รายงานวิจัยฉบับสมบูรณ์

ระบบโปรตีนของเซลล์มนุษย์ที่มีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่

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14 มิถุนายน 2557

สัญญาเลขที่ RSA5480024

รายงานวิจัยฉบับสมบูรณ์

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Project Code : RSA5480024

Project Title : Human Host Cellular Protein System Interacting with the Dengue Virus NS1 Protein

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Project Period : 3 years (15 June 2011 – 14 June 2014)

Dengue virus (DENV) is the important causative agent of dengue hemorrhagic fever and dengue shock syndrome that are potentially life-threatening diseases affecting human population globally. At present, the underlying mechanism of dengue diseases remains unclear and no dengue vaccine is commercially available. One of the crucial viral products conceivably involved in DENV production and dengue pathogenesis is the nonstructural protein 1 (NS1). The NS1 protein expresses in multiple forms in different cellular compartments with possibly distinct functions. This study was carried out to identify host cellular proteins interacting with cell-associated NS1 in both DENV infection and NS1 transfection systems and to investigate their interactive roles in DENV life cycle and host response against DENV infection by using biochemical and proteomic approaches. As evidenced by liquid chromatography tandem-mass spectrometry (LC MS/MS), two separate groups of novel host cellular proteins have been identified from DENV-infected HepG2 cells and NS1-expresing Huh7 cells to be potential NS1-interacting partners. Some of the selected proteins have been confirmed for their association with DENV NS1 in virus-infected cells by co-immunoprecipitation assay. Additionally, hnRNP C1/C2 were found to interact with DENV NS1 and viral RNA and moderately influence DENV life cycle at the steps of viral RNA replication and viral protein synthesis. GRP78 was also another host cellular protein interacting with DENV NS1 and likely played a role in viral protein processing. Taken together, our study provided new evidence for potential DENV NS1-inteacting host cellular proteins in DENV-infected hepatocyte cells and addressed certain aspects of host protein and DENV NS1 association in DENV life cycle. Further investigation on detailed molecular mechanisms required for their specific interactions would be needed to pave the way for future research on anti-viral drug development.

Keywords : Dengue virus, NS1, interaction, LC MS/MS, siRNA-mediated gene knockdown

บทคัดย่อ

รหัสโครงการ : RSA5480024

ชื่อโครงการ : ระบบโปรตีนของเซลล์มนุษย์ที่มีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่

ชื่อนักวิจัย : ดร. ศันสนีย น้อยสคราญ (หัวหน้าโครงการ) หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางการแพทย์ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
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ระยะเวลาโครงการ : 3 ปี (15 มิถุนายน 2554 ถึง 14 มิถุนายน 2557)

เชื้อไวรัสเด็งกี่เป็นต้นเหตุสำคัญของโรคไข้เลือดออกเด็งกี่และไข้เลือดออกเด็งกี่ที่มีภาวะช็อคซึ่งเป็นโรคที่อาจก่อให้เกิดอันตรายถึงชีวิตที่มีผลกระทบต่อประชากรมนุษย์ทั่วโลก ในปัจจุบันนี้กลไกการเกิดโรคไข้เลือดออกเด็งกี่ยังไม่เป็นที่ทราบแน่ชัดและยังไม่มีวัคซีนป้องกันโรควางจำหน่าย โปรตีนที่สำคัญตัวหนึ่งของเชื้อไวรัสดังกี่ที่เชื่อว่ามีส่วนเกี่ยวข้องกับการสร้างเชื้อไวรัสและการเกิดพยาธิสภาพของโรคไข้เลือดออกเด็งกี่คือโปรตีนที่ไม่ใช่ส่วนโครงสร้างของเชื้อไวรัสที่เรียกว่าโปรตีน NS1 โปรตีนตัวนี้ถูกสร้างได้หลายรูปแบบในบริเวณที่ต่างกันของเซลล์และอาจทำหน้าที่ได้แตกต่างกัน งานวิจัยนี้ได้ถูกทำขึ้นเพื่อค้นหาโปรตีนของเซลล์เจ้าบ้านที่มีปฏิสัมพันธ์กับโปรตีน NS1 ในระบบของเซลล์ที่มีการติดเชื้อไวรัสเด็งกี่และในเซลล์ที่มีการแสดงออกของโปรตีน NS1 และเพื่อศึกษาบทบาทของปฏิสัมพันธ์เหล่านั้นที่มีต่อการเพิ่มจำนวนของเชื้อไวรัสและการตอบสนองของเซลล์ที่มีต่อการติดเชื้อโดยอาศัยวิธีการทางชีวเคมีและโปรตีโอมิกส์ จากหลักฐานการวิเคราะห์โดยใช้เครื่องแอลซี เอ็มเอส เอ็มเอส พบว่ามีโปรตีนกลุ่มใหม่ที่แตกต่างกันจำนวน 2 กลุ่มจากเซลล์ที่ติดเชื้อไวรัสเด็งกี่และเซลล์ที่มีการแสดงออกของโปรตีน NS1 ที่อาจเป็นโปรตีนที่มีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่ โปรตีนเหล่านี้บางตัวถูกยันว่ามีปฏิสัมพันธ์กันจริงกับโปรตีน NS1 โดยวิธีการตกตะกอนทางอิมมูนวิทยาร่วมกัน นอกจากนี้โปรตีน hnRNP C1/C2 ยังถูกพบว่ามีปฏิสัมพันธ์กับโปรตีน NS1 และสารพันธุกรรมของเชื้อไวรัสและมีอิทธิพลปานกลางต่อวงจรชีวิตของเชื้อไวรัสในขั้นตอนของการเพิ่มจำนวนสารพันธุกรรมและการสร้างโปรตีนของเชื้อไวรัส โปรตีน GRP78 ยังเป็นโปรตีนของเซลล์เจ้าบ้านอีกหนึ่งตัวที่ถูกพบว่าจับกับโปรตีน NS1 และมีแนวโน้มว่ามีส่วนเกี่ยวกับกระบวนการสร้างโปรตีนของไวรัส โดยสรุปงานวิจัยนี้ได้นำเสนอหลักฐานใหม่เกี่ยวกับกลุ่มโปรตีนของเซลล์เจ้าบ้านที่อาจมีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่และทดสอบบางแง่มุมของปฏิสัมพันธ์ที่เกิดขึ้นซึ่งอาจมีผลต่อวงจรชีวิตของเชื้อไวรัส การศึกษาเพิ่มเติมถึงกลไกทางอณูวิทยาในเชิงละเอียดที่เกี่ยวข้องกับการเกิดปฏิสัมพันธ์ที่จำเพาะของโปรตีนจะช่วยนำทางไปสู่การค้นคว้าวิจัยหายาต้านไวรัสเด็งกี่ได้ในอนาคต

คำหลัก : ไวรัสเด็งกี่, เอ็นเอสวัน, ปฏิสัมพันธ์, แอลซี เอ็มเอส เอ็มเอส, การลดการแสดงออกของยีนโดยใช้เอสไออาร์เอ็นเอ

สัญญาเลขที่ RSA5480024

โครงการ: ระบบโปรตีนของเซลล์มนุษย์ที่มีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่

รายงานสรุปปิดโครงการวิจัย

ชื่อโครงการ ระบบโปรตีนของเซลล์มนุษย์ที่มีปฏิสัมพันธ์กับโปรตีน NS1 ของเชื้อไวรัสเด็งกี่

ระยะเวลาโครงการ 3 ปี (15 มิถุนายน 2554 ถึง 14 มิถุนายน 2557)

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน : ดร. ศันสนีย น้อยสคราญ

รายงานในช่วงตั้งแต่วันที่ 15 มิถุนายน 2554 **ถึงวันที่** 14 มิถุนายน 2557

1. การดำเนินงาน

☒ ได้ดำเนินงานตามแผนที่วางไว้

☐ ได้ดำเนินงานล่าช้ากว่าแผนที่วางไว้

☐ ได้เปลี่ยนแผนงานที่วางไว้ดังนี้.....

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2. สรุปผลการดำเนินงานของโครงการ

The nonstructural protein 1 (NS1) is one of the most important viral proteins of dengue virus (DENV) conceivably involved in virus production and pathogenesis of dengue. It exists in different forms in different host cell compartments with potentially distinct functions. At the cellular level, NS1 may be involved in the formation of replicating complexes required for multiplication of DENV; however, very little is known about the interaction of NS1 with host cellular proteins in DENV-infected cells. Therefore, specific aims of our study were (i) to identify host protein candidates interacting with DENV NS1 in human cell line systems, (ii) to verify the interaction between NS1 and host cellular proteins in DENV-infected human cells, and (iii) to investigate the roles of NS1 and host protein interaction in DENV infection. Human hepatic cell lines (HepG2 and Huh7), which have been well recognized for their high susceptibility to DENV infection and potentially relevant to target cells of DENV, were used as *in vitro* models for DENV infection and NS1 protein expression throughout the entire study. DENV-infected HepG2 cells and NS1-transfected Huh7 cells were employed for immunoprecipitation with anti-NS1 spcific antibodies and immunoprecipitated samples were processed and analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). Two groups of cellular proteins were identified from 2-3 independent experiments to be potential candidates of DENV NS1-interacting partners, i.e, 50 proteins from DENV-infected HepG2 cells and 33 proteins from NS1-transfected Huh7 cells. Some of these candidates were selected based on their peptide intensity for further verification of their interactions with DENV NS1 in virus-infected cells. Co-immunoprecipitation assays with specific antibodies confirmed that NIMA-related kinase 2 (NEK2), component of oligomeric Golgi complex 1 (COG1), thousand and one amino acid

protein kinase 1 (TAO1) and transcription factor IIH basal transcription factor complex helicase XPD subunit interacted with DENV NS1 in virus-infected cells. Their functional contribution to DENV infection and host response will be further examined. In addition, parallel sets of experiments were set up in this study to investigate the interactions of human heterogeneous ribonucleoprotein (hnRNP) C1/C2 and human chaperone proteins with DENV NS1 and their involvement in the process of DENV production by using siRNA-mediated gene knockdown. We found that hnRNP C1/C2 not only associated with DENV NS1 and viral RNA, but also moderately played a role in DENV life cycle, particularly at the steps of viral replication and viral protein synthesis. DENV NS1 was also found to interact with human immunoglobulin binding protein/78 kDa glucose regulated protein (BiP/GRP78) and their association seemed to be important for viral protein synthesis of DENV. Taken together, our study discovered novel DENV NS1-interacting human proteins potentially involved in DENV infection and defined some interactive roles of host proteins in different processes of the DENV life cycle. Further investigation on finding detailed mechanisms of their interacting functions and screening for compounds or inhibitors that specifically affect these events will pave the way for new strategies to help control DENV infection.

Outputs generated from this research project included:

1. Abstracts and Proceedings for national and international meetings

- Thanyaporn Dechtawewat, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. Role of hnRNP C1/C2 and Dengue Virus NS1 Association in Dengue Virus Production. National and International Conference in Medicine and Public Health to Commemorate 150th Anniversary of the Birth of Queen Sri Savarindira. Faculty of Medicine Siriraj Hospital, Mahidol University, 17-21 September 2012. The abstract was published in Siriraj Medical Journal, Volume 64, July-August 2012, page A4.
- Thanyaporn Dechtawewat, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. A Potential Contribution of hnRNP C1/C2 and Dengue Virus NS1 Association to Dengue Virus Infection. The Third International Conference of Dengue and Dengue Haemorrhagic Fever. The Imperial Queen's Park Hotel, Bangkok, Thailand, 21-23 October 2013.
- Thanyaporn Dechtawewat, Suchada Sengsai, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. Association of Dengue Virus NS1 and hnRNP C1/C2 Proteins in Different Human Target Cells for Dengue Virus Infection. Proceedings of the 6th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute Convention Center, 30 August – 2 September 2011, pp. 146-153.

2. Manuscripts for international publications

- Interaction of dengue virus NS1 with hnRNP C1/C2 facilitates dengue virus production. (ready for submission to Virology Journal in June 2014)
- LC MS/MS analysis for host cellular proteins interacting with the non-structural protein 1 in dengue virus-infected cells (Manuscript in preparation for Journal of Proteomics)
- Association of dengue virus NS1 and human GRP78 in dengue virus infection (Manuscript in preparation for Microbes and Infection)

3. Poster presentations

- Poster presentation entitled “Association of Dengue Virus NS1 and hnRNP C1/C2 Proteins in Different Human Target Cells for Dengue Virus Infection” at the 6th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute Convention Center, 30 August – 2 September 2011.
- Poster presentation entitled “A Potential Contribution of hnRNP C1/C2 and Dengue Virus NS1 Association to Dengue Virus Infection" at the Third International Conference of Dengue and Dengue Haemorrhagic Fever. The Imperial Queen’s Park Hotel, Bangkok, Thailand, 21-23 October 2013.

4. Invited speaker for Research and Development program for “Mass Spectrometry and Protein Analysis”

- Presented in the topic of “Interactions of Dengue Virus NS1 Protein with Human Host Cellular Proteins” on 18 March 2014 at Faculty of Medicine Ramathibodi Hospital, Mahidol University.

5. Graduate student training

- Miss Thanyaporn Dechtawewat, M.Sc. (Immunology) 2013, Faculty of Medicine Siriraj Hospital, Mahidol University.

6. Scientific awards

- The second-place award for best oral presentation in the Graduate Research Forum 2012 (Miss Thanyaporn Dechtawewat)
- The L'Oreal Thailand For Women in Science 2012 award in the field of life sciences (Dr. Sansanee Noisakran)

3. ปัญหาและอุปสรรคในการดำเนินงาน

We encountered a difficulty of setting a system to verify DENV NS1 and host protein interaction in human cell lines by using use yellow fluorescent protein-based protein fragment

complementation (YFP-PCA). Cloning of DENV NS1-coding gene into plasmid vectors used in this system was not successful as no bacterial colony was observed on transformation plates after several conditions have been tested. This might be due to the toxicity or instability of dengue virus NS1-coding gene in the context of the YFP-PCA plasmid vectors in bacterial strains used. Therefore, co-immunoprecipitation assay with specific antibodies has been established in DENV infection system as an alternative approach to confirm protein-protein interaction. Due to a budget constraint, trial sizes of antibodies specific to human host proteins were purchased and some of these antibodies did not recognize the host proteins of interest specifically and thus could not be used for the confirmation purpose. Different sources of the specific antibodies have been changed to help in the validation of the interactions between the identified host cellular proteins and DENV NS1 in virus-infected cells. In addition, our initial attempts to knockdown host cellular proteins using two sets of custom-designed siRNA sequences from different companies were not successful. As a result, we had to modify strategies for siRNA transfection by using commercially available siRNA sequences that are guaranteed for specific knockdown of each host protein and a different transfection reagent to continue the experiments. These problems altogether caused a delay in our work progress especially in a time frame of manuscript preparation.

4. ข้อคิดเห็นและข้อเสนออื่น ๆ ต่อ สกว.

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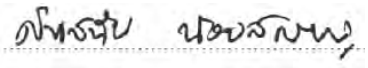
ลงนาม 

ดร. ศันสนีย น้อยสคราญ

(หัวหน้าโครงการวิจัยผู้รับทุน)

Introduction

Dengue virus (DENV) infection is a major leading cause of mosquito-borne viral diseases affecting human population globally, especially in tropical and subtropical countries. Infection with any of four serotypes of DENV (DENV-1, 2, 3, and 4) can lead to different clinical manifestations ranging from asymptomatic or mild febrile illness (dengue fever, DF) to more severe and potentially deadly forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Approximately 50 to 100 million people contract dengue fever annually, and about 200,000 to 500,000 of these are DHF/DSS, which has a mortality rate about 1%–5% (1). DHF/DSS occurs predominantly in children responding secondarily to a heterologous serotype of DENV distinct from previous exposure and it is generally observed after the viremic period when virus is rarely detectable in blood circulation and massive immune responses are rising. Thus, the pathogenesis of DHF/DSS is likely to be immune-mediated; however, its underlying mechanism remains unclear and there is no dengue vaccine or anti-viral drug commercially available.

During DENV infection, one of the DENV proteins, which is detectable in blood circulation of dengue patients at levels closely correlated with the disease severity and therefore serves as a diagnostic marker for DENV infection, is the non-structural protein 1 (NS1). DENV NS1 is an approximately 45-kDa protein with two N-linked glycosylation sites. It exists in multiple forms (i.e., monomer, dimer, and hexamer) within or on the surface of virus-infected cells as well as in the extracellular milieu (2-6). Following DENV, NS1 is synthesized from a long viral polyprotein (NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH), which is directly translated from viral genomic positive-stranded RNA, by cleavage of host signalase and an unknown endoplasmic reticulum (ER)-resident protease at the N-and C-terminus of the protein, respectively (7). This process requires the last C-terminal 28 amino acid sequence of E protein acting as a signal peptide for NS1 translocation across the ER membrane and the last C-terminal 8 amino acid sequence of NS1 for the efficient cleavage between NS1-NS2A (8-10). In the ER, monomeric NS1 protein is modified by the addition of high-mannose type glycans followed by rapid dimerization (11, 12). In addition, a sub-population of NS1 is potentially linked to glycosylphosphatidylinositol (GPI) anchor (2, 13). The mature NS1 is then transported to the Golgi apparatus for further modifications of carbohydrate moieties prior to its presentation on the surface of infected cells and release to the extracellular compartment in a hexameric form (6, 11, 14).

DENV NS1 was first described as soluble complement fixing antigen (15, 16). Its detectable levels are varying in the circulation of patients experiencing primary and secondary DENV infection and correlated with disease severity (17-19). NS1 is an immunogenic protein inducing production of specific antibodies that confer partial protection against dengue virus infection in animal models

(20, 21). Recently, immunodominant B cell epitopes have been identified on DENV NS1 (22, 23). In contrast to the protective role, the anti-NS1 antibodies have been found to cross-react with cell surface molecules containing an arginine-glycine-aspartic acid motif such as ATP synthase β chain, protein disulfide isomerase, vimentin and heat shock protein 60 on human fibrinogen, platelets and endothelial cells and this autoimmune response may result in decreased blood coagulation factors, thrombocytopenia, inhibited platelet aggregation, endothelial cell damage and subsequent vascular leakage (24-28). Circulating NS1 can serve as an important target for complement and antibody attacks, resulting in immune complex formation and subsequent complement activation (15, 29, 30). The soluble NS1 has been found to trigger complement activation leading to formation of terminal C5b-9 complexes, which are detectable in plasma of dengue patients and in pleural fluids of DSS cases and their levels are likely associated with the severity of disease (31). In accordance with this finding, another study demonstrated that the soluble NS1 is capable of binding to complement regulatory protein clusterin and this interaction may affect a putative role of clusterin in inhibiting the formation of terminal complement complex and potentially contribute to plasma leakage and the progression of DHF (32). In addition, NS1 may usurp complement pathway to escape host immune response by binding to complement components C4 and C1s as well as C4-binding protein, thus protecting DENV from complement-dependent neutralization and protecting DENV-infected cells from complement attack (33, 34).

In addition to the secreted form, DENV NS1 existing in the cell-associated form potentially plays important roles in host cellular responses and viral replication. NS1 on the cell surface can activate complements in the presence of anti-NS1 antibody, resulting in deposition of C3 and C9 on the surface of virus-infected cells (35, 36). Cross-linking of the cell surface-associated NS1 with the specific antibody also triggers tyrosine phosphorylation which may in turn stimulate intracellular signalling pathways responsive for DENV infection (2). At present, it is not clear how the NS1 protein associates with the host cell membrane. Possible mechanisms include peripheral attachment of a hydrophobic surface of NS1 generated by dimerization (6), association of NS1 with lipid rafts (13), and binding back of secreted NS1 to the cell surface via interactions with heparan sulfate and chondroitin sulfate E (36).

Although the functional contribution of distinct NS1 forms to DENV infection is not clearly understood, several lines of evidence suggest the involvement of cell-associated NS1 in virus replication (37-41). A genetic approach using NS1 gene deletion in full-length infectious cDNA clone of yellow fever virus, a dengue-related flavivirus, revealed the inhibition of minus-strand viral RNA synthesis which could be reversed by complementing NS1 expression in trans (Lindenbach and Rice 1997). Other studies on immunofluorescence and cryo-immunoelectron microscopy demonstrated co-localization of NS1 with double stranded-viral RNA and its tight association with

intracellular membranes that are presumed sites of virus replication (37, 42). In addition, NS1 was found to localize to some extent in the same cellular compartment as LC3 and LAMP1 (a marker of autophagic vacuoles as well as endosomal and lysosomal membranes, respectively) which may be involved as part of the viral translation/replication complex in production of DENV (43, 44). Endocytosis of NS1 and its subsequent accumulation in late endosomes have also been proposed to facilitate DENV replication in human hepatocytes (45). At present, interactions of cell-associated NS1 with host cellular proteins are not well defined, despite having extensive studies on DENV NS1, possibly due to complexity of conformational structures of this protein being processed under different conditions. Nevertheless, three previous studies have reported potential candidates of NS1-interacting partners (46, 47). Specifically, GAL4 yeast two hybrid system with human bone marrow cDNA library showed that NS1 binds to STAT3β protein and this interaction may result in significant induction of pro-inflammatory cytokines, IL-6 and TNF-α, and in turn influence the pathology of DHF/DSS (46). Employing a similar yeast two hybrid system with human liver cDNA library, complement component C1q was found to bind DENV NS1 and this may help DENV avoid complement activation through classical pathway (48). Another study using human embryonic kidney epithelial HEK-293T cells, which may be irrelevant cells for natural DENV infection, identified another NS1-interacting host protein, heterogeneous ribonucleoprotein C1/C2, and suggested its potential association with replication process of DENV (47).

To date it is still poorly defined as to how dengue virus NS1 is differently processed into various forms existing in different cellular locations, how distinct forms of NS1 exert different functions, and whether these events require interrelationship between host cellular proteins and DENV NS1. Therefore, we hypothesize that NS1 may utilize host cell machinery via its interaction with cellular partners to facilitate viral RNA and viral protein processing and to provide a favorable condition for the virus to replicate and/or evade from host cellular responses during DENV infection.

Materials and Methods

I. Identification of host protein candidates interacting with dengue virus NS1

Dengue virus infection and transfection with NS1-encoding plasmid DNA

Two hepatic cell lines, HepG2 and Huh7, were employed in this study. HepG2 cells were cultured in 10% FBS-DMEM containing 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 2 mM L-glutamine, 36 µg/ml penicillin and 60 µg/ml streptomycin. Huh7 cells were cultured in 10% FBS-RPMI containing 0.1 mM non-essential amino acid, 2 mM L-glutamine, 36 µg/ml penicillin and 60 µg/ml streptomycin. For DENV infection, HepG2 and Huh7 cells were incubated with DENV serotype 2 (strain 16681) at the multiplicity of infection (MOI) of 5 and 0.5, respectively,

at 37°C for 2 hr. Thereafter, virus supernatant was removed and the infected cells were washed with PBS three times and cultured in growth medium as described above at 37°C in an incubator with 5% CO_2 and humidified atmosphere. Mock-infected cells served as a control. For DNA transfection, 8 x 10^5 HepG2 cells or 6 x 10^5 Huh7 cells were seeded onto a 35 mm-tissue culture dish containing growth medium. Twenty-four hr later, the cells were washed with plain medium and incubated with plasmid DNA and lipofectamine complex (a ratio of 1:3, 5 µg DNA and 15 µl of lipofectamine) in serum-free Opti-MEM for 6 hr. Thereafter, the supernatant was replaced with fresh growth medium and cultures were maintained for 24-72 hr. Two plasmid DNA constructs encoding dengue virus NS1 were used in this study: (a) pcDNAHygro E-NS1-His which encodes the last C-terminal 28 amino acids of dengue E (signal sequence for translocation of NS1 into the endoplasmic reticulum), full length of NS1 and 6 x histidine tag at the C-terminus; and (b) pcDNA3.1 His-NS1 which encodes 6 x histidine tag at the N-terminus followed by full-length of NS1. pCDNAHygro and pcDNA3.1/His C were employed as vector controls for DNA transfection and NS1 expression.

Immunoprecipitation of NS1-interacting host proteins with anti-NS1 antibody

To determine candidates of host cellular proteins which interact with dengue virus NS1, mock and virus-infected cells at 48 hr post infection were harvested and incubated with RIPA buffer (20 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, , 0.1% SDS, 0.5% deoxycholate and protease inhibitor cocktail) containing 1% NP-40 or 1% Triton X-100 at 4ºC for 30 min. Total cell lysate was centrifuged at 13,000 rpm for 5 min at 4ºC and clear lysate was collected. The clear lysate from mock and DENV-infected cells was incubated at 4ºC overnight with 50% slurry of protein G sepharose beads binding to anti-NS1 antibodes (clones 1A4 and 1F11) or isotype-matched control antibody (clone UPC). The samples were then centrifuged at 13,000 rpm for 5 min and the beads were washed three times with RIPA buffer and three times with 10 mM Tris buffer pH 7.5. One set of the immunoprecipitated proteins was eluted with 40 µl of 1×sample buffer (50 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue) and the other parallel set was eluted with 40 µl of 0.1% RapidGest in 50 mM ammonium bicarbonate. Aliquots of the immunoprecipitated proteins in the 1×sample buffer were separated by electrophoresis in 10% SDS-polyacrylamide gel followed by immunoblotting analysis using NS1-specific antibody to confirm the presence of DENV NS1 in the eluate. A pre-stained protein ladder of 10-180 kDa was used as a standard protein marker. The immunoprecipitated proteins in the RapidGest buffer were further processed for mass spectrometric analysis.

Pull down assay for DENV NS1

To set up pull down assay for screening of NS1-interacting host proteins, pcDNA3.1/His C (vector) or pcDNA3.1 His-NS1 transfected cells were harvested at 24 or 48 hr post transfection and subjected to lysis process as described above using RIPA buffer in the absence of EDTA. NS1-His and its interacting host partners were pulled down from whole cell lysate by using TALON IMAC system (Clontech laboratories, Inc.) based on metal affinity according to the manufacturer's instruction. Vector-transfected cell lysate, which had been similarly processed, served as a negative control. Bound proteins were eluted with varying concentrations (40-200 mM) of imidazole and subsequently analyzed by immunoblotting. In addition, the eluted samples were dialyzed with 10 mM Tris buffer pH 7.5 and further processed for mass spectrometric analysis.

Analysis of immunoprecipitated and pulled down proteins by mass spectrometry

Immunoprecipitated and pulled down samples were processed for in-solution tryptic digestion prior to peptide analysis. Briefly, the immunoprecipitated and pulled down proteins in the RapidGest buffer were boiled at 100ºC for 5 min followed by successive incubation with 5 mM dithiothreitol at 60ºC for 30 min and 15 mM iodoacetamide at RT in the dark for 30 min. The samples were centrifuged at 13,000 rpm for 5 min and clear supernatants were incubated with 40 ng/µl of trypsin at 37ºC overnight. Thereafter, the samples were incubated with 0.5% trifluoroacetic acid (TFA) at 37ºC for 45 min and centrifuged at 13,000 rpm for 10 min. Clear supernatants were dried using Speed Vac concentrator and dried peptides were resuspended in 10 µl of 0.1% TFA and subjected to liquid chromatography tandem-mass spectrometry (LC-MS/MS, SYNAPT HDMS, Waters Corp., MA, USA) for peptide analysis. DeCyder MS Differential Analysis software (GE Healthcare, USA) was used for the differential quantitation of peptides based on MS signal intensities of individual LC-MS/MS analyses. To evaluate the average abundance ratio of peptides from test and control samples, quantitation of the peptides based on the peptide signal intensities was performed using the PepDetect module for automated detection of peptides and assignment of charge states. Peptides were matched across different signal intensity maps between the test and control samples using the PepMatch module. All ion intensities of test groups were normalized with that of control groups. The matched peptides with an average abundance ratio > 1.5 fold were selected as potential candidates for interacting with DENV NS1 protein. The LC-MS/MS data from DeCyder MS analysis were searched against the NCBInr database (taxonomy: *Homo sapiens*) for protein identification using Mascot software (Matrix Science, London, UK).

II. Verification of the interaction between NS1 and host cellular proteins

Co-immunoprecipitation of NS1 and host cellular proteins

To confirm the NS1 and host protein interaction, mock and DENV-infected cell lysate were subjected to immunoprecipitation using anti-NS1 monoclonal antibodies (clones 1A4 and 1F11) or isotype-matched control antibody (UPC) as described above. The immunoprecipitated samples were separated by 10% SDS-polyacrylamide gel electrophoresis followed by immunoblotting analysis and each set of the samples was probed with both DENV NS1-specific antibody and host protein-specific antibodies i.e., mouse anti-hnRNP C1/C2 monoclonal antibody (clone 4F4), goat anti-GRP78 polyclonal antibody (clone C-20), rabbit anti-calnexin polyclonal antibody (clone H-70) or goat anti-calreticulin polyclonal antibody (clone C-17) from Santa Cruz Biotechnology and rabbit polyclonal antibodies against human NEK2, COG1, TAOK1 and XPD proteins from One World Lab. Results from DENV-infected samples that had been immunoprecipitated with NS1-specific antibody were compared to results from mock-infected samples that had been processed similarly and DENV-infected samples immunoprecipitated with isotype-matched control antibody.

Co-localization between NS1 and host cellular proteins

HepG2 and Huh7 cells were grown on glass coverslips for 24 h and then infected with DENV at the indicated MOI. Mock and DENV-infected cells on the coverslips were harvested at different time points post infection. Cells on the coverslips were washed with PBS three times, fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. The cells were then incubated with a mixture of mouse anti-dengue NS1 monoclonal antibody (clone 1F11) and chaperone-specific antibodies (i.e., goat anti-GRP78 polyclonal antibody, rabbit anti-calnexin polyclonal antibody, goat anti-calreticulin polyclonal antibody; 1:100 dilution) or with PBS for 1 h at room temperature (RT). Following three-time washes, the cells were incubated with a mixture of Alexa 647-conjugated chicken anti-mouse IgG antibody and either Alexa 488-conjugated donkey anti-goat IgG antibody or Alexa 488-conjugated donkey anti-rabbit IgG antibody (Invitrogen, 1:1000 dilution) in the presence of Hoechst 33258 dye (Invitrogen, 1:1000 dilution) for 1 hr at RT in the dark. The stained cells were washed three times and mounted onto the glass slides with Prolong Gold antifade reagent (Invitrogen). The stained cells were observed for co-localization between NS1 and host proteins under a confocal microscope Zeiss LSM 510 META (Carl Zeiss, Jena, Germany) with a 63× oil immersion lens. All images were captured by using an image capture program (LSM 510 software version 3.2, Carl Zeiss). To determine co-localization between DENV NS1 and human hnRNP C1/C2, fixed and permeabilized cells on the coverslips were blocked with 10% human AB serum for 30 min followed by incubation with 1 μg/ml mouse anti-human hnRNP C1/C2 monoclonal antibody or isotype-

matched control antibody for 1 hr at RT. Thereafter, the cells were incubated with Cy3-conjugated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a dilution of 1:2000 for 30 min at RT. The stained cells were washed three times with PBS, then blocked with 10% normal mouse serum in PBS for 30 min and incubated with a mixture of 3.75 µg/ml mouse anti-NS1 monoclonal antibody (clone 1A4), which had been conjugated with Alexa Fluor 488 by using Zenon Mouse IgG2a Alexa 488 labeling kit according to the manufacturer's instruction (Invitrogen), and 10 µg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 30 min. The stained cells were fixed with 1% paraformaldehyde for 30 min at RT. Following washing three times, the glass coverslips were mounted onto glass slides with anti-fade agent (DAKO) and visualized under a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

III. Investigation of the roles of NS1 and host protein interaction in DENV infection

Determination of DENV RNA in NS1 and host protein complexes

To test whether NS1 and host protein complexes associate with DENV RNA, the immunoprecipitated samples on the beads were eluted with 140 µl of TRIzol reagent (Invitrogen) and RNA was isolated according to the manufacture's protocol. Reverse transcription was performed in the 20-µl reaction as follow: (i) incubation of 11.75 µl of RNA template with a mixture of 1 µl of 10 pmole/µl NS1-3477 reverse primer (5'-GATCGATCGCGGCCGCTTAGGCTGTGACC AAGGAGTTAACCAAATTCTCTTCTTTCTC-3'), 1 µl of 10 mM dNTP mix, 4 µl of 5x the first strand buffer (Invitrogen), and 1 µl of 0.1M DTT at 65ºC for 5 min; (ii) addition of 1 µl of RNaseOUT and 0.25 µl of Superscript II Reverse Transcriptase (Invitrogen) and incubation at 55ºC for 60 min; (iii) inactivation at 70ºC for 15 min. The resultant cDNA was subsequently utilized as template for amplification of NS1 fragments by PCR in a 12.5-µl reaction containing 4 µl of DNA template, 1.25 µl of 25 mM $MgCl_2$, 1.25 µl of 10× Taq buffer, 0.25 µl of 10 mM dNTP mix, 0.5 µl of each 10 pmole/µl NS1-2422 forward primer (5'-CCGGCCAGATCTGATAGTGGTTGCGTTGTGAGC-3') and NS1-3477 reverse primer, 4.625 µl of DEPC-treated water, and 0.125 µl of 5 U/µl Taq DNA polymerase (Promega). The PCR reactions were pre-heated at 95ºC for 5 min followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 48ºC for 30 s, and extension at 72ºC for 1 min 30 s, as well as one cycle of final extension at 72ºC for 10 min using GeneAmp PCR system 9700 (Perkin Elmer-Applied Biosystems). The PCR products (12.5 µl) were mixed with 2.5 µl of 6× loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, and 30 % glycerol in deionized water), then separated by 2 % agarose gel electrophoresis, and visualized by ethidium bromide staining and UV-transillumination. PhiX-174 DNA-*Hae* III was used as a standard marker. The DNA bands were photographed by GelDoc imaging system (GeneSnap version 6.05, Synoptic Ltd.).

Involvement of NS1 and host protein interaction in dengue viral RNA synthesis

A role of NS1 and host protein association in viral RNA synthesis was determined by gene silencing. Huh7 cells in 24-well culture plates (50% confluency) were transfected with 114 nM hnRNP C1/C2-specific siRNA (siGENOME SMARTpool, Dharmacon), 66 nM GRP78-specific siRNA (Origene Technologies) or negative control siRNA (Stealth Select RNAi, Invitrogen and Origene Technologies) using Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions with minor modifications. To knockdown hnRNP C1/C2 proteins, two rounds of siRNA transfection were performed. Approximately 24-30 h after siRNA transfection, cells were either infected with DENV at an MOI of 0.03 or transfected with 1 μg of DENV replicon and were cultured for 48-72 h. At different time points post infection/transfection, both RNA and protein were extracted from the harvested cells as described above to determine the levels of knocked down cellular proteins by real-time RT-PCR and immunoblotting analysis using primer sequences and antibodies specific for hnRNPC1/C2, GRP78 and β-actin (internal control for housekeeping gene), respectively. Relative mRNA and protein expression of hnRNP C1/C2 and GRP78 were determined by normalization with expression levels of β-actin (control). In addition, RNA was subjected to quantitative real-time RT-PCR for measurement of the amount of DENV RNA by using Light Cycler 480 II (Roche) with a primer set specific for DENV E or NS1 region and the Light Cycler 480 SYBR Green I Master kit (Roche) according to the manufacturer's instruction. The amounts of total viral RNA from the knockdown and control cells were determined by quantitative analysis comparing with known copy numbers of standard DENV RNA molecules.

Effect of NS1 and host protein interaction on DENV infection and viral protein synthesis

To determine whether hnRNP C1/C2 knockdown interferes with the percentage of DENV infection, immunofluorescence staining assay was performed. Briefly, mock or DENV-infected cells that had been transfected with hnRNP C1/C2-specific siRNA or control siRNA were fixed with 4% paraformaldehyde in PBS and smeared on a glass slide and left to air-dry for 30 min at RT. The cells were subsequently permeabilized with 0.2% Triton-X-100 for 10 min at RT and sequentially incubated with mouse anti-DENV E monoclonal antibody (clone 3H5) for 1 h at RT and Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Invitrogen) at a dilution of 1:1000 for 30 min at RT. The stained cells were visualized under a confocal laser-scanning microscope (LSM 510 Meta).

To determine the effect of gene-specific knockdown on the synthesis of DENV proteins, DENV-infected or DENV replicon-transfected cells that had been transfected with hnRNP C1/C2-specific siRNA, GRP78-specific siRNA, or control siRNA were subjected to immunoblotting analysis for DENV E or NS1 protein Expression levels of viral proteins were quantified by normalization to that of human β-actin using GeneTools software from Syngene (Syngene, Cambridge, UK).

Influence of NS1 and host protein interaction in DENV production

To determine whether the interaction between DENV NS1 and host protein plays a significant role in the production of infectious virus, Huh7 cells were transfected with hnRNP C1/C2-specific siRNA or control siRNA and then infected or left uninfected with DENV. At varying time points post infection, supernatants from knockdown or control cell cultures were collected and assessed for DENV titers by focus forming unit (FFU) assay. Briefly, Vero cells were seeded onto a 96-well plate at a concentration of 2 x 10^4 cells/100 µl/well in growth medium (MEM medium containing 10 % FBS, 2 mM L-glutamine, 36 µg/ml penicillin and 60 µg/ml streptomycin) and incubated at 37^{o}C in a humidified chamber with 5% CO_2 for 24 h. Thereafter, 70 µl of culture medium was removed and 30 µl of DENV-containing supernatants, which had been serially diluted by 10-fold in maintenance medium (MEM medium containing 2% FBS, 2 mM L-glutamine, 36 µg/ml penicillin and 60 µg/ml streptomycin), were added (100 µl/well) onto the cell monolayer and incubated at 37^{o}C for 2 h. The cell culture was then overlaid with overlayer medium containing 2% (w/v) carboxymethylcellulose (Sigma) in MEM (100 µl/well) and incubated for 3 days at the same culture condition. Next, the overlayer medium was removed and the cells were washed with 200 µl of PBS three times, fixed with 3.7% formaldehyde in PBS (100 µl/well) and permeabilized with 1% Triton X-100 in PBS (100 µl/well) at RT (10 min each for the fixation and permeabilization step. Following five-time washes with 200 µl of PBS, mouse anti-dengue E monoclonal antibody (clone 4G2; 50 µl/well) was added onto the cells and incubated in a humidified chamber at 37ºC for 1 h. The cells were washed once again five times and further incubated with rabbit anti-mouse Igs conjugated with horseradish peroxidase (DAKO) at a dilution of 1:1000 in the dark at 37^{o}C in a humidified chamber for 30 min. The stained cells were washed five times and incubated with 50 µl of a substrate solution containing 0.6 mg/ml diaminobenzidine (DAB) in PBS, 5% H_2O_2, and 8%$NiCl_2$ at RT until dark brown stained foci of infected cells were developed. The reaction was terminated by washing with distilled water a few times. The foci of the infected cells were enumerated under a light microscope and their numbers were calculated based on the dilution factors to represent DENV titers as FFU/ml.

Results and Discussion

I. Screening for DENV NS1-interacting host cellular proteins

In this study, we utilized both DENV infection and NS1 transfection systems to screen for potential host cellular proteins that interact with DENV NS1 in target cells containing and lacking other viral components. Mock and DENV-infected HepG2 cells were subjected to immuno-precipitation using DENV NS1-specific antibodies or their isotype-matched control antibody and immunoprecipitated samples were verified for the presence of DENV virus NS1 by immunoblotting

blot analysis and subsequently processed for proteomic analysis using LC-MS/MS. Three independent sets of mock and DENV-infected samples were prepared for the proteomic analysis. In parallel, Huh7 cells were transfected with pcDNA Hygro E-NS1-His, pcDNA3.1 His-NS1 and their vector controls. pcDNA Hygro E-NS1-His contained the last C-terminal 28 amino-acid sequence of DENV E, acting as a signal peptide for translocation of DENV NS1 into the ER lumen, similar to that observed in natural infection. Lacking this sequence in pcDNA3.1 His-NS1 was expected to yield DENV NS1 in the form that cannot enter the ER lumen and thus may serve as another negative control for identification of NS1-interacting host proteins. It should be noted that DENV NS1 generated from the pcDNA Hygro E-NS1-His-transfected cells could not be recognized by anti-His antibody after several attempts have been made (data not shown). Therefore, Huh7 cells transfected with pcDNA Hygro E-NS1-His and its vector control were subjected to immunoprecipitation with DENV NS1-specific antibodies or their isotype-matched control antibody whereas Huh7 cells transfected with pcDNA3.1 His-NS1 and its vector control were subjected to pull down assay using TALON IMAC metal affinity system. The immunoprecipitated samples and the pulled down samples were verified for the presence of dengue virus NS1 by immunoblotting analysis and then processed for the proteomic analysis. Two independent sets of each plasmid DNA-transfected sample were prepared for LC-MS/MS analysis.

Results showed that DENV NS1 could be detected in DENV-infected sample immuno-precipitated with anti-NS1 antibody and no specific protein band was observed in DENV-infected sample immunoprecipitated with isotype-matched control antibody and mock-infected samples immunoprecipitated with either antibody (Figure 1). Likewise, immunoprecipitation with anti-NS1 antibody yielded NS1 band in pcDNA Hygro E-NS1-His-transfected sample but not in vector control-transfected sample whereas immunoprecipitation with isotype-matched control antibody did not show any specific band in both pcDNA Hygro E-NS1-His and vector control-transfected samples (Figure 2). In addition, DENV NS1 could be pulled down specifically from pcDNA3.1 His-NS1-transfeced sample, but not from vector control-transfected sample by using TALON IMAC metal affinity system (Figure 3). Altogether, these results confirmed the presence of DENV NS1 in the samples used for the proteomic analysis of NS1-interacting host proteins.

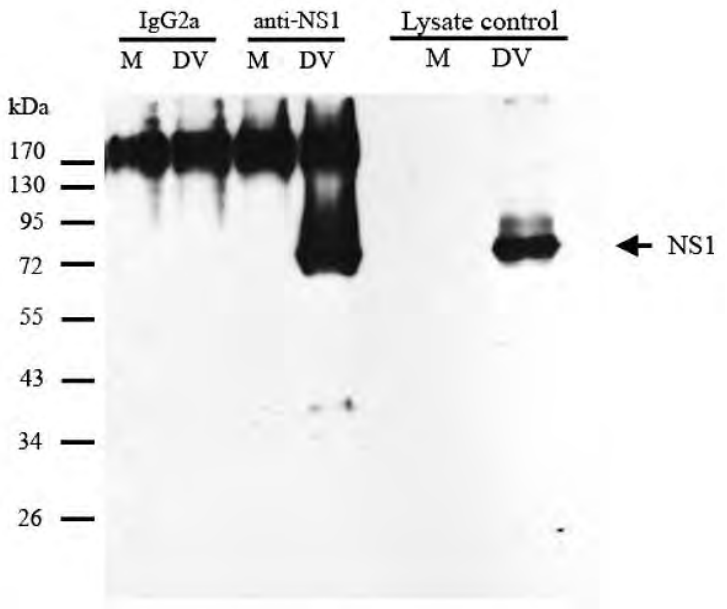


Figure 1. Immunoprecipitation of DENV NS1 from DENV-infected HepG2 cells. HepG2 cells were uninfected (mock control, M) or infected (DV) with DENV at an MOI of 5. On day 2 post infection, the cells were harvested and processed for immunoprecipitation using anti-DENV NS1 or isotype-matched IgG2a control antibodies. The precipitated samples were subjected to immunoblotting analysis for DENV NS1. Mock and DENV-infected cell lysates served as controls.

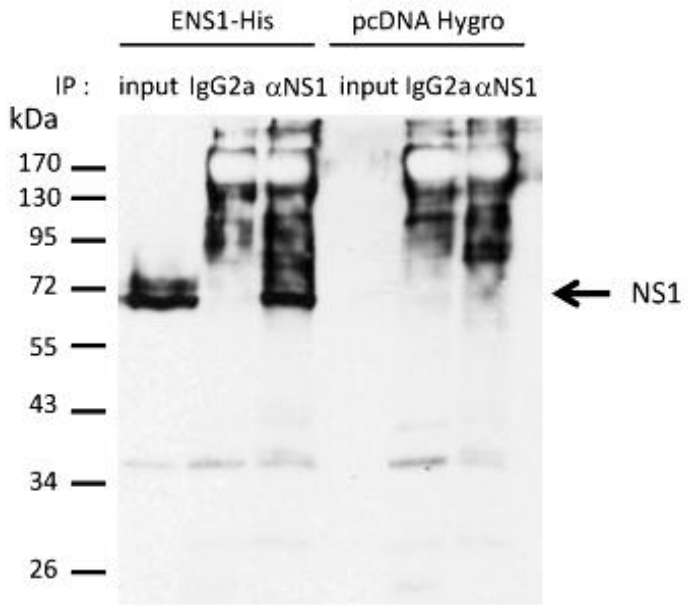


Figure 2. Immunoprecipitation of DENV NS1 from NS1-transfected Huh7 cells. Huh7 cells were transfected with pcDNA Hygro (vector control) or pcDNA Hygro E-NS1-His using Lipofectamine. At 48 hr post transfection, vector or NS1-transfected cells were harvested and processed for immunoprecipitation using anti-DENV NS1 or isotype-matched IgG2a control antibodies. The precipitated samples were subjected to immunoblotting analysis for DENV NS1. Vector or NS1-transfected cell lysates (input) served as controls.

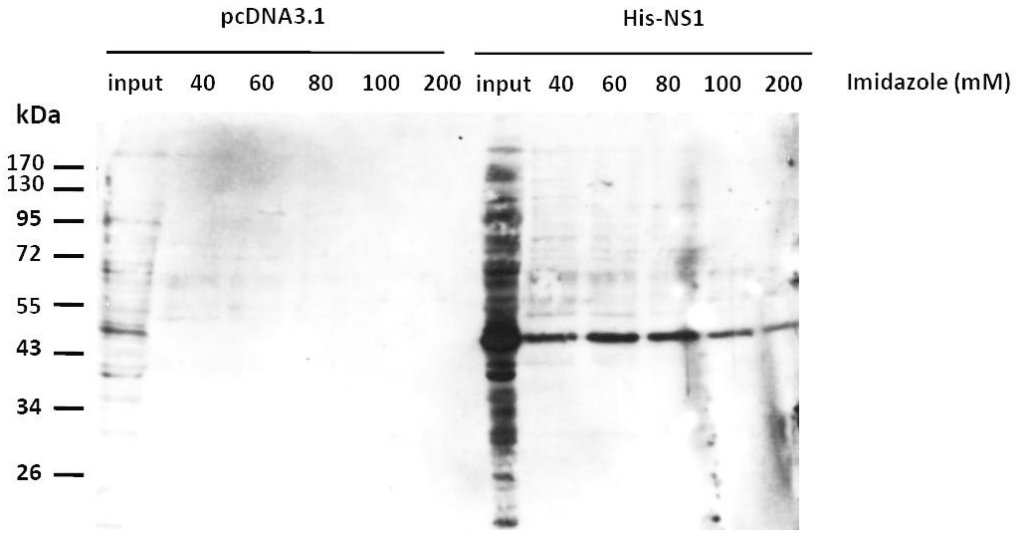


Figure 3. Detection of DENV NS1 from transfected cell lysate by pull down assay. Huh7 cells were transfected with pcDNA3.1/His C (vector control) or pcDNA3.1 His-NS1 using Lipofectamine and harvested on day 2 post transfection. Cell lysate was prepared from the transfected cells and subjected to pull down assay using TALON IMAC metal affinity system. Bound sample was eluted with varying concentrations (40-200 mM) of imidazole. Cell lysate (input) and eluates were analyzed for DENV NS1 by immunoblotting using anti-NS1 specific antibody. Vector-transfected cells that had been similarly processed served as a control.

Each set of processed samples was injected to a mass spectrometer three times. After primary data of mass spectrometry were obtained from both the infection and transfection systems, DeCyder MS program was used for analysis of ion intensities of tryptic peptides from test and control groups. All the ion intensities of the test groups were normalized with that of control groups and the matched peptides with an average ratio of ion intensity (test group/control group) > 1.5 fold were selected as potential candidates for DENV NS1-interacting host proteins. Using DENV-infected cells, 50 host cellular proteins were identified in DENV-infected samples that had been immunoprecipitated with anti-NS1 antibody (DENV-αNS1) when compared with DENV-infected samples that had been immunoprecipitated with isotype-matched control antibody (DENV-UPC) and mock-infected samples that had been immunoprecipitated with anti-NS1 antibody (mock-αNS1) (Figure 4 and Table 1; n=3). In the transfection system, 33 host cellular proteins were identified in pcDNA Hygro E-NS1-His transfected cells that had been immunoprecipitated with anti-NS1 antibody (E-NS1-αNS1) as compared with pcDNA Hygro E-NS1-His or vector control-transfected cells that had been immunoprecipitated with isotype-matched control antibody or anti-NS1 antibody, respectively (control) (Figure 5 and Table 2; n=2). When pcDNA3.1 His-NS1 or vector control-transfected cells were used for pull down assay, 11 host cellular proteins were

identified in the eluate from pcDNA3.1 His-NS1 transfected cell lysate (His-NS1) as compared with the elute from vector control-transfected cell lysate (vector control) (Figure 6 and Table 3; n=2).

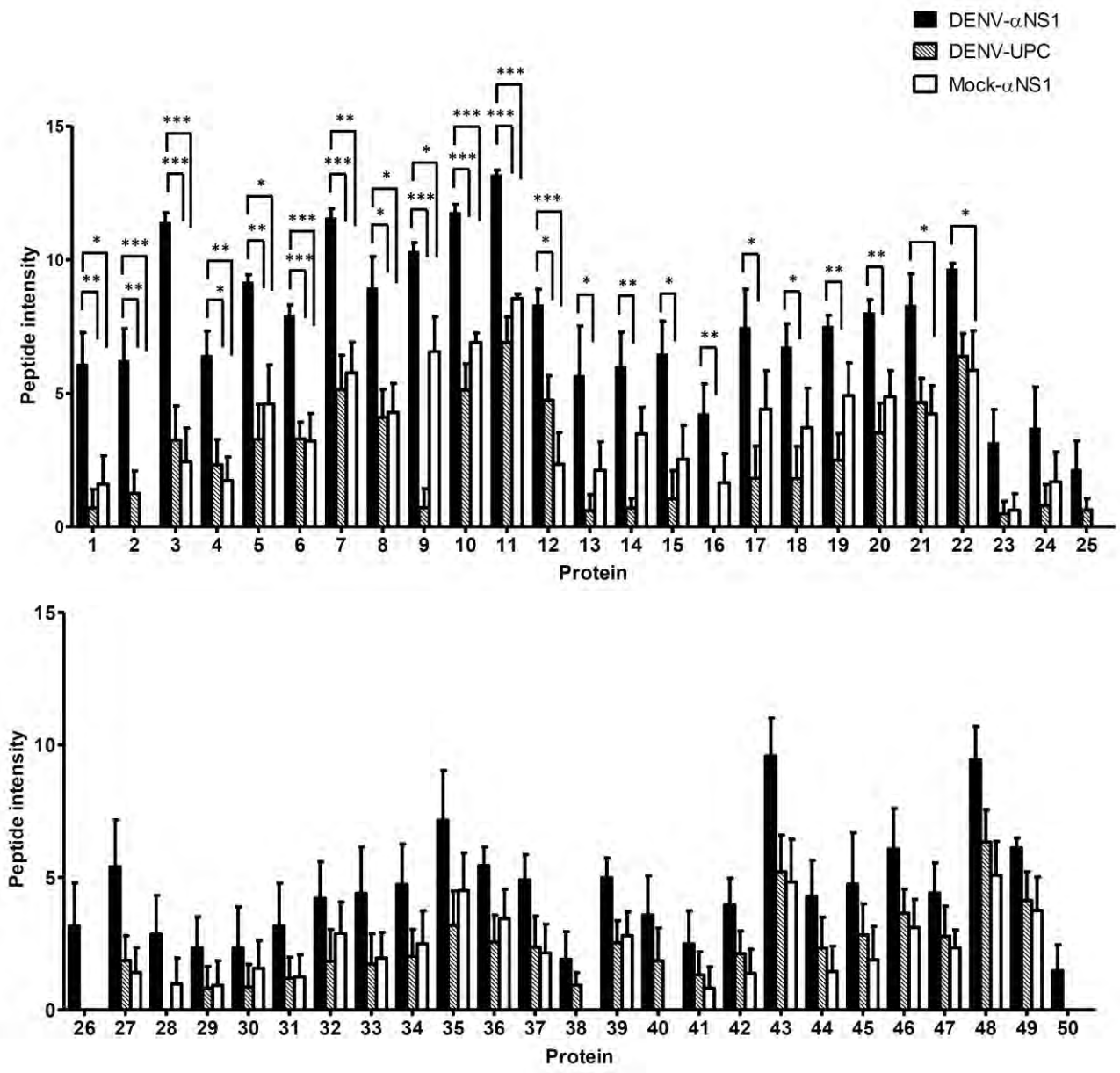


Figure 4. Mass spectrometric analysis of host cellular proteins potentially interacting with DENV NS1 in virus-infected cells. Mock and DENV-infected cells were subjected to immuno-precipitation with anti-NS1 antibody (αNS1) or its isotype-matched control antibody (UPC). The immunoprecipitated samples were processed and analyzed by mass spectrometry. Fifty host cellular proteins were identified in the immunoprecipitated complexes. Data show peptide intensities (mean + SEM) of the identified proteins from three independent experiments. Statistically significant differences in peptide intensities between all sample groups were analyzed by one-way ANOVA (*p<0.05; ** p<0.01; *** p<0.001).

Table 1. Identification of potential DENV NS1-interacting host cellular proteins in DENV-infected cells

Protein	Name	Intensity ratio of DENV-αNS1 to DENV-UPC	Intensity ratio of DENV-αNS1 to mock-αNS1
1	chromosome 21 open reading frame 70 [Homo sapiens]	8.67	3.80
2	serine/threonine-protein kinase Nek2 [Homo sapiens]	4.94	UD(a)
3	serine/threonine-protein kinase TAO1 [Homo sapiens]	3.52	4.68
4	conserved oligomeric Golgi complex subunit 1 (COG1) protein [Homo sapiens]	2.75	3.69
5	protein phosphatase 1M [Homo sapiens]	2.80	1.99
6	folylpolyglutamate synthetase mitochondrial isoform [Homo sapiens]	2.40	2.46
7	protein AHNAK2 [Homo sapiens]	2.25	2.01
8	Melanin-concentrating hormone receptor 1 [Homo sapiens]	2.18	2.09
9	WD repeat domain 78 [Homo sapiens]	14.47	1.57
10	hCG2040936 [Homo sapiens]	2.30	1.71
11	TMCO3 protein [Homo sapiens]	1.91	1.54
12	trifunctional enzyme subunit alpha, mitochondrial precursor [Homo sapiens]	1.75	3.54
13	melanoma-associated antigen E1 [Homo sapiens]	9.38	2.67
14	SERPINA11 type b [Homo sapiens]	8.54	1.72
15	solute carrier family 26 [Homo sapiens]	6.14	2.55
16	putative E1-E2 ATPase [Homo sapiens]	UD(b)	2.57
17	hCG2036719 [Homo sapiens]	4.10	1.69
18	rho guanine nucleotide exchange factor 17 [Homo sapiens]	3.72	1.81
19	zinc finger protein 1 homolog (ZEP1) protein [Homo sapiens]	3.00	1.53
20	hCG1777996 [Homo sapiens]	2.29	1.65
21	hCG1813755 [Homo sapiens]	1.78	1.96
22	repulsive guidance molecule A (RGMA) protein [Homo sapiens]	1.51	1.65
23	protocadherin Fat 4 precursor [Homo sapiens]	6.56	5.06
24	E3 ubiquitin-protein ligase HECW2 [Homo sapiens]	4.67	2.19
25	actin filament associated protein [Homo sapiens]	3.37	UD(c)

UD = Unable to determine the intensity ratio of DENV-αNS1 to control (DENV-UPC or mock-αNS1)

(a) = intensity of DENV-αNS1 = 6.22; intensity of mock-αNS1 = 0

(b) = intensity of DENV-αNS1 = 4.23; intensity of DENV-UPC = 0

(c) = intensity of DENV-αNS1 = 2.13; intensity of mock-αNS1 = 0

Table 1. Identification of potential DENV NS1-interacting host cellular proteins in DENV-infected cells (continued)

Protein	Name	Intensity ratio of DENV-αNS1 to DENV-UPC	Intensity ratio of DENV-αNS1 to mock-αNS1
26	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4 [Homo sapiens]	UD(d)	UD(e)
27	transcription factor IIH basal transcription factor complex helicase XPD subunit [Homo sapiens]	2.90	3.85
28	early endosome antigen 1 [Homo sapiens]	UD(f)	2.92
29	putative teratocarcinoma-derived growth factor 2	2.85	2.52
30	PION protein [Homo sapiens]	2.73	1.49
31	T cell receptor beta chain [Homo sapiens]	2.63	2.53
32	type II inositol-3,4-bisphosphate 4-phosphatase [Homo sapiens]	2.58	1.45
33	THO complex 4 [Homo sapiens]	2.54	2.25
34	suppressor of cytokine signaling 6 [Homo sapiens]	2.35	1.90
35	nascent polypeptide-associated complex subunit alpha isoform a [Homo sapiens]	2.24	1.59
36	Uncharacterized protein FLJ45252	2.12	1.58
37	DLG5 [Homo sapiens]	2.08	2.28
38	zinc finger protein 21 (KOX 14), isoform CRA_c [Homo sapiens]	2.04	UD(g)
39	talin 2 [Homo sapiens]	1.97	1.78
40	SNW1 protein [Homo sapiens]	1.94	UD(h)
41	transmembrane protein vezatin [Homo sapiens]	1.89	3.04
42	transmembrane protein 39B [Homo sapiens]	1.87	2.88
43	chromosome X open reading frame 55 [Homo sapiens]	1.84	1.98
44	UDP-Gal:GlcNAc beta1,3-galactosyltransferase 5 [Homo sapiens]	1.84	2.95
45	FEZ1 [Homo sapiens]	1.68	2.50
46	alpha3 type IV collagen [Homo sapiens]	1.67	1.95
47	titin [Homo sapiens]	1.59	1.88
48	ACSL1 protein [Homo sapiens]	1.49	1.86
49	zinc finger and BTB domain containing 17 [Homo sapiens]	1.48	1.63
50	KIAA1714 protein [Homo sapiens]	UD(i)	UD(j)

UD = Unable to determine the intensity ratio of DENV-αNS1 to control (DENV-UPC or mock-αNS1)

(d) = intensity of DENV-αNS1 = 3.16; intensity of DENV-UPC = 0

(e) = intensity of DENV-αNS1 = 3.16; intensity of mock-αNS1 = 0

(f) = intensity of DENV-αNS1 = 2.85; intensity of DENV-UPC = 0

(g) = intensity of DENV-αNS1 = 1.91; intensity of mock-αNS1 = 0

(h) = intensity of DENV-αNS1 = 3.58; intensity of mock-αNS1 = 0

(i) = intensity of DENV-αNS1 = 1.48; intensity of DENV-UPC = 0

(j) = intensity of DENV-αNS1 = 0; intensity of mock-αNS1 = 0

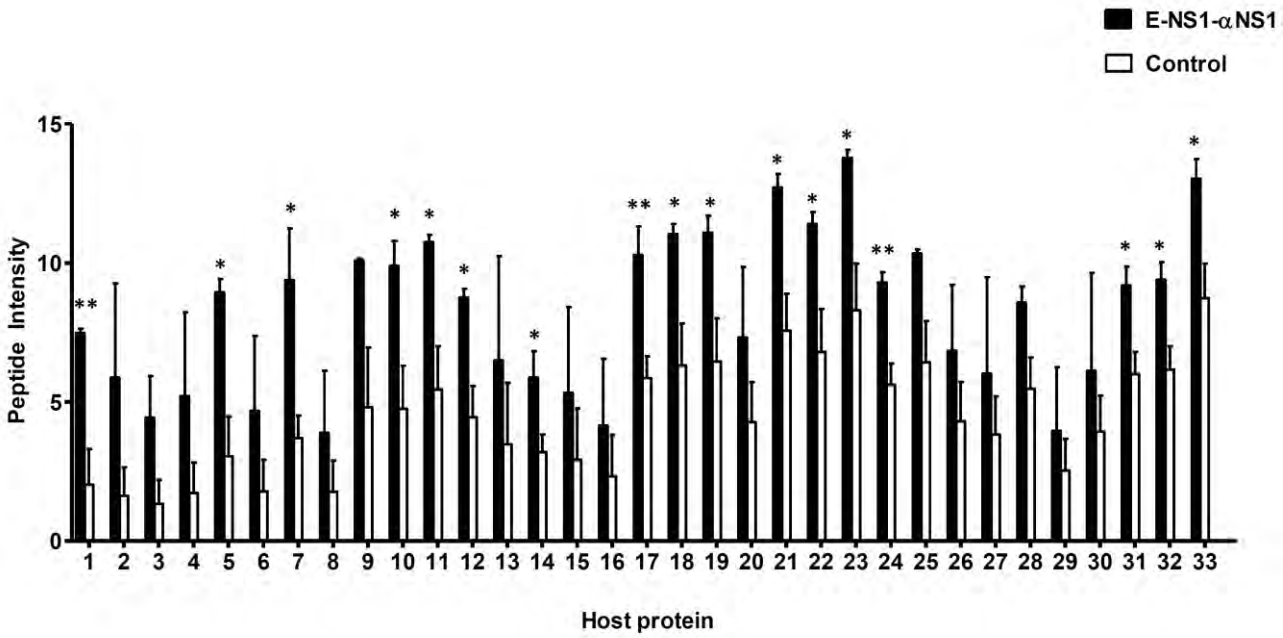


Figure 5. Mass spectrometric analysis of host cellular proteins potentially interacting with DENV NS1 in pcDNA Hygro E-NS1-His-transfected cells. pcDNA Hygro E-NS1-His or vector control-transfected cells were immunoprecipitated with anti-NS1 antibody (αNS1) or its isotype-matched control antibody (UCP). The immunoprecipitated samples were processed and analyzed by mass spectrometry. Thirty-three host cellular proteins were identified in the immunoprecipitated complexes. Data from two independent experiments show peptide intensities (mean + SEM) of the identified proteins in pcDNA Hygro E-NS1-His-transfected samples immunoprecipitated with anti-NS1 antibody (E-NS1-αNS1) and control samples (Control), which included pcDNA Hygro E-NS1-His-transfected samples immunoprecipitated with isotype-matched control antibody and vector-transfected samples immunoprecipitated with anti-NS1 antibody. Statistically significant differences in peptide intensities between two sample groups were analyzed by unpaired *t* test (*$p<0.05$; ** $p<0.01$).

Table 2. Identification of potential DENV NS1-interacting host cellular proteins in pcDNA Hygro E-NS1-His transfected cells

Protein	Name	Intensity ratio of E-NS1-αNS1 to control
1	KIAA1263 protein [Homo sapiens]	3.69
2	C-myc promoter-binding protein isoform 2 [Homo sapiens]	3.62
3	Ribosomal protein L27a [Homo sapiens]	3.32
4	hCG1741982 [Homo sapiens]	3.03
5	ACTN3 protein [Homo sapiens]	2.94
6	6-phosphogluconolactonase [Homo sapiens]	2.62
7	coiled-coil domain-containing protein 113 isoform 1 [Homo sapiens]	2.54
8	armadillo repeat-containing protein 6 [Homo sapiens]	2.19
9	SCAP protein [Homo sapiens]	2.10
10	NAD (H)-specific isocitrate dehydrogenase gamma subunit [Homo sapiens]	2.09
11	death-associated protein kinase 2 [Homo sapiens]	1.97
12	inositol 1,4,5-trisphosphate 3-kinase, isoform B [Homo sapiens]	1.97
13	HLA class I histocompatibility antigen	1.87
14	guanine nucleotide-binding protein subunit alpha-13 [Homo sapiens]	1.84
15	myosin regulatory light chain 12B [Homo sapiens]	1.83
16	coiled-coil domain-containing protein 69 [Homo sapiens]	1.78
17	immunoglobulin heavy chain variable region [Homo sapiens]	1.76
18	Laminin [Homo sapiens]	1.75
19	60S ribosomal protein L34 [Homo sapiens]	1.72
20	Chromosome 3 open reading frame 26 [Homo sapiens]	1.72
21	Chain H, 48g7 Hybridoma Line Fab Complexed With Hapten 5-(Para- Nitrophenyl Phosphonate)-Pentanoic Acid	1.68
22	mitochondrial translation-initiation factor 2 [Homo sapiens]	1.68
23	unnamed protein product [Homo sapiens]	1.66
24	eukaryotic initiation factor 4 gamma [Homo sapiens]	1.66
25	rhodanese thiosulfate sulfurtransferase [Homo sapiens]	1.61
26	anti-tetanus toxoid immunoglobulin heavy chain variable region [Homo sapiens]	1.59
27	BAH and coiled-coil domain-containing protein 1 [Homo sapiens]	1.57
28	KVLQT1 isoform1 [Homo sapiens]	1.57
29	collagen type IV alpha-3-binding protein isoform 3 [Homo sapiens]	1.56
30	bromodomain adjacent to zinc finger domain 2A [Homo sapiens]	1.55
31	hCG2042670 [Homo sapiens]	1.53
32	chymotrypsin inhibitor	1.52
33	regulator of nonsense transcripts 2 [Homo sapiens]	1.49

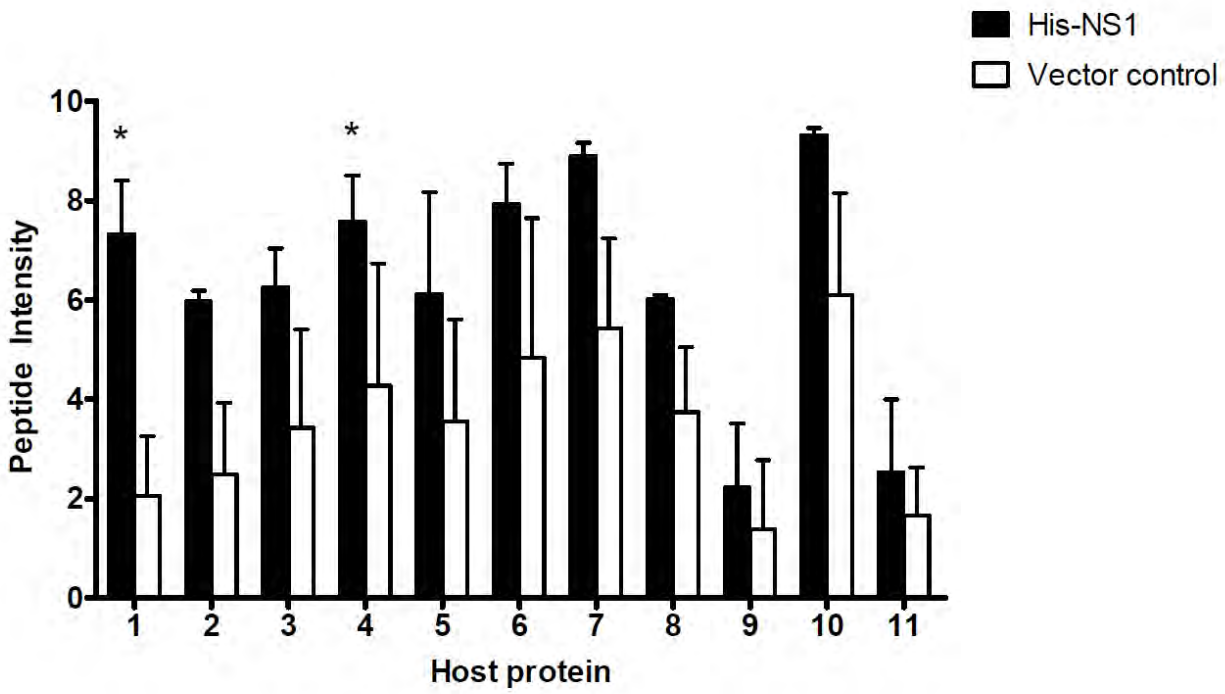


Figure 6. Mass spectrometric analysis of host cellular proteins potentially interacting with dengue virus NS1 in pcDNA3.1 His-NS1-transfected cells. Huh7 cells transfected with pcDNA3.1 His-NS1 and its vector control were subjected to pull down assay using TALON IMAC metal affinity system. The pulled down samples were processed and analyzed by mass spectrometry. Eleven host cellular proteins were identified in the pulled down samples. Data from two independent experiments show peptide intensities (mean + SEM) of the identified proteins in pcDNA3.1 His-NS1-transfected samples (His-NS1) and vector control-transfected samples (Vector control) following tag-based affinity pull down. Statistically significant differences in peptide intensities between two sample groups were analyzed by unpaired *t* test (*$p<0.05$).

Table 3. Identification of potential DENV NS1-interacting host cellular proteins in pcDNA3.1 His-NS1 transfected cells

Protein	Name	Intensity ratio of His-NS1 to vector control
1	Trafficking protein particle complex subunit 9 (NIK- and IKBKB-binding protein) (Tularik gene 1 protein) [Homo sapiens]	3.55
2	Chain A, Crystal Structure Of The C-Terminal Phophotyrosine Interaction Domain Of Human Apbb3 [Homo sapiens]	2.40
3	Ankyrin repeat and LEM domain-containing protein 2 (ANKLE2) [Homo sapiens]	1.82
4	hCG1820442 [Homo sapiens]	1.78
5	hypothetical protein [Homo sapiens]	1.72
6	WAS/WASL-interacting protein family member 3 [Homo sapiens]	1.64
7	KIAA1864 protein [Homo sapiens]	1.64
8	HD domain containing 3 [Homo sapiens]	1.61
9	anti-HIV-1 gp120 V3 loop antibody DO142-10 light chain variable region [Homo sapiens]	1.60
10	protein kinase N2 [Homo sapiens]	1.53
11	filamin A [Homo sapiens]	1.53

The data obtained from mass spectrometry demonstrated completely different sets of potential DENV NS1-interacting host proteins that were identified from DENV-infected cells and NS1-expressing cells. This may be due to a possibility that one set of these host cellular proteins may require the presence of other viral proteins for their association with NS1 in DENV-infected cells whereas the other set may interact with NS1 independently of other DENV components. Another possibility that may cause this difference is the use of different cell lines, i.e., HeG2 for DENV infection and Huh7 for NS1 transfection because, using our transfection method, we found better NS1 transfection efficiency in Huh7 cells than HepG2 cells. In the process of sample preparation for mass spectrometric analysis, the pulled down sample from pcDNA3.1 His-NS1-transfected cell lysate served as a control for any background interaction that might happen in the system because DENV NS1 protein generated in this sample is not targeted to the ER lumen as occurred naturally and, therefore, any cellular proteins that are identified to interact with this form of NS1 may represent artificial interactions. Our results showed that only 11 host proteins have been identified in this control sample and the majority of them (9/11) had no significant differences in the peptide intensities between pcDNA3.1 His-NS1-transfected and control samples (Figure 6 and Table 3). None of these proteins were found on the lists of host cellular proteins identified from DENV-infected cells and pcDNA Hygro E-NS1-His-transfected cells (Tables 1 and 2), thus

suggesting the presence of cellular compartment-specific interactions between DENV NS1 and identified host cellular proteins.

According to the list of host cellular proteins identified in pcDNA Hygro E-NS1-His-transfected cells (Table 2), laminin and eukaryotic translation initiation factor 4 gamma were human proteins that have been found previously to interact with DENV NS1 by using a yeast two-hybrid screening system with a human liver library (49). However, the experimentation to verify the interaction of these host cellular proteins with DENV NS1 has not been performed in that study. Further analysis on the proteomic data for host cellular proteins identified from DENV-infected cells revealed that the majority of the identified proteins with GeneInfo Identifier (GI) number could be mapped to UniProt database to be human proteins with known functions (40/50 = 80%) or with unknown function (3/50 = 6%) although 7 of the identified proteins (14%) were not detectable in the same database (Table 1 and Figure 7). The UniProt-mapped human proteins were further classified into subgroups based on their molecular function, biological function and protein class.

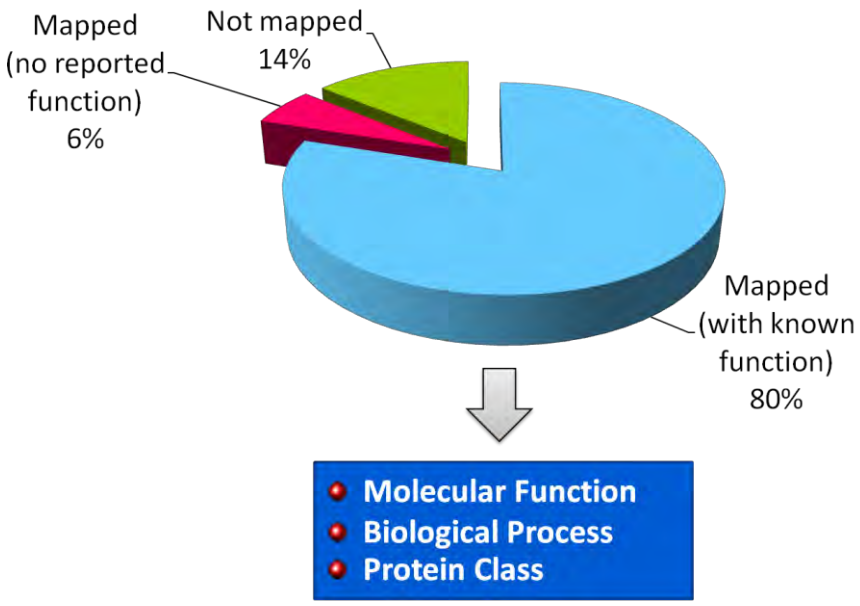


Figure 7. A pie chart illustrating the percentages of potential NS1-interacting host proteins identified from DENV-infected cells that are mapped to UniProt database.

Table 4. Mapping of identified DENV NS1-interacting host proteins from DENV-infected cells to UniProt database

Protein	Name	GI number	UniProt accession number	UniProt entry name
1	chromosome 21 open reading frame 70 [Homo sapiens]	gi|119629776	–	–
2	serine/threonine-protein kinase Nek2 [Homo sapiens]	gi|4505373	P51955	NEK2_HUMAN
3	serine/threonine-protein kinase TAO1 [Homo sapiens]	gi|45439370	Q7L7X3	TAOK1_HUMAN
4	conserved oligomeric Golgi complex subunit 1 (COG1) protein [Homo sapiens]	gi|71052081	Q4G0L8	Q4G0L8_HUMAN
5	protein phosphatase 1M [Homo sapiens]	gi|34526234	Q96MI6	PPM1M_HUMAN
6	folylpolyglutamate synthetase mitochondrial isoform [Homo sapiens]	gi|15911844	Q96LE4	Q96LE4_HUMAN
7	protein AHNAK2 [Homo sapiens]	gi|156766050	Q8IVF2	AHNK2_HUMAN
8	Melanin-concentrating hormone receptor 1 [Homo sapiens]	gi|12804625	Q99705	MCHR1_HUMAN
9	WD repeat domain 78 [Homo sapiens]	gi|55665586	A0AVI9	A0AVI9_HUMAN
10	hCG2040936 [Homo sapiens]	gi|119620382	–	–
11	TMCO3 protein [Homo sapiens]	gi|15214846	Q6UWJ1	TMCO3_HUMAN
12	trifunctional enzyme subunit alpha, mitochondrial precursor [Homo sapiens]	gi|20127408	E9KL44	ECHA_HUMAN
13	melanoma-associated antigen E1 [Homo sapiens]	gi|20143482	Q5JXC7	MAGE1_HUMAN
14	SERPINA11 type b [Homo sapiens]	gi|28207593	Q86WD7	SPA9_HUMAN
15	solute carrier family 26 [Homo sapiens]	gi|18643950	Q8TE54	S26A7_HUMAN
16	putative E1-E2 ATPase [Homo sapiens]	gi|6457268	Q9Y2G3	AT11B_HUMAN
17	hCG2036719 [Homo sapiens]	gi|119571779	–	–
18	rho guanine nucleotide exchange factor 17 [Homo sapiens]	gi|21361458	Q96PE2	ARHGH_HUMAN
19	zinc finger protein 1 homolog (ZEP1) protein [Homo	gi|40352974	Q6P2D0	ZFP1_HUMAN
20	hCG1777996 [Homo sapiens]	gi|119615085	–	–
21	hCG1813755 [Homo sapiens]	gi|119569343	–	–
22	repulsive guidance molecule A (RGMA) protein [Homo sapiens]	gi|111185504	Q96B86	RGMA_HUMAN
23	protocadherin Fat 4 precursor [Homo sapiens]	gi|165932370	B3KU84	FAT4_HUMAN
24	E3 ubiquitin-protein ligase HECW2 [Homo sapiens]	gi|55741473	Q9P2P5	HECW2_HUMAN
25	actin filament associated protein [Homo sapiens]	gi|10441465	Q8N556	AFAP1_HUMAN

Table 4. Mapping of identified DENV NS1-interacting host proteins from DENV-infected cells to UniProt database (continued)

Protein	Name	GI number	UniProt accession number	UniProt entry name
26	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4 [Homo sapiens]	gi|21619494	Q9C0J1	B3GN4_HUMAN
27	transcription factor IIH basal transcription factor complex helicase XPD subunit [Homo sapiens]	gi|296645	P18074	ERCC2_HUMAN
28	early endosome antigen 1 [Homo sapiens]	gi|55770888	Q15075	EEA1_HUMAN
29	putative teratocarcinoma-derived growth factor 2	gi|1706127	P51864	TDGF3_HUMAN
30	PION protein [Homo sapiens]	gi|77415426	A4D1B5	GSAP_HUMAN
31	T cell receptor beta chain [Homo sapiens]	gi|12751201	–	–
32	type II inositol-3,4-bisphosphate 4-phosphatase [Homo sapiens]	gi|156104895	O15327	INP4B_HUMAN
33	THO complex 4 [Homo sapiens]	gi|119610105	Q86V81	THOC4_HUMAN
34	suppressor of cytokine signaling 6 [Homo sapiens]	gi|21450785	O14544	SOCS6_HUMAN
35	nascent polypeptide-associated complex subunit alpha isoform a [Homo sapiens]	gi|163965366	Q13765	NACA_HUMAN
36	Uncharacterized protein FLJ45252	gi|172046183	Q6ZSR9	YJ005_HUMAN
37	DLG5 [Homo sapiens]	gi|19070635	Q8TDM6	DLG5_HUMAN
38	zinc finger protein 21 (KOX 14), isoform CRA_c [Homo sapiens]	gi|119579740	P17025	ZN182_HUMAN
39	talin 2 [Homo sapiens]	gi|21666571	Q9Y4G6	TLN2_HUMAN
40	SNW1 protein [Homo sapiens]	gi|48146983	Q6I9S2	Q6I9S2_HUMAN
41	transmembrane protein vezatin [Homo sapiens]	gi|11596110	Q9HBM0	VEZA_HUMAN
42	transmembrane protein 39B [Homo sapiens]	gi|169234667	Q9BT39	TM39B_HUMAN
43	chromosome X open reading frame 55 [Homo sapiens]	gi|119623025	–	–
44	UDP-Gal:GlcNAc beta1,3-galactosyltransferase 5 [Homo sapiens]	gi|7593021	Q9Y2C3	B3GT5_HUMAN
45	FEZ1 [Homo sapiens]	gi|4572462	Q9Y250	LZTS1_HUMAN
46	alpha3 type IV collagen [Homo sapiens]	gi|13559798	Q01955	CO4A3_HUMAN
47	titin [Homo sapiens]	gi|47060309	Q71S18	Q71S18_HUMAN
48	ACSL1 protein [Homo sapiens]	gi|20072835	P33121	ACSL1_HUMAN
49	zinc finger and BTB domain containing 17 [Homo sapiens]	gi|56202746	Q13105	ZBT17_HUMAN
50	KIAA1714 protein [Homo sapiens]	gi|27529919	Q9BZ76	CNTP3_HUMAN

Using online PANTHER classification system, potential NS1-interacting host proteins identified from DENV-infected cells were categorized into human proteins with different molecular and biological functions as shown in Figure 8. A major group of the identified host proteins (30%) had molecular function in catalytic activity and the similar number of proteins (29%) was also found to have biological function in metabolic process. Other molecular functions of the identified proteins included binding (22%), transporter activity (15%), receptor activity (13%), enzyme regulator activity (10%), transcription regulator activity (5%) and structural molecular activity (5%) (Figure 8). These proteins were likely to play a biological role in cell communication (19%), transport (12%), cell adhesion, cellular component organization and immune system process (8%), response to stimulus and homeostatic process (6%), and cell cycle/apoptosis (2%) (Figure 8). Additionally, the majority of the identified host proteins fell into the same protein classes as receptor, transporter, transferase and cell adhesion molecule (10%) (Figure 9).

To verify whether the identified host proteins interact with DENV NS1 in virus-infected cells, we initially attempted to set up yellow fluorescent protein-based protein fragment complementation assay (YFP-PCA) by cloning genes coding for DENV NS1 and host cellular proteins into two plasmid vectors containing N-terminal and C-terminal sequences of YFP. If the two proteins physically interact with each other in the cells, the fluorescent signal of YFP will expectedly be observed after co-transfection of two plasmid constructs. However, cloning of DENV NS1-coding sequence into the plasmid vectors for the YFP-PCA system was not successful as bacterial colonies were scarcely detected after transformation with the plasmid vectors containing DENV NS1 gene (data not shown). Inability of bacterial transformants to grow on the culture plates might be due to the toxicity or instability of the gene of interest in the context of YFP-PCA plasmid vectors in the bacterial strains (DH5α and Stbl2) used. To overcome this problem, co-immunoprecipitation assay has been carried out in DENV-infected cells using specific antibodies. Results showed that NIMA-related kinase 2 (NEK2), thousand and one amino acid protein kinase 1 (TAO1), component of oligomeric Golgi complex 1 (COG1), which are the top three identified host proteins with the highest significant difference in the peptide intensities between the tested sample and two control groups at the ratio >2.5 (Table 1 and Figure 4), could be co-immunoprecipitated with NS1 in DENV-infected HepG2 cells (Figure 10). Moreover, transcription factor IIH basal transcription factor complex helicase XPD subunit, another identified host protein that had the peptide intensity ratio > 2.5 without statistically significant difference, was also found to associate with NS1 following co-immunoprecipitation in DENV-infected HepG2 cells (Figure 10). Verification of these protein-protein interactions has been performed several times with the similar outcome. Taken together, this study demonstrated our success to identify a number of novel host cellular protein potentially interacting with DENV NS1 in both DENV infection and NS1 transfection

systems by using combined techniques of co-immunoprecipitation and mass spectrometry and to confirm DENVNS1 interactions with some of the selected host proteins in DENV-infected cells. Future investigation would be required to address functional contributions of the identified host proteins in cellular processes that might involve virus replication as well as cell cycle and apoptosis in response to DENV infection.

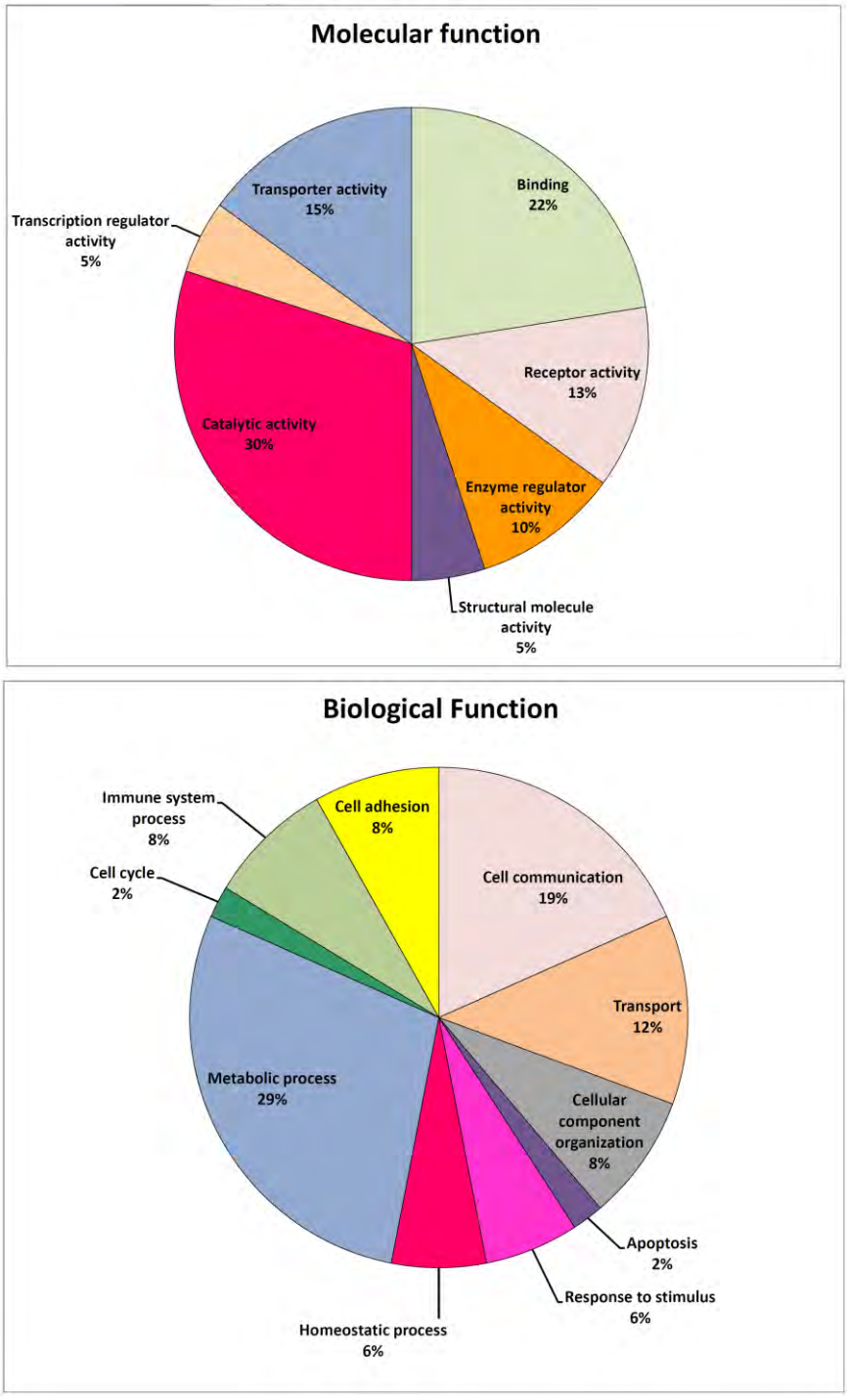


Figure 8. Pie charts illustrating molecular and biological functions of potential NS1-interacting host proteins identified from DENV-infected cells.

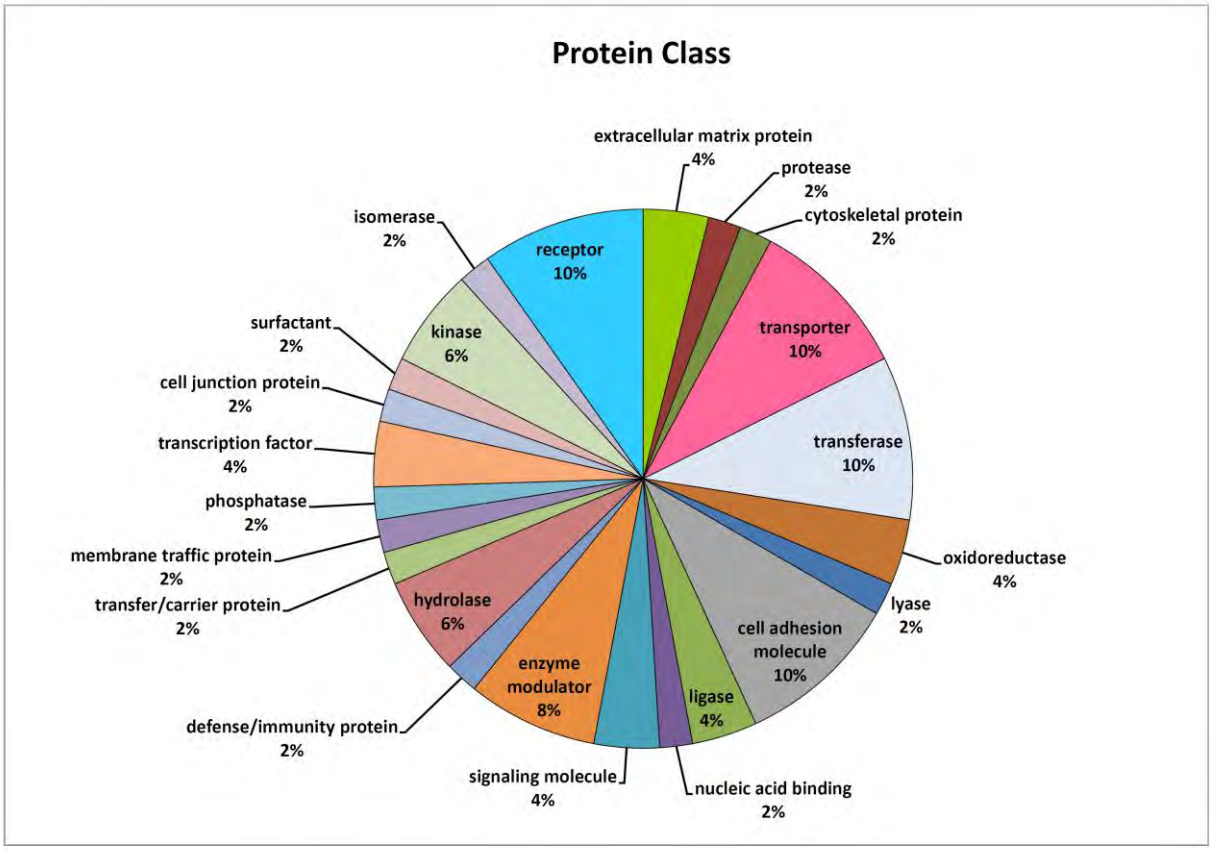


Figure 9. A pie chart demonstrating different classes of potential NS1-interacting host proteins identified from DENV-infected cells.

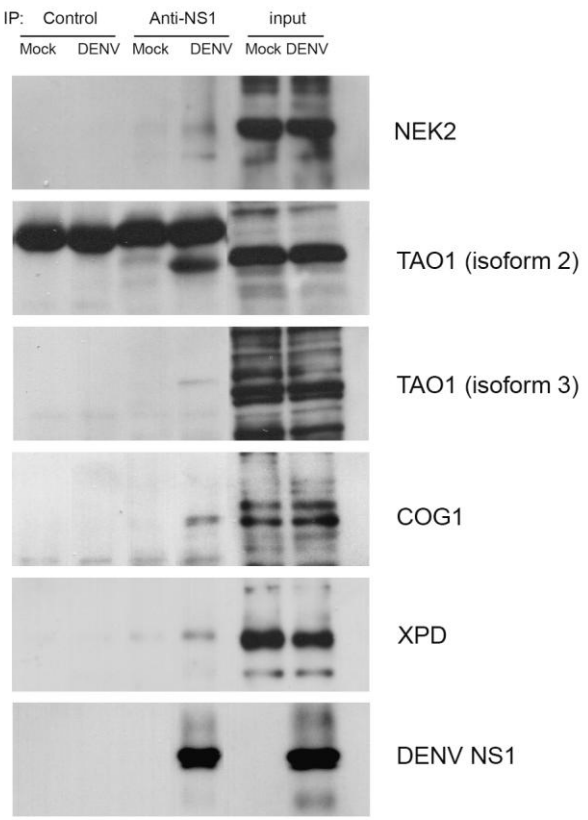


Figure 10. Verification of interactions between DENV NS1 and identified host cellular proteins in DENV-infected HepG2 cells by co-immunoprecipitation assay. Mock and DENV-infected HepG2 cells at 24 hr post infection were processed for immunoprecipitation with anti-NS1 antibody (Anti-NS1) or isotype-matched control antibody (Control). The immunoprecipitated samples were assessed for the presence of human NEK2, TAO1, COG1 and XPD proteins as well as DENV NS1 protein by immunoblotting analysis using specific antibodies. Mock and DENV-infected cell lysates prior to immunoprecipitation served as input control for the immunoblotting analysis.

II. The role of hnRNP C1/C2 and DENV NS1 association in DENV infection

Previously, we demonstrated that hnRNP C1/C2, which are known to be an RNA-binding host cellular protein, interact with DENV NS1 in human embryonic kidney epithelial HEK-293T cells. We next sought to investigate whether this interaction might associate with DENV RNA and involve the process of DENV infection in a more relevant and susceptible hepatocyte cell line. DENV-infected Huh7 cells were subjected to immunoprecipitation using isotype-matched control antibody (IgG1 or IgG2a) or specific antibodies against human hnRNP C1/C2 or DENV NS1 and the immunoprecipitated samples were analyzed by immunoblotting analysis. Results demonstrated co-immunoprecipitation of hnRNP C1/C2 and DENV NS1 following immunoprecipitation with the anti-NS1 antibody but not with the isotype control antibody (Appendix III, Figure 1). However, DENV NS1 was not observed when the virus-infected sample was immunoprecipitated with the hnRNP C1/C2-specific antibody (Appendix III, Figure 1). As hnRNP C1/C2 are abundant host cellular proteins with multiple functions particularly in mRNA biogenesis and processing, it is likely that only a portion of large hnRNP C1/C2 pools interacts with DENV NS1 in the virus-infected cells. In addition, association between DENV NS1 and hnRNP C1/C2 was confirmed by partial co-localization of these two proteins in DENV-infected cells at varying time points post infection (Appendix III, Figure 2). Whether the DENV NS1 and hnRNP C1/C2 complexes associate with DENV RNA was determined from the immunoprecipitated samples by RNA extraction and subsequent RT-PCR for DENV NS1 fragment. Results showed that DENV RNA could be detectable in the DENV-infected Huh7 cell lysates immunoprecipitated with either DENV NS1 specific antibodies or hnRNP C1/C2-specific antibody, but not with their isotype-matched control antibodies (Appendix III, Figure 3). Similar results of co-immunoprecipitation between DENV NS1 and hnRNP C1/C2 as well as their complex association with DENV were also observed in another hepatocyte cell line, HepG2, infected with DENV (data not shown).

Further investigation was performed to determine the role of DENV NS1 and hnRNP C1/C2 association in the process of DENV infection by using siRNA-mediated gene knockdown of hnRNP C1/C2. Initially, several conditions of siRNA transfection and DENV infection have been tested and we found that double transfection of Huh7 cells with hnRNP C1/C2-specific siRNA (40 pmol each) prior to infection with DENV at an MOI of 0.03 is the optimal condition to knockdown hnRNP C1/C2 without any significant effect on the number of cell death and cell proliferation. Thus, this condition has been applied throughout the entire study. Huh7 cells were transfected with 24 hr-interval two doses (40 pmol each) of either irrelevant control siRNA (IR) or hnRNP C/1C2-specific siRNA (SP) and 6 hr after transfection they were left uninfected (mock) or infected with DENV at the MOI of 0.03. Mock and DENV-infected cells and culture supernatants were harvested at 0, 12, 24, 36 and 48 hr post infection. Immunoblotting analysis reveled that transfection with hnRNP C1/C2-specific siRNA resulted in a decrease in hnRNP C1/C2 protein expression approximately by 50% at all time points tested in mock

and DENV-infected cells as compared with transfection with irrelevant control siRNA (Appendix III, Figure 4). A reduction in the percentage of DENV-infected cells was observed as early as 24 hr post infection after hnRNP C1/C2 knockdown as shown by immunofluorescence staining for DENV E antigen (Appendix III Figure 5). Whether hnRNP C1/C2 knockdown has any effect on DENV RNA replication and protein synthesis was determined by real-time RT-PCR and immunoblotting analysis, respectively. Results demonstrated diminished levels of viral RNA as well as viral E and NS1 protein expression at different time points post infection when the cells were transfected with hnRNP C1/C2-specific siRNA as compared with the control siRNA-transfected cells (Appendix III, Figures 6 and 7). Moreover, the hnRNP C1/C2 knockdown cells appeared to produce and release lower titers of infectious virus into the extracellular milieu than the control cells as determined by a focus forming unit assay using culture supernatants collected at varying time points post infection (Appendix III, Figure 8). The effects of hnRNP C1/C2 knockdown on varying steps in DENV life cycle may be derived from the interference of complex formation between hnRNP C1/C2 and DENV NS1 since DENV NS1 was co-immunoprecipitated with hnRNP C1/C2 at lower extents in the hnRNP C1/C2 knockdown samples as compared with the control samples (Appendix III, Figure 9). This set of study has been completed and a draft of manuscript (Appendix III) will be revised for submission to Virology Journal in June 2014.

III. <u>Association between DENV NS1 and their chaperone proteins in DENV infection</u>

As DENV NS1 protein is processed in the ER of host cells during DENV infection, we hypothesize that this viral protein might interact with three important host chaperone proteins (i.e., BiP/GRP78, calnexin and calreticulin) to facilitate viral protein processing, viral replication and viral production. Double immunofluoresence staining of DENV NS1 and chaperone proteins was performed in DENV-infected HepG2 cells. Partial co-localization of the two proteins was observed (Figure 11). In addition, association of DENV NS1 and BiP/GRP78 appeared to be time-dependent in DENV-infected HepG2 and Huh7 cells as shown by double immunofluorescence staining (Figures 12A and 12B) as well as immunoprecipitation and subsequent immunoblotting analysis (Figure 13). Co-immunoprecipitation assay for detection of DENV NS1 and these chaperone proteins were also performed. Results from three independent experiments showed that DENV NS1 could be co-immunoprecipitated with BiP/GRP78 and calnexin, but not calreticulin, in DENV-infected cells (Figure 14). As we did not perform a pulse-chase experiment in our study to study the kinetics of protein-protein interaction over time, we could not rule a possibility that the interaction between calreticulin and DENV NS1 might happen in DENV-infected cells so rapidly that it was out of our detection limit.

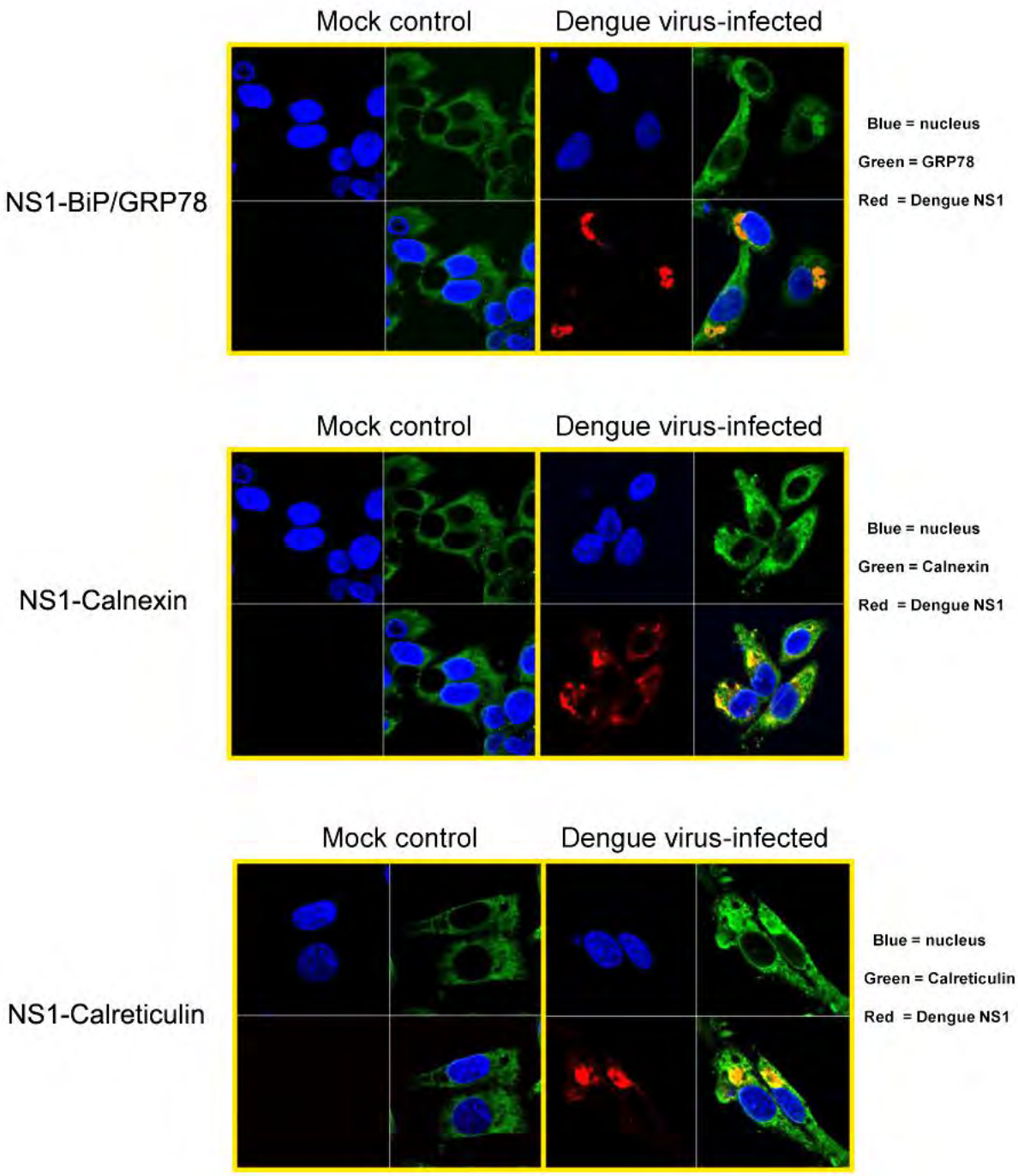
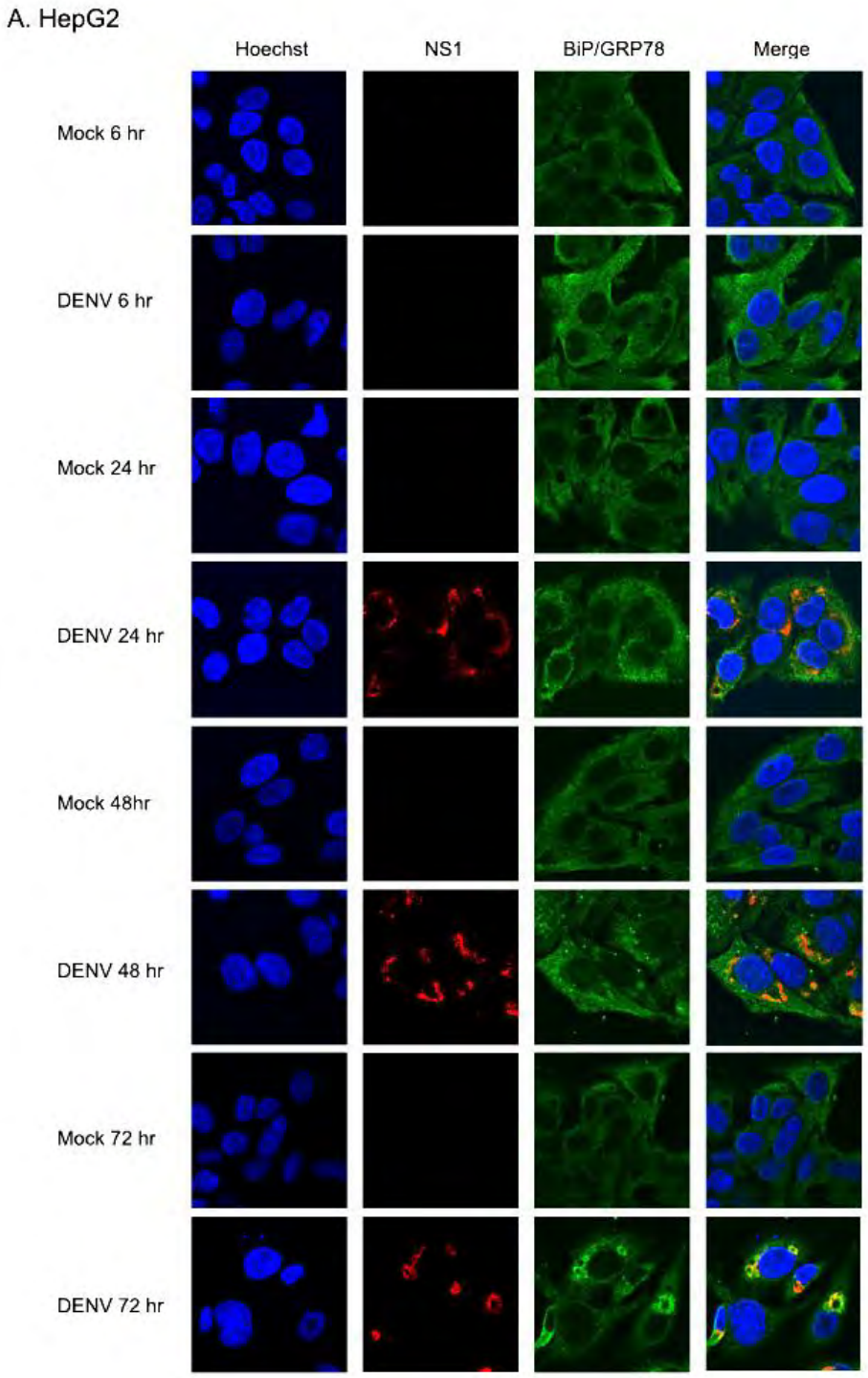


Figure 11. Partial co-localization of DENV NS1 and human chaperone proteins. Mock and DENV-infected HepG2 on day 2 post infection were subjected to double immunofluorescence staining for viral NS1 and human chaperone protein (BiP/GRP78, calreticulin or calnexin) using specific antibodies. Nuclear staining was performed in each sample using Hoechst. The stained cells were observed under a laser scanning confocal microscope at a 63x objective lens. Co-localization of the NS1 and chaperone protein is shown in orange or yellow.


A. HepG2
Hoechst
NS1
BiP/GRP78
Merge
Mock 6 hr
DENV 6 hr
Mock 24 hr
DENV 24 hr
Mock 48hr
DENV 48 hr
Mock 72 hr
DENV 72 hr

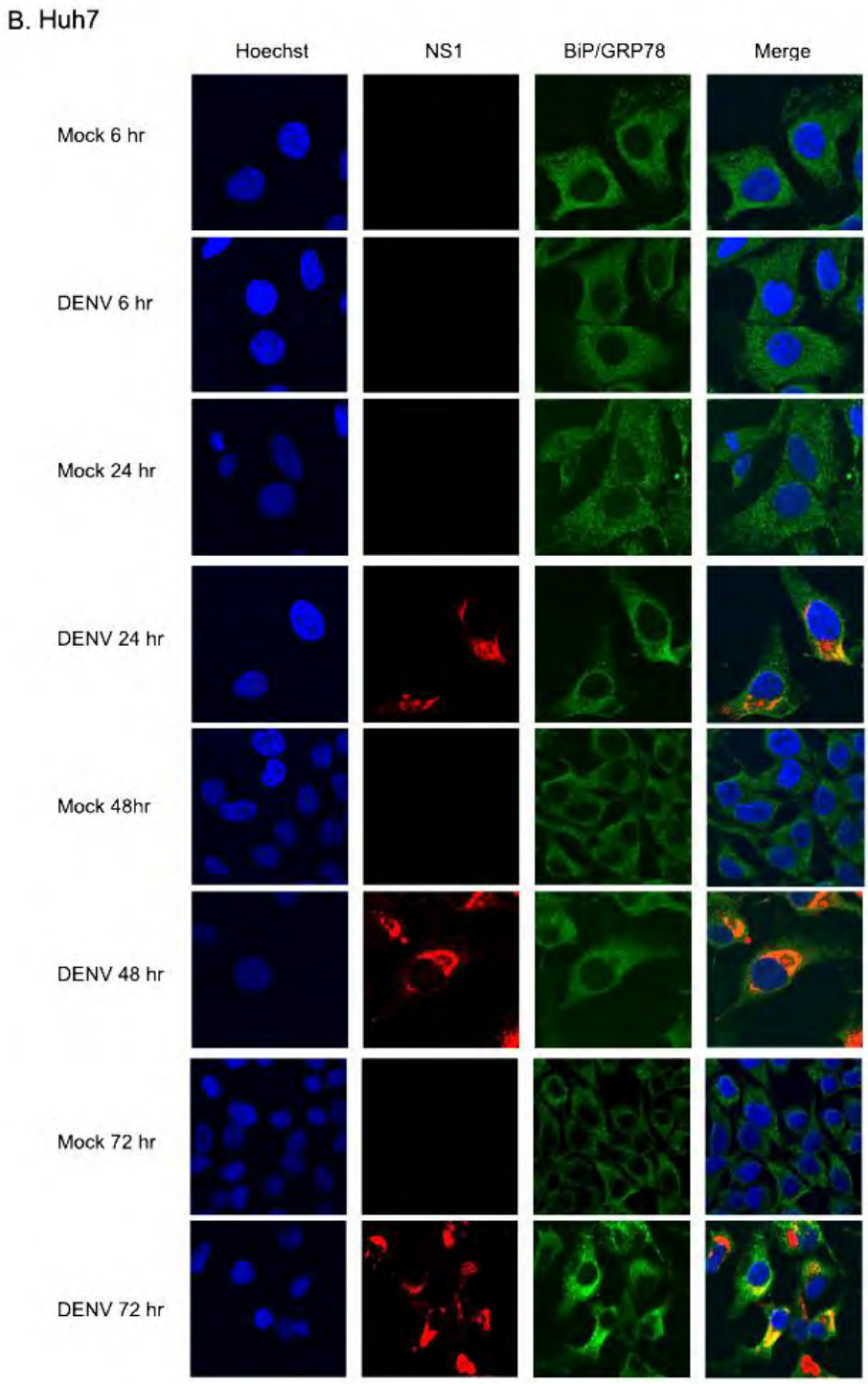


Figure 12. Kinetics of co-localization between DENV NS1 and human BiP/GRP78. Mock and DENV-infected HepG2 (A) or Huh7 (B) cells at the indicated time points were subjected to double immunofluorescence staining for DENV NS1 and human BiP/GRP78 using specific antibodies. Nuclear staining was performed in each sample using Hoechst. The stained cells were observed under a laser scanning confocal microscope at a 63x objective lens. Co-localization of the NS1 and BiP/GRP78 is shown in orange or yellow.

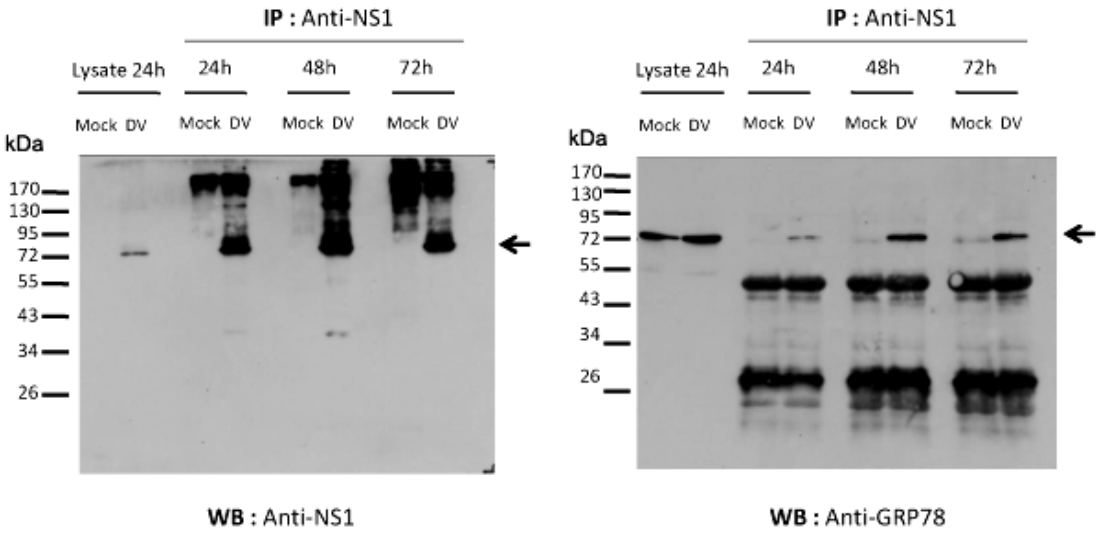


Figure 13. Kinetics of co-immunoprecipitation between DENV NS1 and BiP/GRP78. Mock and DENV-infected Huh7 cells at the indicated time points post infection were processed for immunoprecipitation with anti-NS1 antibodies. The immunoprecipitated samples were assessed for the presence of DENV NS1 (left panel) and BiP/GRP78 (right panel) by immunoblotting analysis using specific antibodies.

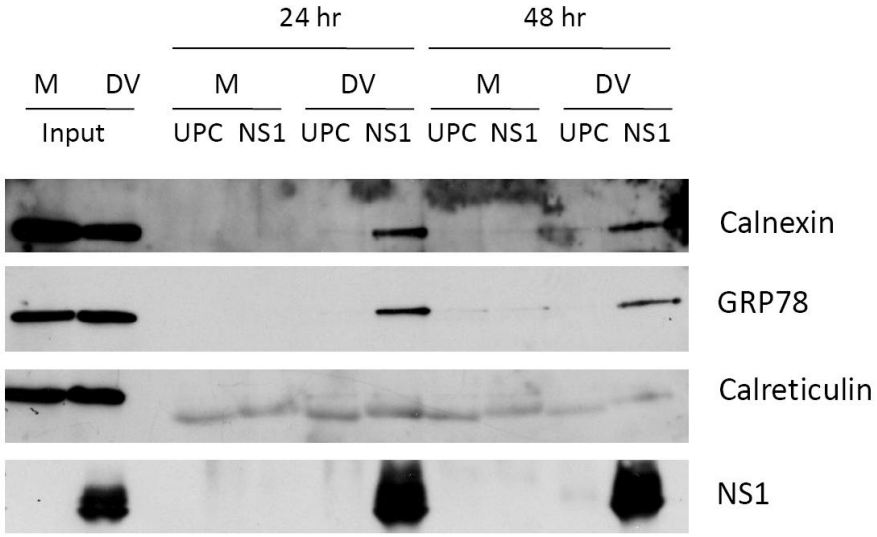


Figure 14. Co-immunoprecipitation between DENV NS1 and ER-resident chaperone proteins. Mock and DENV-infected Huh7 cells at 24 hr post infection were processed for immunoprecipitation with anti-NS1 antibody (αNS1) or isotype-matched control antibody (UPC). The immunoprecipitated samples were assessed for the presence of DENV NS1, BiP/GRP78, calreticulin and calnexin by immunoblotting analysis using specific antibodies.

To investigate the functional contribution of the chaperone and DENV NS1 association in DENV infection by using siRNA-mediated gene knockdown for each chaperone protein. Varying conditions of transfection with two sets of custom-designed siRNA sequences targeting against each chaperone had been applied in attempts to knockdown the specific protein in Huh7 cells; however, in all the conditions tested, the levels of three chaperone proteins remained unchanged (data not shown). Therefore, we optimized the siRNA transfection conditions by using commercially available siRNA sequences with guaranteed gene knockdown and a different transfection reagent to obtain the efficient knockdown of these chaperone proteins. Huh7 cells were transfected with different amounts of siRNA targeted against BiP/GRP78, calnexin and calreticulin (OriGene) using Lipofectamine RNAiMax (Invitrogen) and then determined for expression levels of the corresponding proteins as compared with control siRNA-transfected cells. A successful knockdown of the target protein was observed only in siRNA transfection with BiP/GRP78, but not in calnexin and calreticulin siRNA transfection (data not shown). Based on these results, we pursued our study on the role of BiP/GRP78 in NS1 association. Previous studies have demonstrated that BiP/GRP78 protein interacted with DENV E in DENV-infected cells and knockdown of this protein resulted in reduced DENV production (50, 51). Therefore, to address the involvement of BiP/GRP78 protein interacting with DENV NS1 in viral RNA replication and viral protein processing, we performed siRNA-mediated gene knockdown in DENV replicon-expressing cells that had no expression of DENV structural proteins. Huh7 cells were transfected with BiP/GRP78-specific siRNA or control siRNA followed by DENV replicon transfection. The transfected cells were then assessed for the efficiency of siRNA-mediated BiP/GRP78 knockdown as well as the levels of DENV RNA and protein expression. At 24 hr after DENV replicon transfection, BiP/GRP78 mRNA and protein expression was found to decrease by approximately 65% and 30%, respectively, as evidenced by real-time RT-PCR and immunoblot analysis (Figures 15 and 16). Kinetic studies of DENV replicon-expressing cells within 72 hr after transfection revealed that although about 60-70% reduction in BiP/GRP78 mRNA expression has been observed throughout the entire study period, BiP/GRP78-specific siRNA transfection did not affect DENV RNA replication (Figure 17). Transfection with either BiP/GRP78-specific siRNA or control siRNA yielded the similar pattern of kinetic DENV RNA expression in DENV replicon-expressing cells in that the amount of DENV RNA peaked at 4 hr after DENV replicon transfection, then declined at later time points and finally remained unchanged between 24-72 hr of the study period (Figure 17). Further investigation on the effect of BiP/GRP78-specific siRNA transfection on the kinetic of DENV protein synthesis showed that transfection with BiP/GRP78-specific siRNA not only decreased BiP/GRP78 protein expression, but also caused approximately 50%-70% reduction of DENV NS1 expression protein particularly within 24 hr after DENV replicon transfection as compared with control siRNA transfection (Figures 18 and 19). However, the levels of DENV NS1 protein expression tended to

increase in BiP/GRP78 knockdown cells at 48-72 hr after transfection and this may be correlated with rising levels of BiP/GRP78 protein expression at the late time points (Figures 18 and 19). Altogether, our results demonstrated the association of human BiP/GRP78 with DENV NS1 in DENV-infected cells that was likely to influence the process of viral protein synthesis, but not viral RNA replication. Exploring a detailed mechanism of particular domains of BiP/GRP78 protein required for NS1 interaction might be important to help designing any small molecules that can be used to inhibit this specific interaction and thus result in the inhibition of DENV protein synthesis.

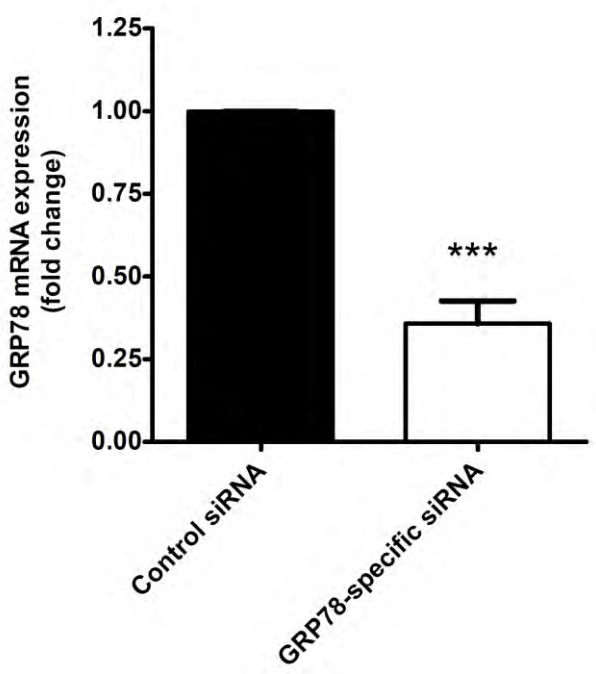


Figure 15. Expression of BiP/GRP78 mRNA following siRNA transfection. Huh7 cells were transfected with 66 nM of control siRNA or BiP/GRP78-specific siRNA using Lipofectamine RNAiMax and cultured for 24 hr. Cells were then transfected with 1 μg of DENV replicon cells and harvested at 24 hr later to determine for the expression of BiP/GRP78 and β-actin (internal control) mRNA by real-time RT-PCR. Relative levels of BiP/GRP78 mRNA expression were assessed by normalization of BiP/GRP78 to β-actin mRNA expression and presented as fold change compared to control siRNA transfection. Data represent mean and SEM of 7 independent experiments. Asterisks indicate statistically significant differences (***$p<0.001$) in relative expression of BiP/GRP78 mRNA between the specific siRNA-transfected and control siRNA-transfected samples by unpaired *t* test.

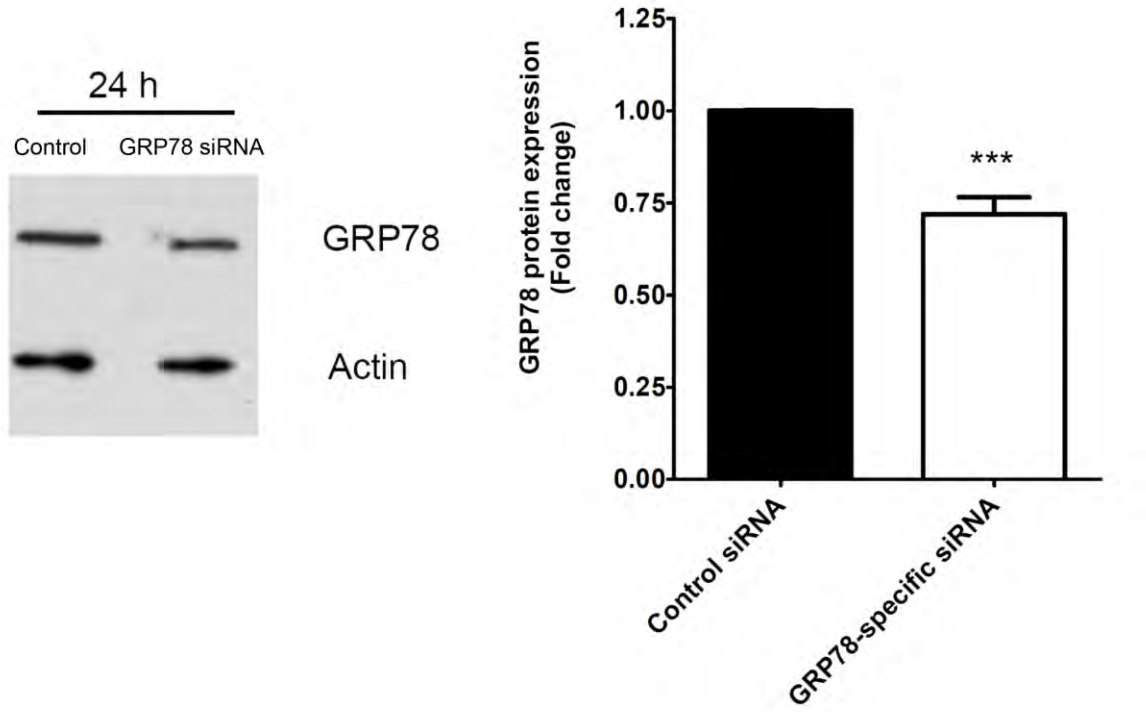


Figure 16. Expression of BiP/GRP78 proteins following siRNA transfection. Huh7 cells were transfected with 66 nM of control siRNA or BiP/GRP78-specific siRNA using Lipofectamine RNAiMax and cultured for 24 hr. Cells were then transfected with 1 μg of DENV replicon cells and harvested at 24 hr later to determine for the expression of BiP/GRP78 and β-actin (internal control) proteins by immunoblotting analysis. A representative image of immunoblotting results from 4 independent experiments is shown (left panel). Relative levels of BiP/GRP78 protein expression were assessed by normalization of BiP/GRP78 to β-actin protein expression and presented as fold change compared to control siRNA transfection (right panel). Data represent mean and SEM of 4 independent experiments. Asterisks indicate statistically significant differences (***$p<0.001$) in relative expression of BiP/GRP78 protein between the specific siRNA-transfected and control siRNA-transfected samples by unpaired *t* test.

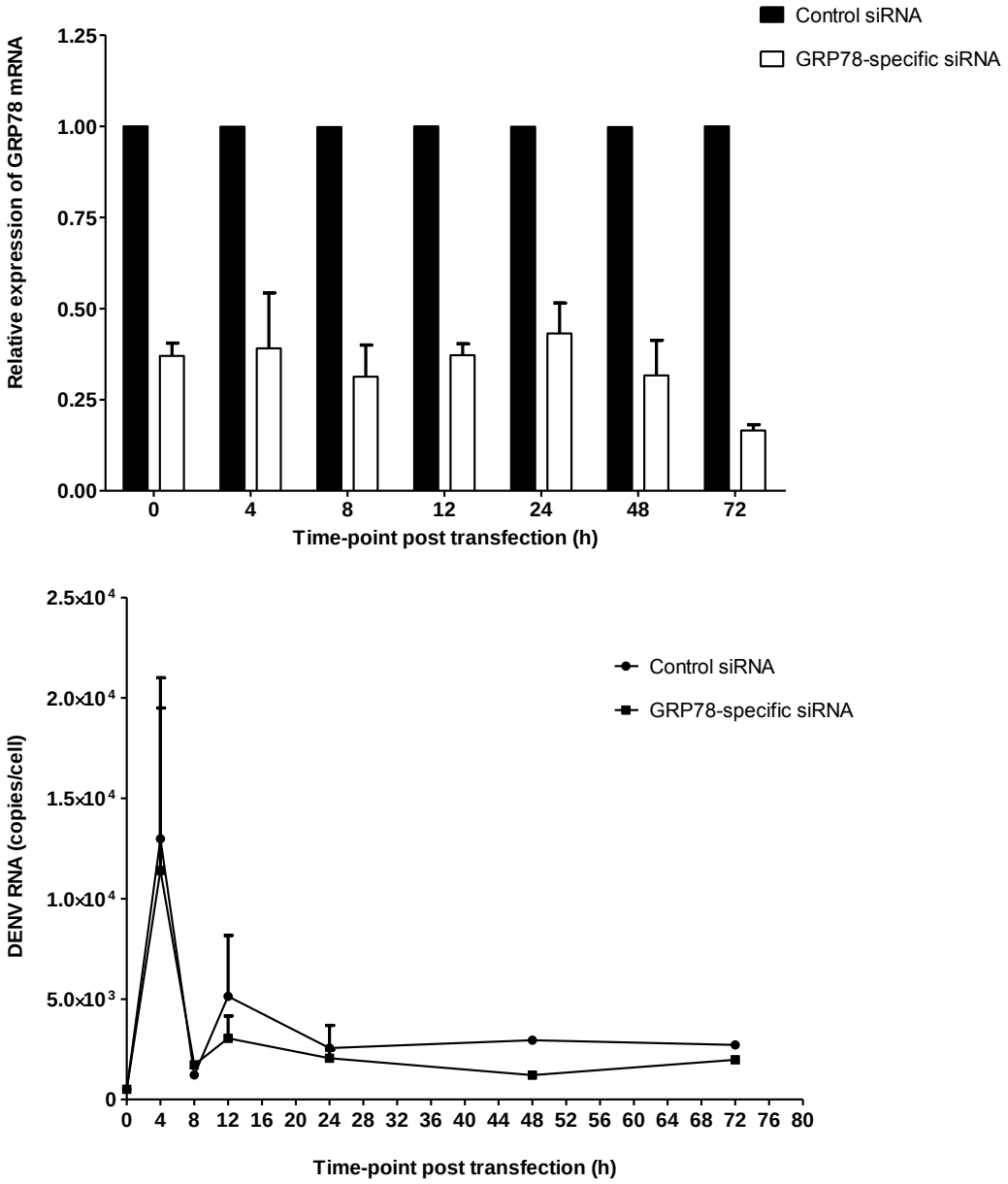


Figure 17. Effect of BiP/GRP78-specific siRNA transfection on BiP/GRP78 mRNA and DENV RNA expression. Huh7 cells were transfected with 66 nM of control siRNA or BiP/GRP78-specific siRNA using Lipofectamine RNAiMax and cultured for 24 hr. Cells were then transfected with 1 μg of DENV replicon cells and harvested at varying time points post transfection to determine BiP/GRP78 mRNA and DENV RNA expression by real-time RT-PCR. Determination of β-actin mRNA expression was also performed as an internal control for normalization of BiP/GRP78 expression. Relative expression of BiP/GRP78 mRNA in control siRNA and GRP78-specific siRNA-transfected cells expressing DENV replicon was shown at indicated time points (top panel). By comparing with known copy number of standard DENV RNA, the amount of DENV RNA in control siRNA and GRP78-specific siRNA-transfected cells expressing DENV replicon was determined and presented as RNA copies per cell at the indicated time points (bottom panel). Data represent mean and SEM of 2-3 independent experiments.

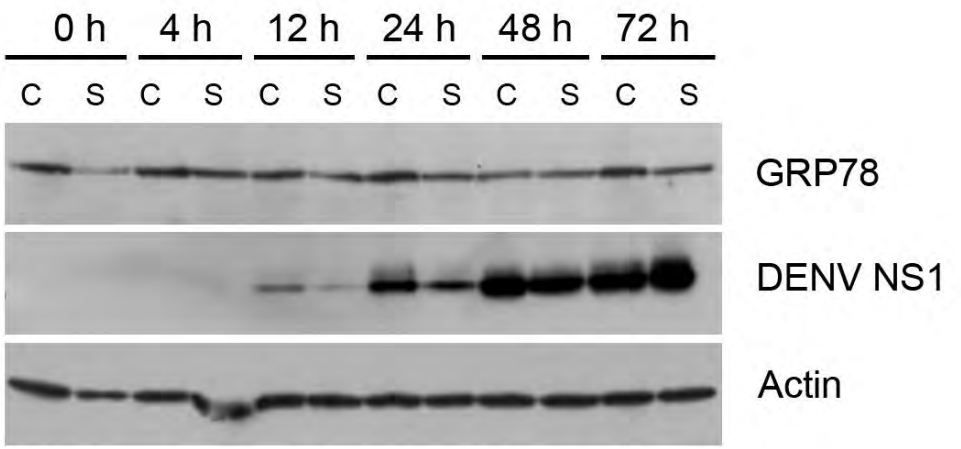


Figure 18. Effect of BiP/GRP78-specific siRNA transfection on BiP/GRP78 and DENV NS1 protein expression. Huh7 cells were transfected with 66 nM of control siRNA (C) or BiP/GRP78-specific siRNA (S) using Lipofectamine RNAiMax and cultured for 24 hr. Cells were then transfected with 1 μg of DENV replicon cells and harvested at varying time points post transfection to determine BiP/GRP78, β-actin (internal control) and DENV NS1 protein expression by immunoblotting analysis. Data show a representative image of immunoblotting results from 2 independent experiments.

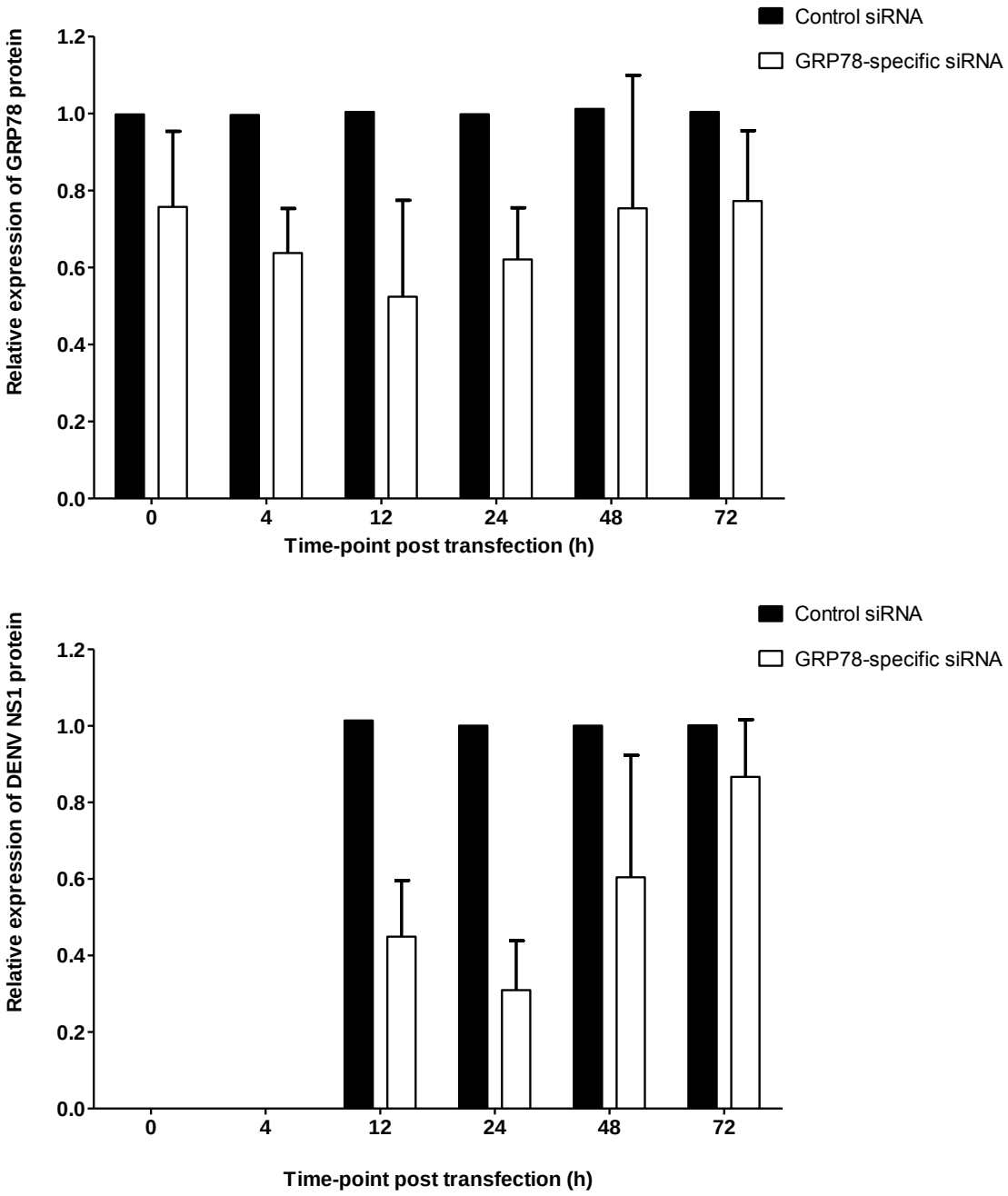


Figure 19. Effect of BiP/GRP78-specific siRNA transfection on BiP/GRP78 and DENV NS1 protein expression. Huh7 cells were transfected with 66 nM of control siRNA or BiP/GRP78-specific siRNA using Lipofectamine RNAiMax and cultured for 24 hr. Cells were then transfected with 1 μg of DENV replicon cells and harvested at varying time points post transfection to determine BiP/GRP78 and DENV NS1 protein expression by immunoblotting analysis. Determination of β-actin mRNA expression was also performed as an internal control for normalization of BiP/GRP78 and DENV NS1 expression. Relative expression of BiP/GRP78 protein (top panel) and DENV NS1 protein (bottom panel) in control siRNA and GRP78-specific siRNA-transfected cells expressing DENV replicon was shown at indicated time points. Data represent mean and SEM of 2 independent experiments.

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Outputs

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการ

Abstracts (Appendix I)

- Thanyaporn Dechtawewat, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. Role of hnRNP C1/C2 and Dengue Virus NS1 Association in Dengue Virus Production. National and International Conference in Medicine and Public Health to Commemorate 150th Anniversary of the Birth of Queen Sri Savarindira. Faculty of Medicine Siriraj Hospital, Mahidol University, 17-21 September 2012. The abstract was published in Siriraj Medical Journal, Volume 64, July-August 2012, page A4.
- Thanyaporn Dechtawewat, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. A Potential Contribution of hnRNP C1/C2 and Dengue Virus NS1 Association to Dengue Virus Infection. The Third International Conference of Dengue and Dengue Haemorrhagic Fever. The Imperial Queen's Park Hotel, Bangkok, Thailand, 21-23 October 2013.

Proceedings (Appendix II)

- Thanyaporn Dechtawewat, Suchada Sengsai, Pucharee Songprakhon, Thawornchai Limjindaporn, Chunya Puttikhunt, Watchara Kasinrerk, Pa-thai Yenchitsomanus, **Sansanee Noisakran**. Association of Dengue Virus NS1 and hnRNP C1/C2 Proteins in Different Human Target Cells for Dengue Virus Infection. Proceedings of the 6th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute Convention Center, 30 August – 2 September 2011, pp. 146-153.

Full Articles (Appendix III)

- Interaction of dengue virus NS1 with hnRNP C1/C2 facilitates dengue virus production. This draft of manuscript will be revised for submission to Virology Journal in June 2014.
- LC MS/MS analysis for host cellular proteins interacting with the non-structural protein 1 in dengue virus-infected cells (Manuscript in preparation for Journal of Proteomics)
- Association of dengue virus NS1 and human GRP78 in dengue virus infection (Manuscript in preparation for Microbes and Infection)

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ โดยเป็นวิทยากรรับเชิญจากหน่วยวิจัยชีวเคมีและเคมีวิเคราะห์ งานห้องปฏิบัติการวิจัย สำนักงานวิจัยวิชาการและนวัตกรรม คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล ที่จัดโครงการพัฒนาบุคลากรเรื่อง “Mass spectrometry and protein analysis” เพื่อการบรรยายในหัวข้อเรื่อง การจับของโปรตีน NS1 จากไวรัสเด็งกี่กับโปรตีนของเซลล์เจ้าบ้าน วันที่ 18 มีนาคม 2557

3. กิจกรรมอื่น ๆ ที่เกี่ยวข้อง ได้แก่

3.1. การไปเสนอผลงานในที่ประชุมวิชาการ

- Poster presentation entitled “Association of Dengue Virus NS1 and hnRNP C1/C2 Proteins in Different Human Target Cells for Dengue Virus Infection” at the 6th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute Convention Center, 30 August – 2 September 2011.

- Poster presentation entitled “A Potential Contribution of hnRNP C1/C2 and Dengue Virus NS1 Association to Dengue Virus Infection" at the Third International Conference of Dengue and Dengue Haemorrhagic Fever. The Imperial Queen’s Park Hotel, Bangkok, Thailand, 21-23 October 2013.

3.2. การได้รับเชิญไปเป็นวิทยากร

- Invited speaker in the topic of “Interactions of Dengue Virus NS1 Protein with Human Host Cellular Proteins” on 18 March 2014 at Faculty of Medicine Ramathibodi Hospital, Mahidol University.

3.3. การเป็นอาจารย์ที่ปรึกษาร่วมตลอดหลักสูตร

- Graduate student training:
  Miss Thanyaporn Dechtawewat graduated from the master's degree program at the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University in August 2013 with the thesis entitled "Role of hnRNP C1/C2 and Dengue Virus NS1 Association in Dengue Virus Infection"

3.4. ผลงานตีพิมพ์ในรูปแบบหนังสือ

ศันสนีย น้อยสคราญ และ พัชรี ทรงประโคน. Confocal Laser Scanning Microscope. ใน: พิไลพันธ์ พุธวัฒนะ, เทคนิคอิมมูนเรืองแสงทางไวรัสวิทยา. พิมพ์ครั้งที่ 1. กรุงเทพฯ: วี.เจ. พริ้นติ้ง; 2556. หน้า 45-60.

3.5. การเชื่อมโยงทางวิชาการกับนักวิชาการอื่น ๆ ทั้งในและต่างประเทศ

Research collaboration with Dr. Sittirak Roytrakul and Ms. Atchara Paemanee at the Proteomics Unit, Genome Institute, National Center for Genetic Engineering and Biotechnology.

3.6. รางวัลที่ได้รับ

- Miss Thanyaporn Dechtawewat, an M.Sc. graduate student (the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University) who worked in part of this project, received a scientific award for the second-place oral presentation entitled “Role of hnRNP C1/C2 and Dengue Virus NS1 Association in Dengue Virus Production” in the Graduate Research Forum 2012 at the Faculty of Medicine Siriraj Hospital, Mahidol University.

- Dr. Sansanee Noisakran was given the L'Oreal Thailand For Women in Science 2012 award in the field of life sciences for investigating the mechanisms of dengue virus infection and host cellular responses for better understanding of dengue pathogenesis.

Appendix I

(Abstracts)

ABSTRACTS

National and International Conference in Medicine and Public Health to Commemorate 150th Anniversary of the Birth of Queen Sri Savarindira

Faculty of Medicine Siriraj Hospital, Mahidol University

17-21 September 2012

O-10

Title: Impaired Survival of Diabetes-Associated Paired box 4 Mutants under a High-Glucose Condition

Authors: Jatuporn Sujjitjoon[1,2], Nattachet Plengvidhya[3], Suwattanee Kooptiwut[4], Wanthanee Hanchang[4], Titikan Chukitrungroat[4], Watip Tangjitipokin[2], Pa-thai Yenchitsomanus[1]

Institutions: *[1]Division of Molecular Medicine, [2]Department of Immunology, [3]Division of Endocrinology and Metabolism, Department of Medicine, [4]Department of Physiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

Objectives: Pair box 4 (PAX4) is essential for survival in rat insulinoma cells (INS-1). Diabetes-associated *PAX4* variations were reported in several ethnic groups. Our group identified *PAX4* R164W and R192Hcause maturity-onset diabetes of the young (MODY) and type 2 diabetes (T2D), respectively. Silencing of *Pax4* in INS-1 increased cell apoptosis, while *PAX4* over-expression promoted human isletsproliferation. Therefore, we aim to examine the cell viability mediated by over-expression of *PAX4* wild-type (WT) and variants related to diabetes in Thais.

Materials and Methods: Human embryonic kidney (HEK293T) or INS-1 cells were transiently transfected with *PAX4* WT or variants and cultured in basal (11.1 mM) and high (33.3 mM) glucose medium for three days. Cell viability was examined by using PrestoBlue dye and MTT assay. Data was derived from three independent experiments and analyzed by student *t'* test. The *p*-value<0.05 was considered as statistically significance.

Results: Viability of HEK293T or INS-1 over-expressing PAX4WT and both variants were similar under basal glucose condition. However, in high glucose medium, cells over-expressing PAX4 R164W exhibited significant reduction of viability compared to PAX4WT over-expressing cells (p=0.014) while PAX4 R192H did not show significant difference from PAX4 WT.

Conclusion: *PAX4* R164W mutation may leads to diabetes via reduced cell survival under high glucose condition, whereas *PAX4* R192H polymorphism does not affect cell viability. However, functional study of these two variants in other condition such as free fatty acid needs further investigation for better understanding the role of PAX4 in pathogenesis of diabetes mellitus.

Keywords: *Paired box 4*, diabetes mellitus, cell survival

O-11

Title: Role of hnRNP C1/C2 and Dengue Virus NS1Association in Dengue Virus Production

Authors: Thanyaporn Dechtawewat[1,2], Pucharee Songprakhon[2,3], Thawornchai Limjindaporn[4], Chunya Puttikhunt[5,6], Watchara Kasinrerk[7,8], Pa-thai Yenchitsomanus[2], Sansanee Noisakran[5,6]

Institutions: *[1]Department of Immunology and Graduate Program in Immunology, Faculty of Medicine Siriraj Hospital, [2]Division of Molecular Medicine, [3]Division of Instruments for Research, Office of Research and Development, Faculty of Medicine Siriraj Hospital, [4]Department of Anatomy, Faculty of Medicine Siriraj Hospital, [5]Division of Dengue Hemorrhagic Fever Research Unit, Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, [6]Medical Biotechnology Research Unit and [7]Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, [8]Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.*

Objectives: Human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 were previously demonstrated to interact with the nonstructural protein 1 (NS1) of dengue virus (DENV); however, the role of this association was not known. This study aimed at investigating the potential contribution of the hnRNP C1/C2 and NS1 interaction to the production of infectious virus in DENV-infected cells.

Materials and Methods: A human hepatocyte cell line, Huh7, was utilized as a target cell for DENV infection. Immunoprecipitation with specific antibodies, RNA extraction and RT-PCR for a DENV NS1 region were employed to determine the association between hnRNP C1/C2 and NS1 proteins with DENV RNA. The function of this complex formation in DENV production was addressed by using hnRNP C1/C2-specific siRNA to knockdown the target protein expression inHuh7 cells prior to DENV infection and monitoring its effects on the synthesis of viral RNA and protein as well as the release of infectious virus.

Results: hnRNP C1/C2 and DENV NS1 proteins associated with viral RNA in DENV-infected cells. Knockdown of hnRNP C1/C2 resulted in a decrease in the number of DENV antigen-expressing cells as well as the relative levels of DENV RNA and protein in DENV-infected cells. A significant reduction of DENV titers was also observed in the culture supernatant following knockdown of hnRNP C1/C2.

Conclusion: These results suggested that hnRNP C1/C2 and DENV NS1, which form complexes with viral RNA, may participate in the early events of DENV production mostly likely at the steps of viral replication and release of infectious virus.

Keywords: hnRNP C1/C2, dengue virus, NS1, virus production

O-12

Title: Human ScFv to M2 Protein that Interferes Influenza Virus Infectious Cycle

Authors: Tippawan Pissawong,[1] Kanyarat Thueng-in,[2] Jeeraphong Thanongsaksrikul,[2] Thaweesak Songserm,[3] Potjanee Srimanote,[4] Santi Maneewatch,[5] Wanpen Chaicumpa[2]

Institutions: *[1]Graduate Program in Immunology, [2]Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700; [3]Department of Veterinary Pathology, Faculty of Veterinary Medicine, Kasetsart University, Kam-paeng-saen Campus, Nakhonpathom; [4]Graduate Program in Biomedical Science, Faculty of Allied Health Sciences, Thammasat University, Rangsit campus, Pathumthani; [5]Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400.*

Objectives: To produce fully human monoclonal single chain antibody fragments (human ScFv) specific to M2 protein of highly pathogenic H5N1 influenza virus by using a human ScFv phage display library which can be developed further into a sole therapeutic agent or adjunctive remedy for influenza immunotherapy.

Materials and Methods: Full length recombinant M2 (rM2) of highly pathogenic avian influenza virus, A/H5N1 was produced. Phage clones displaying HuScFv from a human antibody phage display library using rM2 as an antigen in phage biopanning were selected. The specific binding of HuScFv to rM2 was determined using ELISA and Western blotting. DNA banding patterns of each *huscfv* was determined by using restriction fragment length polymorphism (RFLP) experiment to reveal the diversity of *huscfv*. The microneutralization test was used to determine the ability of HuScFv for inhibiting influenza replicative cycle ex vivo in influenza infected MDCK cells.

Results: Soluble specific HuScFvto rM2 was produced and purified from transformed *E. coli* clones. The RFLP experiments also revealed multiple DNA banding patterns which indicated epitope/affinity diversity of the HuScFv. The selected HuScFv showed inhibition in influenza replicative cycle ex vivo by using described method.

Conclusion: Soluble HuScFv to rM2 of A/H5N1 influenza virus that inhibit influenza replicative cycle were successfully produced from *E. coli* transformants carrying *huscfv*-phagemids. The HuScFv warrants testing further for their *in vivo* protective efficacy in mammalian models of influenza such as mice and ferrets before being proceeded to human clinical trials.

Keywords: Influenza A, A/H5N1, Matrix 2 (M2) protein, ScFv, and phage displayed-HuScFv library

A POTENTIAL CONTRIBUTION OF hnRNP C1/C2 AND DENGUE VIRUS NS1 ASSOCIATION TO DENGUE VIRUS INFECTION

Thanyaporn Dechtawewat[1,2], Pucharee Songprakhon[2,3], Thawornchai Limjindaporn[4], Chunya Puttikhunt[5,6], Watchara Kasinrerk[7,8], Pa-thai Yenchitsomanus[2], Sansanee Noisakran[5,6]

[1]Graduate Program in Immunology, Department of lmmunology, [2]Division of Molecular Medicine, [3]Division of Instruments for Research, Office of Research and Development, [4]Department of Anatomy, [5]Division of Dengue Hemorrhagic Fever Research Unit, Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [6]Medical Biotechnology Research Unit and [7]Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand; [8]Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

BACKGROUND: The nonstructural protein 1 (NS1) is one of the dengue viral products conceivably involved in dengue virus (DENV) infection and pathogenesis of dengue. Cell-associated NS1 has previously been shown to interact with human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 protein in DENV-infected cells with unknown function.

OBJECTIVE: This study aimed at investigating the functional relevance of DENV NS1 and hnRNP C1/C2 association in the life cycle of DENV infection.

METHODS: A human hepatocyte cell line, Huh7, was utilized for DENV infection and hnRNP C1/C2-specific siRNA transfection. Effects of hnRNP C1/C2 knockdown on viral RNA and protein synthesis, release of infectious virus as well as hnRNP C1/C2-DENV NS1 complex formation were assessed in DENV-infected knockdown cells by real-time RT-PCR and immunofluorescence staining, virus titration, and immunoprecipitation, respectively.

RESULTS: DENV NS1 was found to associate with hnRNP C1/C2 and viral RNA in DENV-infected cells. Co-localization of DENV NS1 and hnRNP C1/C2 could be observed in a sub-population of cells, particularly those with undergoing mitosis. Specific siRNA transfection significant reduced hnRNP C1/C2 mRNA and protein expression and disrupted the complex formation between hnRNP C1/C2 and DENV NS1 in the virus-infected cells. In addition, knockdown of hnRNP C1/C2 resulted in a decrease in the percentage of DENV infection, the relative levels of viral RNA and viral protein expression, and the production of infectious virus.

CONCLUSIONS: These findings suggested that the DENV NS1 and hnRNP C1/C2 interaction may participate in the process of DENV infection potentially at the steps of virus replication and viral protein expression.

KEYWORDS: dengue virus, hnRNP C1/C2, NS1, virus infection

Appendix II

(Proceedings)

Proceedings 7

ASSOCIATION OF DENGUE VIRUS NS1 AND hnRNP C1/C2 PROTEINS IN DIFFERENT HUMAN TARGET CELLS FOR DENGUE VIRUS INFECTION

Thanyaporn Dechtawewat[1,2], Suchada Sengsai[2,a], Pucharee Songprakhon[2,3], Thawornchai Limjindaporn[4], Chunya Puttikhunt[5], Watchara Kasinrerk[6,7], Pa-thai Yenchitsomanus[2], Sansanee Noisakran[5]

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ABSTRACT

Dengue virus is a mosquito-borne human pathogen causing dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). At present, the mechanism of DHF/DSS is not clearly understood. One of the key dengue viral proteins possibly involved in dengue pathogenesis and virus production is the nonstructural glycoprotein 1 (NS1). Using a proteomic approach, we previously demonstrated that dengue virus NS1 interacts with human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 in dengue virus-infected human embryonic kidney (HEK) 293T cells. Further study was performed to extend our previous finding in three human cell types which likely serve as target cells for dengue virus infection. These cells were hepatocyte (HepG2), foreskin fibroblast (HF) and endothelial (EA.hy926) cell lines as well as primary umbilical vein endothelial cells (HUVEC). The selected cells were infected with dengue virus and assessed for association of hnRNP C1/C2 and dengue virus NS1. Results from co-immunoprecipitation and co-localization assays using specific antibodies showed that the interaction of human hnRNP C1/C2 and dengue virus NS1 could be observed in all the cell types tested particularly at the perinuclear region. In addition, dengue viral RNA could be detectable in the hnRNP C1/C2 and viral NS1 complexes in dengue virus-infected HepG2 cells. Taken together, our findings suggested that association of hnRNP C1/C2 and dengue virus NS1 may be a cell type-independent phenomenon that possibly takes part in the process of virus replication in dengue virus-infected cells.

INTRODUCTION

Dengue virus is one of the most important mosquito-borne human pathogens that causes a serious public health threat throughout tropical and subtropical countries. It is an enveloped, positive single-stranded RNA virus belonging to the genus *Flavivirus* of the

family *Flaviviridae* and consists of four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Infection with any of the four serotypes leads to a spectrum of diseases ranging from mild febrile illness of dengue fever (DF) to severe and potentially fatal diseases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). However, the mechanisms involved in the pathogenesis of DHF/DSS remain unclear [1]. Dengue virus enters human body through the skin via the bite of an infected mosquito, then proliferates locally and spreads to become generalized within a short period of time usually with significant viremia. The route of progression through the vertebrate host has not been clearly established, but it appears that the virus progresses initally from the site of the bite to draining lymph nodes where it replicates and is amplified [2]. Several human cell types have been found to be susceptible to dengue virus infection and potentially serve as targets cell for dengue virus. These include monocytes, dendritic cells, fibroblasts, hepatocytes and endothelial cells [3].

Following dengue virus infection, dengue virus RNA genome is translated into a single polyprotein which is then cleaved into three structural proteins (C, prM/M, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by virus- and host-encoded proteases [4]. These proteins exert different functions such as virus replication, assembly and maturation as well as inhibition of host immune response against dengue virus infection [5]. Dengue virus NS1 exists in different forms (monomer, dimer and hexamer) inside the cells, on the cell surface and in the extracellular milieu. It is detectable in blood circulation of dengue patients at levels correlated with viremia and disease severity. The level of NS1 was higher in DHF patients than DF patients and also present in a large amount in pleural fluids from DSS patients [6]. To date, the functions of dengue virus NS1 are not clearly understood. Several lines of evidence demonstrated its involvement in immune-mediated pathogenesis of dengue disease and in virus replication. Previous studies demonstrated that dengue virus NS1 associates with intracellular membrane structures, which are presumed sites of virus replication, and possibly with NS2A, NS3, NS4A, and NS5 to form viral replication complexes in virus-infected cells [7, 8]. In addition, dengue virus NS1 has been found to interact with three host cellular proteins, STAT3β, clusterin and hnRNP C1/C2 [9-11]. Nevertheless, the interplay between dengue virus NS1, host proteins and cellular responses remain elusive. Our previous study revealed the interaction of dengue virus NS1 and hnRNP C1/C2 in human embryonic kidney HEK 293T cells infected with dengue virus. However, whether this interaction could be observed in other human cell types which may be more susceptible to dengue virus infection and pathogenesis has not been addressed. This study, therefore, utilized three human cell types (i.e., hepatocytes, fibroblasts and endothelial cells) potentially being the target cells for dengue virus infection to study the interactions between the hnRNP C1/C2 and viral NS1 as well as its possible association with dengue virus replication.

MATERIALS AND METHODS

Cell line, virus, and antibodies

HepG2, a human hepatoma epithelial cell line, and HF, a human fibroblast cell line, were cultured in Minimum Essential Medium (MEM, GIBCO, Grand Island, NY, USA) containing 10% FBS, 2 mM L-glutamine, 36 μg/ml penicillin and 60 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. EA.hy926, a human endothelial cell line, and HUVEC, primary human umbilical cord vein endothelial cells were cultured in Dulbecco's Modified Eagle

Medium Nutrient Mixture F-12 (DMEM-F12, GIBCO) and Medium 199 (M-199, GIBCO) containing 10% FBS, 36 μg/ml penicillin, and 60 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Dengue virus (DENV) serotype 2 virus strain 16681, isolated from a DHF patient in Thailand in 1964, was originally provided by Drs. Bruce Innis and Ananda Nisalak, Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Mouse monoclonal antibodies recognizing linear epitopes (1B2, NS1-1F, NS1-3F, and NS1-4F) or conformational epitopes (NS1-8.2 and 1A4) on dengue virus NS1 were obtained as described previously [12]. Mouse anti-human hnRNP C1/C2 monoclonal antibody (clone 4F4) was purchased from Santa Cruz Biotechnology, Inc., CA, USA.

Dengue virus infection and immunoprecipitation

Human cell lines were infected with dengue virus at a multiplicity of infection (MOI) of 1 (HepG2), 5 (HF and EA.hy926) or 10 (HUVEC). Dengue virus-infected cells were harvested at 24 h post infection (HUVEC) or at 48 h post infection (HepG2, HF and EA.hy926). Uninfected cells collected at the same time points were used to serve as mock control. Clear lysates were prepared from mock and dengue virus-infected cells and then subjected to immunoprecipitation using a mouse isotype-matched control IgG2a antibody (UPC 10, Sigma, St. Loius, MO, USA) and a mouse anti-NS1 monoclonal antibody (1A4, IgG2a) in the presence of protein G sepharose beads (GE Healthcare, Uppsala, Sweden) as previously described [11, 13].

Immunoblot analysis

Immunoprecipitated proteins were separated by electrophoresis in 10% SDS–polyacrylamide gel under a non-reducing and unheating condition and subsequently transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA) using a SemiPhor semi-dry transphor unit (Amersham Bioscience, Piscataway, NJ, USA). The membrane was processed as described previously [13, 14], and then incubated with a mixture of mouse anti-NS1 monoclonal antibodies described above or a mouse anti-human hnRNP C1/C2 monoclonal antibody (4F4) followed by HRP-conjugated rabbit anti-mouse immunoglobulin antibody (DAKO, Glostrup, Denmark). The immunoreactive proteins were visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Co-localization of dengue NS1 and hnRNP C1/C2 protein

Mock and dengue virus-infected cells grown on glass coverslips were harvested at the indicated time point as described above. The cells were fixed with 3.7% formaldehyde in PBS for 7 min and permeabilized with absolute methanol for 10 min at room temperature (RT). Thereafter, the cells were incubated with a mouse anti-human hnRNP C1/C2 monoclonal antibody (4F4) at the dilution of 1:500 for 1 h at RT followed by Cy3-conjugated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at the dilution of 1:4000 for 30 min at RT. Thereafter, the stained cells were blocked with 10% normal mouse serum in PBS and then incubated with 20 μg/ml of FITC-conjugated mouse anti-NS1 monoclonal antibody (1A4). The stained cells were visualized under a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

Detection of dengue viral RNA in immunoprecipitated complexes

RNA was extracted from immunoprecipitated complexes captured on protein G sepharose beads by TRIzol reagent (Invitrogen Corporation, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed by using reverse transcriptase

superscript III (Invitrogen Corporation, CA, USA) in the 20 µl reaction according to the manufacturer's protocol by using the primer D containing a nucleotide sequence as previously described [13]. The resultant cDNA was subsequently utilized as a template for amplification of a dengue virus NS1 fragment with the primer pair C-D using Biometra TGradient Thermal Cycler (Biometra GmbH, Goettingen, Germany) as previous described [13]. PCR products (12.5 µl) were mixed with 2.5 µl of 6× loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in deionized water) and electrophoresed in 1.5 % agarose gel. The gel was subjected to ethidium bromide staining and PCR products were visualized by Gene Genius Bio Imaging system (Syngene, Cambridge, UK)

RESULTS

As hepatocytes, fibroblasts and endothelial cells have been reported to be highly susceptible to dengue virus infection and possibly related to the immunopathogenesis of DHF/DSS [15]. We chose these cells to investigate the interactions between human hnRNP C1/C2 and dengue virus NS1 in a virus infection system. Human hepatocyte (HepG2), fibroblast (HF) and endothelial (EA.hy926) cell lines, as well as primary human umbilical vein endothelial cells (HUVEC) were infected with dengue virus and harvested at 24 h or 48 h post infection as previously described in the Materials and Methods. Mock (control) and dengue virus-infected cell lysates were subjected to immunoprecipitation using an isotype-matched control antibody or an anti-NS1 monoclonal antibody. Precipitated proteins were analyzed by immunoblotting with anti-hnRNP C1/C2 and anti-NS1 monoclonal antibodies. Results showed that precipitation with anti-NS1 antibody could pull down both dengue virus NS1 and hnRNP C1/C2 from virus-infected cell lysate derived from all cell types tested as compared with mock-infected control (Figure 1 A-D; Anti-NS1). No specific protein band was detected in mock and virus-infected samples precipitated with the control antibody (Figure A-D; UPC10). Further investigation was performed to verify sub-cellular distributions of hnRNP C1/C2 and dengue virus NS1 by double immunofluorescence and confocal microscopy. Dengue virus NS1 was observed exclusively in the cytoplasm whilst hnRNP C1/C2 was present predominantly in the nucleus as well as, to certain extents, in the cytoplasm of all types of virus-infected cells tested (Figure 2) as compared with mock-infected controls (data not shown). Co-localization of the two proteins could be detected in the cytoplasm of these cell lines following dengue virus infection, particularly in the perinuclear regions (Figure 2). The results from the co-immunoprecipitation and co-localization assays were consistent and, therefore, suggested existing interaction between hnRNP C1/C2 and dengue virus NS1 in all the three cell types tested.

Dengue virus NS1 has been proposed to be part of dengue virus replication complexes which may consist of other viral nonstructural proteins and viral RNA as well as host cellular proteins [7]. It is possible that the interaction of the NS1 protein with hnRNP C1/C2, which is known as an RNA binding protein, may be involved in the process of virus replication. Therefore, we selected HepG2 cells as a model to test the presence of dengue viral RNA in hnRNP C1/C2 and viral NS1 complexes. Mock and dengue virus-infected HepG2 cells were subjected to immunoprecipitation using an isotype-matched control antibody or an anti-NS1 monoclonal antibody. Following immunoprecipitation with the anti-NS1 antibody, dengue virus NS1 and hnRNP C1/C2 could be observed in the precipitated complexes as evidenced by immunoblot analysis (data not shown). The immunoprecipitated complexes were then utilized for RT-PCR to amplify a specific viral NS1 fragment. Results demonstrated that an amplicon band of about 1,056-bp in size corresponding to the viral NS1 fragment was clearly

detectable in dengue virus-infected sample immunoprecipitated with the anti-NS1 monoclonal antibody (Figure 3, Anti-NS1) as compared with that immunoprecipitated with the control antibody (Figure 3, UPC10). No DNA band was observed in mock-infected samples immunoprecipitated with either the anti-NS1 or isotype-matched control antibody (Figure 3; Anti-NS1 and UPC10). Our findings, therefore, suggested that the hnRNP C1/C2 and NS1 complexes may associate with dengue viral RNA in virus-infected cells.

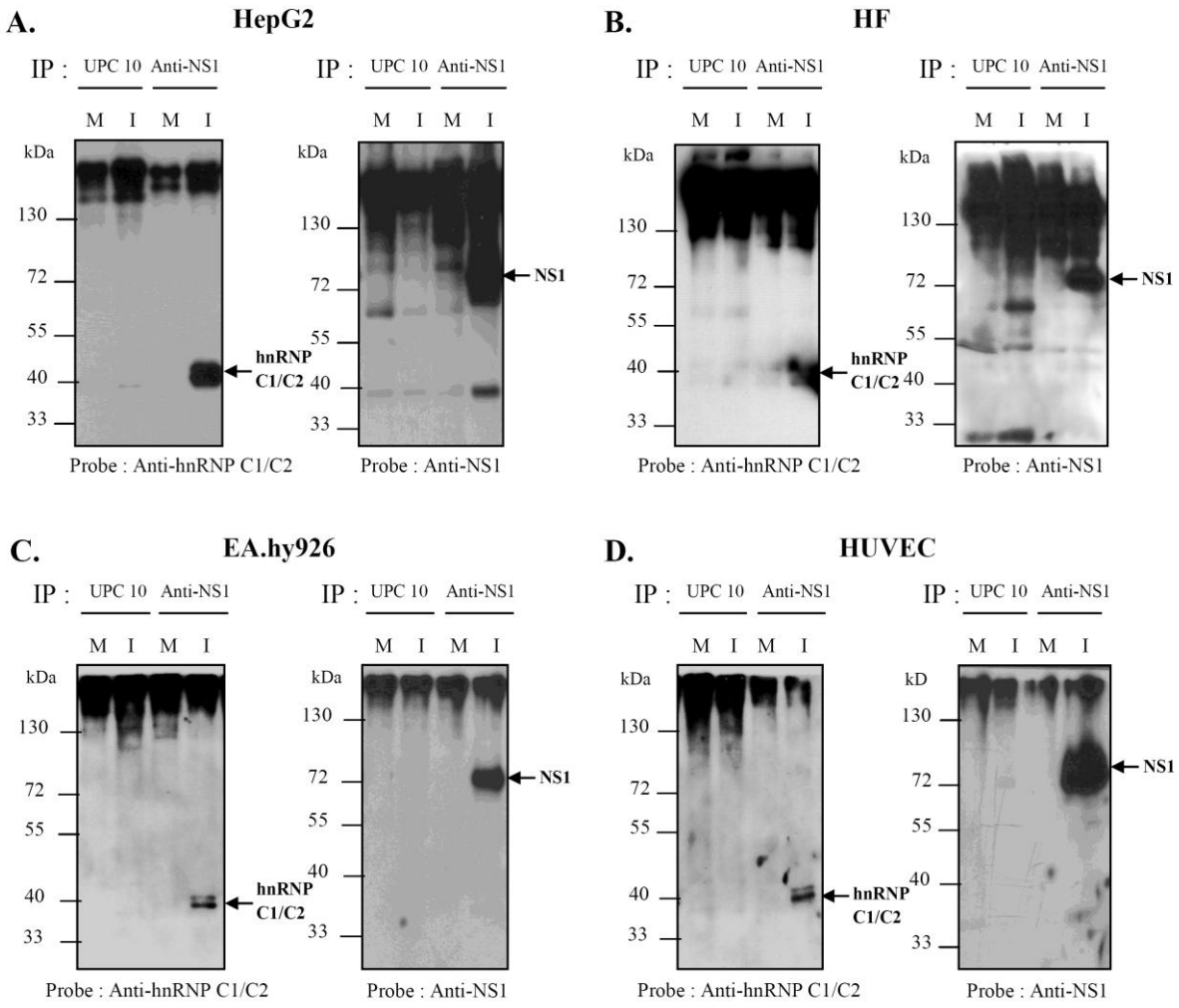

Figure 1 Co-immunoprecipitation of dengue virus NS1 and hnRNP C1/C2 in different human target cells. HepG2 (A), HF (B), EA.hy926 (C) and HUVEC (D) were utilized in this study. Mock (M) and dengue virus-infected (I) cells were processed for immunoprecipitation with an isotype-matched control IgG2a (UPC 10) antibody or an anti-NS1 (1A4) monoclonal antibody. Precipitated proteins were then subjected to immunoblot analysis by using anti-hnRNP C1/C2 and anti-NS1 monoclonal antibodies.

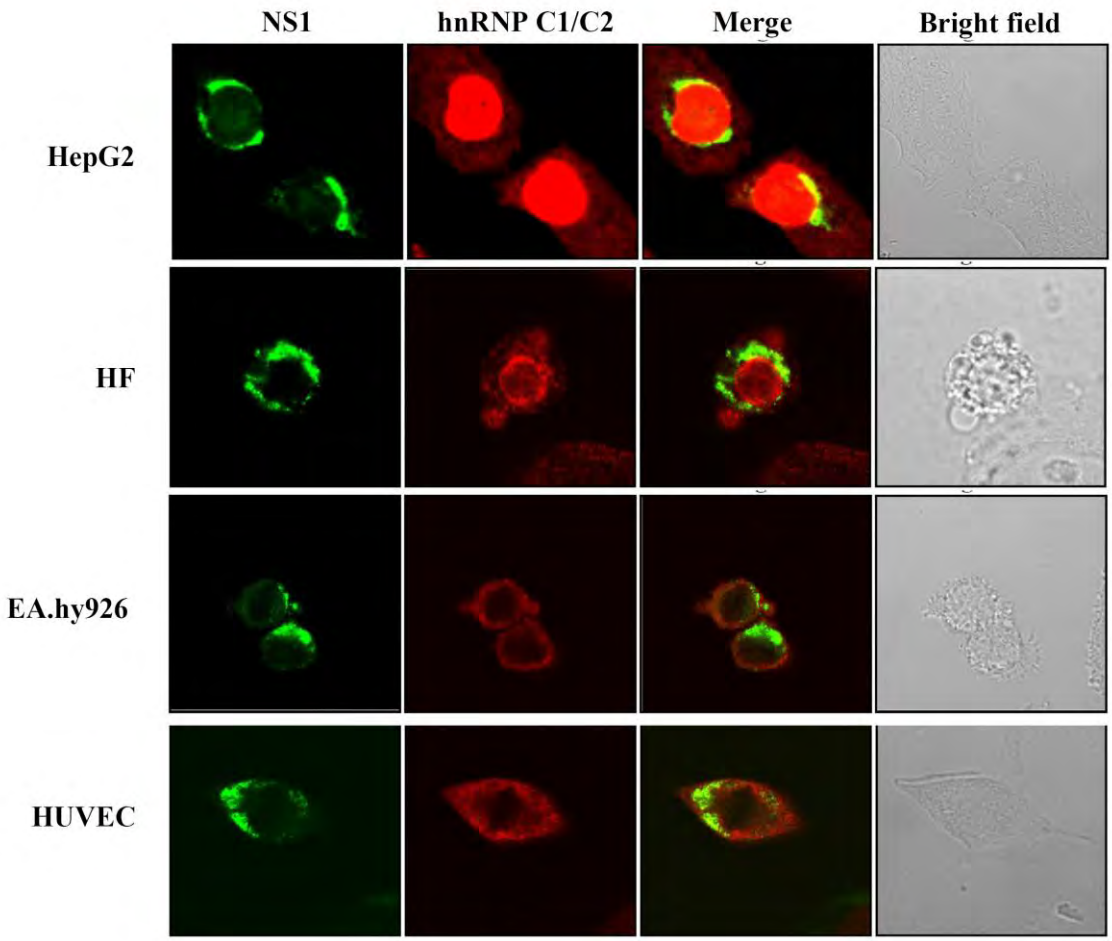

Figure 2 Localization of dengue virus NS1 and hnRNP C1/C2 in dengue virus-infected cells. HepG2, HF, EA.hy926 and HUVEC grown on glass coverslips were infected with dengue virus and harvested at 24 h or 48 h post infection as described in the Materials and Methods. The infected cells were subjected to double immunofluorescence staining for hnRNP C1/C2 (red) and dengue virus NS1 (green). The stained cells were observed by a confocal laser-scanning microscope with a 63 × objective lenses and digitally enlarged by 3×. Images captured in the same field are merged and areas where co-localization of both the proteins occurs are shown in yellow color.

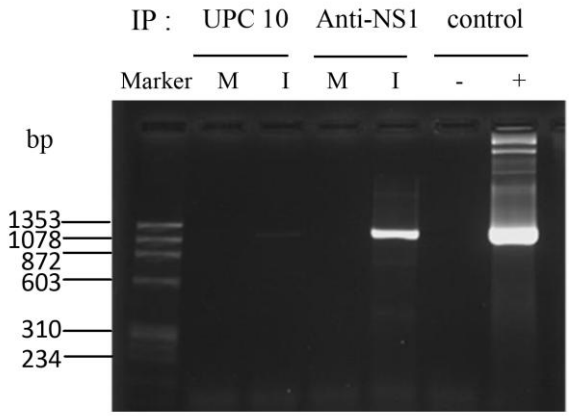

Figure 3 Association of dengue virus NS1 and hnRNP C1/C2 complexes with dengue viral RNA. Mock (M) and dengue virus-infected (I) HepG2 cells were subjected to immunoprecipitation using an isotype-matched control IgG2a antibody (UPC 10) or an anti-NS1 (1A4) monoclonal antibody. RNA was isolated from precipitated complexes and utilized as a template for RT-PCR using a primer pair specific for dengue NS1 fragment. An expected size of the NS1 fragment is approximately 1056 bp as compared with standard DNA markers, *Hae*III-digested ØX174 DNA fragments (Marker). Reactions performed in the absence of cDNA and in the presence of pcDNAhygro containing dengue virus NS1 gene were also included as negative (-) and positive (+) controls for PCR, respectively.

CONCLUSION AND DISCUSSIONS

In this study, we found that dengue virus NS1 interacts with hnRNP C1/C2 in three potential target cells (i.e., hepatocytes, fibroblasts and endothelial cells) for dengue virus infection as evidenced by co-immunoprecipitation and co-localization assays. This finding, therefore, suggested that the interaction of the two proteins is not cell-type specific as this phenomenon can occur in various types of dengue virus-infected cells. In addition, the hnRNP C1/C2 and viral NS1 complexes were found to associate with dengue viral RNA. However, it remains unclear how these complexes are formed. Dengue virus NS1 may interact with hnRNP C1/C2 directly or indirectly through binding with other host or viral proteins that are in association with dengue viral RNA. Alternatively, dengue viral RNA itself may be required as a direct linker between the dengue NS1 and hnRNP C1/C2 in the complexes. Potential roles of the hnRNP C1/C2 and viral NS1 interaction have yet to be determined whether it has any effect on other cellular processes essential for survival of the infectious virus or it is involved in the process of virus production in dengue-virus infected cells.

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Appendix III

(Manuscript)

Interaction of Dengue Virus NS1 with hnRNP C1/C2 Facilitates Dengue Virus Production

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Keywords: hnRNP C1/C2, dengue virus, NS1, virus production

Abstract

Dengue virus (DENV) infects host cells to replicate by using the host cellular machinery. Although DENV and host protein-protein interactions have been extensively studied, very little is known about the functional significance of their interactions in the virus replication. The nonstructural protein 1 (NS1) of DENV was demonstrated previously to interact with human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2; however, the role of this interaction was not known. This study aimed to investigate the potential contribution of the hnRNP C1/C2 and NS1 interaction to DENV production. Using a hepatocyte cell line (Huh7) infected with DENV, the NS1 protein was found to co-immunoprecipitate and co-localize, to a certain extent, with hnRNP C1/C2 proteins. Immunoprecipitation with specific antibodies and subsequent RT-PCR revealed the presence of DENV RNA in the immunoprecipitated complex containing hnRNP C1/C2 and NS1 proteins. Effects of this association on different stages of DENV life cycle were assessed by exploiting siRNA-mediated knockdown of hnRNP C1/C2. Reduced hnRNP C1/C2 expression tended to decrease the percentage of DENV antigen-positive cells as well as the amount of DENV RNA and the relative levels of DENV E and NS1 proteins in DENV-infected cells. In addition, a significant reduction of DENV titers was observed in the supernatant from DENV-infected culture following knockdown of hnRNP C1/C2. These results were consistent with decreased interaction between hnRNP C1/C2 and DENV NS1 in the hnRNP C1/C2 knockdown cells. Our findings, therefore, suggested that the hnRNP C1/C2 and DENV NS1 association may take part in the life cycle of DENV production most likely at the steps of viral replication and viral protein synthesis.

1. Introduction

Dengue virus (DENV) infection poses a major public health threat that affects about half of the world's population (World Health Organization Fact Sheet N^{O} 117, 2012). All four serotypes of DENV (i.e., DENV-1, -2, -3 and -4) can cause a spectrum of diseases ranging from asymptomatic, self-limiting mild febrile (dengue fever, DF) to life-threatening illness, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (52). At present, the mechanism of DENV infection that leads to pathogenesis of DHF/DSS is not clearly understood.

Dengue virus is a member of flaviviruses in the family Flaviviridae. It is a positive-sense, single-stranded, enveloped RNA virus that has previously been reported to enter different types of host cells, e.g., dendritic cells, monocytes, endothelial cells and hepatocytes through receptor-dependent mechanisms or antibody-dependent enhancement (53-55). The positive-stranded viral RNA genome encodes a long polyprotein that is subsequently processed by viral and cellular proteases to generate three structural proteins (capsid, C; pre-membrane, prM; envelope, E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (56). Of all the DENV proteins, the nonstructural protein 1 (NS1) is of particular interest owing to its potential contribution to DENV replication (37, 41, 45, 57, 58) and pathogenesis of DHF/DSS (25, 59-61).

DENV NS1 could be detected inside and on the surface of virus-infected cells and secreted in the extracellular milieu as lipoprotein (2, 6, 11, 13, 14, 62). Detectable levels of circulating NS1 in patients experiencing primary and secondary DENV infection was found to correlate with viremia and disease severity (17-19).

Several lines of evidence suggest the involvement of cell-associated NS1 in the replication, assembly and maturation of flaviviruses (37-41). A genetic approach using NS1 gene deletion in full-length infectious cDNA clone of yellow fever virus revealed the inhibition of minus-strand viral RNA synthesis which could be reversed by complementing NS1 expression in *trans* (Lindenbach and Rice 1997). Other studies on immunofluorescence and cryo-immunoelectron microscopy demonstrated co-localization of NS1 with double stranded-viral RNA in tight association with intracellular membranes that are presumed sites of virus replication (37, 42). Moreover, NS1 localized to some extent in the same cellular compartment as the autophagic vacuole marker LC3 and the endosomal and lysosomal marker LAMP1 that may be involved, as part of the viral translation and replication complex, in DENV production (37, 43, 44). Endocytosis of NS1 and its subsequent accumulation in late endosomes have also been shown to facilitate DENV replication in human hepatocytes (45). In addition to the relevance in virus amplification, the NS1 protein has been proposed to be a contributing factor for the pathogenesis of dengue disease through complement-mediated mechanisms (33-36, 60, 63).

Although a number of studies on DENV and host protein interaction have been reported previously (49, 64-66), it is not clear to date how DENV NS1 interacts with host cellular proteins and what their functional contributions to DENV infection and dengue pathogenesis are. Exploiting biochemical and proteomic approaches, we previously identified an NS1-interacting host protein, heterogeneous ribonucleoprotein (hnRNP) C1/C2, in DENV-infected human embryonic kidney epithelial (HEK) 293T cells and other human cell lines that are susceptible to DENV infection (47); however, the functional importance of this interaction has not been determined. Therefore, further investigation was carried out in the present study to explore a possible role of DENV NS1 and hnRNP C1/C2 interaction in supporting DENV infection.

2. Materials and Methods

2.1. Cell lines, virus and antibodies

Human hepatocellular carcinoma (Huh7) cells were cultured in RPMI 1640 (GIBCO, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO, NY, USA), 2 mM L-glutamine (Sigma Chemicals, MO, USA), 1% non-essential amino acid (NEAA; GIBCO), 37 μg/ml of penicillin (Sigma) and 60 μg/ml of streptomycin (Sigma) at 37ºC in a 5% CO_2 incubator with humidified atmosphere. Dengue virus (DENV) serotype 2 (strain 16681) was propagated in C6/36 mosquito cells. Mouse monoclonal antibodies recognizing a linear epitope (NS1-3F.1) or a conformational epitope (1A4) on DENV NS1 were obtained as described previously (Puttikhunt et al., 2003; Sittisiri, 1994). Mouse anti-dengue E monoclonal antibodies (clone 3H5, (67) and clone 4G2, Henchal et al., 1982) were produced in our laboratory. Mouse monoclonal antibodies specific for human hnRNP C1/C2 (clone 4F4) and human actin (clone C4) were purchased from Santa Cruz Biotechnology, Inc., CA, USA. Mouse isotype-matched control antibodies, IgG1 (clone MOPC21) and IgG2a (clone UPC) were purchased from Sigma.

2.2. Co-immunoprecipitation of DENV NS1 and hnRNP C1/C2 proteins

Huh7 cells (5×10^6) were seeded into a T-75cm^2 flask (Costar, MA, USA) and cultured for 24 hr. The cells were then incubated with DENV-2 at an MOI of 0.5 in the culture medium at 37ºC in a 5% CO_2 incubator for 2 hr. Thereafter, the supernatant containing DENV was replaced with the fresh culture medium and the culture was maintained under the same condition as described above. At 48 hr post infection, uninfected cells (mock control) and DENV-infected Huh7cells were harvested and clear lysates were prepared by resuspending cell pellets with 500 μl of RIPA buffer containing 20 mM Tris-Cl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate with protease inhibitor cocktail (Roche, Mannheim, Germany), then incubated on ice for 30 min and centrifuged at 9,100g, 4°C for 5 min. The clear lysates (125 μl) were subjected to immunoprecipitation by incubating with 5 μg of mouse isotype-matched control antibodies (IgG1, clone MOPC21 and IgG2a, clone UPC 10), mouse anti-NS1 monoclonal antibody (clone 1A4, IgG2a) or mouse anti-hnRNP C1/C2 antibody (clone 4F4, IgG1) in RIPA buffer (875 μl) at 4°C overnight. Thereafter, 15 μl of 50% slurry protein G–conjugated sepharose 4B beads (GE Healthcare, Uppsala, Sweden) in RIPA buffer were added to each sample (500 μl) and then the mixture was incubated at 4°C for another 2 hr. Immunoprecipitated samples were centrifuged at 15,300 g, 4°C for 5 min and washed three times with RIPA buffer. The immunoprecipitated complexes were eluted from the beads by incubation with 20 μl of non-reducing buffer on ice for 30 min and subjected to immunoblot analysis to determine the presence of hnRNP C1/C2 and DENV NS1 proteins. Alternatively, the immunoprecipitated complexes on the beads were directly processed for RNA extraction and subsequent RT-PCR for the determination of DENV RNA.

2.3. Co-localization of DENV NS1 and hnRNP C1/C2 proteins

Huh7 cells (9×10^4) were grown in a 24-well plate containing glass coverslips for 24 hr. The cells were then infected with DENV-2 at an MOI 0.5 or left uninfected (mock control). The cells were harvested at 12, 24, 36 and 48 hr post infection,fixed with 0.5 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with 0.5ml of 0.2% Triton X-100 in PBS for 10 min at room temperature (RT). After washing three times with PBS, the cells were incubated successively with 10% human AB serum for 30 min at RT and with 1 μg/ml of either a mouse anti-human hnRNP C1/C2 monoclonal antibody (4F4) or its isotype

match control IgG1 antibody (MOPC) for 1 hr at RT. Thereafter, the cells were incubated with Cy3-conjugated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a dilution of 1:2000 for 30 min at RT followed by three-time washes with PBS. The stained cells were incubated with 10% normal mouse serum in PBS for 30 min and with a mixture of 3.75 μg/ml of mouse anti-NS1 monoclonal antibody (clone 1A4) conjugated with Alexa Fluor 488 and 10 μg/ml of Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 30 min. After complete staining, the cells were fixed with 1% paraformaldehyde in PBS for 30 min RT and washed three times with PBS. The glass coverslips containing stained cells were mounted onto the glass slides with anti-fade agent (DAKO) and visualized under a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

2.4. siRNA-mediated knockdown of hnRNP C1/C2

Huh7 cells were seeded onto a 24-well plate in the culture medium without antibiotics (maintenance medium) at a concentration of 9×10^4 cells/ml/well. Fifteen hours later when the cells reached about 50% confluence, the cell culture was replaced with fresh RPMI medium and then transfected with either duplex hnRNP C1/C2-specific siRNA (siGENOME SMARTpool HNRNPC M-011869-01, Dharmachon, CO, USA) or duplex irrelevant siRNA (Stealth RNAi negative control medium GC, Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction with a fixed ratio of siRNA to Lipofectamine 2000 (40 pmol: 1 μl). After 6 hr incubation with siRNA (114 nM), the cells were supplemented with the maintenance medium and further incubated for 24 hr. Thereafter, the second round of siRNA transfection was performed using a similar approach described above. To test the effects of hnRNP C1/C2 knockdown on dengue virus infection, the cells that had been subjected to the second round of siRNA transfection for 6 hr were incubated with DENV-2 at an MOI of 0.03 or the maintenance medium (mock control) for 2 hr. Thereafter, the cells were washed twice and cultured in the maintenance medium. At 0, 12, 24, 36 and 48 hr post infection, mock and DENV-infected cells and their culture supernatants were collected. The total number of viable cells was determined by trypan blue (GIBCO) exclusion dye assay. The percentage of cell death was assessed by staining total cells with propidium iodide (PI) at a final concentration of 2 ng/μl for 15 min at $4^{\circ}C$ and subsequent flow cytometric analysis. Aliquots of the cells were processed for immunoblot analysis, real-time RT-PCR and immunofluorescence staining and the culture supernatants were subjected to a focus forming unit assay. To investigate the effect of hnRNP C1/C2 knockdown on the formation of hnRNP C1/C2 and DENV NS1 complexes, cells that had been transfected with irrelevant siRNA (control) or hnRNP C1/C2-specific siRNA were infected with DENV-2 at an MOI of 0.03 and harvested at 36 hr for preparation of cell lysate. An equal amount of total proteins in the cell lysates was subjected to immunoprecipitation and immunoprecipitated complexes were analyzed by immunoblot analysis using specific antibodies.

2.5. Immunoblot analysis

Clear lysates or immunoprecipitated samples prepared from mock and DENV-infected cells that had been untransfected or transfected with siRNA were mixed with 4 × loading buffer [50 mM Tris/HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue and 10% glycerol] with or without 5% β-mercaptoethanol and heat treatment at $95^{\circ}C$ for 5 min. Proteins in the samples were separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Corporation, MA, USA) using a SemiPhor semi-dry Transphor unit

(Amersham Bioscience, NJ, USA). Non-specific binding sites on the membrane were blocked with 5% skimmed milk in PBS or in TBST for 1 hr and the membrane was incubated with mouse monoclonal antibodies specific for DENV NS1 (clone NS1-3F.1), DENV E (clone 4G2), human hnRNP C1/C2 (clone 4F4) and human β-actin (clone C4) at 4 $^{\circ}$C overnight. Following three washes with PBS or TBST, rabbit anti-mouse immunoglobulin antibody conjugated with horseradish peroxidase (HRP) (DAKO, Glostrup, Denmark) at a dilution of 1:1000 was applied for 1 hr at RT. The membrane was washed three times and immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Western Lightning Chemiluminescence Reagent Plus; Perkin Elmer Applied Biosystems, CA, USA). Relative levels of human hnRNP C1/C2, DENV NS1 and DENV E protein expression were assessed by normalization of their protein band intensities to human β-actin intensity using GeneTools software from Syngene (Cambridge, UK).

2.6. Determination of DENV RNA in immunoprecipitated complexes

RNA was extracted from immunoprecipitated complexes captured on protein G sepharose beads by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription was performed by using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol with the NS1-F primer (Table 1). The resultant cDNA was utilized as a template for amplification of DENV NS1 region with the NS1-F and NS1-R primers (Table 1) using Biometra TGradient Thermal Cycler (Biometra GmbH, Goettingen, Germany). Briefly, a 25-μl reaction contained 1 μl of cDNA template, 5 μl of 5× Green GoTaq Flexi Reaction Buffer (Promega), 2.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 1 μl each of 10 μM primers NS1-F and NS1R, 13.9 μl of DEPC-treated deionized water, and 0.1 μl of 5 U/μl of Taq DNA polymerase (Promega). The PCR reaction was preheated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 min 30 sec, with the final extension at 72°C for 10 min. The PCR products (12.5 μl) were mixed with 2.5 μl of 6× loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in deionized water) and electrophoresed in 1.5 % SeaKem LE Agarose gel (Cambrex Bio Science Rockland, ME, USA). The gel was stained with ethidium bromide and the PCR products were visualized by Gene Genius Bio Imaging system (Syngene, Cambridge, UK).

2.7. Real-time RT-PCR for measurement of hnRNP C1/C2 mRNA and DENV RNA

RNA was extracted from DENV-infected cells that had been transfected with irrelevant siRNA or hnRNP C1/C2-specific siRNA by TRIzol reagent (Invitrogen) and 62.5 ng of total RNA was subjected to reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) or AMV Reverse Transcriptase (Promega, WI, USA) according to the manufactures' instructions with minor modifications. Oligo(dT) 20 primer and DEUR primer (Table 1) were used to synthesize cDNA templates for determination of human hnRNP C1/C2 and β-actin mRNA as well as dengue viral RNA, respectively. The resultant cDNA (1 μl) was utilized as a template for real-time PCR according to the manufacturer's instruction of Light Cycler 480 SYBR Green I master mix (Roche, Mannheim, Germany) using primer pairs specific for human hnRNP C1/C2 and β-actin as well as dengue viral E (D2L and D2R) as listed in Table 1. The real-time PCR was performed by LightCycler 480 II (Roche) with (i) pre-incubation at 95°C for 10 min, (ii) 45 amplification cycles of denaturation at 95°C for 10 sec, annealing at 62°C for 10 sec and extension at 72°C for 20 sec, (iii) melting curve and cooling steps as recommended by the manufacturer. Relative levels of human hnRNP C1/C2 mRNA expression were determined by normalization to

the expression levels of human actin according to the $2^{-\Delta\Delta Ct}$ method (68). Standard DENV RNA with known concentration (copy number/μl) that had been subjected to the same RT and real-time PCR processes was utilized as a control for determining the amount of viral RNA in DENV-infected cells.

2.8. Immunofluorescence staining for the detection of DENV E antigen

To determine the percentage of DENV infection, mock and DENV-infected cells that had been transfected with irrelevant siRNA or hnRNP C1/C2-specific siRNA were fixed with 4% paraformaldehyde in PBS, smeared on a glass slide and left to air-dry for 30 min at RT. Thereafter, the cells were permeabilized with 0.2% Triton-X-100 for 10 min at RT and incubated successively with mouse anti-DENV E monoclonal antibody (clone 3H5) for 1 hr at RT and with Alexa Fluor 488-conjugated rabbit anti-mouse IgG antibody (Invitrogen) at a dilution of 1:1,000 for 30 min at RT. The stained cells were visualized under a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) with a 40× objective lens.

2.9. Focus forming unit (FFU) assay for the determination of DENV production

Supernatants collected from DENV-infected cell cultures that had been transfected with irrelevant siRNA or hnRNP C1/C2-specific siRNA as described above were assessed for the production of infectious DENV as follows. Vero cells were seeded onto a 96-well plate (Costar) at a concentration of $2.5x10^4$ cells/100 μl/well in MEM medium containing 10% FBS, 2 mM L-glutamine, 36 μg/ml penicillin and 60 μg/ml streptomycin, and cultured at 37°C in a 5% CO_2 incubator for 24 hr to obtain approximately 90% confluence of the cell monolayer. Thereafter, the medium (80 μl) was removed from each well. DENV was serially diluted by 10-folds in MEM medium containing 3% FBS, 2 mM L-glutamine, 36 μg/ml penicillin and 60 μg/ml streptomycin, added to each well (100 μl/well) and incubated at 37°C in a 5% CO_2 incubator for 2 hr. Overlay medium (MEM containing 3% FBS, 10% tryptose phosphate broth and 1.5% gum tragacanth) was then added to each well (100 μl/well) and the culture was further incubated for 3 days at the same condition. On the third day post infection, the medium was discarded from DENV-infected cell culture and the adherent cells were washed three times with 200 μl of PBS (pH 7.4). Thereafter, the cells were fixed with 3.7% formaldehyde (BDH Laboratory Supplies, Poole, England) in PBS and permeabilized with 1% Triton X-100 (Fluka, Steinheim, Switzerland) in PBS at RT for 10 min in each step (100 μl/well). The cells were then incubated sequentially with mouse anti-DENV E monoclonal antibody (clone 4G2; 50 μl/well) at RT for 1 hr and HRP-conjugated rabbit anti-mouse Igs (DAKO) at a dilution of 1:500 in PBS containing 2% FBS and 0.05% Tween-20 in the dark at RT for 30 min. To develop an enzymatic reaction, the cells were incubated with a substrate solution (50 μl/well) containing 0.6 mg/ml diaminobenzidine (DAB), 0.03% H_2O_2 and 0.08% $NiCl_2$ in PBS at RT in the dark for 5 min. The enzymatic reaction was terminated by washing with PBS three times. Dark brown foci of the DENV-infected cells were enumerated under a light microscope. Virus titers were calculated from the duplicated samples and reported as focus forming unit (FFU)/ml.

3. Results

3.1. Interaction of DENV NS1 and hnRNP C1/C2 proteins in DENV-infected Huh7 cells

DENV NS1 has been shown previously to interact with human hnRNP C1/C2 proteins in DENV-infected HEK 293T cells (47). The present study was, therefore, conducted to further investigate a potential contribution

of this interaction to the production of infectious DENV using a hepatocyte cell line, Huh7, which has proven a frequently used *in vitro* model to study DENV infection and pathogenesis (45, 69-71). Initially, interaction between DENV NS1 and hnRNP C1/C2 was confirmed in Huh7 cells by performing co-immunoprecipitation and co-localization assays. Prior to immunoprecipitation, immunoblot analysis demonstrated that hnRNP C1/C2 was present in both mock and DENV-infected cell lysates whereas DENV NS1 was detectable only in DENV-infected cell lysate (Figure 1, Input). Immunoprecipitation with anti-hnRNP C1/C2 antibody resulted in the detection of hnRNP C1/C2 proteins in mock and DENV-infected samples while no NS1 protein band was discernible in these immunoprecipitated samples (Figure 1, αhnRNP C). However, in the reciprocal immunoprecipitation, both hnRNP C1/C2 and DENV NS1 proteins could be detected in the DENV-infected samples immunoprecipitated with anti-NS1 antibody (Figure 1, αNS1). The observed protein bands appeared to be specific for the anti-hnRNP C1/C2 and anti-NS1 antibodies as no specific protein band was noticeable in mock and DENV-infected samples immunoprecipitated with isotype-matched control antibodies (Figure 1, IgG1 and IgG2a).

Further validation of DENV NS1 and hnRNP C1/C2 interaction was performed by double immunfluorescence staining of mock and DENV-infected cells at different time points post infection. As shown in Figure 2, hnRNP C1/C2 were dispersed mainly in the nucleus and, to a lesser extent, in the cytoplasm of both mock and DENV-infected cells with similar staining patterns observed at all time points tested. In contrast, DENV NS1 was detected only in the cytoplasm of DENV-infected cells and its punctate foci distributed predominantly at the perinuclear region at varying degrees in a time-dependent manner (Figure 2). Nevertheless, both the DENV NS1 and hnRNP C1/C2 proteins could be observed within the same cellular locations around the nucleus in a sub-population of DENV-infected cells — virtually all of them exhibited very intense nuclear staining pattern of condensed chromosomes and some were in the stage of cell division (Figure 2). Consistent findings of co-localization and co-immunoprecipitation indicated the existence of the DENV NS1 and hnRNP C1/C2 protein interaction in DENV-infected Huh7 cells.

3.2. Association of dengue viral RNA with DENV NS1 and hnRNP C1/C2 complexes

hnRNP C1/C2 are cellular proteins belonging to the heterogeneous ribonucleoprotein (hnRNP) family that comprises at least 20 protein members namely hnRNP A-U with RNA-binding properties. It is thus plausible that hnRNP C1/C2 may associate not only with DENV NS1 protein, but also with viral RNA in DENV-infected cells. To address this possibility, immunoprecipitation of mock and DENV-infected cell lysate was performed as previously described using specific antibodies against hnRNP C1/C2 and DENV NS1 or their isotype-matched control antibodies. Obtained immunoprecipitated complexes were subjected to RNA extraction and subsequent RT-PCR for detection of DENV NS1 amplicon. Results showed that an amplicon band of about 1,056-bp in size corresponding to DENV NS1 region was detected clearly in DENV-infected sample immunoprecipitated with anti-NS1 antibody and, to much lesser extent, in the same set of sample immunoprecipitated with anti-hnRNP C1/C2 antibody (Figure 3, αhnRNP C and αNS1: I) while mock-infected samples that had been immunoprecipitated with either specific antibody did not yield any amplicon band (Figure 3, αhnRNP C and αNS1: M). In addition, no specific band was evident in both mock and DENV-infected samples immunoprecipitated with isotype-matched control antibodies (Figure 3, IgG1 and IgG2a). Our results indicated the specific detection of viral RNA in the context of hnRNP C1/C2 and DENV NS1 complexes in DENV-infected cells.

3.3. Knockdown of hnRNP C1/C2 proteins reduced DENV infection

Since hnRNP C1/C2 proteins were demonstrated to associate with both DENV NS1 and viral RNA in DENV-infected cells, we next sought to address whether this association may be involved in the infection process of DENV by using siRNA transfection to knockdown hnRNP C1/C2 proteins prior to DENV infection. Huh7 cells were transfected with either hnRNP C1/C2-specific siRNA or irrelevant control siRNA and subsequently infected with DENV or left uninfected (mock control). The cells were then harvested at varying time points post infection to determine the expression of hnRNP C1/C2 proteins and the percentage of DENV infection. As evidenced by immunoblot analysis, transfection with hnRNP C1/C2-specific siRNA resulted in decreased expression of hnRNP C1/C2 protein bands in mock and DENV-infected cells as compared to control siRNA transfection at all time points tested while equivalent expression of β-actin protein bands (internal control) was observed in the same set of samples following transfection with either siRNA (Figure 4A). Normalization of hnRNP C1/C2 to β-actin protein demonstrated that relative expression of hnRNP C1/C2 proteins was reduced by approximately 40-60% during the whole study period in both mock and DENV-infected cells that had been transfected with hnRNP C1/C2-specific siRNA as compared with control siRNA-transfected cells (Figure 4B). This hnRNP C1/C2 knockdown effect could still be observed even at 48 hr post infection (Figure 4B). A parallel set of siRNA transfected cells was subjected to immunofluorescence staining for DENV E antigen and assessed for the frequency of DENV E expression, an indication of DENV infection. Relatively low percentage of control and hnRNP C1/C2 siRNA-transfected cells (about 4-6%) expressed detectable DENV-E antigen at the early time point post infection (12hr); however, the number of DENV E-antigen expressing cells rapidly increased over time following DENV infection (Figure 5A and 5B). Interestingly, hnRNP C1/C2 siRNA-transfected cells tended to yield lower percentage of DENV E antigen expression than control siRNA-transfected cells, i.e., 25%, 37% and 73% vs. 39%, 56% and 86% at 24, 36 and 48 hr post infection, respectively (Figure 5A and 5B). This decrease in the proportion of DENV E-positive cells, thus, reflected a consequence of hnRNP C1/C2 knockdown on DENV infection.

3.4. Effects of hnRNP C1/C2 knockdown on DENV RNA and protein synthesis as well as infectious DENV production

Interplays between virus-host proteins have previously been shown to play important roles in several virus infections (65, 72-76). The outcome of these interactions either provides favorable environments for viruses to survive and propagate in the host cells, or boosts host responses to protect the cells from virus infection. The process of DENV infection encompasses critical steps for generation of viral machineries required for amplification of virus in the host cell, including viral RNA replication, viral protein synthesis and virus assembly and release. To investigate possible effects of hnRNP C1/C2 and DENV NS1 interaction on these different stages of DENV life cycle, control and hnRNP C1/C2 siRNA-transfected cells were infected with DENV and then harvested at varying time points post infection to determine dengue viral RNA and DENV E and NS1 proteins by employing real-time RT-PCR and immunoblot analysis, respectively. In addition, supernatants from the corresponding cultures were collected and assessed for infectious virus titers by a focus forming unit (FFU) assay.

As shown in Figure 6A, results of real-time RT-PCR demonstrated that DENV RNA could be detected in both control and hnRNP C1/C2 siRNA-transfected cells at different levels in a time-dependent manner following DENV infection. Whilst the levels of DENV RNA in the control siRNA-transfected cells rapidly increased and

peaked within 36 hr post infection, the kinetics of DENV RNA appeared to be delayed in hnRNP C1/C2 siRNA-transfected cells and its expression levels were lower than that in the control cells at 24, 36 and 48 hr post infection. Particularly, a substantial difference of approximately 6 folds in DENV RNA levels between the control and hnRNP C1/C2 siRNA-transfected cells was observed at 36 hr post infection. Moreover, real-time RT-PCR using the same set of RNA samples also revealed a significant decrease of hnRNP C1/C2 mRNA expression in hnRNP C1/C2 siRNA-transfected cells as compared with control cells throughout the entire study period, thereby confirming the efficiency of hnRNP C1/C2 knockdown during DENV infection (Figure 6B).

In line with the real-time RT-PCR, immunoblot analysis of DENV E and NS1 proteins in control and hnRNP C1/C2 siRNA-transfected cells showed that both DENV protein bands were initially visible at 24 hr post infection and their expression levels increased over time in both the siRNA-transfected samples after DENV infection (Figure 7A). Nevertheless, the intensities of DENV E and NS1 protein bands were less in hnRNP C1/C siRNA-transfected cells (SP) than control cells (IR) at all time points tested (Figure 7A). After normalization with β-actin (internal control), relative levels of DENV E and NS1 protein expression in hnRNP C1/C2 siRNA-transfected cells were found to decrease by approximately 30-50% and 20-40%, respectively, as compared with those in control cells observed within 24-48 hr post infection (Figure 7B). Altogether, our findings based on real-time RT-PCR and immunoblot analysis suggested that hnRNP C1/C2 knockdown had negative influences on viral RNA and protein synthesis during DENV infection.

Further investigation of DENV production in HuH7 cells revealed that the amount of extracellular infectious DENV was detectable at very low levels (less than 10 FFU/ml) in culture supernatants collected at 12 hr post infection from control and hnRNP C1/C2 siRNA-transfected cultures and became rising continuously at later time points in both the cultures (Figure 8). However, DENV titers in the hnRNP C1/C2 siRNA-transfected culture were significantly lower than that in the control siRNA-transfected culture by approximately 3-4 folds at 24-48 hr post infection. A reduction of infectious DENV production in the cultures following specific siRNA transfection implied that hnRNP C1/C2 knockdown may interfere with the upstream processes of virus maturation and/or release of DENV into the extracellular milieu.

3.5. Disruption of hnRNP C1/C2 association with DENV NS1 protein in DENV-infected knockdown cells

To verify whether the effects of hnRNP C1/C2 knockdown on different processes in DENV infection resulted from disturbance of the complex association between hnRNP C1/C2 and DENV NS1 proteins following specific siRNA transfection, DENV-infected cells that had been transfected with irrelevant control or hnRNP C1/C2-specific siRNA were subjected to immunoprecipitation and subsequent immunoblot analysis. Lysates of irrelevant control siRNA- and hnRNP C1/C2-specific siRNA-transfected cells prior to immunoprecipitation served as input controls for detection of DENV NS1 and hnRNP C1/C2. Results showed that the DENV NS1 and hnRNP C1/C2 proteins were present in both input cell lysates; however, their expression levels in the hnRNP C1/C2-specific siRNA-transfected cell lysate (SP) were lower than that in the irrelevant control siRNA-transfected cell lysate (IR) when compared to equivalent amounts of β-actin detected in the samples (Figure 9, Input). Immunoprecipitation with anti-NS1 antibody could pull down DENV NS1 as well as hnRNP C1/C2 from control and hnRNP C1/C2-specific siRNA-transfected samples, albeit at different degrees (Figure 9, αNS1). Specifically, comparable amounts of DENV NS1 were observed in both the control and hnRNP C1/C2-specific siRNA-transfected cell lysates immunoprecipitated with the anti-NS1 antibody. However, the same set of

immunoprecipitated samples exhibited much lower hnRNP C1/C2 content in the hnRNP C1/C2 knockdown cells (SP) than the control sample (IR) (Figure 9, αNS1). No specific band was evident in either siRNA-transfected cell lysates immunoprecipitated with isotype-matched control antibody (Figure 9, IgG2a). The finding that relative proportion of hnRNP C1/C2 to DENV NS1 obtained from the immunoprecipitated samples diminished following hnRNP C1/C2 knockdown indicated potential disruption of the complex formation between DENV NS1 and hnRNP C1/C2 in DENV-infected cells.

4. Discussion

Understanding the interactive role of DENV NS1 and host cellular proteins in DENV infection and dengue pathogenesis is of particular importance. However, only a small number of studies on the identification of DENV NS1-interacting partners have been reported so far (46-48). A study employing recombinant DENV NS1 produced in *E. coli* and secreted NS1 from DENV-infected cells showed binding of DENV NS1 to complement regulatory protein clusterin in human serum, potentially affecting the regulation of terminal complement pathway that may contribute to plasma leakage and progression of severe disease (32). Yeast two-hybrid systems with human bone marrow and liver cDNA libraries demonstrated the interaction of DENV NS1 with human signal transducer and activator of transcription 3β (STAT3β) and complement component 1 (C1q), respectively, which may influence the pathogenesis of DENV infection through significant induction of proinflammatory cytokines, TNF-α and IL-6, or evasion of complement activation (46, 48). Our previous work focused on the investigation of host cellular proteins that interact with cell-associated form of DENV NS1 by performing immunoprecipitation of DENV-infected cell lysate with NS1-specific antibodies and mass spectrometry (47). hnRNP C1/C2 proteins were identified as DENV NS1-interacting partners in DENV-infected HEK 293T cells and this interaction was also confirmed in other three human cell lines, i.e., hepatocyte HepG2, fibroblast HF and endothelial EAhy926 and in primary human umbilical vain endothelial cells (47). Whether the hnRNP C1/C2 and DENV NS1 interaction plays a significant role in DENV infection was further addressed.

The present study exploited an *in vitro* model of hepatocyte Huh7 cell line, which has been frequently used as an effective target for DENV infection and specific siRNA-mediated gene knockdown, to investigate the functional relevance of hnRNP C1/C2 and DENV NS1 association in DENV production. DENV NS1 was found to interact with hnRNP C1/C2 and viral RNA in DENV-infected cells. A significant knockdown of hnRNP C1/C2 proteins affected DENV infection by decreasing viral RNA replication and viral protein synthesis as well as subsequent production of infectious virus. These events likely resulted from the interference of the complex formation between DENV NS1 and hnRNP C1/C2 proteins by hnRNP C1/C2 knockdown.

hnRNP C1/C2 are abundant host cellular proteins among at least 20 members of the hnRNP family that are involved in mRNA biogenesis, transport and stability as well as protein translation (77, 78). In spite of sharing some common characteristics, the hnRNPs still possess a variety of domain compositions and functional properties. hnRNP C1/C2 differ by 13-amino acid residues after glycine 106 or serine 107, which are present only in the latter isoform, as a result of alternative mRNA splicing and the longer isoform (C2) comprises about 25% of the total hnRNP C in the cells (79, 80). Both the hnRNP C isoforms contain an RNA recognition motif (residues 8-87) and a basic leucine zipper (bZIP)-like RNA binding motif (residues 140-207) that bind RNA in a highly cooperative manner as heterotetramers in the ratio of C1(3)C2 (81, 82).

hnRNP C1/C2 resided mainly in the nucleus (83) and functioned as key components of 40S core particles in association with hnRNP A1, B1, A2 and B2 proteins to package nascent RNA transcripts in a non-sequence-specific manner (84). Moreover, hnRNP C1/C2 committed mRNA export to the cytoplasm via either mRNA or spliceosomal U small nuclear RNA (snRNA) pathways based on their recognition of different RNA length (85). Certain cellular conditions, e.g., apoptosis, mitosis and virus infection caused translocation of hnRNP C1/C2 from the nucleus to the cytoplasm (86-90). Active export of hnRNP C1/C2 to the cytoplasm was triggered by TNF-α or phorbol 12-myristate 13-acetate (PMA)-mediated apoptosis through activation of Rho-associated kinase (ROCK) (87). The presence of hnRNP C1/C2 in the cytoplasm was also observed during mitosis upon nuclear membrane breakdown in late G2-M phase of the cell cycle (90). Consistent with this finding, our immunofluorescence staining revealed that hnRNP C1/C2 could be observed in the cytoplasm and partially co-localized with DENV NS1 in a subpopulation of DENV-infected cells, especially those with characteristics of ongoing mitosis. A previous study using subcellular fractionation assay also demonstrated the existence of hnRNP C1/C2 predominantly in the nuclear fraction and, to a lesser extent, in the cytoplamic fraction of DENV-infected EAhy926 cells (47, 91). Cytoplasmic relocalization of hnRNP C1/C2 has previously been found to occur in different types of virus infection via distinct mechanisms. Infection with rhinovirus and poliovirus resulted in redistribution of hnRNP C1/C2 to the cytoplasm of virus-infected cells due to inhibition of nuclear import and alteration of nuclear pore complex composition (86, 88). Vesicular stomatitis virus infection induced translocation of hnRNP C1/C2 by enhanced nuclear export of the proteins (89). Nevertheless, our previous work (47) and present study (Figure 2) did not observe any difference in the pattern of subcellular localization of hnRNP C1/C2 between mock and DENV-infected cells.

In our study, co-localization of hnRNP C1/C2 and DENV NS1 was observed in the perinuclear regions of DENV-infected cells and this protein complex was also found to associate with DENV RNA (Figures 1-3). Thus, it may be possible that the interaction of hnRNP C1/C2 with DENV NS1 and RNA is confined to specialized areas required for DENV replication. In support of this notion, a previous study showed that DENV NS1 co-localized with dsRNA, a replicative form of DENV genome, in vesicle packets that are induced membrane structures originated from the endoplasmic reticulum (ER) during DENV infection (Mackenzie 1996). These membrane structures may serve as platforms, possibly with association of NS2A, NS3, NS4A and NS5, for the formation of viral replication complex in flavivirus-infected cells (42, 92, 93). Although the mechanism of the complex formation between hnRNP C1/C2, DENV NS1 and viral RNA is not yet determined, it is tempting to speculate that hnRNP C1/C2 might be recruited into the vesicle packets to interact with DENV NS1 and viral RNA in DENV-infected cells through their inherent RNA binding activity. Based on these findings, we hypothesized that the association of hnRNP C1/C2 and DENV NS1 may take part in the life cycle of DENV infection.

To address this hypothesis, Huh7 cells were transfected with specific siRNA to knockdown hnRNP C1/C2 followed by DENV infection and three important processes within the DENV life cycle, including viral RNA replication, viral protein expression, and DENV production, were assessed in DENV-infected knockdown cells. Specific-siRNA transfection resulted in a significant decrease in hnRNP C1/C2 at both mRNA and protein levels (Figures 4 and 6B) as well as a disruption of the complex formation between hnRNP C1/C2 and DENV NS1 (Figure 9); however, it had no profound effects on the mortality and proliferation of DENV-infected cells during the entire studied period (data not shown). It should be noted that although the total numbers of mock and DENV-infected cells following hnRNP C1/C2 knockdown were slightly lower than that of control cells at 48 hr

post infection, they did not exhibit a statistically significant difference. These observations were in line with a previous report in HEK 293T cells showing that hnRNP C1/C2 knockdown did not enhance cell death but affected cell growth at late time points, i.e., 72 and 96 hr, post infection by impaired cell cycle progression with the cell accumulation in G2/M phase (94). As demonstrated by immunofluorescence staining of DENV E antigen, we found that hnRNP C1/C2 knockdown reduced the percentage of DENV infection (Figures 5A and 5B). This effect was unlikely due to direct hindrance at the initial step of DENV entry into the cells as a result of reduced hnRNP C1/C2 expression, but rather due to multiple downstream processes within the DENV life cycle that led to a signification decrease in DENV production (Figure 8). The fact that hnRNP C1/C2 were not detectable on the cell surface (data not shown) helped to exclude their involvement in DENV binding on the plasma membrane.

Using real-time RT-PCR, delayed kinetics of viral RNA synthesis was observed in DENV-infected cells following hnRNP C1/C2 knockdown particularly with a significant reduction of viral RNA accumulation at 36 hr post infection (Figure 6A). This suggested a potential contribution of hnRNP C1/C2, in association with DENV NS1 and viral RNA, to facilitate the replication process of DENV. Similar findings on the role of hnRNP C1/C2 and viral protein interaction in virus replication have previously been reported in other positive strand RNA viruses such as poliovirus and hepatitis C virus (HCV) (95-98). hnRNP C1/C2 interacted with 5'- and 3'-ends of poliovirus negative strand RNA intermediate and with poliovirus protein precursors essential for virus replication, i.e., 3CD (a precursor of viral RNA-dependent RNA polymerase), P2 and P3 (precursors of nonstructural proteins), hence promoting viral replication complex assembly and viral RNA synthesis of poliovirus (96-98). Moreover, hnRNP C1/C2, accompanied by polypyrimidine-tract binding protein (PTB or hnRNP I), was found to bind pyrimidine-rich region within 3'-untranslated region (UTR) of HCV RNA and, therefore, played an important role in initiation and/or regulation of HCV replication (95). Even though a detailed mechanism by which the hnRNP C1/C2 and DENV NS1 association participates in viral replication has not been demonstrated in our study, it might be possible that this interaction functions, in concert with other viral and host proteins, to assist in the structural formation of viral replication complex required for DENV replication. Supporting evidence showed the association of NS1 with NS4A that was not only a component of viral replication complex involved in the induction of membrane alterations to form ER-derived vesicles, where are proposed sites of virus replication (41, 92, 93, 99), but also interacted with PTB and DENV genome to influence viral RNA replication at the step of negative strand RNA synthesis of DENV (100, 101). Therefore, the association of hnRNP C1/C2 and DENV NS1 may act, together with the PTB and DENV NS4A complex, to interact with DENV genome in a higher order of viral replication complex formation to promote efficient DENV replication. Further investigation would be needed to address this possibility.

In addition to decreased viral RNA replication, the present study revealed that hnRNP C1/C2 knockdown diminished the expression of DENV E and NS1 proteins in DENV-infected cells (Figure 7). This reduced viral protein expression may result from a reduction of viral RNA templates generated during DENV replication and/or a direct effect of hnRNP C1/2 knockdown on DENV protein synthesis. It was possible that the interaction between hnRNP C1/C2 and DENV NS1 may help support viral RNA stability and viral protein translation, leading to efficient DENV protein expression. Previous studies demonstrated that binding of hnRNP C1/C2 with 3'-UTR of amyloid precursor protein mRNA and urokinase receptor mRNA was found to increase the stability of mRNAs (102, 103). Furthermore, hnRNP C1/C2 have been known to function as translational modulators to regulate the expression of several host cellular and viral proteins most likely through their specific binding to polyuridine-rich

regions of RNA, some of which may contain internal ribosome entry site (IRES) activity (77). Interaction of hnRNP C1/C2 with IRES of c-myc mRNA, c-cis mRNA, upstream of N-Ras (UNR) mRNA and X-linked inhibitor of apoptosis (XIAP) mRNA stimulated IRES-mediated translation, thus resulting in increased protein expression (90, 94, 104, 105). This event took place in the cytoplasm of cells during mitosis at which RNAs containing an active IRES sequence were preferentially translated whereas those with cap-dependent translation were suppressed (106). Unlike poliovirus and HCV, 5'-UTR of DENV genome did not possess IRES activity and translation of DENV occurred by non-IRES-medicated mechanisms through both canonical cap-dependent and noncanonical cap-independent processes (107). Since the interaction between hnRNP C1/C2 and DENV NS1 could be observed in the perinuclear regions of mitotic cells (Figure 2), it was possible that the potential involvement of hnRNP C1/C2, along with binding of DENV NS1 and viral RNA, in protein translation of DENV may be mediated by noncanonical cap-independent process. Although the IRES region mostly located upstream of the open reading frame (ORF) of viral and cellular origins, certain sequences within the ORF may serve as IRES elements. As evidenced in human immunodeficiency virus (HIV), an IRES was identified in the gag ORF and potentially functioned in usual backward translation initiation from the gag initiation codon (108). Whether the downstream sequence of the 5'-UTR within the DENV genome contains IRES activity that may be a potential site for interaction with the hnRNP C1/C2 and DENV NS1 complex remains to be explored.

Following viral replication and viral protein translation, assembly of DENV particles occurs through encapsidation of viral genome and budding of immature virion into the ER lumen and this process is followed by virion transport to the Golgi complex where the virus becomes mature and release of virus progeny into the extracellular milieu (93). The results obtained from our study did not provide evidence on the involvement of the hnRNP C1/C2 and DENV NS1 interaction in the process of DENV assembly. Nonetheless, determination of DENV titers in the culture supernatant demonstrated a significant reduction of infectious virus production from DENV-infected cells upon hnRNP C1/C2 knockdown (Figure 9). This inhibitory effect on DENV production was, therefore, likely attributable to decreased levels of viral RNA and viral protein synthesis as a consequence of the disturbance of hnRNP C1/C2 and DENV NS1 interaction in DENV-infected knockdown cells.

In conclusion, the present study demonstrated a potential important role of the DENV NS1 and hnRNP C1/C2 interaction in facilitating the production of infectious DENV. This protein complex may participate, by itself or with association of other viral or host proteins, in the life cycle of DENV infection most possibly at the steps of viral RNA replication and viral protein synthesis. Our findings provided a novel aspect of DENV NS1 interaction with host cellular proteins during DENV infection. Further study would be warranted to investigate detailed molecular mechanisms of this viral and host protein association in DENV-infected cells.

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Figure legends

Figure 1. Co-immunoprecipitation of DENV NS1 and hnRNP C1/C2 in DENV-infected cells. Mock (M) and DENV-infected (I) cells were immunoprecipitated with anti-hnRNPC1/C2 (αhnRNP C), anti-DENV NS1 (αNS1) monoclonal antibody or their isotype-matched control antibody (IgG1 or IgG2a). The immunoprecipitated samples were subjected to immunoblot analysis with specific antibodies for detection of hnRNP C1/C2 and DENV NS1. Mock and DENV-infected cell lysate prior to the immunoprecipitation were included as controls for immunoblot analysis (Input).

Figure 2. Co-localization of DENV NS1 and hnRNP C1/C2 in DENV-infected cells. Huh7 cells grown on glass coverslips were left uninfected (mock control) or infected with DENV at an MOI of 0.5 and harvested at the indicated time points post infection. The cells on coverslips were subjected to double immunofluorescence staining for DENV NS1 (green) and hnRNP C1/C2 (red), together with nuclear staining (blue). The stained cells were observed under a confocal laser-scanning microscope with a 63 × objective lenses. Fluorescent images captured in the same field were merged and areas where co-localization of the DENV NS1 and hnRNP C1/C2 proteins occurs are shown in yellow. Results show two representative fields of each stained samples: mock-infected cells at 48 hr post infection and DENV-infected cells at 12, 24, 36 and 48 hr post infection.

Figure 3. Association of DENV NS1 and hnRNP C1/C2 complexes with dengue viral RNA. Mock (M) and DENV-infected (D) cells were subjected to immunoprecipitation using anti-hnRNPC1/C2 (αhnRNP C) or anti-DENV NS1 (αNS1) monoclonal antibody and their isotype-matched control antibody (IgG1 or IgG2a). RNA was extracted from the immunoprecipitated samples and utilized as a template for RT-PCR with a primer pair specific for DENV NS1 region. The expected size of the amplicon was approximately 1,056 bp. PCR that was performed in parallel in the absence of cDNA and in the presence of pcDNAhygro containing DENV NS1 gene were included as negative (-) and positive (+) controls, respectively.

Figure 4. siRNA-mediated knockdown of hnRNP C1/C2 in mock and DENV-infected cells.
Huh7 cells were transfected with either hnRNP C1/C2-specific siRNA (SP) or irrelevant negative control siRNA (IR) followed by mock or DENV infection at an MOI of 0.03. Cells were collected at the indicated time points post infection and subjected to immunoblot analysis using monoclonal antibodies specific against human hnRNP C1/C2 and β-actin (internal protein control). (A) Immunoblot analysis of hnRNP C1/C2 and β-actin in mock and DENV-infected cells at indicated time points. Results are representative of three independent experiments. (B) The intensity of hnRNP C1/C2 bands was normalized to that of β-actin bands and shown as fold change of hnRNP C1/C2 protein expression. Data represent mean and SEM of three independent experiments. Asterisks indicate statistically significant differences (*, $p<0.05$; **, $p<0.01$) in relative expression of hnRNP C1/C2 proteins between the specific siRNA-transfected and control siRNA-transfected samples by Unpaired t-test.

Figure 5. Effect of hnRNP C1/C2 knockdown on the percentage of DENV infection. Huh7 cells that had been transfected with either hnRNP C1/C2-specific siRNA or control siRNA were infected with DENV at an MOI of 0.03. The cells were collected at 12, 24, 36 and 48 hr post infection and subjected to immunofluorescence staining for DENV E antigen. The percentage of DENV infection was determined based on the enumerated numbers of DENV E antigen-positive cells. (A) Representative images of immunofluorescently stained cells at

indicated time points from three independent experiments. Dengue E antigen (green); Nucleus (blue); Magnification (63×). (B) Percentages of infection shown as mean +/- SEM from three independent experiments. Asterisks indicate statistically significant differences (**, $p<0.01$) in the percentage of dengue virus infection between the specific siRNA-transfected and control siRNA-transfected groups by Unpaired t-test.

Figure 6. Effect of hnRNP C1/C2 knockdown on dengue viral RNA. Huh7 cells that had been transfected with either hnRNP C1/C2-specific or control siRNA were infected with DENV at an MOI of 0.03. Cells were then collected at 12, 24, 36 and 48 hr post infection and subjected to total RNA extraction and subsequent reverse transcription and real-time PCR for determination of DENV RNA and hnRNP C1/C2 mRNA expression. (A) The amount of DENV RNA in virus-infected cells was reported as viral RNA copies per cell. (B) Relative hnRNP C1/C2 mRNA expression in siRNA-transfected cells following DENV infection. Fold change of the hnRNP C1/C2 mRNA expression between the specific siRNA and control siRNA-transfected samples was compared at each time point tested. Data represent mean +/- SEM from three independent experiments. Asterisks indicate statistically significant differences (* $p<0.05$; **, $p<0.01$) in DENV viral RNA or hnRNP C1/C2 levels between the specific siRNA-transfected and control siRNA-transfected groups by Unpaired t-test.

Figure 7. Effect of hnRNP C1/C2 knockdown on DENV viral protein expression. Huh7 cells that had been transfected with either hnRNP C1/C2-specific siRNA (SP) or irrelevant negative control siRNA (IR) were infected with DENV at an MOI of 0.03. The cells were then collected at 12, 24, 36 and 48 hr post infection and subjected to immunoblot analysis using monoclonal antibodies specific against DENV E, DENV NS1 and β-actin (internal control). (A) Protein bands of DENV E, DENV NS1 and β-actin in DENV-infected cells that had been transfected with the specific siRNA (SP) or control siRNA (IR) at indicated time points (B) The intensity of the DENV E and NS1 protein bands were normalized to that of β-actin bands. Results show fold change of relative DENV E and NS1 protein expression in the specific siRNA-transfected samples in comparison to the control siRNA-transfected samples. Asterisks indicate statistically significant differences (*, $p<0.05$) in DENV E and NS1 protein expression between the specific siRNA-transfected and control siRNA-transfected groups by Unpaired t-test.

Figure 8. Effect of hnRNP C1/C2 knockdown on release of infectious DENV. Huh7 cells that had been transfected with either hnRNP C1/C2-specific siRNA or control siRNA were infected with DENV at an MOI of 0.03. Supernatant from DENV-infected culture was collected at 0, 12, 24, 36 and 48 h post infection and assessed for titers of infectious DENV by focus forming unit (FFU) assay. Results are derived from three independent experiments — each with three times of virus titration in duplicate. Asterisks indicate statistically significant differences (**, $p<0.01$; ***, $p<0.005$) in DENV viral RNA or hnRNP C1/C2 levels between the specific siRNA-transfected and control siRNA-transfected groups by Unpaired t-test.

Figure 9. hnRNP C1/C2 knockdown reduced the association between DENV NS1 and hnRNP C1/C2 complexes. DENV-infected (I) cells that had been transfected with either hnRNP C1/C2-specific siRNA (SP) or irrelevant negative control siRNA (IR) were subjected to immunoprecipitation using anti-DENV NS1 (αNS1) monoclonal antibody and its isotype-matched control antibody (IgG2a). The immunoprecipitated samples were analyzed for the presence of hnRNP C1/C2 and DENV NS1 proteins by immunoblotting. Mock and DENV-infected cell lysate prior to the immunoprecipitation (Input) were included in the immunoblot analysis using specific antibodies for detection of DENV NS1, hnRNP C1/C2 and β-actin (an internal loading control).

Table 1. Oligonucleotide Primers for Reverse Transcription-PCR

Primer	Orientation	Sequence
NS1-F	Sense	5′ CCGGCCAGATCTGATAGTGGTTGCGTTGTGAGC 3′
NS1-R	Antisense	5′ GATCGATCGCGGCCGCTTAGGCTGTGACCAAGG AGTTAACCAAATTCTCTTCTTTCTC 3′
hnRNP C1/C2-F	Sense	5′ TCGAAACGTCAGCGTGTATC 3′
hnRNP C1/C2-R	Antisense	5′ TCCAGGTTTTCCAGGAGAGA 3′
DEUR	Antisense	5′ GCTGTGTCACCCAGAATGGCCAT 3′
D2L	Sense	5′ ATCCAGATGTCATCAGGAAAC 3′
D2R	Antisense	5′ CCGGCTCTACTCCTATGATG 3′
actin-F	Sense	5′ AGAAAATCTGGCACCACAAA 3′
actin-R	Antisense	5′ CTCCTTAATGCTACGCACGA 3′

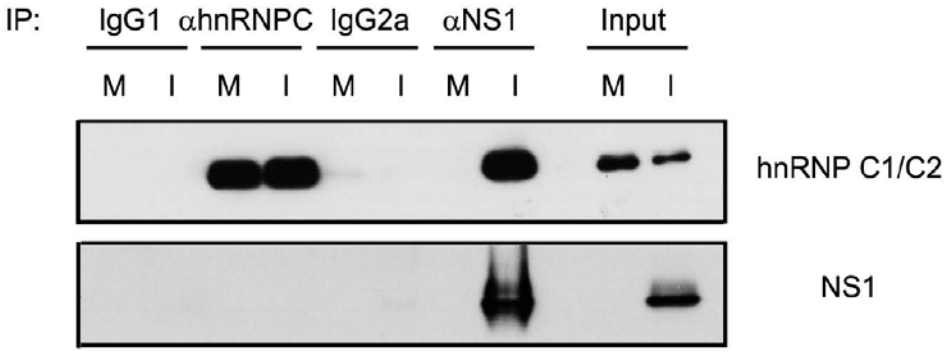


Figure 1

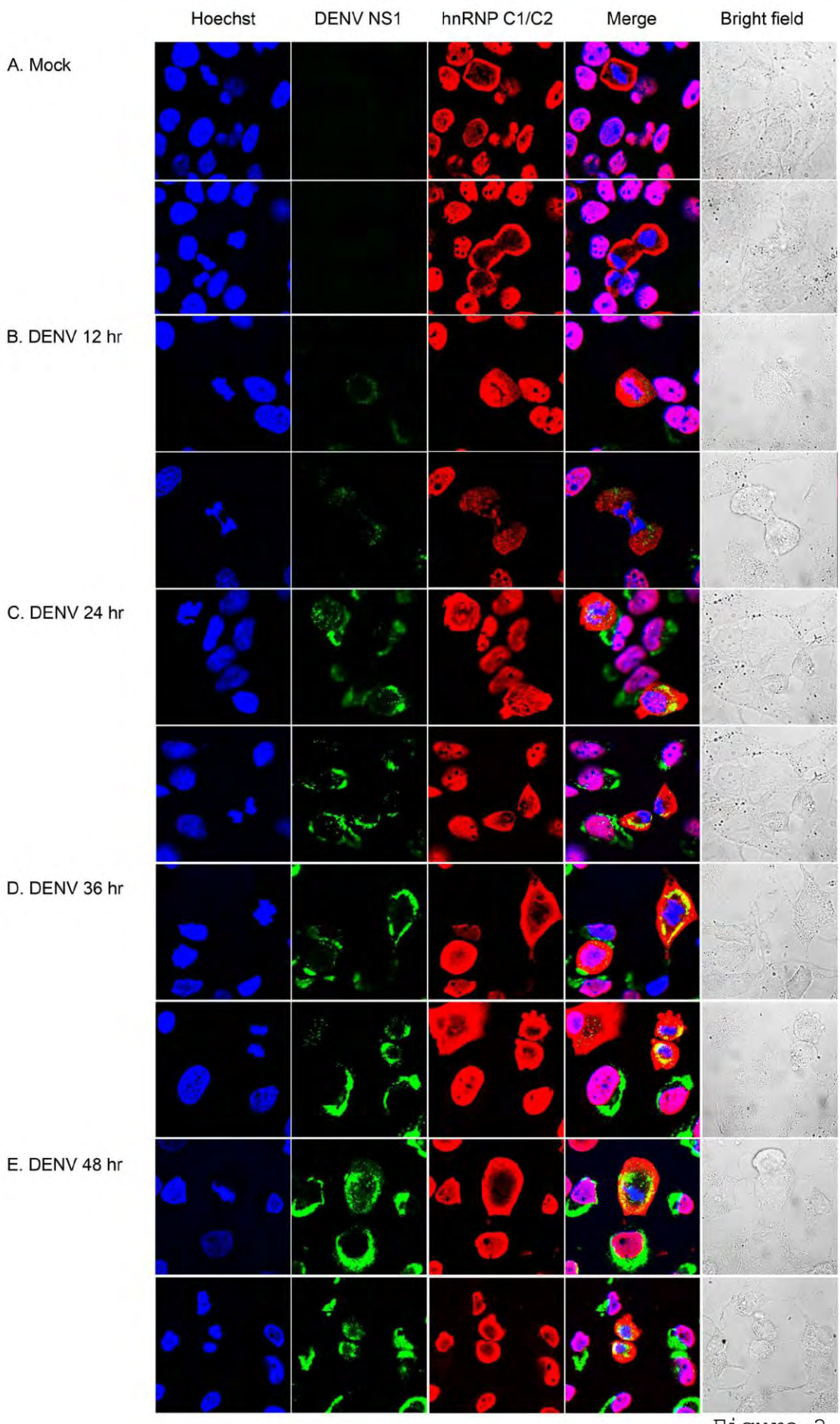

Hoechst
DENV NS1
hnRNP C1/C2
Merge
Bright field
A. Mock
B. DENV 12 hr
C. DENV 24 hr
D. DENV 36 hr
E. DENV 48 hr

Figure 2

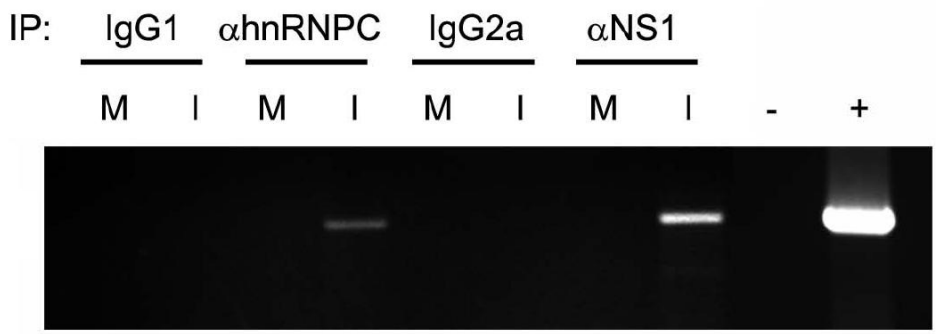


Figure 3

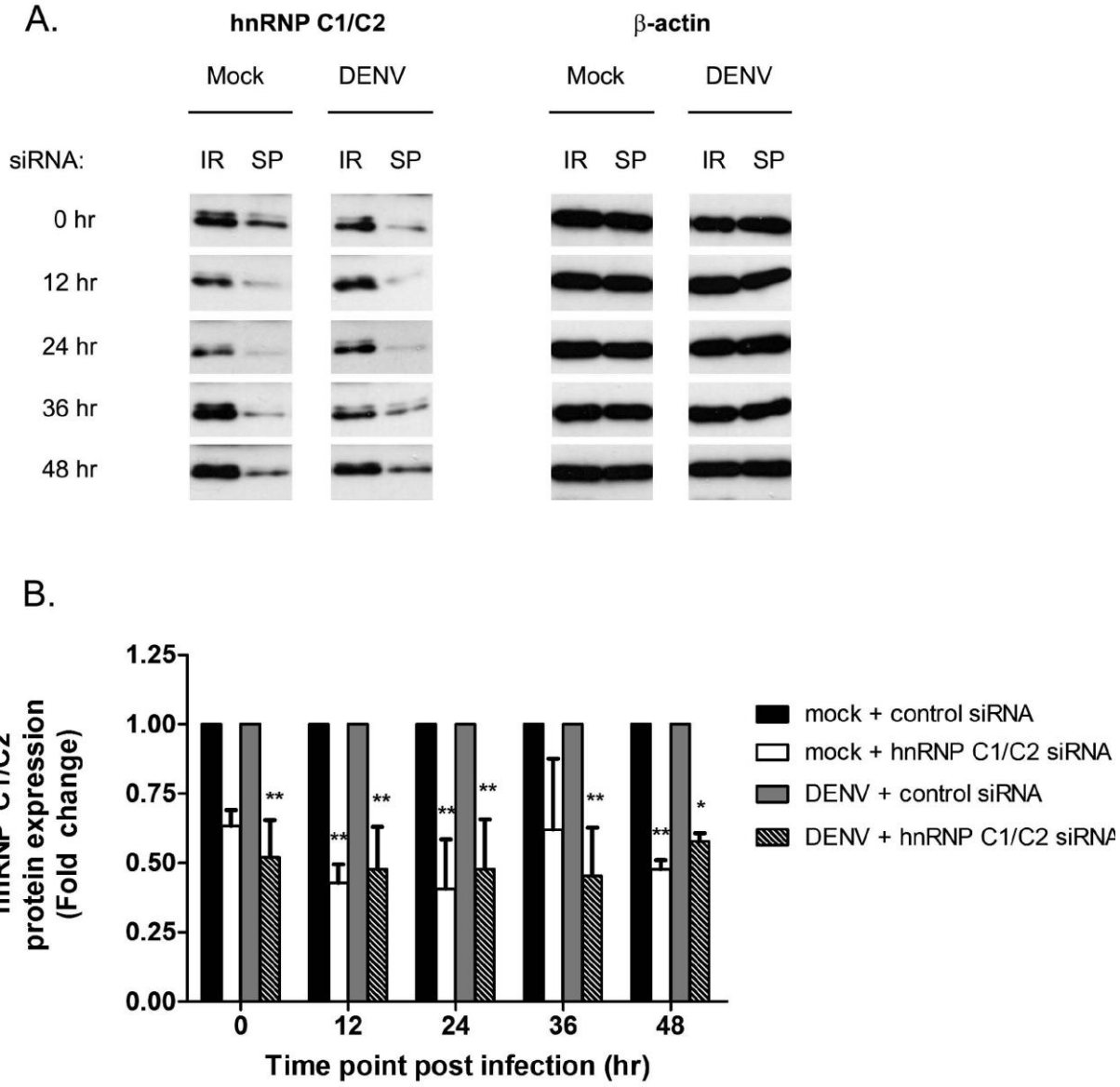
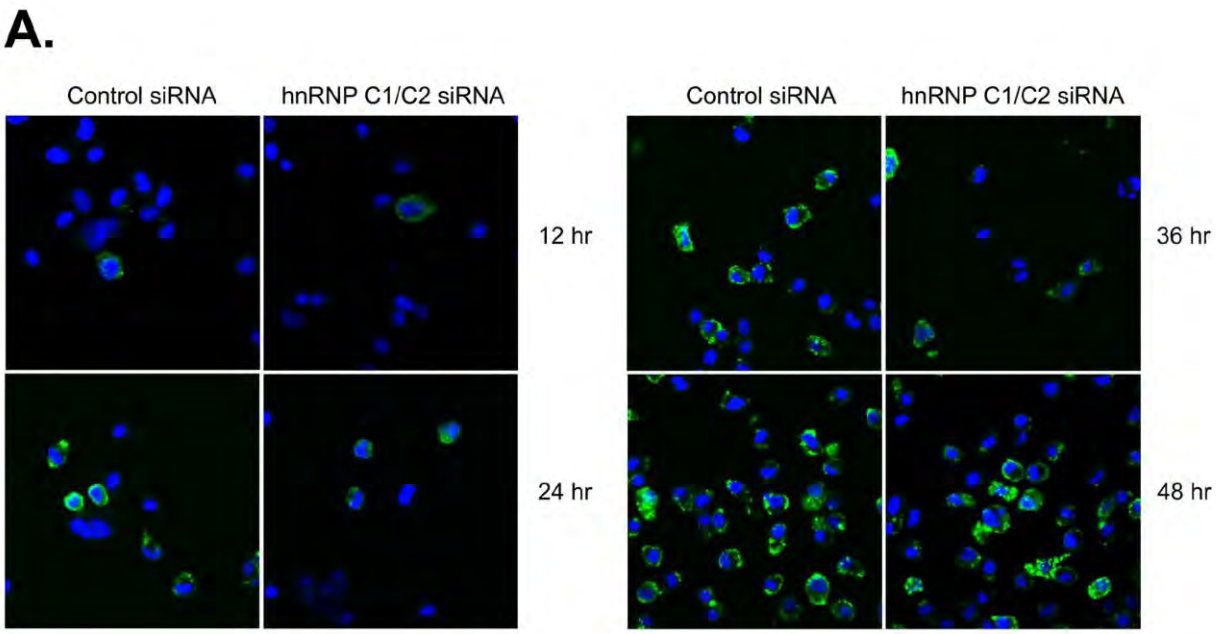


Figure 4



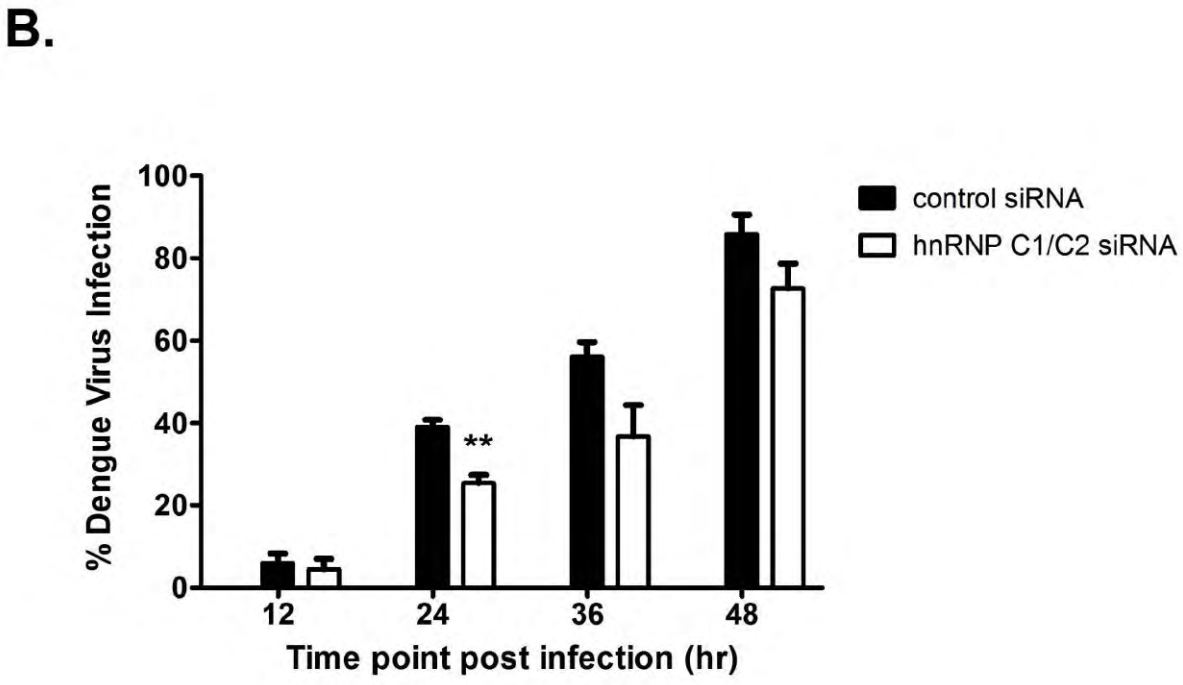
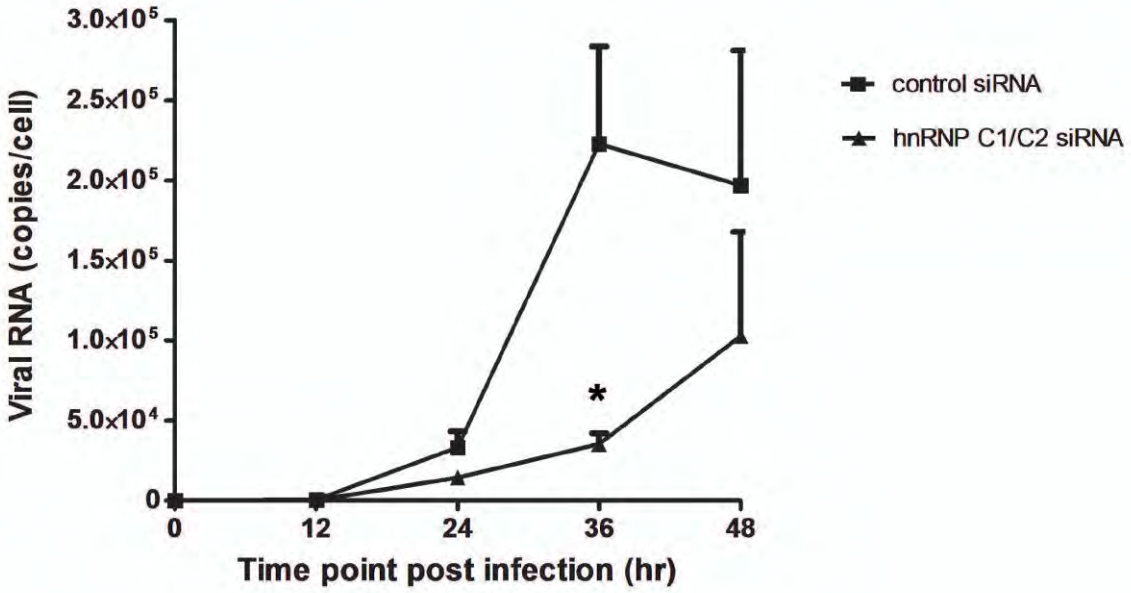


Figure 5

A. DENV viral RNA



B. hnRNP C1/C2 mRNA

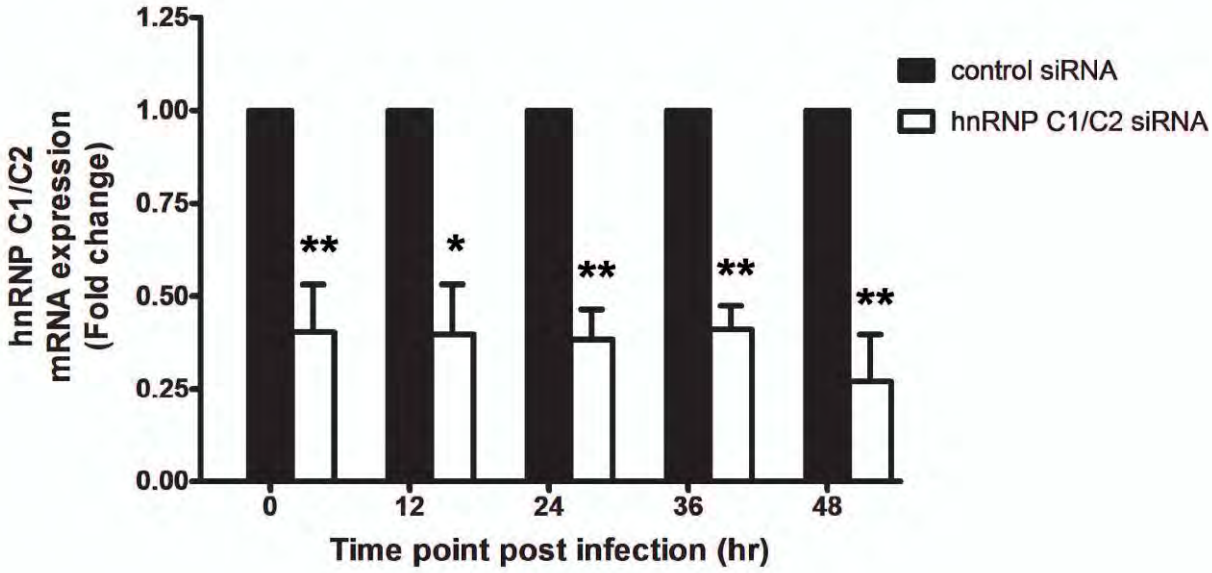


Figure 6

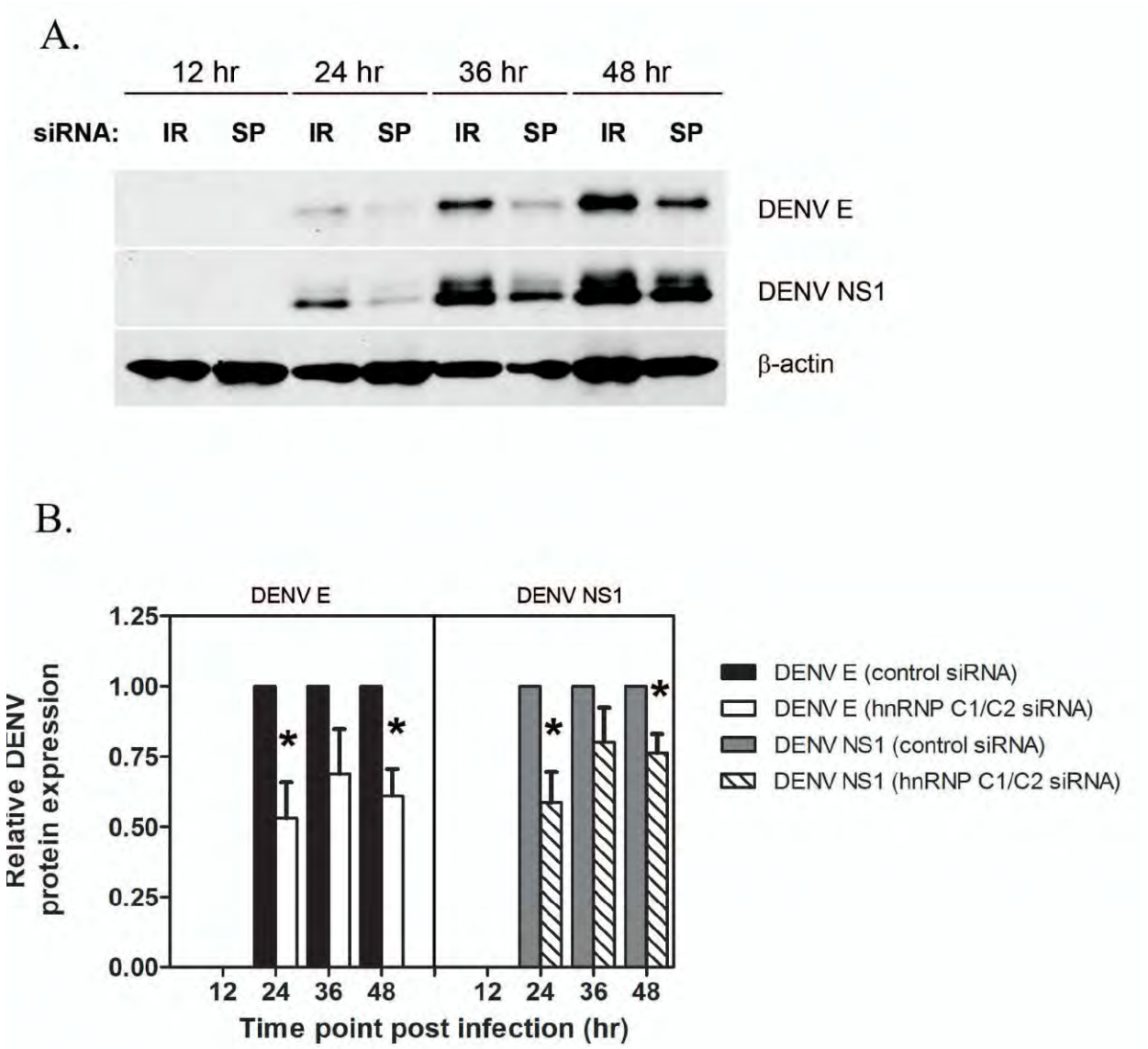


Figure 7

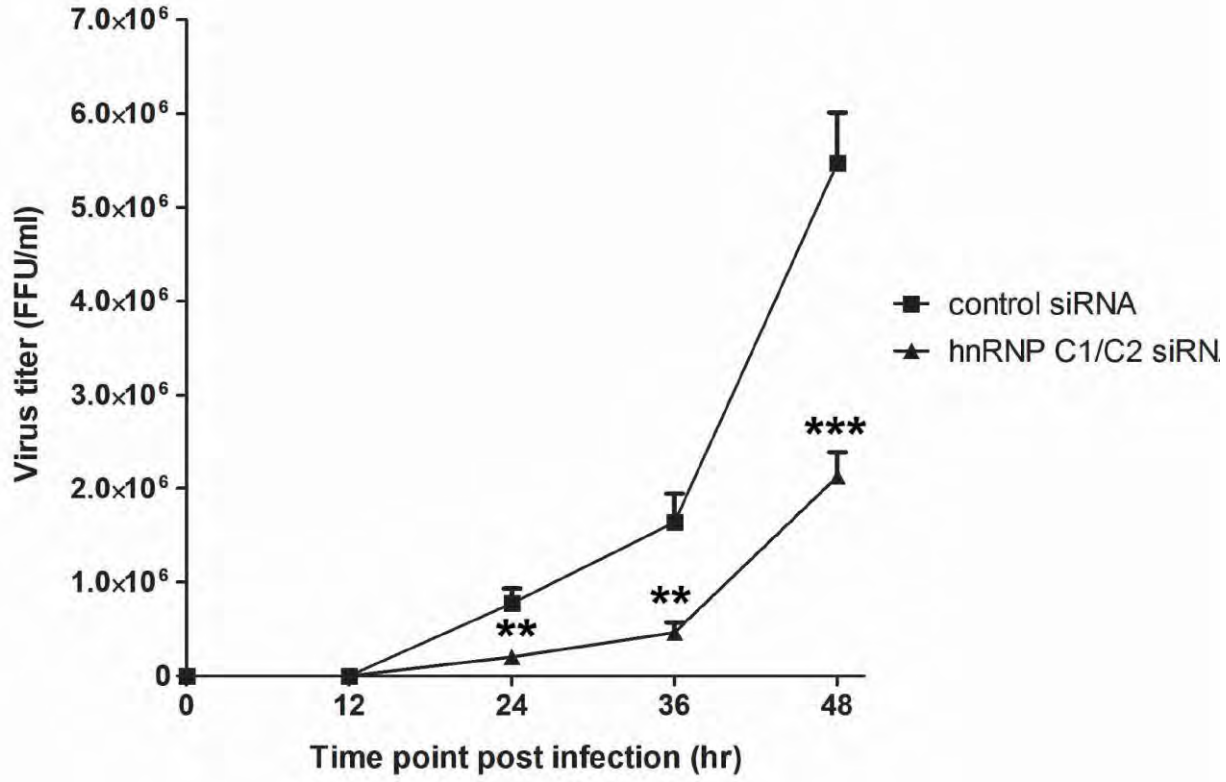


Figure 8

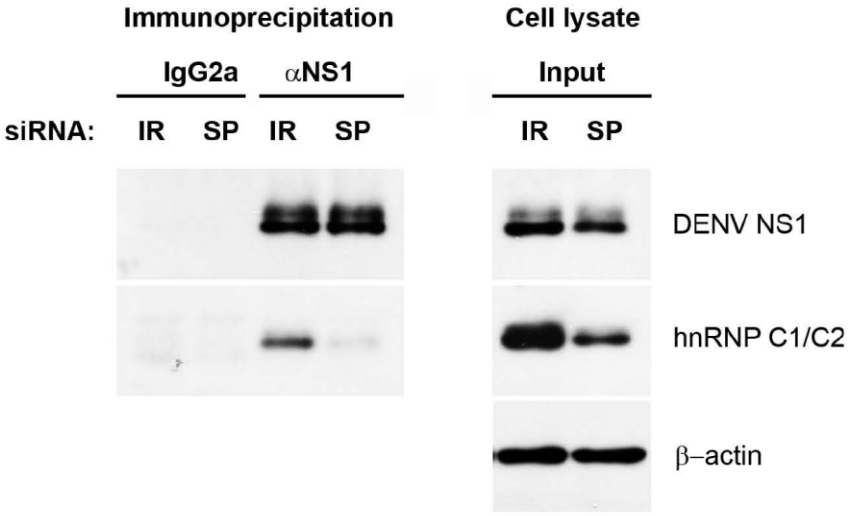


Figure 9