes, can be exploited to mimic the natural cellular milieu of infection [19]. Utilizing retroviral technology, infectious viral particles that bear the assay elements can be
produced and used to transduce mammalian cells in order to produce cell lines that constitutively and stably express the elements of the assay [34].

Previously in the laboratory two assays have been developed to monitor protease activity. The first assay monitors cleavage by the viral protease in the cytosol and was initially developed for HIV-1 protease (Figure 3) [31]. The assay relies on the highly characterized yeast transcription factor Gal4 and its upstream activation sequence (UAS) driving the expression of the green fluorescent protein (GFP) reporter gene. Gal4 is comprised of the DNA binding domain (DBD) and the transcription activation domain (TAD). The DBD has a strong nuclear localization signal (NLS) and will bind the UAS in the nucleus. If fused with TAD, TAD will then drive the expression of the downstream reporter GFP. In this assay (depicted in Figure 4, pathway 1) protease was fused between the two domains, so it auto-catalytically cleaves itself out of the chimera if active. The resulting disrupted fusion leads to the nuclear localization of DBD, which, in the absence of TAD, is unable to activate GFP. In contrast, if the protease is mutated or inhibited, the intact fusion will travel to the nucleus and activate GFP expression.

Figure 3. Representation of the inducible Gal4 based system. The top panel shows the system without induction. With no doxycycline, the TRE does not drive expression of the downstream Gal4 fusion, which in turn, cannot active the UAS-GFP reporter, and the cells remain unchanged. The bottom panel shows that with induction by doxycycline, the rtTA is activated, and able to bind the TRE, driving expression of the downstream Gal4 fusion. The intact Gal4 (containing both the DBD and TAD) travels to the nucleus where DBD will bind the UAS, and TAD will drive expression of the GFP reporter. The cells will turn green.
Figure 4. Depiction of relevant cell based assays: The first construct depicts monitoring cleavage in the cytosol utilizing the Gal4/UAS system. This assay is dependent on the autocatalytic cleavage abilities of protease. The second depicts monitoring cleavage in the classical secretory pathway. This assay is dependent on the ability of the scaffold to signal and embed in the ER membrane and travel to the cell surface. Through the detection of one or two tags, indicating cleavage or no cleavage, respectively, host proteolytic processing is monitored. The third depicts an assay that combines the cytosolic cleavage of one and the ER anchoring of two. Further depicted in detail in Figure 5.

Importantly, the assay was engineered in an inducible off/on system. The system relies on activation with Doxycycline (Dox), which binds to the reverse-tetracycline Trans-Activator (rtTA) causing a conformational change. This allows the rtTA to bind the Tetracycline Response Element (TRE), which serves as the Gal4 promoter in the assay. When rtTA binds to TRE and activates Gal4 expression, Gal4, which with no protease serves as positive control (Figure 3), travels to the nucleus and activates GFP expression.

The second assay (depicted in Figure 4, pathway 2) monitors cleavage in the classical secretory pathway. In the assay, a scaffold protein targeted to the ER travels to the cell surface through the Golgi/Trans-Golgi network [35]. The scaffold is comprised of two tags with a cleavable substrate fused between them. If cleaved during transport, only one tag will be recognized on the cell surface by fluorescently coupled antibodies. In contrast, if cleavage is blocked, the two tags will be presented on the surface.
In 2007, Breiman, *et al* have developed an assay in order to detect and isolate Hepatitis C Virus (HCV) infected cells [36]. In this assay a HCV cleavage site (CS) was inserted between the human ER membrane-resident, protein kinase RNA-like endoplasmic reticulum kinase (PERK), and Gal4 (depicted in Figure 4, pathway 3). This assay thus allows to specifically discriminate between HCV infected and non-infected cells, as infected cells will lead to cleavage and expression of the GFP reporter.

Here, we have combined certain aspects of each of the above-mentioned assays. Our new assay exploits, on one hand, the ER-anchoring properties of specific viral proteins, with, on the other hand, the monitoring of cytosolic cleavage exploiting Gal4 (Figure 5). The assay thus combines the strong ER-anchoring domain of a viral protein with the strong NLS of Gal4. This platform has been engineered to specifically monitor cleavage events of the DenV NS2B/3 protease, utilizing the natural NS2B/3 cleavage site.

As shown in Figure 2, NS2B contains four transmembrane domains, as well as a signal sequence, making it an ideal anchoring domain (AD) for our assay [23]. By combining ER-anchoring properties of an array viral proteins or domains with Gal4 ability to transactivate a fluorescent reporter gene we wished to develop an assay that couples the ER with the nucleus. In the assay, stable expression in mammalian cells will allow for monitoring protease activity, in search for novel protease inhibitors, a priority for the fight against DenV.

**MAIN GOAL: DEVELOPMENT OF A CELL-BASED ASSAY THAT COUPLES CYTOSOLIC PROTEASE ACTIVITY WITH ENDOPlasMIC RETICULUM VIRAL PROTEIN LOCALIZATION**

**Identifying Anchoring Domains (AD):** By exploiting the natural topology of the various proteins of the DenV proteome, we can identify proteins that act as strong anchoring domains to the ER and/or contain a strong ER signal sequence, outcompeting the nuclear localization signal of DBD/TAD. By analyzing the topology of the virus, the assay has been initially engineered to utilize NS2B, which is known to be strongly associated to the ER. NS2B contains four transmembrane domains, including one that acts as a signal sequence.
Figure 5. Depiction of the proposed assay: A viral protein anchored to the ER (red shape) fused to a known CS of the same virus (yellow oval) and DBD/TAD of Gal4, can be used to monitor cleavage. The left panel shows an either inactive or inhibited protease, where cleavage will not occur, and DBD/TAD will remain anchored to the ER. The right panel shows an active, uninhibited protease that cleaves the centrally located CS, releasing DBD/TAD and allowing it to travel to the nucleus where it will activate the upstream activation sequence (UAS) fused to a green fluorescent protein (GFP). When GFP is expressed the cells will fluoresce green, indicating cleavage.

**Monitoring Protease Activity**: In order to provide the source of protease *in cis*, the catalytic domain of NS3 was added to the AD, containing the natural CS boundary between the two proteins NS2B and NS3. In the assay, the AD is fused to the DBD and TAD of Gal4 through a glycine linker. The assay will thus be ultimately used to monitor cytoplasmic viral protease activity via the NS2B/3 CS, in the AD. By expressing the assay in an inducible system, when induced, DBD/TAD will be released when cleavage occurs and will travel to the nucleus where it will bind UAS and activate transcription of GFP. The assay will thus provide a platform for high-throughput screening of DenV specific antivirals against the viral protease (Figure 5).
CHAPTER 2

MATERIALS AND METHODS

GENERAL CLONING

Vectors constructed for this project were digested using restriction enzymes purchased from Fermentas (Glen Burnie, MD), and primers synthesized by Integrated DNA Technologies (San Diego, CA). Conventional primers contain 21bp of homology to the sequence being amplified with a TATATA tail flanking terminal restriction sites. Primers used to introduce a mutation with overlapping PCR contain the amino acid codon desired, flanked by 21bp of homology on each side.

PCR products were amplified using .5µL of each forward and reverse primer at 20pMol and 1U Pfu polymerase (Fermentas) in a 25µl reaction. For PCR reactions the initial denaturation step was carried out at 95° for 5min. Following the initial step, 32 cycles were carried out beginning with 0:30 sec denaturation at 98°C, followed by 0:30 sec annealing at a temperature specified by the Tm of the homologous sequence of the primer, typically around 58°C, and an elongation at 72°C lasting 0:60 sec for each 1Kb being amplified. After 32 cycles a final elongation step was carried out for 10min followed by cooling to 4°C.

PCR products were run on a 1% agarose gel, and the desired bands were gel extracted and purified using an adaption of the Patterson Protocol [37]. Vectors and inserts were digested using restriction enzymes from Fermentas, and following their protocols. Ligations were performed using 1U T4 DNA Ligase (Fermentas) in a 10µl reaction with approximately 50-200ng of total DNA with a molar ratio of 1:5, vector to insert. Ligation products were transformed into chemically competent E. coli XL-1 Blue Cells from Invitrogen. After plating transformations on LB/Ampicillin (100ug/mL) plates, cells transformed with retroviral and non-retroviral vectors were grown at 30° C and 37°C, respectively. The transformed plasmids from Ampicillin resistant colonies were purified using GeneJET™ Plasmid Miniprep Kits.
Constructs were assembled in the transient mammalian expression vector, pcDNA 3.1 Zeo (+) multiple cloning site. The DenV2 New Guinea cDNA template used was kindly provided from Dr. Mohana of Universidade Federal do Rio de Janeiro. The HCV products were amplified using JFH1 template provided from Dr. Wakita, Department of Virology II, National Institute of Infectious Diseases, Tokyo.

Once assembled and analyzed by transfection, relevant constructs in pcDNA were amplified by primers flanked with AscI sites on both ends. These were then digested with AscI in preparation for transfer to the inducible pH TRE plasmid with a selectable IRES-Neptune fluorescent marker. This plasmid was also digested with AscI, but was further blunted utilizing Fast AP (Fermentas). The expression vectors for the reporters 5xUAS-GFP and for rtTA were developed previously in the Wolkowicz Lab [31].

**FLOW CYTOMETRY ANALYSIS AND SORTING**

Trypsinized adherent cells (see “Cell Maintenance”) were centrifuged at 1500 rpm for 5 min, decanted, washed twice with 1xPBS, and resuspended in 1xPBS before being loaded into a flow cytometer. Flow cytometry analysis and sorting was performed on a BD FACSARia (with 405nm, 488nm, and 633nm lasers). Flow cytometry analysis was also performed on a BD FACS Canto (with 488nm and 633nm lasers). Data was collected on FACSDiva 6.1.1 software and then exported to FlowJo for analysis.

**CELL MAINTENANCE**

HEK293T and Phoenix GP were provided by Dr. Gary Nolan, from Stanford University. Huh7.5.1 hepatocytes were generously provided by Dr. Frank Chisari (Scripps Research Institute, La Jolla, CA). Adherent cell lines are maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro), supplemented with 10% fetal calf serum, penicillin-streptomycin, and 2mM L-glutamine. Another cell line engineered in the lab stably expressing rtTA and UAS-GFP, referred to as 293T RUGs, are maintained the same way.

For cell passaging, the cells were incubated in Trypsin-EDTA (Invitrogen) for 5 minutes at 37°C to detach from the plates. Trypsin was promptly neutralized by diluting the mixture 1:5 with complete DMEM.
The non-adherent cell line, also provided by Dr. Nolan, SupT1 cells, were maintained at 37°C and 5% CO₂ in RPMI (Cellgro) supplanted with 10% fetal calf serum, penicillin-streptomycin, and 2mM L-glutamine. Cells were routinely screened for mycoplasma contamination.

**TRANSIENT TRANSFECTIONS**

For transient expression of constructs in HEK 293T or Huh 7.5.1 cells, 1.5 x 10⁵ cells were seeded per well in a 12-well plate 24 hours prior to transfection. For the transfection mixture, 125µL DMEM without supplements was mixed with 2mg/mL linear 25kDa polyethylenimine (PEI) (Polysciences, Inc.) and each construct at a 6µL of PEI: 1µg DNA ratio. This mixture was incubated at room temperature for 20min, and then added drop-wise to cells in the 12-well plate reaching 50-60% confluency. For inducible vectors, Dox is added at 1ug/mL. Transfections in 10cm plates were seeded with 2.5x10⁶ cells 24 hours prior to transfection and 15uL of PEI: 3ug DNA ratio was used for the transfection mixture.

**GENERATION OF INFECTIOUS VIRAL PARTICLES**

For production of MLV, the Phoenix GP cell-line at 60–70% confluence was transfected with 6µg of the transfer vector (pBMN) and 3µg of a vector expressing the Envelope glycoprotein of the Vesicular Stomatitis Virus (pCI-VSVg). DMEM media was replaced 24 hours post-transfection and viral supernatant was collected 48 hours and at 72 hours after transfection. For the production of HIV based virus particles, HEK293T cells were transfected with 6µg transfer vector (pH vectors), 2µg pCI-VSVg, 1µg of Viral Protein R-expressing vector (pRSV-Vpr), and 3µg of pCMVΔ8.2 (kindly provided by Didier Trono, EPFL, Switzerland). DMEM was replaced 24 hours post-transfection and viral supernatant was collected 48 hours post transfection. All viral stocks were filtered with 0.45 micron PTFE filters (Pall Corporation) and frozen at −80°C.

**TRANSDUCTIONS**

HEK293T cells grown in DMEM at 250,000 cells/well in a six well plate were prepared for transduction. 24 hours after plating cells were treated with 5µg/mL Polybrene (Hexadimethrene Bromide, Sigma) and transduced with viral stocks by hanging bucket rotors centrifuge (Becton Dickinson) at1500G’s, for 120 minutes at 32°C. 24 hours post
transduction media was changed. Cells were then analyzed for expression 72 hours post-infection.

**FLUORESCENT MICROSCOPY**

Cells were checked for fluorescence on a Zeiss Observer D1 microscope with a 50x lens connected to an AxioCam MRm camera, and analyzed on Axio-Vision software.

**WESTERN BLOTS**

Trypsinized adherent cells were lysed in NP-40 lysis buffer (150 mM NaCl, Tris-Cl 50 mM, 10% Glycerol, 0.25% NP-40) supplemented with Complete Protease Inhibitor cocktail (Roche, Indianapolis, IN) on ice for 30 min. Cellular extracts centrifuged at 14,000rpm for 10 min. The supernatant fraction was then boiled in Laemmli’s 4X loading buffer with 10% BME at 100°C for 5 min and run on either 8 or 12% SDS-PAGE Tris-Glycine gels at 120V for 90 minutes. Proteins were transferred to PVDF membranes at 110mA for 90min, or 15V overnight. Membranes were blocked with 5% skim milk in PBST (PBS+ 0.05% Tween 20) at pH 7.5 (with 1M HCl) for 60 minutes at room temperature. The primary antibody was 1:500 rabbit anti-HA (Cell Signaling). Membranes were washed in PBST three times for 5 min at room temperature following primary and secondary stains. Anti-rabbit IgG conjugated to HRP (Cell Signaling) was used as secondary antibody at 1:2000 dilution staining for 30 minutes at room temperature. Antibody staining was detected by an Enhanced Chemiluminescence kit (Pierce).

**IMMUNOFLUORESCENCE**

For confocal microscopy 2x10^4 Huh 7.5.1 cells were seeded per well on glass cover slips in a 24 well plate 24 hours before transfection. Cells were transfected as described previously. 48 hours post transfection the cells were fixed in 4% PFA for 10min and permeabilized in 100% ice cold methanol. Cells were washed three times in 1xPBS and blocked with 5% BSA in 1xPBS before staining with primary antibodies: anti-PDI (1:75)(Santa Cruz), anti-DBD(1:500)(Santa Cruz). Cells were washed three more times with 1xPBS before staining with AlexaFluor 488 and 555 conjugated secondary antibodies at a 1:1000 dilution. Cells were again washed three times with 1xPBS then stained with DAPI at 1:5000 dilution. After a final set of PBS washes the glass cover slips were then mounted on
microscope slides and visualized using the ZEISS LSM 710 confocal microscope. Analysis was accomplished using Zen lite 2012 copyright Carl Zeiss Microscopy.
CHAPTER 3

RESULTS

DEVELOPMENT OF THE ASSAY IN TRANSIENT EXPRESSION VECTORS

As stated, the assay is designed to mimic, as much as possible, the natural viral lifecycle. Thus, the constructs of the assay have been divided into two domains, which have been linked: the anchoring domain (AD) and the protease-monitoring domain. AD is required to ensure the scaffold protein embeds itself into the appropriate cellular compartment where natural viral protease activity occurs: the ER membrane. The protease-monitoring domain relies on the Gal4 protein, which, when released from the anchored scaffold, travels to the nucleus and activates the UAS-GFP element. Depending on the nature of the AD, protease can be either supplied in cis, where it is a part of the AD, or added in trans from an independent source.

Investigation of the viral proteome of several Flaviviridae lead us to chose the single transmembrane domain of the HCV viral protease cofactor NS4A as the initial anchoring domain for the implementation of the assay (Figure 6A) [38, 39]. Previous studies have suggested that NS4A embeds and anchors fusion proteins to the ER membrane [40]. NS4A was thus linked to Gal4 through a cleavage site recognized by the HCV viral protease NS3. The cleavage site chosen was the adjacent cleavage site between NS4A and NS4B (Figure 6B). The chimera was expected to be targeted to the ER membrane through NS4A and in such a way expose the CS and Gal4 in the cytosol. A co-transfection experiment was performed with this construct in conjunction with three different sources of protease (PR): NS3, NS3/4A, and NS2/3/4A. The preliminary results (Figure 6C) suggested that NS4A was unable to retain the chimera in the ER, probably due to the strong nucleophylic properties of Gal4. Had the protein been retained in the ER, the level of GFP expression
Figure 6. Utilization of NS4A of HCV as initial AD of the assay: A. Depiction of the HCV proteome, showing only the relevant proteins of the construct: NS3/4A/4B. The teal arrows show the cleavage sites for the NS3/4A Serine protease between the NS3/4A and NS4A/B boundaries. B. Linear depiction of the assay construct. In yellow, the NS4A transmembrane cofactor serving as the AD; in blue, the NS4A/B cleavable site by the PR if added in trans. The linker and Gal4 are in white and grey, respectively. C. Results of transfection in naïve 293T cells: All wells transfected with the UAS-GFP reporter. Top panel: Control with UAS-GFP only, control with Gal4, and the NS4A-CS-Gal4 construct. Bottom Panel: Co-transfection with various sources of PR, in order to show cleavage.

would have decreased in comparison to the Gal4 only control. If NS4A had embedded in the chimera in the ER, addition of sources of PR would have reconstituted GFP expression. No change in GFP expression was observed, suggesting that NS4A might not be the ideal protein of choice as an AD.

According to literature, the viral protease cofactor of DenV, NS2B, encodes for an ER signal sequence at its N-terminus, necessary for proper topology of the viral proteome (Figure 2) [23]. Importantly, the NS2B/3 CS is cleaved in cis as the first cleavage event by
the NS2B/3 complex [28]. We have thus decided to engineer a new scaffold that contains the natural NS2B protein through the catalytic domain of NS3 (186 amino acids) as the putative AD for the assay [41]. This domain was fused to Gal4 through a Glycine linker (Figure 7). Furthermore, as previous studies in the lab have shown a possible cleavage site by NS2B/3 in the C-terminus of DBD we have also deleted the last ten amino acids of DBD. This shorter version of Gal4 did not have any effect on the nuclear localization or binding activity to the UAS. This version of Gal4 was thus used in the engineering of all constructs further developed to study DenV PR.

**Figure 7.** Linear representation of assay constructs: Top: The N-terminus (yellow) contains the AD, and in these constructs, both the source of protease and the CS are arranged in the AD. The C-terminus (blue) contains the protease monitoring domain, specifically DBD/TAD and an HA tag. Bottom: Specific protein boundaries used as ADs. The Linker-DBD-TAD-HA remain unchanged in the constructs. The ADs include NS2B/3cat, NS2B/3full and NS3cat only, as described above.

Since there are currently no inhibitors of DenV that directly target PR, mutated constructs were made to serve as controls. In order to make NS3 catalytically inactive, a unique amino acid change is required within the catalytic triad of the enzyme. The Serine at position 135 was thus mutated to an Alanine, resulting in a mutated protease (mPR) [41]. A second control was engineered with active WTPR but with no CS, by destroying the CS between NS2B/3, making it unrecognizable to PR. Typical Serine PRs recognize a very specific pattern of amino acids, as previously mentioned. The cleavage site of NS2B/3 is comprised of the C-terminal three amino acids of NS2B followed by the N-terminal three
amino acids of NS3 [28]. In previous studies, the mutation of the P1 position from a basic amino acid to a small, neutral one, destroys the ability of a Serine PR to cleave the CS [42]. The NS2B/3 CS was thus mutated at the last amino acid of NS2B, converting an Arginine into a Serine.

Further, literature has shown in several Flaviviridae that the Helicase domain (the C-terminal two-thirds of NS3) enhances the efficiency of proteolytic cleavage by NS3 [29]. Interestingly, there is also a CS in the helicase domain [28]. Constructs were thus also engineered to contain the full-length NS2B/3 proteins as putative ADs (Figure 7). Both the WT and catalytically inactive mutant versions were engineered for comparison.

Finally, to confirm that NS2B is the domain responsible for anchoring, constructs containing only NS3cat with no NS2B were engineered for comparison (Figure 7). NS3cat was again, with both WT and mutant forms, linked to Gal4. If NS2B is indeed the domain responsible for signaling and anchoring to the ER, these new constructs, which lack NS2B, should activate GFP. This is even more so, considering that literature suggests that NS3 alone is catalytically inactive without the NS2B cofactor.

These fusions were engineered in the transient mammalian expression vector, pcDNA, where transfections of the plasmids into human immortal cell lines in order to understand and study the activity of the protein inside the cell. This allowed us to identify the ideal viral anchoring domains, which will be ultimately transferred into a Lentiviral plasmid, that will allow for stable expression in the relevant cell lines.

CONFIRMATION OF AN ACTIVE PROTEASE

The assay fundamentally relies on the ability to monitor GFP expression in an inducible system; however, the constructs required engineering in a transient expression vector to identify the relevant trends, as described previously. In order to analyze the constructs, we have thus utilized a cell line previously developed in the lab, referred to as RUGs, because they contain the rtTA (R) element, and the UAS-GFP (UG) element. An initial transfection experiment was performed in the 293T RUGs with the constructs to monitor whether GFP expression is observed. While GFP expression as detected by flow cytometry can be the results of Gal4 only or Gal4 fused to the entire non-anchored fusion protein, it was crucial to exploit classical western blotting for determining the size of the
protein products. Therefore, in order to confirm that protease actively cleaves the NS2B/3 boundary, and does not simply trans-locate to the nucleus as part of the intact fusion, we performed flow cytometry analysis, and then lysed the cells for western blot analysis.

These preliminary results were promising as they showed similar level of GFP expression when compared to the Gal4 positive control (Figure 8A). The transfection control (pBMN IRES-GFP) showed a much higher GFP level, probably due to a higher rate of transfection. The RUG cells, when the UG element was activated, were not extremely robust. We therefore, transduced with UAS-GFP again, and re-sorted the population that had high GFP expression, when activated by Gal4. Naïve cells and WT construct-transfected cells were lysed and prepared for western blotting. The results (Figure 8B) show a band of around 52kD, which is the expected size of the cleaved product; NS3 Linked to Gal4. Interestingly, several other faint smaller bands appeared on the blot, and not in the negative control of naïve cells only. There also appeared to be two bands very close together at the expected 64kD size of the full length, uncleaved fusion, which were further separated using a lower percentage acrylamide gel in later experiments.

A second transfection was performed in naïve 293Ts for western blotting purposes only (Figure 9A); in order to further separate the top bands that were extremely close in size. Eight percent acrylamide gels successfully separated these, and were used for all further experiments unless specified otherwise. As stated previously, according to literature, the first of the four transmembrane domains of NS2B is a signal sequence that targets the protein to the ER membrane required for proper topology [23]. Typically intramembrane host signal peptidases cleave at these types of protein boundaries [43]. Thus, utilizing a transmembrane prediction generator, the amino acid sequence of NS2B was studied in silico (Figure 9B) to pinpoint the exact position of the first transmembrane domain [44]. Identified to be within the first twenty amino acids the sequence was then analyzed utilizing the SignalP 4.1 Server (Figure 9C), which identifies potential cleavage sites of signal peptides [45]. At the nineteenth amino acid, the sequence analysis program predicted a potential cleavage site, which could explain the less prominent band observed at around 64kD. The more prominent band at 63kD is most likely the WT fusion, with the signal peptide being cleaved by the host.

The catalytically inactive mutant, mPR, as described previously, was designed as an additional negative control, as it serves as a proteolytic inactive protein. This mutant was
Figure 8. Transfection analysis with flow cytometry and western blot: A. Flow cytometry analysis with very low background GFP in naïve cells, and a high transfection efficiency shown in column two. There was low GFP expression from the Gal4 only control, similar expression in the WT. B. A 12% Gel was used and stained with αHA antibody, as previously described. The arrow points to the cleaved product.

transfected into 293Ts alongside the WT version, and Gal4 HA. Once again, a western blot was performed to identify if the mutant, with a unique amino acid change, was indeed catalytically inactive (Figure 10). Significantly, the results show a complete inhibition of not only the primarily cleaved product (at the NS2B/3 CS), but also of the previously noticed smaller bands. Western blotting thus suggested that no cleavage activity was observed with this mutant. Interestingly, there were two high bands, similar to the WT, further suggesting that this is host protein modification.

In order to explain the suggested promiscuity of the WT protease in regards to the multiple faint bands, the sequence of the entire chimera was scanned in search of any potential cleavage motifs utilizing Serial Cloner 2.6 [46]. As previously described, the P2 and P1 positions contain highly basic amino acids, followed by a small one at the P1’ position. Combinations of Lysine, Arginine and Glutamine in the P2 and P1 positions were first identified. Those sites were then analyzed to have a Serine, Alanine, Glycine or Threonine at the P1’ position. Two regions within the entire fusion were found that fit this
Figure 9. Western blot analysis with transmembrane and cleavage predictions: A. The western blot: Separating the top bands, and showing two distinct bands at two different intensities. Across from the western blot is a linear reference of the WT construct that was transfected with the red arrow showing where the viral protease cleaves, creating the lowest band on the western blot. B. The transmembrane predictor of the NS2B sequence, identifying the first transmembrane domain in the first twenty amino acids. C. The signal peptide cleavage site predictor at amino acid 19.

Figure 10. Western blot analysis of naïve 293T transfection: The first three lanes are controls, with an untransfected negative control, an HA positive control from a previous experiment, and a transfection Gal4 HA control in lane 1. Lane 2 shows the WT active protease, and lane 3 shows the catalytically inactive mutant with no cleaved products.
motif, which may explain the presence of additional multiple cleaved products observed in the WT lysates.

Although western blotting shows a completely active an inactive PR through the WT and mPR constructs, respectively, the flow cytometry data showing GFP activation is not as robust. A transfection experiment was performed to compare GFP expression with the addition of alternative sources of PR (Figure 11). The three sources that were cotransfected include NS2B/3cat WT, NS2B/3full, and NS2B/3cat mPR. Interestingly, the WT construct, alone, activated 12.8% GFP, but with the sources of PR added in trans, GFP expression definitely increased, especially with the NS2B/3cat WT and mPR plasmids. The NS2B/3full PR source did not have as robust of an increase as the others (Figure 11A).

Also studied in this transfection, were two other constructs that had been engineered. The NS2B/3full WT linked to Gal4, as well as the NS2B/3cat mCS linked to Gal4 constructs. The NS2B/3full as AD showed no GFP expression, and interestingly, no HA expression in the western blot, leading us to believe there may be a mutation in the construct. The mCS chimera, however, activated 9.4% GFP, which was lower than the WT, but higher than the mPR. The single amino acid mutation at the P1 position was probably not enough to completely disguise the recognition site from the active protease; however, it significantly reduced cleavage. The cleaved product was only visible at the twelve-minute exposure (shown by the orange arrow) (Figure 11B).

**IDENTIFYING ANCHORING PROPERTIES**

The assay specifically relies on the presence of a perfect ER anchoring domain to ensure that proteolytic activity of the DenV protease is monitored in the proper cellular compartment: cytosolic cleavage adjacent to the ER. Thus, two constructs were engineered to compare anchoring properties in the presence and absence of NS2B. Again, for these experiments a non-inducible system was used, and we therefore utilized 293T UGs, which contain only the UAS-GFP element. Thus, this cell population only relies on a source of Gal4 from the pcDNA mammalian expression system. The Gal4 control in the transfection reached 31.8% (Figure 12A). The other constructs tested contained the catalytic domains of NS3 in both WT and mutant versions, with or without NS2B. The robustness of the NS2B/3cat WT was still very low in the transient transfection, but will probably be improved
Figure 11. Transfection experiment of Protease in trans: A, The first panel shows the necessary controls of the experiment, column 1 is the untransfected control, column 2 shows the activation of UAS-GFP by a Gal4 control, and columns 3 and 4 show the mPR and WT NS2B/3cat constructs linked to Gal4, respectively. The second panel shows the WT NS2B/3cat cotransfected with different sources of protease in columns 1-3: NS2B/3cat, NS2B/3full and NS2B/3cat mPR, showing that NS2B/3cat enhanced cleavage the most. Additionally columns 4 and 5 show transfections of two previously mentioned constructs, but not shown yet. NS2B/3full fused to Gal4 and the mCS version of NS2B/3cat. The NS2B/3full chimera was not made, confirmed by western blotting. The mCS chimera showed a reduction of cleavage at the NS2B/3 CS, but not complete inhibition. B, This shows a western blot prepared from the lysates of the cells shown in part A. Lanes 1 and 2 show the mPR and WT constructs as controls. Lanes 3-5 show the WT construct with the addition of PR in trans. Lane 6 shows the NS2B/3full construct, which was completely negative. Lane 7 shows the mCS construct, and 7* shows the same construct exposed at twelve minutes, showing a drastic reduction of cleavage at the NS2B/3 CS (shown by the orange arrow).
Figure 12. Transfection analysis with flow cytometry and western blot: A. Flow cytometry analysis. Top panel shows controls, with very low background GFP in naïve cells, and a high transfection efficiency shown in column two, and high Gal4 activation of GFP. Bottom panel shows comparable GFP expression between WT and mPR constructs of NS2B/3cat as AD constructs. Followed by NS3cat only as AD constructs, with more GFP in the WT than the mPR. B. Western blot utilizing the same cells as analyzed by flow cytometry. Positive HA control using a previous lysate, and negative HA control using untransfected cells. Lane one shows the Gal4 control. Lanes two and three show WT and mPR constructs with NS2B/3cat as AD, respectively. Lanes 4 and 5 show WT and mPR constructs with NS3cat only as AD, respectively. Importantly, both WT constructs have a very active protease, with or without the cofactor.

in cell lines stably expressing the assay. Importantly, the trend of reduction of GFP expression was observed with the NS2B/3cat mutant. Interestingly, the NS3cat only constructs have increased GFP expression; although, not as high as the Gal4 control. It is thus possible that NS3cat is still attached to Gal4 hindering its ability to translocate to the nucleus or the ability of DBD to bind UAS. However, the WT NS3cat shows more GFP than
the mutant, also confirming other potential cleavable sites within the construct that further separate Gal4 from the entire chimera. Analysis by western blotting with the NS3cat constructs show that the cleaved product in the NS2B/3cat WT construct is in fact the cleaved product at the NS2B/3 protein boundary (Figure 12B). Contradictory to literature, which suggests that NS3 is only catalytically active when it is associated with the NS2B cofactor, the WT NS3cat construct does show activity, as observed by the several bands, whereas the mutant version of the construct has no subsequent bands [28].

It was important to further prove protein localization. In order to visualize where the proteins were in the cell, we thus performed an additional experiment for confocal microscopy. An adherent hepatocytic cell line called Huh 7.5.1 was transfected with the WT, mPR and mCS versions of the NS2B/3cat-L-Gal4 constructs, in addition to the Gal4-only control. DAPI staining was used to visualize nuclei (blue), as seen throughout all images (Figure 13). The top panel shows an anti-DBD stain (red), and the bottom panel shows both anti-DBD and anti-PDI, used as ER marker. A naïve, untransfected control was used to show non-specific DBD stain. In the Gal4 positive control, DBD is localized to the nucleus as expected. In all three fusions with NS2B/3cat (WT, mPR and mCS constructs), showed a very strong DBD signal not only perinuclear, but specifically in the ER. This confirms our previous results that the NS2B protein has indeed a strong signal sequence that targets and embeds the protein in the ER. Interestingly, we did not observe DBD in the nucleus with WT, as we did observed by flow cytometry.

**Inhibition of Host Cell Proteases**

The results suggest that the active NS2B/3 viral protease is the enzyme responsible for cleaving several sites within the protein fusion; however, to confirm that all cleavage events observed were originated from viral protease, and not cellular proteases, experiments were performed in which the cells were treated with a cellular Serine and Cysteine Protease Inhibitor (PI) Cocktail Set 1 (Merck). The cells were treated with increasing concentrations of the stock inhibitor immediately after transfection with the WT NS2B/3cat constructs. The negative control for HA staining was untransfected 293T RUGs, and the positive control was an HA positive lysate from a previous western blot. Figure 14 shows the western blot of this analysis. Lane one contains the Gal4 control, lane two contains the NS2B/3cat mPR
Figure 13. Confocal microscopy, showing protein localization: Column one shows the naïve (untransfected) Huh 7.5.1 cells, stained. Column two depicts the Gal4 positive control. Columns three, four and five depict the WT, catalytically inactive protease (mPR), and the mutated NS2B/3 CS (mCS) versions of NS2B/3cat linked to Gal4, respectively. The top panel shows the nuclear DAPI stain (blue) in tandem with the anti-DBD stain (red). The bottom panel shows both DAPI and anti-DBD with the addition of the anti-PDI ER marker (green).

Figure 14. Western blot analysis of transfection treated with a host protease inhibitor: The band at 52kD is the cleaved product by the viral protease (shown by the arrow); all of the lower bands were being tested to confirm that the viral protease was cleaving these boundaries. Inhibition of cellular proteases was performed with a cocktail of PIs.

- + 1 2 3 4 5 6 7 8 9

construct, and lane three contains the NS2B/3cat WT construct, uninhibited to serve as controls. Lanes four through nine contain lysates of cells that were transfected with the NS2B/3cat WT construct, but were treated with the protease inhibitor cocktail, at increasing concentrations from 0.4x, increasing at 0.4x increments, up to 2.4x, seen in lane nine. Importantly, the cells did not exhibit cytotoxicity at any concentrations. Overall, the host
protease inhibitor cocktail showed no effect on the appearance of the lower faint bands observed with the constructs in the absence of inhibitors.

Interestingly, in Figures 11, 13 and 15 two very close bands were always observed in the Gal4 only control. An additional experiment was performed to treat the Gal4 transfected cells with the same PI cocktail (at 800x), to study if host proteases are actually cleaving within Gal4 alone (Figure 15). There was very little change in GFP expression when cells were incubated with PI, and importantly, the change observed was a decrease in GFP rather than an increase; although, this could be due to transfection efficiency. When the cells were lysed for western blotting, both the untreated and treated cells had the same two bands, leading us to believe that host cell proteases are unlikely to be cleaving Gal4, but it does not disprove host post-translational modification of the Gal4 protein.

Transfection of 293T-RUGs

Figure 15. Flow cytometry and western blot examining Gal4 processing: A. The flow cytometry analysis showing GFP expression. The first plot shows the untransfected negative control, and the activation of GFP by Gal4, without and with incubation of PI cocktail, respectively. B. The western blot analysis of the same cells run for flow cytometry. The positive HA control has bands around 55 and 70kD, and the untransfected negative HA control has none. Lanes 1 and 2 show two bands in Gal4, treated without and with the PI cocktail.
CHAPTER 4

DISCUSSION

CONCLUSIONS

An assay was developed for the purpose of monitoring proteolytic activity of the DenV protease. The nature of the assay was also exploited to analyze ER targeting and ER anchoring capabilities of viral proteins. Originally, an HCV ER (protease cofactor NS4A) bound protein was used in an attempt to anchor the assay to the ER; however, the single transmembrane protein was unable to localize the fusion to the proper cellular compartment. The assay was then adapted to DenV, and engineered to exploit the natural topology and intracellular localization of the viral life cycle during infection. The platform relies on the highly studied Gal4-UAS system, which activates GFP expression in the presence of an active protease. Protease activity should be then detected by flow cytometry and/or fluorescent microscopy. With a convenient HA tag at the C-terminus of the protein chimeras; proteolytic activity and protein localization can also be monitored through western blotting and confocal microscopy. A single Serine-to-Alanine mutation at the catalytic triad of the NS3 protease completely abolished the catalytic activity, as expected from a mutated protease at the catalytic triad. Although the single Arginine-to-Serine substitution within the cleavage site was not enough to abolish cleavage completely, cleavage was severely hindered.

The choice of NS2B as a putative AD proved right. Utilization of NS2B to signal and anchor the fusion to the ER was incredibly robust due, most probably, to the four transmembrane domains present in the protein. Deletion of this protein, completely abrogated localization to the ER, as seen by the inability of the NS3cat constructs to anchor. Cells transfected with this construct were indeed very positive for GFP expression, further corroborating this finding. However, Western Blot analysis revealed that the absence of the required protease cofactor did not completely revoke the cleavage ability of NS3cat. Also, interestingly, in silico analysis of the first transmembrane domain of NS2B revealed a signal
sequence that can serve as a putative cleavage site of host signal peptidases. These membrane-localized enzymes serve to free signal sequences from their proteins immediately following protein insertion within the ER membrane. If proven so, this is an uncharacterized cleavage boundary not yet described in literature.

Addition of the helicase domain to the AD did not appear to enhance proteolysis; however, further studies must be performed to further corroborate these findings. Interestingly, cotransfection with a second source of protease increased cleavage events, *in trans*, as seen by higher GFP expression in the cell. Inhibition of cellular proteases further proved that all cleavage events observed in our assay could be attributed specifically to the activity of the DenV NS2B/3 protease.

Moreover, although Huh 7.5.1 cells are ideal not only as the natural milieu of DenV infection, but also as an adherent cell type for confocal microscopy, they are not as readily transfected. In comparison to 293Ts, which have a very large nucleus and a relatively smaller cytosol, the Huh 7.5.1 cells allow for better visualization of cytosolic proteins, but are not optimized for cell uptake of DNA. Thus, much lower transfection efficiencies are achieved and thus much less protein expression. This also corroborates the hypothesis that the remaining NS3 that is still fused to Gal4 may also hinder the ability of the protein to translocate to the nucleus.

**FUTURE DIRECTIONS: CELL LINE DEVELOPMENT, ADDITION OF A SECOND CLEAVAGE SITE AND EXPLORING OTHER ANCHORING DOMAINS**

By utilizing NS2B as the optimum AD, the entire fusion will be transferred into the inducible, lentiviral vector, pH-TRE IRES-Neptune. Through retroviral technology, the assay can be then transduced into mammalian cell lines containing the other necessary elements of the assay, which include rtTA and UAS-GFP, as stated previously. The internal ribosome entry site (IRES) Neptune component on the plasmid serves as a selectable marker for flow cytometry analysis and sorting, as cells containing the assay will be fluorescent in a channel different to the reporter (GFP). Due to the potential toxic effects that viral proteases often have inside the cell, the cells that have been successfully transduced can be sorted based on Neptune expression without the need to induce protease expression with Dox [47].
Other constructs will be engineered in order to further understand the topology of the proteome as well as to increase the robustness of the assay (Figure 16). The NS2B/3cat can be replaced by other proteins of the virus that can serve as putative ADs. The extremely strong signal sequence at the 5’ of the Capsid protein within the viral proteome serves as the natural signal for anchoring of the entire polyprotein to the ER as it is translated during an infection [23]. By engineering an AD that contains the entire Capsid through NS3cat segment, we envision to increase the anchoring capabilities of scaffold protein of the assay. In this way, the assay will increasingly mimic the natural DenV infection inside the cell, as well as help us understand the topology of the polyprotein.

![Figure 16. Linear representation of future assay constructs: Top: The top construct lengthens the AD to contain DenV Capsid through NS3cat. The red arrow indicates the initial cleavage event in cis. The viral protease will further cleave all cytosolic facing boundaries upstream NS2B. Middle: The middle construct depicts the addition of a second cleavage site, further releasing Gal4 from the fusion. Bottom: The bottom construct shows exploitation of NS2B’s anchoring properties to monitor cleavage events of any substrate of interest.](image)

Due to the potential inability of Gal4 to travel to the nucleus with the NS3cat still fused to it, or potential blockage of the DBD binding to UAS, a second cleavage site will be added within the linker. This cleavage site will contain the boundary of NS4B/5, shown in literature to be efficiently cleaved by the viral protease in trans. In the context of the assay, active PR will initially cleave the NS2B/3 boundary, then freely cleave the second CS within the linker between the AD and Gal4. With the cleavage of this boundary, Gal4 will be further separated from NS3cat, allowing it to easily travel to the nucleus to activate GFP.

Ultimately, this assay can be used to identify novel cleavable substrates by any viral protease of interest. Through these experiments, NS2B has been shown to efficiently signal and anchor proteins to the ER; therefore, it is an ideal AD. Utilizing only NS2B as an AD, a
cellular cDNA library can be inserted within the Linker. With the addition of a viral PR in trans, novel cleavable substrates can be identified. Cleavage events by the viral PR will be monitored, as in the original assay, through the activation of GFP. Importantly, the substrate library may contain protein boundaries that are cleaved by proteases of the host; therefore, a control must be utilized where the viral PR is not present to identify the baseline of cleavage before in trans activation of viral PR.

**ADAPTATIONS TO OTHER VIRUSES AND DRUG DISCOVERY**

The assay exploits the natural topology and localization of DenV, and can further be adapted to monitor viral proteolytic cleavage of many other viral proteases. All of the members of the *Flavivirdae* family have a similar life cycle, in which they translate and process a single polyprotein that is then anchored and woven in and out of the ER membrane [28, 48]. Simply by replacing the AD of the current assay (DenV NS2B/3cat) with the same protein segment in any other *Flavivirus*, such as West Nile Virus, the assay will be applicable to that virus. Importantly, since these viruses are extremely similar, the assay can be multiplexed to search for inhibitors of many viruses, or viral serotypes, in a single well, exploiting the fluorescent genetic barcoding methodology developed in the laboratory. This tool is ideal to adapt the assay to high-throughput screenings with various chemical libraries.
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REFERENCES


APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS

AD: Anchoring Domain
APC: Allophycocyanin
BD: Becton Dickinson
BME: β-Mercaptoethanol
BSA: Bovine serum albumen
cat: catalytic domain
cDNA: Complementary DNA
cm: centimeter
CMV: Cytomegalovirus
CS: Cleavage Site
C-terminus: carboxyl-terminus
DAPI: 4’,6-diamidino-2-phenylindole
DBD: DNA-binding domain
DenV: Dengue Virus
DF: Dengue Fever
DHF: Dengue Hemorrhagic Fever
DMEM: Dulbecco’s Modified Eagle Medium
DNA: Deoxyribonucleic Acid
Dox: Doxycycline
DSS: Dengue Shock Syndrome
E: Flaviviral envelope protein
EDTA: Ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ER: Endoplasmic Reticulum
FACS: Fluorescence Activated Cell Sorting
FITC: Fluorescein isothiocyanate
FSC: Forward Scatter
GFP: Green Fluorescent Protein
HA: Hemagglutinin
HCV: Hepatitis C Virus
HEK 293T: Human Embryonic Kidney
HIV: Human Immunodeficiency Virus
HRP: horseradish peroxidase
hrs: hours
HTS: High throughput screening
Huh 7.5.1: hepatocellular carcinoma cells
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IRES: Internal Ribosome Entry Site
Kb: kilobase
kD: kilodalton
M: Flaviviral membrane protein
mCS: mutant cleavage site
mL: milliliter
MLV: Moloney Leukemia Virus
min: minutes
mPR: mutant protease
mRNA: messenger ribonucleic acid
ng: nanogram
NLS: Nuclear localization signal
NS: Non-structural
N-terminus: amino-terminus
PBS: Phosphate Buffer Saline
PBST: Phosphate Buffer Saline Tween
PCR: Polymerase chain reaction
PDI: Protein disulfide isomerase
PEI: Polyethylenimine
PERK: Protein kinase RNA-like endoplasmic reticulum kinase
PFA: Paraformaldehyde
Phoenix GP: Phoenix gag-pol
PI: Protease inhibitor
PR: Protease
pr-M: Flaviviral pre-membrane protein
PTFE: Polytetrafluoroethylene
PVDF: Polyvinylidene fluoride
RNA: Ribonucleic Acid
RPMI: Roswell Park Memorial Institute media
rt-PCR: real-time polymerase chain reaction
rtTA: Reverse tetracycline Transcription Activator
SDS-PAGE: Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SP: signal peptidase
ssRNA: single stranded ribonucleic acid
TAD: Trans-activation domain
Tm: Melting temperature
TRE: Tetracycline response element
UAS: Upstream-activation sequence
µg: microgram
µl: microliter
µM: micromolar
Vpr: Viral protein R
VSVg: Vesicular Stomatitis Virus glycoprotein
WHO: World Health Organization
WT: Wild type
YFV: Yellow Fever Virus