PROTEIN-PROTEIN INTERACTIONS OF THE UNSTRUCTURED DOMAIN II OF HEPATITIS C VIRUS NS5A

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ABSTRACT

Infection with hepatitis C virus (HCV) is globally highly prevalent. It is the leading cause of hepatocellular carcinoma, and can lead to liver failure and ultimately death. Virally encoded proteins involved in HCV replication have been identified as valid targets for the development of antivirals. One such protein is the non-structural 5A protein (NS5A). The HCV NS5A protein is a multi-functional, RNA binding protein and an essential component of the viral replication complex. It is subdivided into 3 domains (DI, DII and DIII) and is involved in several crucial interactions with viral and cellular factors within the replication complex. Unlike DI, NS5A DII and DIII are intrinsically unstructured yet seem to interact specifically with an ever-growing number of with viral and cellular host factors. As such, NS5A is also a target for the development of host-targeted antivirals (HTAs) that inhibit specific host factor protein interactions. The work outlined in this thesis describes the mechanisms of emergent resistance towards host-targeted antivirals (HTAs) within the NS5A-DII protein. This work also provides an in-depth biochemical characterization of molecular determinants of protein-protein and nucleoprotein interactions, specifically mediated through the intrinsically disordered domain II (NS5A-DII). The NS5A-DII protein mediates the specific interaction with Cyclophilin A (CypA), an essential cellular factor for viral replication. Cyclosporine A (CsA), an immunosuppressive inhibitor of CypA, inhibits this interaction and suppresses HCV replication. Potent and pangenotypic CsA analogs lacking in immunosuppression traits, like Alisporivir, have advanced into clinical trials, expanding the repertoire of potential HCV therapeutics. HTAs such as these that target and inhibit this interaction select for resistance within NS5A-DII; however, the underlying mechanism remains elusive.

I developed biochemical assays, including a Förster Resonance Energy Transfer (FRET)-based approach that allows for the rapid and precise analysis of intermolecular protein interactions

between NS5A-DII and CypA. Using this technique, combined with binding kinetic analysis, I studied binding properties and the rates of association and dissociation of the NS5A-DII complex with CypA under various conditions. I determined that the rate of the complex dissociation in the presence of CsA correlated with the level of resistance conferred by single or double resistance-conferring mutations. In this model, by prolonging the half-life of the protein-protein complex, these mutations specifically limit the inhibitory effect of CsA on the complex of NS5A-DII with CypA. This is achieved by limiting accessibility of the inhibitor to the protein-protein complex.

Apart from its RNA and CypA binding properties, NS5A-DII also interacts with the viral polymerase, NS5B; however, the functional relevance of this interaction remains unclear. The unstructured nature of this protein has hampered structural studies, and no crystal structure has as yet been described. I utilized a mass spectrometry (MS)-assisted foot-printing approach to characterize the nucleoprotein and protein-protein binding interfaces between HCV NS5A-DII, CypA, NS5B and RNA. Overlapping but definitive binding sites for each of the three macromolecules were determined. I identified the conserved residue W316 as a principle mediator of protein-protein interaction (CypA and NS5B) while an arginine-rich region of NS5A-DII was crucial for RNA binding. A novel binding site of NS5A-DII on the NS5B polymerase was mapped predominantly to a region within the NS5B fingers domain associated with RNA binding. Using gel-based activity assays combined with fluorescence binding assays, we found that NS5A-DII inhibits the RNA binding and RNA synthesis activity of NS5B. The results of this study highlight the potential regulatory role of NS5A on the NS5B polymerase activity. Taken together, the combined work of this thesis pinpoints key residues within the intrinsically disordered NS5A-DII that necessitate specific interactions with CypA, NS5B and model RNA substrates. This also provides a possible biochemical mechanism of resistance to CypA inhibitors. The novel

mechanism for the acquisition of resistance, whereby the effect of CsA is delayed rather than prevented, potentially highlight the certain structure constrains that are crucial to protein function. This finding may be exploited for rational drug design.

The identification of the overlapping binding sites described within NS5A-DII points to the highly resilient and adaptive nature of this domain. NS5A-DII has been plucked from obscurity as we begin to understand the biochemical functional relevance for these interactions, and their significance to HCV replication. Lastly, the highly sensitive robust FRET-based assay provides a powerful approach that can be extended into a high-throughput system to expedite the discovery of potent specific NS5A inhibitors.

RÉSUMÉ

L'infection par le virus de l'hépatite C (VHC) est globalement très répandue. Il est la principale cause de carcinome hépatocellulaire, et peut conduire à l'insuffisance hépatique et finalement à la mort. Les protéines à codage viral impliquées dans la réplication du VHC ont été identifiées comme des cibles valables pour le développement d'antiviraux. Une telle protéine est la protéine 5A non structurale (NS5A). La protéine NS5A du VHC est une protéine de liaison ARN multifonctionnelle et une composante essentielle du complexe de réplication virale. Il est subdivisé en 3 domaines (DI, DII et DIII) et est impliqué dans plusieurs interactions cruciales avec des facteurs viraux et cellulaires dans le complexe de réplication. Contrairement à DI, NS5A DII et DIII sont par defaut non structurés mais semblent interagir spécifiquement avec un nombre toujours croissant de facteurs de l'hôte viral et cellulaire. En tant que tel, le NS5A est également une cible pour le développement d'antiviraux ciblés par l'hôte (HTA) qui inhibent les interactions spécifiques des protéines du facteur hôte. Les travaux décrits dans cette thèse décrivent les mécanismes de résistance émergente envers les antiviraux ciblés par l'hôte (HTA) dans la protéine NS5A-DII. Ce travail fournit également une caractérisation biochimique approfondie des déterminants moléculaires des interactions protéine-protéine et nucléoprotéine, spécifiquement à médiation par le domaine intrinsèquement désordonné II (NS5A-DII). La protéine NS5A-DII agit comme médiateur de l'interaction spécifique avec la cyclophiline A (CypA), un facteur cellulaire essentiel pour la réplication virale. La cyclosporine A (CsA), un inhibiteur immunosuppressif de CypA, inhibe cette interaction et supprime la réplication du VHC. Des analogues de CsA puissants et pangénotypiques dépourvus de traits d'immunosuppression, comme Alisporivir, ont avancé dans des essais cliniques, élargissant le répertoire des thérapeutiques potentielles contre le VHC. Les

HTA telles que celles qui ciblent et inhibent cette interaction choisissent la résistance dans NS5A-DII; Cependant, le mécanisme sous-jacent reste difficile à atteindre.

J'ai développé des analyses biochimiques, incluant une approche basée sur le transfert d'énergie de résonance de Förster (FRET) qui permet l'analyse rapide et précise des interactions de protéine intermoléculaire entre NS5A-DII et CypA. En utilisant cette technique, combinée à une analyse cinétique de liaison, j'ai étudié les propriétés de liaison et les taux d'association et de dissociation du complexe NS5A-DII avec CypA dans diverses conditions. J'ai déterminé que la vitesse de la dissociation du complexe en présence de CsA peut être corrélée avec le niveau de résistance conféré par des mutations conférant une résistance simple ou double. Dans ce modèle, en prolongant la demi-vie du complexe protéine-protéine, ces mutations limitent spécifiquement l'effet inhibiteur de CsA sur le complexe de NS5A-DII avec CypA. Ceci est obtenu en limitant l'accessibilité de l'inhibiteur au complexe protéine-protéine.

Outre ses propriétés de liaison à l'ARN et au CypA, NS5A-DII interagit également avec la polymérase virale NS5B; cependant, la pertinence fonctionnelle de cette interaction reste incertaine. La nature non structurée de cette protéine a entravé les études structurelles, et aucune structure cristalline n'a encore été décrite. J'ai utilisé une spectrométrie de masse (MS)-assisté approche de l'empreinte pour caractériser les interfaces nucléoprotéine et protéine-liaison de la protéine entre le VHC NS5A-DII, CypA, NS5B et ARN. Des sites de liaison se chevauchant mais définitifs pour chacune des trois macromolécules ont été déterminés. J'ai identifié le résidu W316 conservé comme un médiateur principal de l'interaction protéine-protéine (CypA et NS5B) tandis qu'une région riche en arginine de NS5A-DII était cruciale pour la liaison de l'ARN. Un nouveau site de liaison de NS5A-DII sur la polymérase NS5B a été cartographié de manière prédominante dans une région dans le domaine des doigts NS5B associé à la liaison de l'ARN. En utilisant des

essais d'activité à base de gel combinés avec des essais de liaison de fluorescence, nous avons constaté que NS5A-DII inhibe la liaison d'ARN et l'activité de synthèse d'ARN de NS5B. Les résultats de cette étude mettent en évidence le rôle potentiel de régulation de la NS5A sur l'activité de la polymérase NS5B. Pris ensemble, le travail combiné de cette thèse identifie les résidus clés dans le NS5A-DII intrinsèquement désordonné qui nécessitent des interactions spécifiques avec CypA, NS5B et des substrats d'ARN modèle. Ceci fournit également un mécanisme biochimique possible de résistance aux inhibiteurs de CypA. Le nouveau mécanisme d'acquisition de la résistance, par lequel l'effet de CsA est retardé plutôt que prévenu, peut mettre en évidence certaines contraintes de structure qui sont cruciales pour la fonction des protéines. Cette constatation peut être exploitée pour la conception rationnelle des médicaments.

L'identification des sites de liaison se chevauchant décrits dans NS5A-DII montre la nature hautement résiliente et adaptative de ce domaine. NS5A-DII a été arraché de l'obscurité alors que nous commençons à comprendre la pertinence fonctionnelle biochimique de ces interactions et leur signification pour la réplication du VHC. Enfin, le test de haute fiabilité basé sur FRET offre une approche puissante qui peut être étendue à un système à haut débit pour accélérer la découverte de puissants NS5A spécifiques.

PREFACE

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation." The format of this thesis conforms to the "Manuscript-based thesis" option, which states that as an alternative to the traditional thesis format, the thesis research may be presented as a collection of scholarly papers of which the student is the author or co-author; that is, it can include the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles reformatted according to the requirements described below. A manuscript-based thesis must be presented with uniform font size, line spacing, and margin sizes, and contain additional text that will connect the manuscripts in a logical progression from one chapter to the next, to function as an integrated whole.

The contribution of co-authors to published or submitted articles appears in the section "AUTHORS' CONTRIBUTIONS". The journal of submission and information from published articles can be found on the title page of the concerned chapters. Other manuscripts not included in this thesis, but to which significant contribution was made by the candidate, are listed as follows:

Balzarini, J., Das, K., Bernatchez, J. A., Martinez, S. E., **Ngure, M.,** Keane, S., Ford, A., Maguire, N., Mullins, N., John, J., Kim, Y., Dehaen, W., Vande Voorde, J., Liekens, S., Naesens, L., Götte, M., Maguire, A. R., and Arnold, E. (2015) Alpha-carboxy nucleoside phosphonates as universal nucleoside triphosphate mimics, Proceedings of the National Academy of Sciences of the United States of America 112, 3475-3480.

Bernatchez, J. A., Paul, R., Tchesnokov, E. P., **Ngure, M.,** Beilhartz, G. L., Berghuis, A. M., Lavoie, R., Li, L., Auger, A., Melnyk, R. A., Grobler, J. A., Miller, M. D., Hazuda, D. J., Hecht, S. M., and Götte, M. (2015) Derivatives of Mesoxalic Acid Block Translocation of HIV-1 Reverse Transcriptase, The Journal of Biological Chemistry 290, 1474-1484.

*Beilhartz, G. L., ***Ngure, M.,** Johns, B. A., DeAnda, F., Gerondelis, P., and Götte, M. (2014) Inhibition of the Ribonuclease H Activity of HIV-1 Reverse Transcriptase by GSK5750 Correlates with Slow Enzyme-Inhibitor Dissociation, The Journal of Biological Chemistry 289, 16270-16277.

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AUTHORS' CONTRIBUTIONS

There are two manuscripts included in this thesis as Chapters 2 and 3. The manuscript in Chapter 2 entitled "*Decreased Dissociation of HCV NS5A and Cyclophilin A provides a Mechanism of Resistance to Cyclosporine A*" was written in collaboration and with M. Issur. M. Ngure, M. Issur and M. Götte designed the research; M. Issur and J. Rickard prepared the protein expression constructs; M. Issur designed the protein expression systems for the NS5A-DII and CypA proteins; M. Issur, H.-W. Liu and G. Cosa developed the FRET-based protein binding assays; M. Ngure and M. Issur performed the experiments; M. Ngure, M. Issur and H.-W. Liu analyzed the data; M. Ngure, M. Issur and M. Götte wrote the paper; M. Ngure, M. Issur, H.-W. Liu, G. Cosa and M. Götte reviewed the manuscript.

In the manuscript entitled "*Interactions of the Disordered Domain II of Hepatitis C Virus NS5A with viral RNA, NS5B and Cyclophilin A Show Extensive Overlap*" in Chapter 3, M. Ngure, M. Issur and M. Götte designed the research; M. Ngure, M. Issur and N. Shkriabai performed experiments; M. Ngure, M. Issur, and H.-W. Liu analyzed the data; M. Ngure, and M. Götte wrote the paper; M. Ngure, M. Issur, N. Shkriabai, H.-W. Liu, G. Cosa, M. Kvaratskhelia, and M. Götte reviewed the manuscript. In addition, Suzanne McCormick and Dr. A. Kulkarni a former research assistant and PhD student in our laboratory, respectively, assisted with the generation of the HCV NS5B polymerase enzyme.

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LIST OF ABBREVIATIONS

HCV: Hepatitis C virus

3'UTR: untranslated region of the 3' end of the genome

5'UTR: untranslated region of the 5' end of the genome

GT: genotype

RNA: ribonucleic acid

MS: mass spectrometry

aa: amino acid

NS5A: non-structural protein five A

NS5A-DII: domain II of non-structural protein five A

NS5B RdRp: non-structural protein five B RNA dependent RNA polymerase

WT: wildtype

CypA: Cyclophilin A

CsA: Cyclosporine A

CsA^{res}: Cyclosporine A resistant variant

DAA: direct-acting antiviral

HTA: host-targeted antiviral

IC_{50}: half-maximal inhibitory concentration

FRET: Förster / Fluorescence Resonance Energy Transfer

HPG: *p*-hydroxyphenylglyoxal

NHS-biotin: *N*-hydroxysuccinimidobiotin

K_D: Dissociation constant

CHAPTER 1: INTRODUCTION

1.1. HEPATITIS C: HISTORY AND GLOBAL BURDEN

Hepatitis C is a global pandemic and a leading cause of liver failure. The infection can range from a brief, acutely severe, to a life-long chronic infection. There are over 170 million chronically infected worldwide (3% of the global populous) and stand a high risk of developing liver cirrhosis, hepatocellular carcinoma and death[1]. It wasn't until 1989 that the hepatitis C virus (HCV) was discovered as the major cause of non-A, non-B hepatitis infections[2]. Although research studies have been underway, the HCV life cycle is not fully understood. The identification in a cell culture-adapted sub-genomic virus and more infectious clones has made possible extensive studies that have unravelled key steps of the complete HCV life cycle from entry to exit, and the major players critical in each step[3, 4].

1.1.1. Origins and Life Cycle of Hepatitis C Virus (HCV)

1.1.1.1. Classification

HCV belongs to the *Flaviviridae* family which is characterized by vector-borne positive-sense, single stranded RNA viruses, such as Dengue fever, West Nile and Zika virus[5]. Many of these viruses are responsible for considerable human and animal morbidity and mortality worldwide, and the global distribution is primarily determined by the principal vectors. The *Flaviviridae* family comprises of four genera: Hepacivirus, Flavivirus, Pestivirus and Pegivirus[6]. Flavivirus genus including yellow fever and Zika viruses are insect-borne, while Pestivirus genus including swine fever and bovine viral diarrhea viruses are important livestock pathogens. Human Pegivirus, originally classed under the Hepacivirus genus, was assigned a new genus Pegivirus. It represents persistent viruses that infect humans, non-human-pirates and other mammals, but not associated with any acute disease[7-9]. HCV is the sole member of the Hepacivirus genera and differs

in its mode of transmission and infection pathology. Transmission is human-to-human through blood or bodily fluids, resulting in both acute and chronic infections. HCV exhibits a high sequence heterogeneity, and is further subdivided into 7 genotypes with 30-35% nucleotide variability, and 67 identified subtypes with up to 25% RNA sequence divergence [10-12]. As such, HCV-infected individuals often host a complex quasispecies of distinct but related variants, generating escape mutants able to overcome host immune responses and treatment therapies[6]. The development of research methods including cell culture and animal models have gone to considerable length to demystify how HCV infection occurs, giving valuable insights into various steps of the HCV lifecycle (Fig. 1.1).

1.1.1.2. Viral cell entry

A quick snapshot of the HCV particle reveals its external lipid envelope and icosahedral capsid encapsulating a single-stranded positive RNA[13]. Glycoproteins expressed on the exterior surface of the envelope mediate binding and entry into host cells. HCV primarily targets and replicates in human hepatocytes. The virus circulates in the blood where it binds to several receptors present on the basolateral surface of hepatocytes[13]. Initial attachment of HCV to hepatocytes occurs via low-affinity interaction with the low-density-lipoprotein receptor (LDLR) and glycosaminoglycans (GAGs)[14]. Five additional cell surface molecules are essential for HCV particle entry: CD81, scavenger receptor class B member 1 (SRB1), claudin 1 (CLDN1), occludin (OCLN) and the cholesterol absorption receptor Niemann–Pick C1-like 1 (NPC1L1). CB81 was the first HCV co-receptor to be identified and is a ubiquitously expressed cell surface receptor[15, 16]. Its interaction with CLDN1 contributes to post-binding steps of HCV entry, leading to a clathrin-mediated internalization of the virus into the cell[14, 17]. SRB1 is also highly expressed on

the surface of hepatocytes which might help to define the tropism for HCV entry into hepatocytes[14, 18]. SRB1 plays multiple roles that contribute to virus attachment and effective virus entry[19]. Together with CD81, OCLN was identified as a factor that helps define the species tropism of HCV entry; mice liver cells expressing human CD81 and OCLN were found to be permissive to HCV infection[20, 21]. Lastly, NPC1L1 is a transmembrane protein that functions in the re-uptake of cholesterol from bile[22]. While the specific role of NPC1L1 in HCV entry is unknown, the knockdown of NPC1L1 inhibits HCV entry in cell culture, confirming it to be an essential HCV entry factor[23]. After attachment, HCV is internalized via clathrin-mediated endocytosis, forming an endosome in the cell cytoplasm[14]. The low pH of the endosomal compartment induces fusion of the HCV envelope with the endosomal membrane, releasing the HCV RNA genome into the cytoplasm where it can undergo direct translation to produce viral proteins, or initiate RNA replication[14, 24, 25].

1.1.1.3. HCV genome translation

Translation can occur directly from the positive-sense HCV genomic RNA. This occurs via a cap-independent mechanism mediated by the HCV internal ribosomal entry site (IRES) located in the viral RNA 5' non-coding region[26-28]. HCV recruits host intracellular machinery by binding of the HCV IRES to the 40s ribosomal subunit[29]. IRES binding causes conformational changes in the 40s subunit, closing its mRNA binding cleft to initiate the translation process. Additional translation factors are then recruited to the IRES-40S complex, including the eukaryotic initiation factor (eIF) 3 and the ternary complex of Met-tRNA–eIF2–GTP, which are essential for recruiting of 60S subunit to form the active 80S ribosomal complexes[30].

Translation of the HCV RNA results in the formation of a long, precursor polyprotein[31, 32]. Proteolytic cleavage is required by both cellular endoplasmic reticulum (ER) signal peptidases and viral proteases to produce structural and non-structural viral proteins, respectively[33]. The amino-terminal end of the polyprotein encodes the virion structural proteins. Following is a small ion channel protein, and the non-structural (NS) proteins that are responsible for the intracellular processes of the virus life cycle, including genome replication and subsequent steps leading to virus assembly and release[33].

1.1.1.4. HCV genome replication

HCV RNA replication occurs within a membrane-associated replication complex composed of the viral NS proteins and several cellular factors[33]. The ER undergoes changes in its structural conformation to form membranous webs where RNA replication occurs[34]. Replication of the HCV genome occurs by the initial production of an intermediate negative-sense HCV RNA. This is used as a template to generate positive-sense RNA, which is reported to be synthesized in five- to ten-fold molar excess over the negative-strand RNA[3].

1.1.1.5. Virus assembly and release

Virion assembly occurs in the lipid droplet (LD)-associated membranous web. Initiation of the virion assembly process is thought to initiate on the ER membrane, where viral replication complexes are recruited to the LD surfaces by several HCV structural and NS proteins[35]. This enables transfer and encapsidation of the newly synthesized copies of the RNA genome. The lipid envelope is acquired from the ER and incorporated together with HCV structural proteins into virions[36]. Nascent virus particles then combine with lipoprotein components and engage their

assembly pathway which facilitates virion maturation[37-39]. This is achieved through trafficking of the nascent virion particles across the ER lumen and into secretory pathways. Here, they undergo complete maturation, after which the virion is released from the cell or can infect neighbouring cells through tight junctions[14, 40]. The process by which HCV particles exit from the cell has also been proposed to utilize clathrin-mediated anterograde trafficking during viral egress, leading to exocytosis and secretion of mature virions from the infected cell[41].

1.2. HCV GENOME ORGANIZATION AND FUNCTION

The HCV RNA genome is 9.6Kb in length, composed of a 5'-untranslated region (UTR), a ~3,000 amino acids long open reading frame encoding structural and non-structural proteins, and a 3'-UTR (Fig. 1.2)[33]. The structural proteins form the viral particle, and include the core protein and envelope glycoproteins E1 and E2. The non-structural proteins are involved mainly in HCV replication and assembly. These include the p7 ion channel, NS2-3 protease, NS3 serine protease and RNA helicase, NS4A co-factor, NS4B, NS5A proteins and the NS5B RNA-dependent RNA polymerase (RdRp)[33].

1.2.1. 5’ UTR & 3’ UTR

The non-coding 5'- untranslated region (UTR) of the HCV RNA genome is 341 nucleotides in length, highly conserved across HCV genotypes and is extensively structured (Fig. 1.2)[42, 43]. The 3'-UTR, on the other hand, can vary between 200 and 235 nucleotides long, and is typically composed of a short variable region, a poly(U/UC) tract, and a highly conserved 98 nucleotide X region (Fig. 1.2)[43]. Regions of the 5′ and 3′ UTR are assumed to contain sequences required for the initiation and potential regulation of RNA replication translation. The 5’ UTR has four major

structural domains (domains I–IV) with conserved extensive secondary and tertiary structures, including multiple stem-loops and a pseudo-knot essential for HCV translation[43]. Domains II-IV feature an internal ribosome entry site (IRES) required for cap-independent HCV translation. In addition, the interaction between the 5'-UTR and the liver-specific microRNA (miRNA), miR-122, has been shown to enhance viral RNA translation and replication[44]. Conserved elements in the 3' UTR poly(U) tract are essential for HCV replication[33], while the X region is involved in the regulation of HCV translation[43].

1.2.2. Structural proteins

1.2.2.1. Core

HCV core is a 21 kDa highly basic, RNA binding protein that forms the viral capsid[45]. Mature core has 2 distinct domains: N-terminal hydrophilic domain and a C-terminal hydrophobic domain. In the precursor immature core protein, an extreme C terminus is present[46]. This is cleaved soon after core is translated to form a mature core protein[47]. The N-terminal domain 1 is principally involved in RNA binding and nuclear localization, and interacts with several cellular proteins and mediates capsid assembly[45]. Domain II mediates interactions with membrane proteins and is responsible for association with the ER[48]. So far, only a short N-terminal fragment of the core protein (residues 25-40) has been successfully crystalized (Fig. 1.3)[49]. This fragment was resolved in complex with a monoclonal antibody, implying that this is a recognized epitope able to illicit highly specific antibodies against HCV.

The core protein has been shown to transcriptionally upregulate growth-related genes to stimulate hepatocyte growth[50]. In addition, it also induces LD formation which are required for viral assembly[35, 48, 51-53].

1.2.2.2. E1 and E2 glycoproteins

HCV encodes two envelope proteins, E1 and E2, that are type I transmembrane glycoproteins essential for viral entry and fusion with the host cellular membrane[54]. They are composed of a highly glycosylated N-terminal ectodomain and C-terminal transmembrane domain. These glycoproteins assemble as noncovalent heterodimers, mediated by their C-terminal transmembrane domain[55, 56]. Additionally, both E1 and E2 are highly glycosylated, and this feature has been shown to be required for HCV infectivity[57, 58].

Both glycoproteins are crucial for HCV cell entry. Specifically, E2 is thought to initiate viral attachment to the host cell via its N-terminal interaction with the CD81 cell surface receptor[59-61]. E2 contains an N-terminal hypervariable region (HVR1) required for SR-BI interaction, and this region can differ by up to 80% between HCV genotypes, evolving rapidly within infected individuals[62, 63]. Two other internal hypervariable regions (HVR2 and HVR3) exist with the E2 ectodomain. The overall structural conformation of the E2 ectodomain is otherwise maintained and possibly plays a role in the interaction with the host cell during HCV entry[61, 64]. Studies to determine high-resolution structures of these proteins have only been partially successful. Crystal structures of the ectodomain of E2 revealed high structural similarities between HCV genotypes 1a and 2a (Fig. 1.4). The complex structure of E2 core ectodomain revealed a compact yet globular protein composed mainly of β-sheets and a few short α-helices[61, 65]. Several conserved residues have been implicated in interactions with cellular co-factors SR-BI and CD81 receptors. In addition, an E1 helical glycopeptide comprised of residues 314-324 has been crystalized in complex with a broadly neutralizing antibody[66]. These findings define possible antigenic template epitopes within E1 and E2 for vaccine design.

1.2.2.3. Frameshift protein

Little is known of the function of the HCV frameshift (F) protein. It is a 16-17 kDa protein generated by a -2/+1 ribosomal frameshift in the N-terminal core-encoding region[67, 68]. The F protein was originally thought to be a truncated version of the core protein. Multiple frameshifting events are proposed to occur in the HCV core sequence derived from genotype (GT) 1a and 1b[69-71]. In GT 1a, HCV F protein was shown to result from a -2/+1 ribosomal frameshift at or around codon 11, while in GT 1b, a +1 frameshift was detected at codon 42. While these results point to genotype-specific differences in the F proteins, it is found expressed during natural HCV infection and possesses high conservation across various HCV isolates[72]. Antibodies against F protein have been identified in chronic patients, suggesting a link between the presence of the F protein and a possible involvement in viral persistence infection[71, 73-75]. However, the exact role of the F protein is unclear, with respect to each HCV genotype.

1.2.3. Non-structural proteins

1.2.3.1. P7

P7 is a small 67 amino acid hydrophobic polypeptide and an integral membrane protein that forms a viroporin ion channel[76]. Interestingly, p7 is selective for cations, and has no eukaryotic homology, making it potential target for HCV drug development[77-79]. Approved influenza M2 ion channel inhibitors, amantadine and rimantadine, and iminosugar derivatives have been shown to inhibitor p7 activity with varying efficacy across HCV genotypes[77, 80-82]. Amino-acid variations between various genotypes may play a role in altering inhibitor interaction with the ion channel, or the ion channel structure. Initial structural modeling and NMR studies suggested that p7 consists

for 2 transmembrane domains, forming a hair-pin structure linked by a cytoplasmic loop[83-86]. Recent studies involving electron microscopy revealed the map of a 42kDa, flower-shaped, hexameric p7 channel from HCV GT 2a, different from other known ion channel structures[87]. This is supported by the NMR structure of p7 from GT 5a indicating an unusual p7 hexamer architecture, forming a largely hydrophilic channel (Fig. 1.5)[88]. In its monomeric state, p7 consists of 3 helical segments, namely H1 (residues 5-16), H2 (residues 20-41), and H3 (residues 48-58). Several conserved residues within each helical region have been identified that help stabilize the compacted channel. The location of conserved polar residues at the C- (R35) and N-terminal (N9) ends of the channel also suggests a unidirectional cation transport across the p7 channel (C-to-N terminus)[88]. The exact conformation of p7 during natural HCV infection remains to be determined. Sequence variation is evident across HCV genotypes, but the structural integrity of p7 appears to be conserved based on the physio-chemical residue properties[86, 88].

Although dispensable for HCV replication, p7 plays a role in viral assembly and is essential for production and release of infectious HCV virions from an infected cell[83, 86, 89]. Interaction between HCV p7 and NS5B polymerase was found to be important for virion infectivity in JFH-1, by decreasing sphingomyelin within the virion[90]. Mutational analysis indicated that residues within the N-terminal H1 are involved in modulating channel activity as well as cleavage between E2 and p7[86].

1.2.3.2. NS2

NS2 is another transmembrane protein, and has 2 internal signal sequences that are responsible for its ER membrane association[91, 92]. Prior to complete polyprotein cleavage, NS2 forms a zinc-dependent cysteine protease together with the N-terminus of NS3 (NS2-3 protease)[93-

[95]. This protease is responsible for the autocatalytic cleavage of the site between NS2 and NS3[96]. A crystal structure of the post-cleavage catalytic domain of the NS2-3 protease, composed of NS2-protease ($NS2^{pro}$) residues 94-217, describes a unique dimeric interface (Fig. 1.6)[97]. Each NS2 monomer consists of 2 subdomains, with an intra-domain linker. As a dimer, $NS2^{pro}$ contains 2 composite active sites with 3 catalytic residues: H143 and E163 contributed by one monomer, C184 from the other (Fig. 1.6)[97]. Thus, from this novel protein fold, it is proposed that each dimeric $NS2^{pro}$ conformation is able to catalyze two cleavage events at the NS2/NS3 junction. It is unclear the function of NS3 in the NS2-3 protease, considering that this complete cysteine-protease catalytic active site is encoded solely within NS2[97]. However, this NS2 structure was resolved post-cleavage, thus the actual NS2-3 protease structure during natural HCV polyprotein processing needs to be defined. NS2 is a short-lived protein in the cell (subsequently degradation by the host proteasome in a phosphorylation dependent manner), however, additional studies suggest that NS2 also plays a role in modulating cellular gene expression as well as facilitating viral persistence by interfering with cellular apoptosis pathways[98, 99].

1.2.3.3. NS3 helicase and NS3-NS4A Protease

NS3 possesses a N-terminal serine protease domain and a C-terminal helicase/NTPase domain, hence serves several functions in the HCV life cycle[100]. NS3 helicase domain has RNA-stimulated NTPase activity which is required for the hydrolysis of natural nucleoside triphosphates[101]. In addition, NS3 helicase binds to RNA and is responsible for the ATP-dependent unwinding of the double-stranded RNA (dsRNA) intermediates during RNA replication[102-104]. NS3 helicase (NS3h) from GT 1b was crystalized in complex with a single-stranded DNA substrate in various conformations, demonstrating a “ratchet-like” unidirectional mode of

translocation during unwinding (Fig. 1.7A)[105]. NS3h was found to translocate one base per every NTP hydrolysis cycle. Further characterization studies identified a "spring" α-helix within the active site involved in coordinating multiple conformational changes during the transitional states of helicase activity[105, 106].

NS3 protease domain is responsible for the processing of downstream cleavage sites of the remaining HCV polypeptide comprised of the NS4A, NS4B, NS5A and NS5B proteins[107]. This is achieved together with NS4A protein which acts as a cofactor for both the protease and helicase activities of NS3[108, 109]. As such, the NS3-4A protease presented as an attractive target for anti-HCV drug development. Crystal structures using a truncated NS3 protease domain, 181 amino-acids (aa), in complex with a NS4A peptide (18 aa essential for NS3 stimulation) revealed a shallow substrate-binding groove, making structure-based drug design of small inhibitors challenging[110 111, 112]. Nonetheless, the first HCV DAAs approved, Boceprevir and Telaprevir (Table 1.1), were targeted against the NS3/4A protease, binding covalently in the active site[113]. Due to the emergence of cross-reactive resistant mutants towards these inhibitors, the development of new generation of HCV protease inhibitors should be geared towards DAAs with alternate mechanisms of action[114]. More recent structural studies have been successful in resolving the structure of the full-length NS3 helicase/protease (GT 1b) bound to the NS4A peptide (Fig. 1.7B)[115]. Residues located in the C-terminal end of NS3h domain were found to bind and interact in the NS3 protease catalytic triad (Ser139, His57, Asp81), stabilizing a closed conformation of the active site[115, 116]. An allosteric site at the NS3 helicase -protease interface is thought to regulator the dual functions of NS3[115]. This is near but distinct from the NS3 protease active site, and possibly represents a novel allosteric inhibitor binding site. This exemplifies the use of structure-guided drug design to develop a new class of NS3/4A inhibitors. The proposed mechanism of

action would include stabilizing this inactive conformation, rendering the protease unable to perform HCV polyprotein processing[115, 117].

Besides its function as a cofactor in the NS3/4A protease complex, the NS4A membrane-bound protein is integral to the reorganization of the ER membrane, and thought to form a scaffold for the formation of the HCV replicase[34]. The NS3-4A protease also functions as an antagonist of dsRNA-dependent immune response by blocking various interferon signalling pathways.

1.2.3.4. NS4B

NS4B (27 kDa) is an integral membrane protein, hydrophobic in nature[118]. Topology studies point to the presence of cytosolic N- and C-terminal tails flanking a central transmembrane domain[119, 120]. Fragments of the NS4B have been resolved, including the NMR structure of the N-terminal amphipathic α-helix, which mediates ER membrane association[119, 121]. Structure-function analysis reveal the importance of several conserved residues within this helix in rearrangement of the ER to form the membranous web[119]. As such, NS4B plays a major role in the establishment and initiation of the membranous web[34, 119, 122]. Additional roles in RNA replication and virus production are also suggested for the NS4B protein. Adjacent to the N-terminal amphipathic helix is a basic leucine zipper motif, which mediates NS4B interaction with ER-stress induced transcription factors, implying a role of NS4B in modulation the ER-stress response[123, 124]. Taken together, NS4B is the key to the organization of the HCV replication complex within the ER, and serves as a membrane anchor for the HCV replication complex[119]. NS4B interacts with other NS proteins as well, such as NS5A and NS5B, modulating their function and activity[125, 126].

1.2.3.5. NS5A RNA-binding, phosphoprotein

Second to last in the polypeptide chain, and the focus of this thesis, is the RNA-binding, multifunctional phosphoprotein NS5A. The NS5A protein is subdivided into 3 domains (Domains I-III), separated by low complexity sequences (LCS I and II) (Fig. 1.8)[127]. Domain I has been shown to be highly structured, whereas domains II and III represent relatively disordered domains[128, 129]. All three domains possess RNA binding properties and are able to bind to the poly U/UC tract in the 3' UTR, and to sites within the 5' UTR, as well[130, 131]. While no activity has been ascribed to NS5A, this protein is required for RNA replication and is implicated in multiple steps of the viral cycle, including viral assembly[132].

Multiple phosphorylation sites exist within NS5A, leading to the existence of two major species termed basally phosphorylated (~56 kDa) and hyperphosphorylated (58 kDa)[133]. Basal phosphorylation is mediated by several host serine/threonine kinases such as casein kinase (CK) I and II, and mitogen-activated protein kinases (MAPKs), while hyperphosphorylation requires the presence of several other NS proteins, including NS2, NS4A, NS5B and the protease activity of NS3[134, 135]. The LCSI and II, as well as domains I and III, contain several sites of phosphorylation, but key phosphoacceptors specific to either the basally or hyperphosphorylated NS5A species remain to be defined[133, 136]. A major site for hyperphosphorylation resides within a serine-rich region within LCS1, with evidence pointing to a sequential cascade of phosphorylation[133, 137]. Several serine residues identified as major phosphoacceptors within this region are highly conserved across all HCV genotypes, which implies an essential role in the virus life cycle. So, one is tempted to ask what is the function relevance of each NS5A phosphorylated species. Perhaps phosphorylation is of functional importance; it is suggested that this is linked to the different roles

of NS5A in the HCV lifecycle[132]. The mechanism by which NS5A protein function is modulated by this posttranslational modification remains to be elucidated.

Further elucidation into the structures and functions ascribed to each NS5A domain will be discussed in depth later in this chapter.

1.2.3.6. NS5B RNA-dependent RNA polymerase (RdRp)

Last in the polyprotein chain is the NS5B RdRp. NS5B (64 kDa) is responsible for the synthesis of complementary negative-strand RNA from the genomic RNA template, and the subsequent replication of HCV RNA genome[33]. NS5B is anchored to the ER via a C-terminal transmembrane domain (21 aa), and it is capable of performing *de novo* synthesis of RNA, as well as primed RNA replication[138-140]. It should be noted that primer-mediated RNA synthesis is observed to be more robust than *de novo* synthesis *in vitro*[139-142]. This protein has been extensively studied and crystal structures revealed the classical fingers, thumb and palm subdomains shared by other viral RdRps (Fig. 1.9)[33, 143-145]. Numerous crystal structures (>70) of truncated NS5B RdRp from GTs 1a, 1b, 2a and 2b depict a structurally similar closed active site with extensive contacts between the fingers and thumb subdomains (Fig. 1.9A)[146-153]. The palm subdomain serves as a base for a deep RNA binding groove, with several conserved residues validated to be essential for RNA binding and RNA synthesis activity[154]. A unique structural feature is the presence of a β-hairpin loop (residues 443–455), extending into the polymerase active site (Fig. 1.9A). In addition, part of the C-terminus tail also interacts with the residues within the β-hairpin loop and thumb subdomain (Fig. 1.9A)[153]. Lastly, the encoded C-terminal 21 aa transmembrane anchor, usually deleted in available crystal structures, is essential for RNA replication in cells, but not required for polymerase activity in *in vitro* biochemical assays[144, 155, 156]. Positioning of the β-hairpin loop inhibits exit of nascent RNA strands, but not binding of nucleotides or short RNA substrates[153].

Incoming nucleotides have been shown to interact with several residues in the β-hairpin loop and others that define the RNA binding groove. Therefore, it is postulated that this "gated" mechanism facilitates the initiation of *de novo* RNA synthesis[157, 158]. Conformation changes would be need to accommodate dsRNA and transition into the elongation phase of RNA product formation. The structure of NS5B from GT 2a (with a β-hairpin loop deletion) bound to a duplex RNA molecule points to structural rearrangement in the thumb and fingers subdomains (Fig. 1.9B)[150]. Deletion of the auto-inhibitory β-hairpin loop has been shown to increase RNA binding and enzymatic activity by more than 100-fold[150, 153]. Taken together, the HCV NS5B RdRp contains key regulatory features which undergo structural changes to modulate the transition between initiation and elongation of RNA product synthesis.

HCV NS5B lacks in proof reading capabilities, making it highly error-prone and contributes heavily to the establishment of HCV quasispecies in an infected individual[159, 160]. Presence of replication-competent resistant mutant species allows for rapid selection under drug pressure. Extensive structural and biochemical studies only partially described here have aided immensely in the development of highly potent inhibitors of NS5B polymerase activity. Sofosbuvir and Dasabuvir, as examples, show high efficacy for the treatment and cure of HCV infection[114, 161]. The current standard of care includes pangenotypic NS5B inhibitors able to achieve a 95-100% sustained virological response (SVR), potentiating the value-added benefits in structure-guided drug development[114].

1.3. NS5A: STRUCTURE AND FUNCTION

1.3.1. Evolutionary relationship within Flaviviridae family

As a point to note, viruses in the Flaviviridae family maintain an evolutionary conservation of a similar organization of the structural and NS proteins, in the N-terminal and C-terminal region of the polyprotein, respectively. However, some differences exist, including the existence of a cleavage site in the NS5 region of HCV and pestiviruses, but not flaviviruses[135]. Processing of this cleavage site within HCV yields the NS5A and NS5B proteins.

NS5A is an essential component of the HCV replication complex, and here acts as an adaptor protein through interaction and recruitment of several factors to this complex[132, 134]. Colocalization of NS5A with the HCV RNA has been observed *in vitro*, as well as the direct interaction with other NS proteins[162-165]. NS5A interaction with the NS4A protein had been associated with hyper-phosphorylation, while the interaction with NS4B is essential for the proper localization of NS5A to the replication complex. NS5A also serves as a modulator of RNA replication via its interaction with the NS5B RdRp, an interaction that is crucial for HCV replication[164, 166-171]. In addition, the core protein and the apolipoprotein host factor have also been shown to colocalize with NS5A at the surface of LDs, and this is integral to the HCV virion assembly process[172-174].

HCV replication is ineffective without the recruitment of host factors[175-177]. True to its multifunctional nature, NS5A mediates protein interactions with an ever-growing number of host factors essential for replication, including human vesicle-associated membrane protein-associated proteins (VAPs), and CypA[128, 178-181]. The multifunctional role of NS5A also extends to the modulation of cellular signaling pathways as well as other physiological processes[134]. NS5A protein-protein interactions include a host of cellular factors to dysregulate processes involved in

IFN signaling pathways, cell growth and apoptosis[132, 134]. Up to 130 cellular factors are purported to interact with the NS5A protein, but how all these interactions are modulated and controlled remains unclear.

1.3.2. Roles of Domain I; aa 1-213

Domain I of NS5A (NS5A-DI) contains a N-terminal amphipathic α-helix, 30 aa long, that is highly conserved and mediates the localization and association of NS5A with the ER membrane (Fig. 1.10A)[134, 182-184]. NS5A membrane association is indispensable during HCV RNA replication. This domain also contains a zinc-binding site, a structural feature that has also been shown to be essential for viral replication[127, 185]. Crystallographic studies reveal NS5A-DI to be highly structured and can exist in various dimeric and multimeric conformations, while still maintaining an identical monomeric structure[132, 152, 185, 186]. One dimer conformation revealed a basic RNA-binding groove at the NS5A-DI (GT 1b) monomer interface able to accommodate an RNA substrate (Fig. 1.10B). This "open" conformation is proposed to be relevant for NS5A RNA binding in the HCV life cycle[22, 131, 187]. Another "closed" dimer conformation has also been described, which suggests the re-assortment capability of these dimers into various conformations (Fig. 1.10C)[186]. The location of the N-terminal region of both NS5A-DI dimers implies that membrane anchoring is mediate by both N-terminal amphipathic α-helices[185, 186]. Other oligomeric states of the NS5A-DI proteins derived from GT 1a describe a novel multimeric state, possibly serving as a scaffold for an expansive network in the membranous web (Fig. 1.10D)[152]. The formation of higher order oligomeric states is also proposed to occur with purified NS5A-DI from GT 1b[186]. This is supported by other studies illustrating increased NS5A protein expression levels within infected cells, implying a functional requirement for larger oligomeric NS5A conformations

during HCV replication[188, 189]. Whether these oligomeric conformations are maintained in the full-length NS5A protein during infection is unknown.

1.3.3. Roles of Domain II; aa 250-342

Domain II of NS5A (NS5A-DII) has been described as an intrinsically unstructured protein[129, 190]. This has made it difficult to study the structure and functions related to this domain. Functions attributed to intrinsically unstructured proteins or regions have often times been related to protein-protein and protein-nucleic acid interactions in numerous human diseases[191-193]. NS5A-DII possesses RNA binding properties, although less efficiently than domain I[130]. This domain also plays a role in HCV viral replication and translation. Several residues in the C terminal region have been identified as essential for viral replication[194]. In addition, NS5A-DII has been implicated in down-regulation of HCV translation, possibly through interactions with the polyU/UC sequence[130, 187, 195, 196]. Several protein-protein interactions are mediated through this domain, including interactions with the NS5B RNA-dependent RNA polymerase[170, 171, 197]. It is unclear the exact function of this interaction. A number of studies have shown the interaction between full-length NS5A and NS5B to have a variable modulatory effect on the RNA synthesis activity of the NS5B polymerase, this being under varying assay conditions with respect to the choice of RNA template and protein concentrations assessed[164, 166, 168, 169, 171, 198]. However, the exact role of NS5A-DII in this modulation has yet to determined. NS5A-DII also interacts with CypA host factor through a similar binding site, and this has been shown to stimulate the RNA-binding properties of NS5A-DII[170, 179, 199, 200]. CypA is an essential host factor for the viral replication of HCV[181, 201-203]. Several CypA inhibitors have been developed that block CypA interaction with NS5A-DII, and subsequently abrogate HCV replication[204-209]. Another cellular interacting partner is the IFN-induced double-stranded RNA activated protein kinase (PKR). PKR is an essential component of

the innate immune response, and NS5A-DII interaction is mediated through its PKR binding domain (aa 237-309), a region that encompasses the interferon sensitivity-determining region (ISDR, aa 237-276)[197, 210, 211]. Mutations within this region seem to enhance sensitivity to IFN therapy, supporting the NS5A involvement in viral resistance to IFN therapy[134, 211, 212]. Other binding partners of NS5A-DII protein that have been identified include the pro-apoptotic Bcl2 protein, Bax, and phosphatidylinositol 3-kinase (PI3K), which may explain the role of NS5A as an agonist of apoptosis signalling pathways[134, 213-215]. Thus, as an intrinsically disordered protein, it packs a punch in its contribution to HCV RNA replication.

1.3.4. Roles of Domain III; aa 356-447

Domain III was also determined to be a highly disordered region of the NS5A protein[216, 217]. Although shown to bear RNA binding properties, toleration of major deletions and insertion of a GFP protein within this domain suggest that this domain is dispensable for RNA replication[130, 218, 219]. Functional studies point to domain III of NS5A (NS5A-DIII) as a key component of HCV infectious particle formation and production[172, 220-222]. Internal deletions within NS5A-DIII affect the colocalization of NS5A with the HCV core protein on the surface of LDs, and also impair production of infectious virus. The association of the HCV core protein with NS5A-DIII is essential for virion production and allows for recruitment of NS5A to LDs as part of the initiation event in virion assembly[35, 223]. NS5A-DIII phosphorylation has also been suggested to be functionally relevant for virion production. A serine cluster that serves as a phosphorylation site of CKII was identified within NS5A-DIII through low-resolution mapping studies[224]. Mutational studies limiting phosphorylation at a single serine in this site suggest that there is a phosphorylation dependent regulation of particle assembly[223, 225]. Fyn tyrosine kinase interacts via its SH3 domain with a proline-rich region extending from LCSII into NS5A-DIII, inhibiting oxidative-stress-

mediated induction of apoptosis[132, 226]. Mutational studies also point to a modulatory function of this domain on HCV translation [195, 196].

NS5A-DIII binds several host factors, including the membrane protein VAPB protein that localizes to the ER where it plays a role in anchoring the HCV RNA replication complex[128, 178, 227, 228]. Mutations that confer low-level resistance to CypA inhibitors have been mapped to this domain[202, 229]. *In vitro* NMR studies reveal a direct interaction between CypA and NS5A-DIII which may modulate the efficiency of proteolytic cleavage between NS5A and NS5B[217]. Overall, the major role of NS5A-DIII remains predominantly in HCV viral assembly.

1.4. ADVANCES IN HCV THERAPEUTICS

Now that the HCV life cycle and its components has been summarized, let's delve into the advances in HCV treatment development. First treatment options for chronic HCV infections were initiated with the FDA-approved interferon (IFN)-α. This only attained 2-16% SVR24 rates, which is characterized as the lack of HCV RNA detection 24 weeks after the end of treatment[230]. Later, the combination of IFN-α with another drug, Ribavirin, boosted SVR rates to 30% in the less responsive GT 1, and up to 50% for other chronic HCV genotypes, as well as reduced the risk of relapse after treatment termination[231, 232]. Ribavirin, a guanosine analogue, was initially used to treat infants and immunocompromised patients suffering from severe respiratory syncytial virus (RSV) infections[233]. It also showed *in vitro* antiviral activity against several HCV related viruses, including Bovine viral diarrhea virus (BVDV) and GB virus of the *Flaviviridae* family[234-238]. Generation of two pegylated forms of IFN-α (pegIFN- α) enhanced its half-life allowing for once-per-week versus the previous 3 times-per-week dosage[239]. In addition, SVR rates increased to approximately 44% and 80% for GT 1 and non-GT 1 infections, respectively[239, 240]. This treatment

was FDA-approved in 2001 and remained the standard of care for the next 10 years before the first direct acting antivirals (DAAs) were introduced.

1.4.1. Direct acting antivirals (DAAs)

The former standard of care with pegIFN- α and ribavirin had varied success for different HCV genotypes, and was poorly tolerated[241]. Multiple side-effects also limited its long-term use. Major advances in the development of HCV antivirals have resulted in treatment success rates of >90% for many patient groups[114]. This also paved the way for the potential of IFN-free HCV therapy that would limit the risk of major side effects and enhance tolerance. To this end, DAAs are designed to specifically target and inhibit HCV proteins essential for various stages in the viral life cycle, the major players being the NS3 protease, NS5A and NS5B proteins (Table 1.1).

1.4.1.1. NS3/4A Protease Inhibitors

A substrate-based approach was used in early drug development to design the first active site inhibitor against the NS3 protease (BILN 2061)[242]. BILN 2061 is a peptidomimetic inhibitor (mimics the natural substrate of the target enzyme) that proved effective in inhibiting the polyprotein processing activity of the NS3/4A protease *in vitro*, as well as effective in clinical trials for HCV GT 1a and 1b infections. This validated NS3 as a therapeutic drug target. Further development and improvement in pharmacokinetics led to the FDA approval of structurally related NS3/4A inhibitors, telaprevir and boceprevir, in 2011[114, 243]. This was a major turning point for HCV therapy: the 1st class of DAAs successfully implemented in clinical treatment. Addition of these new drugs to ribavirin and pegIFN-α treatment regimens increases SVR rates by 20-39% for previously untreated as well as treated HCV patients who had not achieved SVR, especially with chronic HCV GT 1 infections[244-248]. Boceprevir and telaprevir bioavailability can be affected since

they act as substrates for host cytochrome P-450 (CYP) isoenzyme 3A4 and P-glycoprotein proteins, and thus are associated with adverse reactions and variable efficacy[245, 249]. Other disadvantages included a narrow genotype specificity and a low barrier to emergence of resistant mutants that disrupt inhibitor binding but maintain replicative fitness[114, 250]. Similar protease inhibitors introduced as a second wave of 1st generation NS3/4A inhibitors were found to have similar problems, including Asunaprevir, Paristaprevir and Simeprevir [243, 251-254]. In addition, clinically relevant cross-reactive resistance conferring mutations to the first wave were observed, with some single mutations like the NS3 R155 mutant conferring up to 1000-fold loss in drug potency[255]. To maximize SVR rates, the development of 2nd generation proteases inhibitors like Grazoprevir and ACH-2684 was aimed at improving resistance profiles against the pre-existing replicatively fit mutants, and increasing pangenotypic activity. The evaluation of these and several next-generation protease inhibitors in combination therapy with other DAAs show promising early clinical profiles, with SVR12 rates of up 95% and 100% for select GT 1 and 4 patients, respectively[255].

1.4.1.2. NS5B polymerase Inhibitors

Another validated therapeutic drug target is the NS5B RdRp. Two classes of clinically relevant NS5B inhibitors have been in development: nucleos(t)ide analogs and non-nucleoside inhibitors (NNIs and NIs)[114, 160].

Nucleos(t)ide analogs mimic natural nucleos(t)ides and characteristically bind to the catalytic active site of the enzyme[256]. These are considered prodrugs as they are converted to the active 5'-triphosphorylated form in the cell, and act through competitive inhibition, causing chain

termination in the newly synthesized HCV RNA. They exhibit potent antiviral activity with a high barrier for resistance, and are pangenotypic across the various HCV genotypes[257]. The concept for nucleoside analogs came from the success observed in treating HIV, hepatitis B and herpes virus infections[258]. As these viruses encode polymerase enzymes with DNA synthesis activity, their respective clinically relevant nucleoside drugs mimic deoxynucleosides. For greater specificity for the HCV RdRp, structural modifications such as changes at the ribose 2'-position would advantageous in the development of HCV nucleoside analogs[259]. Following screening of various nucleoside analog libraries, the class of 2'-C-methyl nucleosides were identified in cell culture to be potent inhibitors of HCV replication[260-264]. Sofosbuvir, a uridine analog prodrug, was the 1st NS5B polymerase inhibitor approved for clinical treatment[256]. This achieved 80-90% SVR in treatment naïve HCV GT 1, 2 and 3 infected patients, in the absence of interferon. This is a major improvement due to the adverse effects that had limited tolerance of IFN-based treatment therapies.

Non-nucleoside analogs differ in that they bind to other allosteric sites of NS5B, and are thought to exert their inhibitory effect by altering conformational dynamics of NS5B that govern the transition from initiation to elongation steps in RNA synthesis[114]. While these inhibitors tend to be specific to HCV GT 1, they only offer a low barrier to the emergence of resistance[161]. There are 4 types of NNIs that are under development: Thumb I and II, Palm I and II inhibitors[114]. Each type is represented by different classes of chemical compounds. Thumb site I and II inhibitors are characterized by benzimidazole and thiopene-based scaffold, respectively, while benzothiadiazine and benzofuran-based inhibitors dominate the Palm site I and II inhibitors, respectively[114, 161]. Crystal structures of NS5B in complex with thumb site I and II inhibitors, like BMS-791325

(Beclabuvir; FDA-approved) and GS-9669, respectively, confirm that they bind at different sites distal to the polymerase active site and subsequently elicit predominantly distinct resistant conferring mutations[265-268]. Palm site inhibitors bind in close proximity to the active site, as evidenced by their associated resistance conferring mutations[269, 270]. Thus, they are suggested to exert their inhibitory affect at the level of initiation by interfering with nucleotide incorporation. Clinical trials are on-going for several of these potent NNIs, and Dasabuvir (ABT-333) is currently the only clinically-approved NS5B palm inhibitor (site II), specifically for genotype I infections[114, 161].

1.4.1.3. NS5A-DI Inhibitors

Unlike the NS3 protease and NS5B RdRp, NS5A lacks any enzymatic activity, hampering the development of assays for drug discovery. Cell culture based *in vitro* assays allowed for the identification of specific NS5A inhibitors that target domain I[271]. This led to the development and approval for clinical use of the first pangenotypic NS5A inhibitor, Daclatasvir, and others in this family[272-274]. Several models predict that inhibitors like Daclatasvir adopts a symmetric binding mode to a dimeric protein structure[275, 276]. The high potency is likely due to the multifunctionality of NS5A at various stages of the HCV life cycle, as well as inhibition of the membranous web formation[277, 278]. However, these inhibitors have a low barrier to resistance and emergent resistant variants have been identified that overcome this inhibitory effect[279, 280]. Multi-drug therapy offers a higher barrier to resistance and broader activity across all HCV genotypes[114]. In addition, combination of synergistic inhibitors has been shown to resensitize resistant NS5A variants to Daclatasvir inhibition, an unprecedented activity offering new insight into treatment options of resistant viruses[189, 281]. Taken together, the important future goals for treatment therapy continue

to be the development of pangenotypic regimens. No vaccine is yet available, thus other strategies are also needed that address retreatment of therapy failures and reinfection.

1.4.2. Host-targeting antivirals (HTAs)

HCV viruses exhibit high replication rates which result in a highly diverse quasispecies population, unique to each infected individual. Combination of a very high viral replication rate and a high mutational frequency can lead to the rapid selection of drug-resistance conferring mutations in the presence of DAAs, which is observed both *in vitro* and in patients[282]. An alternative approach to DAAs is the targeting of host-derived co-factors essential for the replication of the virus. Over time, HCV evolved to harness the host cell machinery and host factors for efficient viral replication. Host factors that are identified to be indispensable for viral replication make for attractive drug targets[283, 284]. HTAs display a higher barrier to resistance and pangenotypic activity, favourable features shared by the more potent clinically approved DAAs (i.e. Sofosbuvir)[114]. Targeting host factors offer hope for salvage therapy for patient treatment failures since they offer an alternate or complementary mechanism of action to DAAs, as well as possible cost-effective treatments[285, 286]. Caution is needed as limitations can exist due to potential adverse effects of inhibiting host factors required for normal cellular functions[283]. Several HTAs have been developed from preclinical to clinical phases, targeting factors involved in HCV cell entry, translation, replication and assembly[284].

HTAs targeting HCV entry. Host-targeting entry inhibitors have been developed that target factors expressed on the cell surface. A proof-of-concept was established with peptides that bind and inhibit interaction with heparan sulfate moieties that represent the initial site of HCV

attachment to the host cell[284]. Small molecules like ITX5061 that targets the SR-BI cell surface receptors as well as antibodies against several receptors used for viral cell entry were found to be effective at clearing persistent HCV infection[287-290]. EGFR is an essential co-factor for the formation of CD81-CLDN1 co-receptor complex, and HCV phase I and II clinical trials are underway to evaluate the efficacy of erlotinib, a clinically licensed EGFR inhibitor in cancer therapy[284].

HTAs targeting HCV translation. The HCV translation process represents a novel target for HTA development because HCV translation does not utilize canonical factors. For instance, HCV translation is mediated through the 5' UTR IRES interaction with the 40s ribosomal subunit, and factors involved in initiation of this process are limited to eIF2, eIF3 and eIF5[30, 291-295]. A small RNA analogous to the specific regions of the IRES stem loop III that mediate interaction with the 40s subunit were effective at inhibiting HCV translation *in vitro*, however additional studies are needed to validate their efficacy *in vivo* [296]. The microRNA miR-122 has been targeted for HTA development as it is a crucial component for HCV translation and replication. Miravirsen, an antisense nucleic-acid modified oligonucleotide complementary to the 5'end of miR-122, was shown to effectively sequester miR-122, leading to a prolonged decrease in HCV viremia[297-299]. However, treatment responses were varied. In one study, a resistance-conferring mutation arising in the 5' UTR led to a rebound in HCV infection amongst all the patients treated[298, 300]. This points to a low barrier to resistance, suggesting that additional studies are required to establish the clinical implications of the use of miR-122 specific inhibitors.

HTAs targeting HCV replication. Cyclophilins (Cyps) have been implicated in a diverse number of clinically relevant pathogens, including HCV[301]. Cyps are characterized as a group of proteins with peptidyl-prolyl *cis-trans* isomerase activity that bind to proline-rich regions and catalyze the *cis-trans* isomerization of peptide bonds preceding a proline residue[302-304]. Cyps therefore play a crucial role in the proper folding of cellular proteins, and act as protein chaperones within the cellular matrix. Although a total of 16 isoforms have been identified in humans[305], CypA was found to be an essential host factor for HCV replication that interacts with NS5A-DII[179, 202]. Additionally, interaction with NS5A-DII is mediated through the CypA active site[202, 306]. Inhibitors of this interact suppress viral replication and are potential treatment options for HCV-induced hepatocellular carcinoma[307, 308]. The immunosuppressive drug Cyclosporine A (CsA), a cyclic peptide, was found to bind to Cyps and effectively inhibit their isomerase activity (Fig. 1.11)[309]. Further studies revealed that by inhibiting CypA activity, CsA could successfully inhibit HCV replication. However, the Cyp-CsA complex formed also binds to and inhibits calcineurin phosphatase activity, an important component in the signal transduction pathway for T-cell activation, thus suppresses the proliferation of T-cells[310]. This necessitated the development of CsA analogs lacking immunosuppressive properties, all the while able to exhibit highly potent *in vitro* and *in vivo* activity (Fig. 1.11)[205-209, 285, 311, 312]. They inhibit the interaction of CypA with NS5A-DII, as well as potentially restore host innate immunity[313]. While these inhibitors bind to CypA, mutations that confer the highest level of resistance to CypA inhibitors were identified within NS5A-DII: D320E and Y321N in HCV GT 1b[314, 315]. The presence of these mutations was shown to rescue the replication of the virus in *in vitro* cell culture studies[314]. It is often a common phenomenal that the emergence of resistance conferring mutations within a site of protein-protein interaction render the inhibitor ineffective and restore protein binding. Previous studies into the

mechanism of resistance of these mutations showed that they did not render the interaction between CypA and NS5A-DII resistant to inhibition by CsA and analog inhibitors[316]. The exact mechanism of action has yet to be determined. Alisporivir, the most advanced CypA inhibitor in development, was shown to drastically diminish HCV viral loads in patients, with low levels of viral outbreak[317]. Alisporivir monotherapy and alisporivir + RBV therapy were able to achieve SVR12 in 81% and 83%, respectively[318]. However, due to several serious adverse events of acute pancreatitis in phase 2 and 3 clinical trials, including one fatal case documented where patients were treated with Alisporivir in combination with pegIFN and ribavirin, these clinical studies were placed on hold in 2012[307]. It is well worth noting that in these studies, safety profiles were markedly improved in pegIFN-free versus pegIFN + alisporivir combination treatment regimens[319]. In light of this, extensive preclinical and clinical analyses subsequently demonstrated that although pancreatitis was observed, Alisporivir was not the causative agent[320]. To date, no pancreatitis has been observed in patients treated with Alisporivir-containing treatment regimens, in the absence of pegIFN. New classes of small-molecule CypA inhibitors have also been developed, with increased specificity to limit off-target effects[321].

Structurally distinct CypA inhibitors have been developed that exert potent antiviral effect against HCV. Unlike CsA, molecules of the Sanglifehrin (SF) family -SFA to SFD - are non-cyclical, but naturally occurring CypA-binding polyketides produced by the soil bacterium Streptomyces sp. A92-308110 which effectively inhibit CypA isomerase activity[207, 322]. Amide derivatives, like Sangamides, have been shown to exhibit potent pangenotypic antiviral activity *in vitro* against HCV infection, as well as synergistic activity in combination with DAAs[323]. In addition, fragment-based drug design is effective in the generation of small non-peptide inhibitors

that specifically target CypA *cis-trans* isomerase activity, and inhibit Con1b HCV replication *in vitro* with limited cytotoxicity[321, 324].

HTAs against HCV assembly. Other HTAs are in clinical development that target host factors involved at the level of viral assembly. Use of specific CKII inhibitors to target the phosphorylation-mediated interaction of NS5A with the core protein on LDs was shown to drastically inhibit HCV JFH1 virion production (GT 2a) *in vitro*[325]. On the contrary, CKII inhibition by siRNA or chemical inhibitors led to minimal effect or enhanced H77S virus production (GT 1a), respectively[326]. Understanding specific differences among the HCV genotypes should be considered in subsequent clinical application for this class of inhibitors. Other avenues include (but not limited to) antiviral agents that target and inhibit factors involved in host cell lipid metabolism[327], and transcription factors like hepatocyte nuclear factor 4α (HNF4α) essential for the very low density lipoprotein (VLDL) secretion pathway that is hijacked for HCV egress[38, 39, 328]. This is just the tip of the iceberg, and HTAs still remain a viable option. Potential synergistic and additive effects of HTA/DAA combination therapy offer a proof-of-concept for promising pangenotypic treatment options for HCV infection[284, 329].

1.5. OBJECTIVES

While vaccines do not currently exist for a myriad of virus-mediated infections, the development of antiviral therapies has flourished. The aim has been to initiate early treatment with antiviral therapies to prevent rapid deterioration and enable effective management of the infection. When I started my doctoral research in 2011, the first DAAs targeting the NS3/4A protease were approved for the treatment of GT 1 HCV infections, in combination with pegIFN-α and ribavirin.

This greatly improved cure rates to 70%, but single resistance conferring mutations rapidly emerged that suppressed the inhibitory effect of these first generation DAA treatment[330, 331]. Also, the lack of efficacy across all HCV genotypes meant that not all patients responded equally well to the treatment. Fast forward 6 years and major discoveries in structure-function analysis of various HCV proteins have facilitated breakthroughs in the targeted design of HCV therapeutics. Current generations of DAAs feature pangenotypic activity and are able to achieve SVR rates of >90%, in IFN-free therapy regimens for a growing patient population. While treatment options have drastically improved and multiplied, there are still many aspects of the HCV life cycle that remain unknown. NS5A is an essential component of the HCV replication complex, whose numerous protein functions still need to be elucidated. In comparison to the highly structured domain I, the unstructured nature of domains II and III remain an obstacle in understanding their function in the HCV life cycle. As such, extensive studies have been hampered which limit our knowledge of the full functional roles of this protein and its respective interactions. In the case of domain II of NS5A, its importance in various steps of the viral cycle and multiple NS5A protein interactions is irrefutable, but there are many questions that have yet to be explored.

Host factors are attractive drug targets, particularly on account of their higher genetic barrier for the development of resistance. Such a candidate co-factor is CypA, inhibitors of which have been shown to completely abrogate the replication of the virus. A direct association has been shown between NS5A and CypA. This interaction is sensitive to Cyclosporine-A (CsA) and CsA-analogues, which include Alisporivir (Debi025) and NIM811. Low-level resistance-conferring mutations have been identified both *in vitro* and in clinical samples within the NS5A protein, as well as in other HCV proteins. The mechanism of resistance and mechanism of inhibition both still

remain unclear. Insights into linkage between drug resistance mutations and their effects on viral fitness would shed light on the pathways employed by HCV for the emergence of resistance, as well as the efficacy of host-targeting antivirals in future multi-drug treatment regimens.

In Chapter 2, we hypothesize that CypA- inhibitors may not adopt a classical mode of inhibition exhibited by DAAs. We also hypothesize that the D320E and Y321N mutants may alter the interactions of NS5A with other HCV viral proteins. NS5A also interacts with several core components of the HCV replication complex, including the NS3 protease/helicase and NS5B polymerase. Presence of CsA resistance mutations within NS5A may modulate the functions of NS5A and its interactions with the viral RNA. Hence, our objective was to investigate into the binding kinetics between NS5A-DII resistant variants and CypA, and to evaluate the effect that CsA and CsA-analog inhibitors have on NS5A-CypA interaction. Next, we sought to evaluate the effect of the D320E and Y321N resistance mutations on the interactions of NS5A with other core components of the HCV replication complex.

Once the mechanism of resistance to CypA inhibitors had been established, we then wanted to determine whether the residues involved in resistance to CypA-inhibitors play a role in modulating the dynamic interactions between NS5A-DII and crucial viral and cellular components of the replication complex. In Chapter 3, the initial objective was to focus on the interaction between NS5A-DII and CypA, as well as model RNA molecules. Next, we explored the complex intermolecular interactions between NS5A-DII and NS5B, a viral protein that shares a common binding site with CypA on NS5A-DII. Ultimately, we aim to develop a detailed picture of the NS5A-DII interactions with various components involved in HCV replication, and expounded on

our understanding of the functional importance of these interactions, in relation to HCV replication.

Table 1. 1: Table of clinically approved antivirals for HCV treatment, up until 2017

Year of approval for clinical use	pegIFNα (P) + Ribavirin (R)	DAAs:			Host Targeted Antivirals	GTs treated
		NS3 protease	NS5B	NS5A		
2011	+ [a]	Boceprevir + [a]				1
"	+	Telaprevir +				1
2013	+	Simeprevir +				1
"	+		Sofosbuvir +			1, 4 (+P, +R), 2, 3 (+R)
2014 - 2015			Sofosbuvir +	Ledipasvir		1, 4, 5, 6
2014		Simeprevir +	Sofosbuvir			1
"		Paritaprevir +	Dasabuvir +	Ombitasvir +	Ritonavir	1
2015			Sofosbuvir +	Daclatasvir +		1, 3
"		Paritaprevir +		Ombitasvir +	Ritonavir	4
2016		Grazoprevir		Elbasvir		1,4
"			Sofosbuvir	Velpatasvir		1, 2, 3, 4, 5, 6

[a] Used in combination with other antivirals

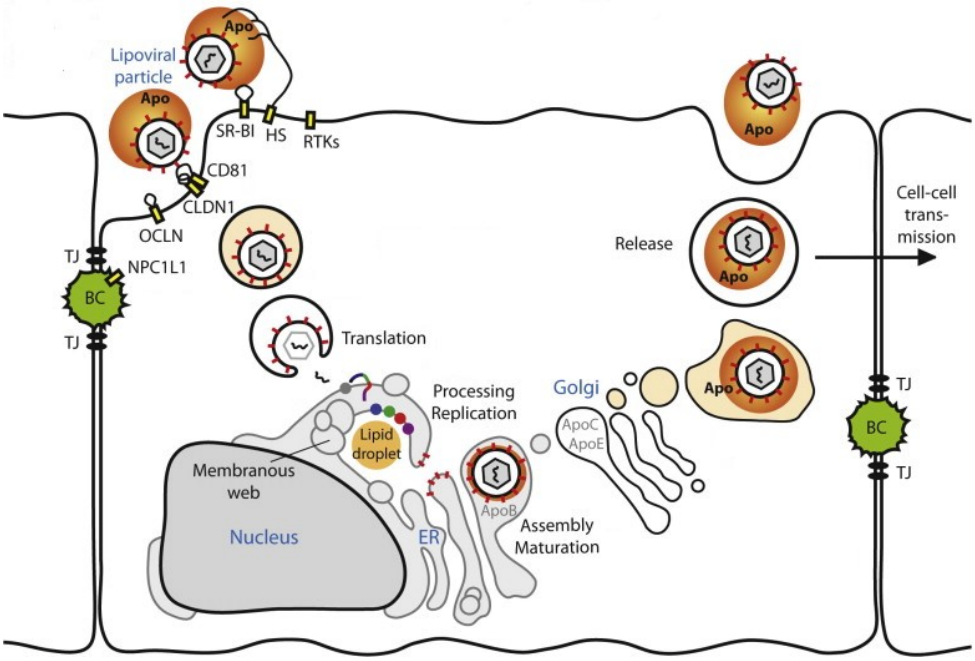


Figure 1. 1: Life Cycle of HCV adapted from[313].

HCV cell entry via several cell surface receptors and host factors (RTKs, receptor tyrosine kinases; SR-BI, scavenger receptor BI; CD81, cluster of differentiation 81; CLDN1, claudin-1; OCLN, occludin; NPC1L1, Niemann-Pick C1-like 1 cholesterol absorption receptor), translation, replication in the endoplasmic reticulum (ER), and viral assembly and release using host factors (apo, apolipoprotein; BC, bile canaliculus; TJ, tight junction) are depicted.

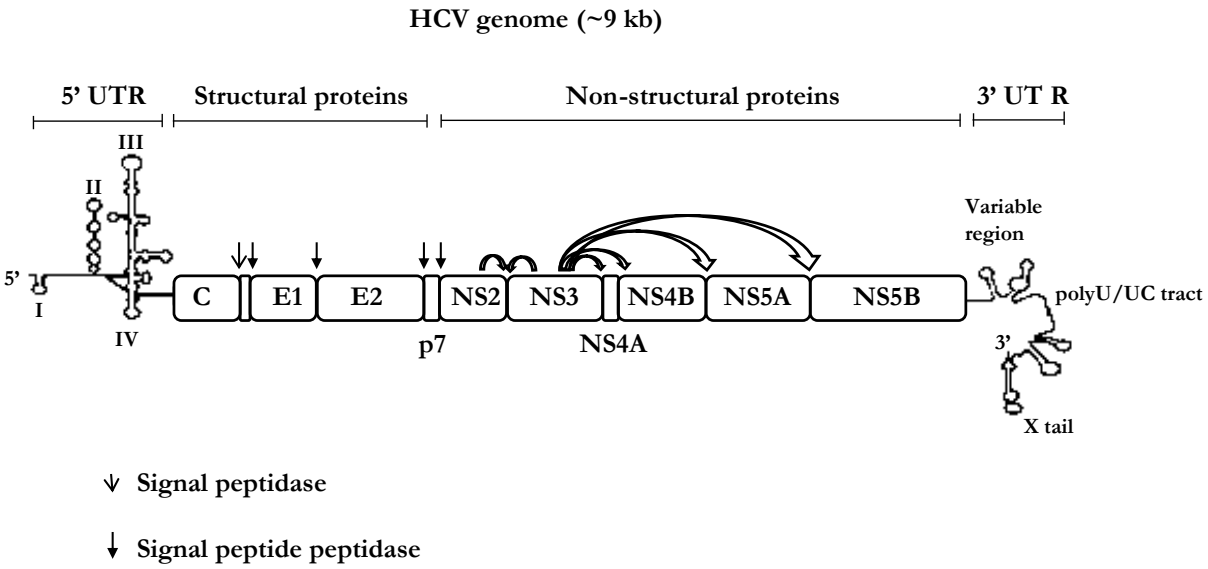


Figure 1. 2: HCV Genome organization adapted from[332].

The HCV genome encoding a single polyprotein, and the predicted secondary structures of the 5′ and 3′ UTRs. Polyprotein cleavage is achieved by cellular signal peptidases (open arrow), signal peptide peptidase (closed arrow), and viral proteases (curved arrows).

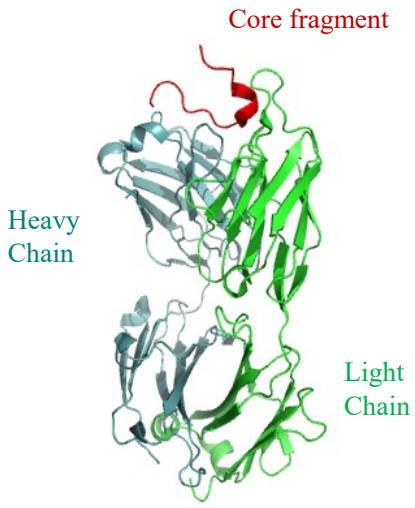


Figure 1. 3: Crystal structure of residues 25-40 of the HCV core protein in complex with a monoclonal antibody (PDB 1N64).

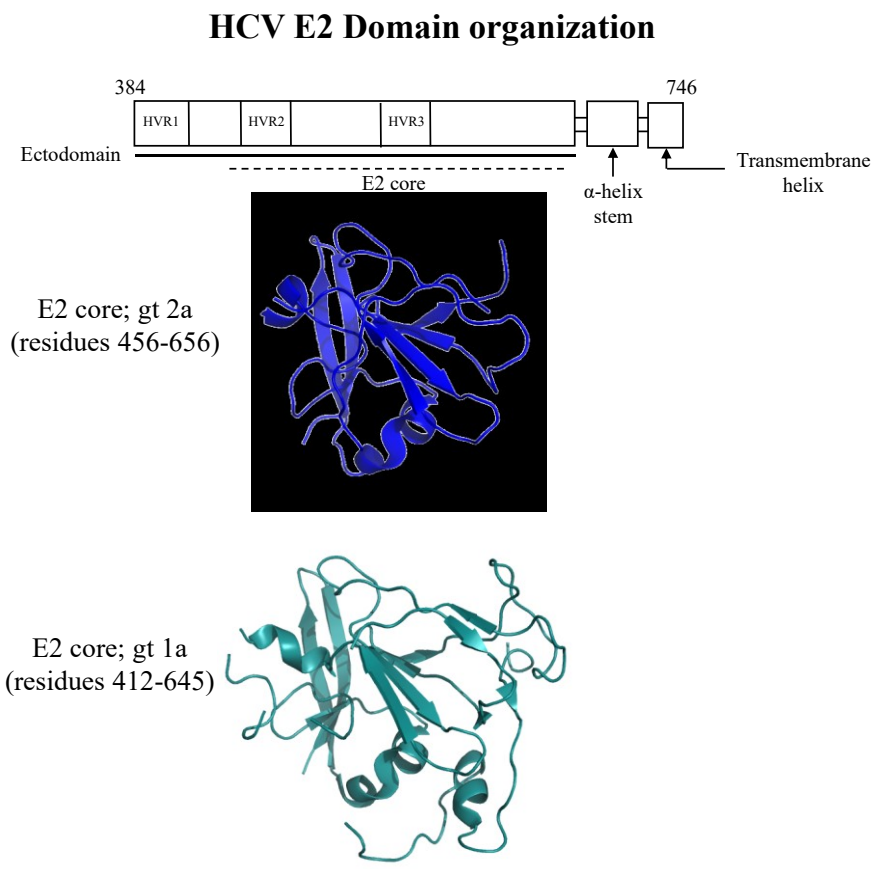


Figure 1. 4: HCV E2 domain organization, and symmetrical, partial crystal structures from GT 1a (PDB 4WMF) and GT 2a (PDB 4WEB).

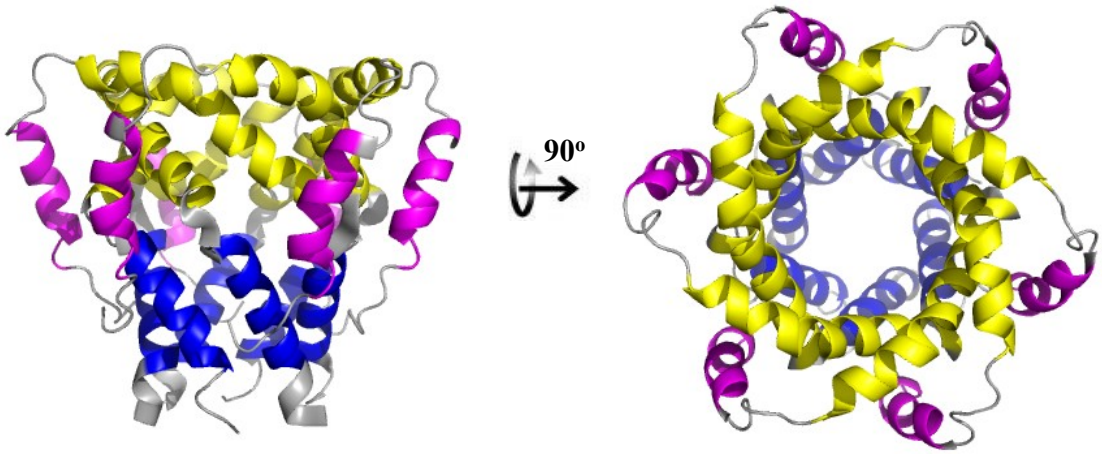


Figure 1. 5: NMR structure of HCV p7 hexamer structure (PDB 2M6X)[88].

In each monomer, the N-terminal, middle and C-terminal helices are shown in blue (H1; residues 5-16), yellow (H2; residues 20-41) and magenta (H3; residues 48-58), respectively.

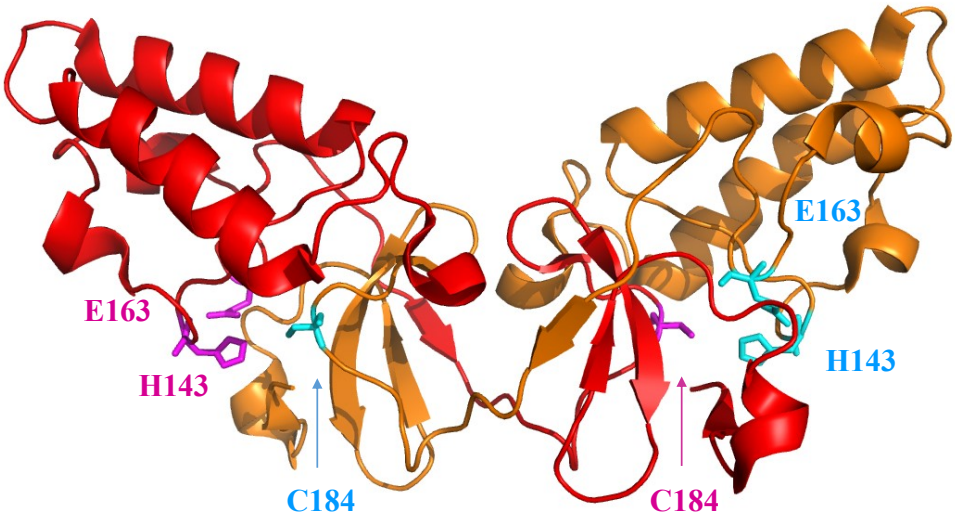
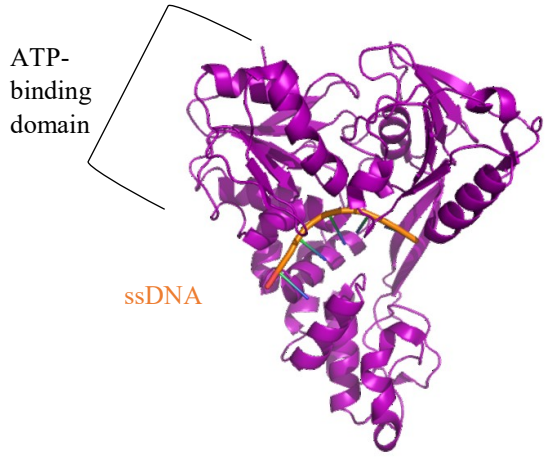


Figure 1. 6: Crystal structure of the catalytic domain of HCV NS2 protease (PDB 2HD0). NS2 protease ($NS2^{pro}$) dimer is shown, with one monomer in red and the other in orange. The protease catalytic residues H143, E163 and C184 encoded by the first and second monomer structure are indicated in magenta and cyan, respectively.

A.



B.

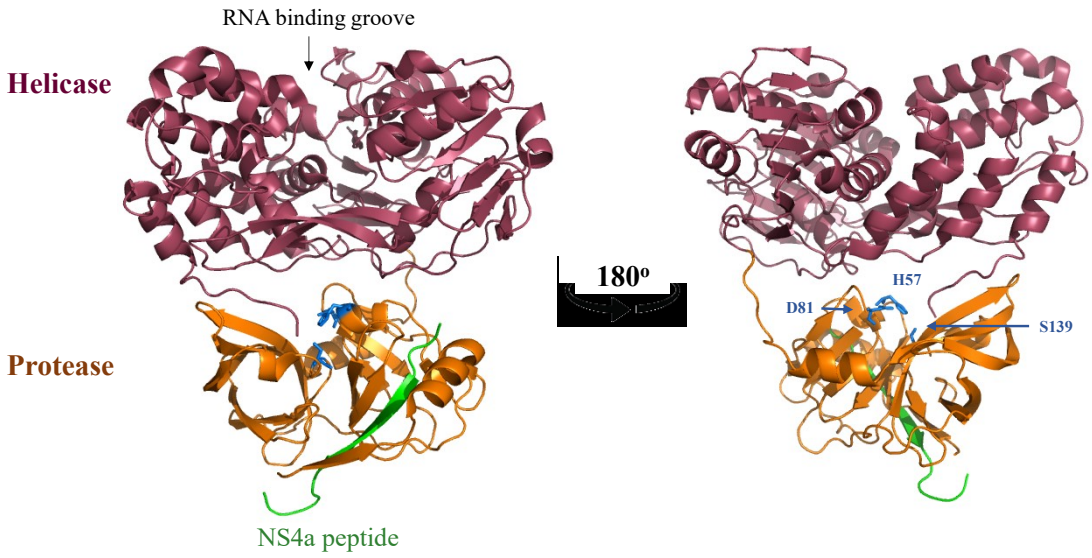


Figure 1. 7: NS3 helicase-protease crystal structures.

(A). Superposition of three NS3 helicase crystal structures (PDB 3KQK, 3KQN, 3KQL), in complex with a single-stranded DNA substrate (ssDNA). The arrow indicates the helicase ratchet translocation mechanism. The ATP-binding domain, required for NS3h activity, is shown. ***(B).*** Crystal structure of the full-length NS3 protein, with the NS4A peptide (shown in green) bound in the protease domain (PDB 4B6E). The catalytic residues that form the NS3 protease active site (H57, D81 and S139) are labeled.

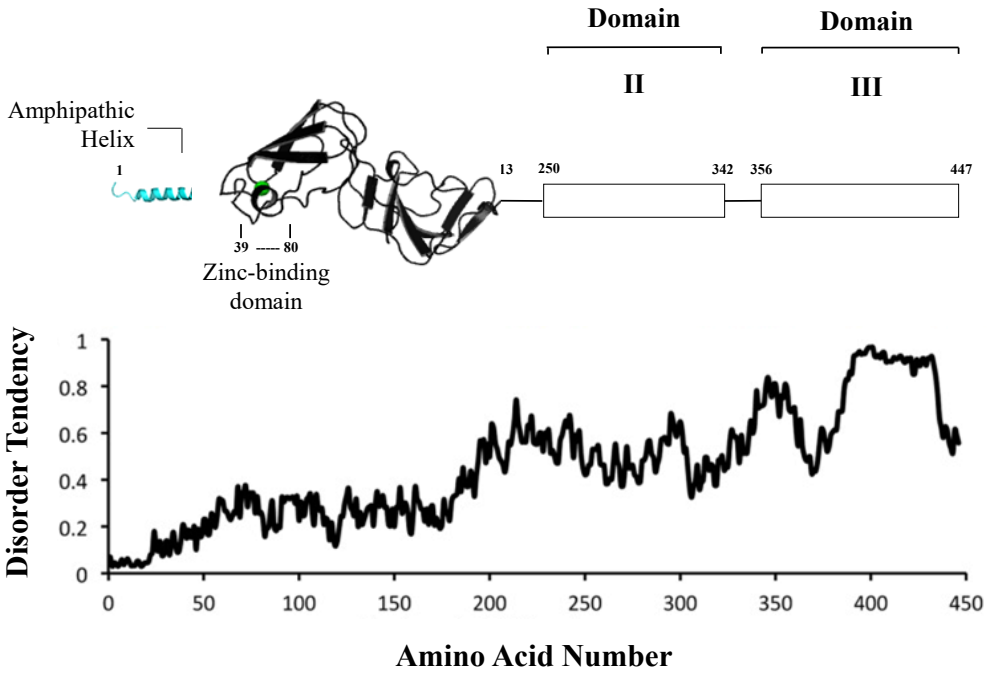


Figure 1. 8: Domain organization of HCV NS5A.

The propensity for disorder within each domain is adapted from[333].

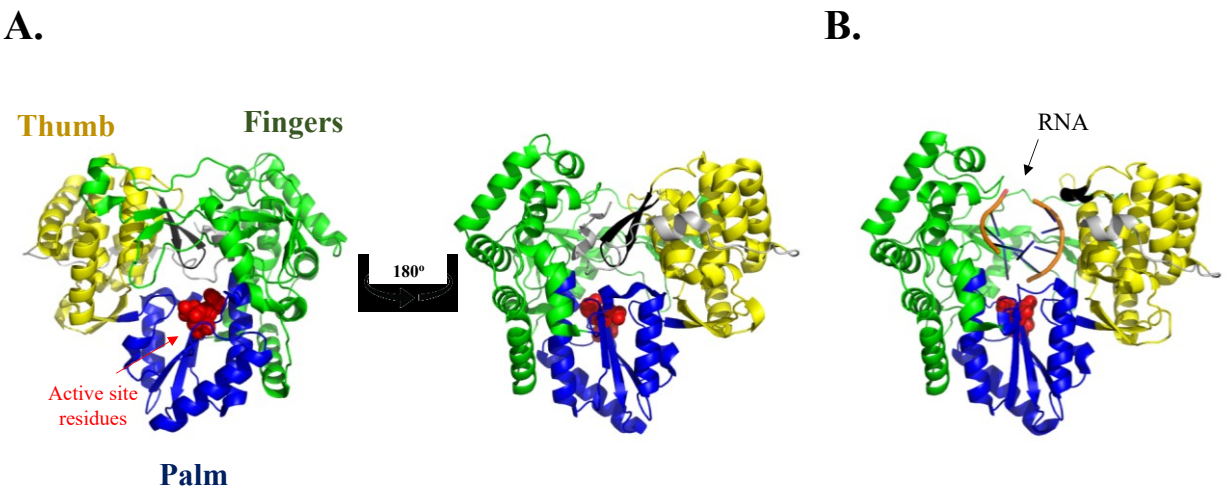


Figure 1. 9: Crystal structure of NS5B RNA-dependent RNA polymerase from GT 1b and 2a (RdRp).

(A). Crystal structure of the NS5B (Δ21) derived from GT 1b (PDB 1C2P). Classical fingers thumb and palm subdomains are depicted in green, yellow and blue, respectively, and the C-terminus in grey. Active site residues D220, D319 and D220 are located in the palm subdomain (shown in red). A unique feature of HCV NS5B is the β-hairpin loop (shown in black), which partially blocks the exit site of nascent RNA templates. ***(B).*** Crystal structure of NS5B (Δ21) derived from GT 2a, in complex with a short RNA substrate (PDB 4E78). For crystallization, the β-hairpin loop was replaced with a glycine-glycine linker.

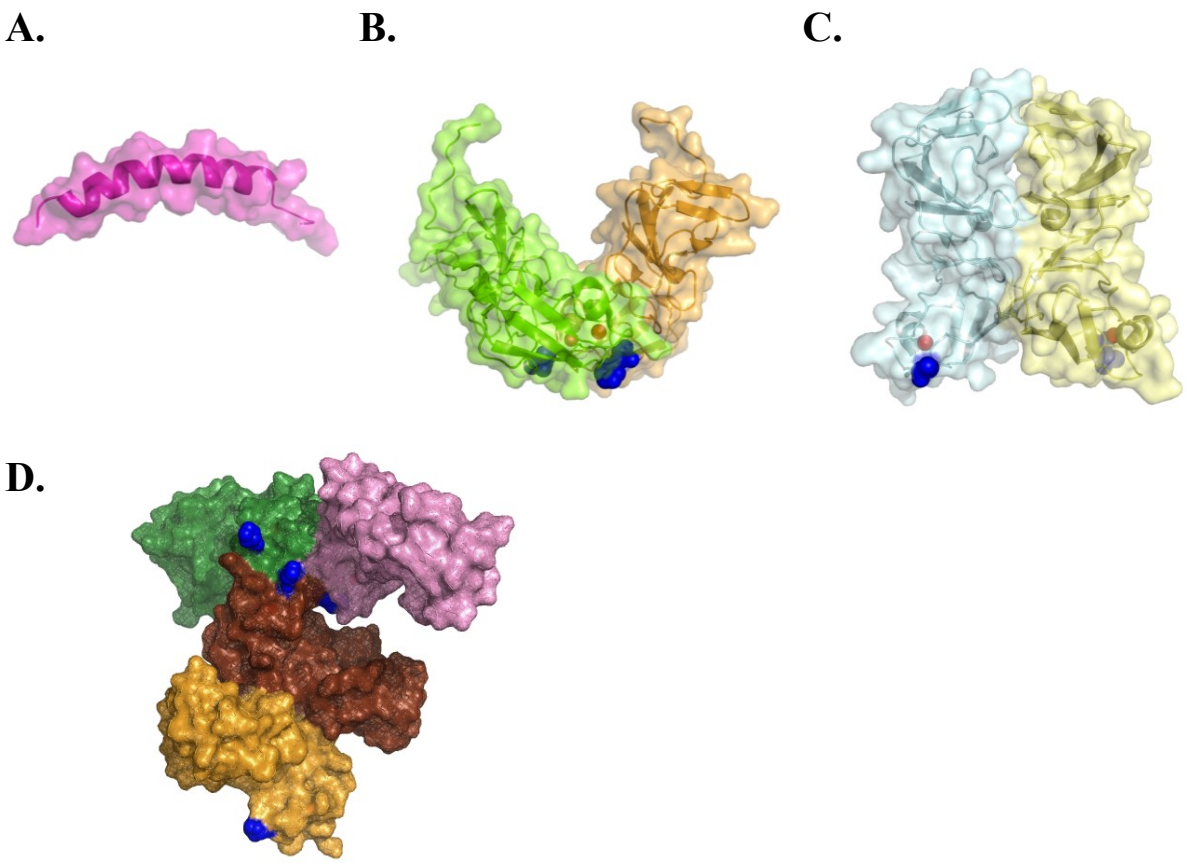


Figure 1. 10: Comparative crystal structures of NS5A DI oligomeric states.

(A). Crystal structure of the N-terminal amphipathic helix of NS5A (1–31) (PDB 1R7G). ***(B).*** The "open" conformation of NS5A-DI, residues 36–198, from GT 1b, depicting an RNA-binding groove (PDB 1ZH1). ***(C).*** The "closed" conformation of NS5A-DI, residues 32–191, from GT 1b (PDB 3FQQ). ***(D).*** The multimeric conformation of NS5A-DI, residues 33–202, from GT 1a (PDB 4CL1). In each crystal structure, the coordinated zinc ions (shown in red) and the N-terminal amino acid (shown in blue) in each monomer are indicated.

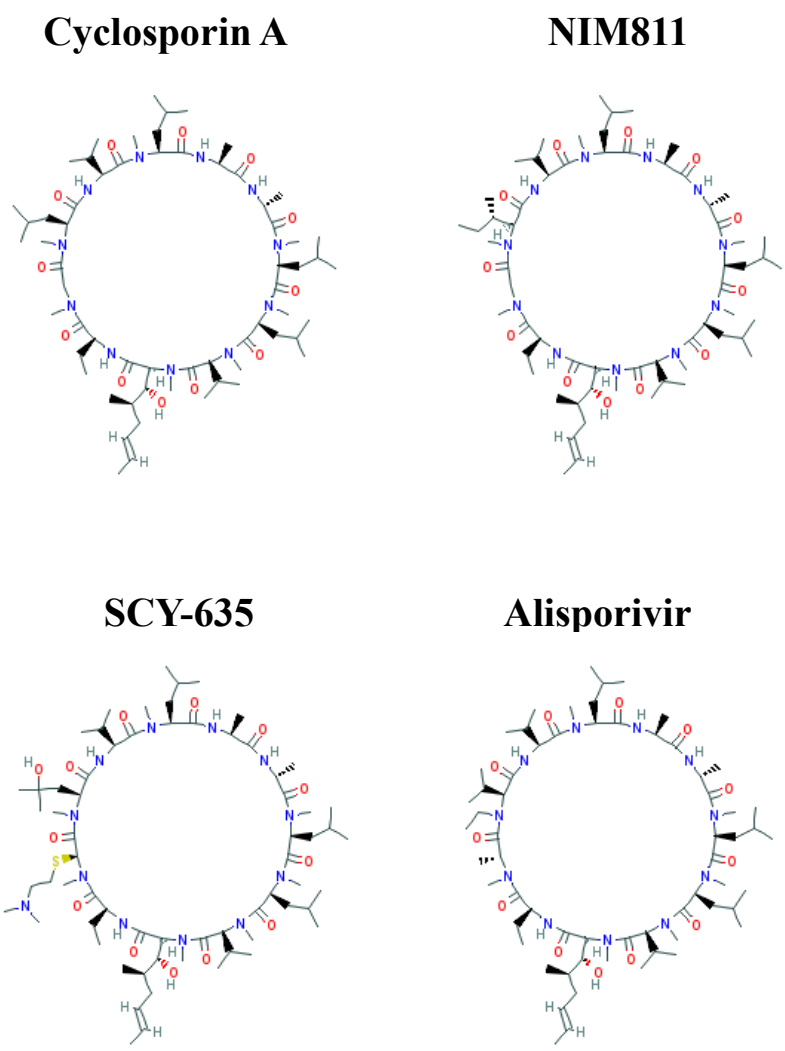


Figure 1. 11: Structures of CypA Inhibitors.

2D Chemical Structures for Cyclosporine A[334] and three non-immunosuppressive derivatives; NIM811[335], Alisporivir[336], and SCY-635[337].

CHAPTER 2: DECREASED DISSOCIATION OF HCV NS5A AND CYCLOPHILIN A PROVIDES A MECHANISM OF RESISTANCE TO CYCLOSPORINE A

This chapter was adapted from an article authored by M. Ngure, M. Issur, J. Rickard, H.-W. Liu, G. Cosa and M. Götte that is to be submitted to ***Journal of Biological Chemistry.***

2.1. Abstract

CypA is a cellular factor that is essential to hepatitis C virus (HCV) replication. Cyclosporine A (CsA) and derivatives can form a complex with CypA, which, in turn, mediate antiviral activity. CsA and related compounds select for resistance-conferring mutations D320E and Y321N in domain II of the HCV NS5A protein (NS5A-DII). However, the underlying mechanism of resistance is unknown. Previous data have indicated that CsA-resistant (CsA^{res}) NS5A-DII variants bind as efficiently to CypA as the wild-type protein. Here we devised a Förster Resonance Energy Transfer-based (FRET) assay to study real-time binding and dissociation of the NS5A-DII complex with CypA. Our data show that the addition of CsA causes signal reductions, which is indicative of complex dissociation. CypA and the HCV NS5B RNA-dependent RNA polymerase are known to bind to overlapping regions of NS5A-DII and we demonstrate that the presence of NS5B also leads to complex dissociation. Most importantly, the D320E and Y321N mutations in CsA^{res} NS5A-DII show slower dissociation kinetics of the complex with CypA when compared with the wild-type. The observed decrease in the rate of complex dissociation correlated with an increase in the level of resistance conferred by D320E, Y321N, and the double mutant, respectively. Together, these data provide a possible biochemical mechanism of resistance to CypA inhibitors.

2.2. Introduction

HCV is a positive-strand RNA-virus and an important human pathogen. Chronic infection with the virus is associated with an increased risk of severe liver disease [338]. The discovery of DAAs that target viral proteins has markedly improved the success in treatment of the infection [167, 339]. Host factors are alternative drug targets that are often associated with a high barrier to the development of resistance [340].

CypA is a 23 kDa ubiquitously expressed cellular peptidyl-prolyl isomerase (PPiase) that catalyzes the *cis/trans* isomerization of peptide bonds at proline residues [341]. Mutations within the active site of CypA, e.g. H126Q, render the enzyme inactive and block HCV replication, which points to an important role of the isomerase activity [342]. Previous studies have shown that CypA plays crucial roles in the life cycles of HCV and the human immunodeficiency virus (HIV) [343]. CypA interacts with the capsid (CA) domain of HIV Gag, while in HCV CypA interacts with the HCV NS5A [301, 344, 345].

NS5A is an RNA-binding protein that is part of the HCV replication complex. It is divided into three domains, separated by low-complexity linker regions (Fig. 2.1A). While domains I and II are essential for HCV genome replication, domain III is required for the production and release of new virus particles [172, 221]. Domain I of NS5A has been characterized in great detail and represents a target for the first in-class NS5A inhibitor daclatasvir [271]. This domain is structured and binds RNA [185, 187]. Although domains II and III remain largely unstructured [129, 346], CypA was shown to bind to proline-rich regions of domain II (NS5A-DII). Specifically, proline residue P314

in HCV GT 1b has been identified as a critical residue in this regard [347]. CypA was also shown to enhance the RNA binding properties of NS5A, presumably via NS5A-DII [200].

CypA inhibitors such as CsA and CsA analogues interfere with the NS5A-CypA interaction [348, 349]. Several CsA analogues, namely alisporivir, NIM811 and SCY-635 among others, show potent *in vitro* and *in vivo* antiviral activity, highlighting their potential for use in HCV treatment [206-208, 350, 351]. Although the main target of these inhibitors is CypA, the most relevant resistance-conferring mutations are located at positions 320 and 321 in HCV GT 1b NS5A-DII [203]. In GT 2a NS5A, resistance to CypA inhibitors has been mapped to the corresponding residues 316 and 317 [203].

Mutations D320E and Y321N in Con 1b NS5A-DII were shown to reduce susceptibility to CypA-inhibitors in *in vitro* cell-based replicon systems [203, 352]. However, these mutations did not render the interaction between CsA^{res} NS5A-DII and CypA resistant to CsA, as observed in pull-down studies where a similar inhibition in binding to CypA was observed for wild-type and CsA^{res} NS5A-DII [353]. This indicates that the interaction between CypA and NS5A-DII remained sensitive to CsA. Thus far, previously described protein binding assays have not been able to capture the resistance phenotype observed in cell culture-based studies. In attempts to shed light on the mechanism of HCV resistance to CypA inhibitors, we developed an *in vitro* FRET-based assay to monitor binding and dissociation of the NS5A-DII complex with CypA. Our FRET-based binding assay enables the use of a scaled-down approach to investigate into the real-time protein-protein interaction. This data provides more specific qualitative information related to changes to the protein-protein complex in the presence of inhibitor, CsA, which have not been previously described. We show that in the presence of CsA, the CsA-resistance conferring mutations D320E

and Y321N in NS5A-DII decrease the rate of dissociation of the protein-protein complex. Decreases in drug sensitivity correlate with decreases in the rate of protein-protein complex dissociation, which provides a plausible mechanism of HCV resistance to CypA inhibitors.

2.3. Experimental procedure

Materials. Cy3 and Cy5 mono-reactive maleimide fluorescent dyes were purchased from GE Healthcare Life Sciences (Quebec, Canada). Cyclosporine A (CsA) was purchased from Enzo Life Sciences (New York, USA). CsA analogues NIM811 and Alisporivir were kindly provided by Novartis (Basel, Switzerland). CsA and analogue compounds were stored at −20°C as 20 mM ethanol stock solutions until used in the assay. Daclatasvir was purchased from Selleck Chemicals (Texas, USA).

Expression and purification of NS5A-DII and CypA. Amino acid residues 250-342 of the NS5A protein from the HCV Con1b strain were cloned into a modified pET-SUMO expression vector, with a N-terminal small ubiquitin-related modifier (SUMO) peptide and a C-Terminal peptide containing 6 tandem histidine residues. This region is effectively all of domain II of NS5A. The expression of SUMO-tagged NS5A-DII was carried out in Rosetta BL21 (DE3) *E. coli*. A custom-made DNA sequence encoding for human CypA (NCBI Reference Sequence: NM_021130.3) was purchased from IDT and cloned into the pet28b expression vector. CypA was expressed in the BL21-DEA3 *E. coli*. A 1L culture of transformed *E. coli* Rosetta/pET-SUMO-NS5A-DII and BL21-DEA3/pET-28b-CypA cells were grown at 37 ºC in Terrific Broth and LB media, respectively. When A_{600} reached 0.6, protein expression was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 3hrs at 22 °C. 2% ethanol was added for

NS5A-DII protein expression. Cells were harvested by centrifugation and suspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM BME and 0.1% NP-40). Cell lysis was achieved with the addition of lysozyme (50µg/ml), followed by sonication. Both proteins were purified by Ni^{2+} affinity chromatography and eluted with buffer containing increasing concentrations of imidazole. All fractions were analyzed on a SDS-PAGE for purity. NS5A-DII fractions were pooled and buffer exchanged into a phosphate buffer (10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0) using a HiTrap desalting column (GE Healthcare). A second purification was performed for NS5A-DII by affinity purification with a HiTrap Heparin HP column (GE Healthcare) and proteins were eluted with phosphate buffer containing increasing concentrations of NaCl (maximum 1M). Final protein fractions were pooled and concentrated with an Amicon concentrator (10 kDa cutoff) and stored at -20°C in 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM DTT, 50% glycerol. When SUMO-cleaved NS5A-DII was required, NS5A-DII was incubated with the CoolCutter SUMO protease (GeneCopoeia) at 4ºC for 18-20h, prior to the heparin affinity purification. Following heparin affinity purification, both the cleaved SUMO tag and the SUMO protease were eliminated to yield only NS5A-DII.

Expression and purification of NS5B. Expression and purification of recombinant NS5BΔ21 were performed as described previously [154].

Generation of mutant enzymes of NS5A-DII and CypA. Mutant enzymes were generated through site-directed mutagenesis with the Stratagene Quick-Change kit, according to the manufacturer's directions. The presence of mutations was confirmed by sequencing at the Genome Quebec Innovation Center.

Fluorescent Labelling of NS5A-DII and CypA. 8 nmoles of protein were incubated with 2 mM TCEP for 1hr at 4ºC. 32 nmoles of Cy3 and Cy5 maleimide dye were suspended in DMSO and added to NS5A-DII and CypA, respectively, in a labelling buffer containing 50 mM sodium phosphate at pH 7.0, 10 mM NaCl and 1 mM TCEP. The labelling reaction was performed for 1hr at 4ºC to allow for conjugation of the maleimide reactive group of the fluorescent dye to the sulfhydryl group of surface exposed cysteine residues. The excess dye was captured using P-6 micro-spin size-exclusion columns (Bio-Rad). We verified the fluorescently-labelled protein on SDS-PAGE as well as the absence of any residual dye. Subsequent protein and dye concentrations were quantified with the Nanodrop Spectrophotometer, and the efficiency of protein labelling was obtained by calculating the ratio of dye to protein concentration. The labelling efficiencies for Cy3-labelled NS5A-DII (^{cy3}NS5A-DII) and Cy5-labelled CypA (^{cy5}CypA) enzymes were 90-100% and 50-70%, respectively. Proteins were stored at -20°C in 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM DTT and 50% glycerol.

Monitoring Binding through FRET. All FRET assays were performed in a binding buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl and 2 mM DTT. ^{cy3}NS5A-DII (0.2µM) was incubated in the presence of ^{cy5}CypA enzyme concentration gradient for 15 mins at 4ºC. Reactions were prepared in a 96-well plate format and visualized with a SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices). Sunnyvale, CA). The excitation wavelength was set to 520 nm, with a cutoff of 550 nm, and the emission spectra obtained from 550 nm to 710 nm. The relative binding between ^{cy3}NS5A-DII and ^{cy5}CypA was determined as a measure of the energy

transfer (*FRET*) between the Cy3 (donor) and Cy5 (acceptor) fluorophores. This was calculated as follows in *Equation I*:

$$FRET = \left(\frac{F_D - F_{DA}}{F_D}\right)\frac{1}{f_A} \qquad (1)$$

Where (F_D) is the relative fluorescence intensity of the donor in the absence of the acceptor; (F_{DA}) is the relative fluorescence intensity of the donor when the acceptor is present; (f_A) is the percent labelling efficiency of the acceptor. Subsequent FRET values were plotted as a function of the total CypA protein concentration, and fitted to the following quadratic equation, *Equation II*:

$$y = K\frac{(X+n[R_t]+K_d)-\sqrt{(x+n[R_t]+K_d)^2-4(n[R_t]x)}}{2[R_t]} \qquad (I1)$$

Where K is a constant, $[R_t]$ refers to total NS5A-DII receptor concentration used in the assay, n refers to the total number of receptor binding sites, and K_d is the equilibrium dissociation constant.

Evaluating Inhibition of Binding by CsA and CsA derivatives. Equilibrium dissociation experiments were performed at 4ºC with equimolar concentrations of ^{cy3}NS5A-DII and ^{cy5}CypA (0.5 μM), in the presence of a concentration gradient of CsA. Initial experiments showed that maximal change in FRET indicative of inhibition of the interaction of NS5A-DII with CypA was achieved after 15 mins, therefore this time point was chosen to assess the inhibition of binding by various CypA inhibitors. FRET was calculated as indicated to determine the relative dissociation for each inhibitor concentration.

Evaluating the dissociation of the NS5A-DII complex with CypA. Dissociation experiments were performed in 50 μL reactions of equimolar concentrations of ^{cy3}NS5A-DII and

^{cy5}CypA. The components were preincubated in binding buffer at 4ºC for 15 mins. Measurements were obtained with the excitation wavelength set at 525 nm and emission wavelength set at 665 nm to obtain initial FRET values for the preformed complex. 10 µL of 0.5, 1 or 3 µM CsA were then injected into the wells, and the reactions were allowed to proceed for 30 mins at room temperature. Measurements were obtained every 15s. The same was done with 0.1 and 0.5 µM NS5B. FRET values were calculated and graphed using GraphPad Prism 5.0. To calculate the half-life for the dissociation of the NS5A-DII complex with CypA, experiments were performed using the EnSpire® Multimode Plate Reader (Perkin Elmer). The Enspire plate reader consists of a quad monochromator and a channel dispenser that allows for real-time dispensing of reagents to enable monitoring of enzyme kinetics over time. After preincubation, plates were transferred to the Enspire plate reader and 10 µL of 0.5, 0.75, 1, 2 or 5 µM CsA were dispensed into the well at t = 30s. The same was done with 0.05, 0.1 and 0.5 µM NS5B. FRET values were fitted by non-linear regression (Graphpad Prism) to a one-phase decay curve preceded by a plateau.

Evaluating the association of NS5A-DII with CypA. Association experiments were performed using the EnSpire® Multimode Plate Reader (Perkin Elmer). 0.5 µM ^{cy3}NS5A-DII was prepared in binding buffer in 96-well plates and equilibrated at 4ºC for 5mins. Plates were transferred to the Enspire plate reader and 10 µL of 0.5 µM ^{cy5}CypA was dispensed into the well at t = 30s. Measurements were taken as described above.

2.4. Results

2.4.1. Expression, Purification and labelling of CypA and NS5A-DII

In efforts to study the dynamic interactions between CypA and NS5A-DII, we developed a FRET-based assay. SUMO-tagged NS5A-DII (genotype 1b) (residues 250-342) and human CypA were over-expressed in *E. coli* and purified to near homogeneity. The purified proteins were fluorescently labelled at solvent accessible cysteines with either Cy3 or Cy5 using maleimide coupling chemistry [354]. C342 is the only cysteine residue in NS5A-DII; no cysteines are present in the SUMO tag (Fig. 2.1A). CypA encodes four cysteine residues; C52, C62, C115 and C161. The crystal structure of CypA (PDB 1CWC) shows that C62, C115 and C161 are embedded within the protein, and C52 is the only residue accessible for Cy5 labelling [355]. The integrity of the labelled proteins was evaluated by SDS-PAGE analysis (Fig. 2.1B). Fluorescent scanning of the SDS-PAGE indicates that the fluorescent labels are indeed attached to NS5A-DII and CypA (Fig. 2.1B). Cleavage of the N-terminal SUMO-tag on NS5A-DII indicates that the fluorescent label is on the NS5A-DII portion of the recombinant protein (Fig. 2.1B, lanes 1 and 2).

2.4.2. Interaction between CypA with NS5A-DII

The interaction between fluorescently labeled NS5A-DII and CypA was evaluated by titrating increasing amounts of ^{cy5}CypA to a fixed concentration of ^{cy3}NS5A-DII. Typical emission spectra obtained from these titration experiments are shown in Fig. 2.2A. The addition of increasing concentrations of ^{cy5}CypA led to a decrease in Cy3 emission (emission maximum at 570 nm) with a concomitant increase in Cy5 emission (emission maximum at 670 nm). This is indicative of FRET. The corresponding saturation binding curve obtained by plotting FRET (measured as the change in Cy3 fluorescence intensity at 570 nm, see Equation I) as a function of added ^{cy5}CypA is shown in Fig. 2.2B. A Cy3-labelled NS5A-DII from which the SUMO tag has been cleaved yields a similar binding curve when titrated with increasing ^{cy5}CypA (Fig. 2.2B). The

binding data was fitted to a quadratic equation (see Equation II) and the equilibrium dissociation constants (K_d) obtained for NS5A-DII with and without the SUMO tag were 0.042 ± 0.01 μM and 0.038 ± 0.01 μM, respectively. We therefore conclude that the SUMO tag does not significantly affect binding to CypA. To simplify the protocol, all further assays were carried out with tagged proteins, unless otherwise stated.

In attempts to validate the assay, we mutated residues that are known to alter the protein-protein interaction. Previously, use of peptide arrays to investigate protein-protein interaction revealed proline 310 in NS5A-DII derived from GT 2a to be essential for CypA binding [347]. Here, we demonstrate that ^{cy3}NS5A-DII derived from GT 1b and harboring an alanine mutation at the corresponding residue P314 leads to a lower FRET signal when incubated with ^{cy5}CypA (Fig. 2.2B). The K_d obtained for P314A NS5A-DII was 0.297 ± 0.12 μM. This data is likewise indicative for diminished binding. Additionally, ^{cy5}CypA harboring the active site H126Q mutation also leads to low FRET when incubated with ^{cy3}NS5A-DII (Fig. 2.2B). Here the K_d obtained was 0.067 ± 0.01 μM.

We next evaluated proteins with single or double mutations D320E and Y321N associated with resistance to CypA inhibitors (Fig. 2.2C). At equilibrium, we did not observe a significant difference between the observed FRET value recorded for the mutants and wild-type. The extrapolated K_d of binding also did not exceed a 2-fold difference as compared to the wild-type (D320E, 0.056 ± 0.02 μM; Y321N, 0.062 ± 0.02 μM; D320E-Y321N, 0.064 ± 0.02 μM). This is in agreement with previous findings which demonstrate through ELISA binding assays that the CsA^{res} NS5A-DII mutant binds as efficiently to CypA as wild-type, and suggests that these mutations do not affect protein-protein binding [353].

2.4.3. *Effects of mutations at residues D320 and Y321 on CypA binding*

The CsA resistant D320 and Y321 amino acids have been suggested to lie near or within the CypA binding site. In order to gain a better insight into the contribution of each residue towards binding to CypA, a structure function analysis was carried out. The effect of single mutations at each position were tested. Each mutation was introduced into NS5A-DII and expressed as N-terminal SUMO tagged derivatives in *E. coli* in parallel with the wild-type version of the protein. The recombinant proteins were purified from soluble bacterial extracts and SDS-PAGE analysis showed that the N-terminal SUMO tagged NS5A-DII was the predominant polypeptide in each case (Fig. 2.3A). The purified proteins were subsequently successfully labelled via Cy3-maleimide coupling chemistry. NS5A-DII harboring an alanine substitution at position 320 retained similar binding to CypA as the wild-type protein (Fig. 2.3B). However, the presence of the Y321A mutation caused marked reductions in the FRET signal, implying diminished binding to CypA (Fig. 2.3C). The K_d obtained for D320A and Y321A NS5A-DII were 0.039 ± 0.01 μM and 0.138 ± 0.06 μM, respectively. Each position was further characterized by additional structure function analysis. At position 320, the Asp residue was substituted with a positively charged residue (Lys), a hydrophobic residue (Leu) and a polar residue (Asn). None of the substitutions affected the binding to CypA (Fig. 2.3B). We surmised that residue 320 in NS5A of GT 1b is not a major contributor for binding to CypA.

With regards to Y321, a conservative mutation to phenylalanine amino-acid severely decreased binding to CypA, on par with the alanine mutation (Fig. 2 3C). However, the CsA-resistant mutation to Asn rescued binding to the same level as wild type. We conclude that Y321 is a major contributor for the binding of NS5A to CypA, and our data suggests that a polar residue is required at this position for binding.

2.4.4. *Effects of mutations at residues D320 and Y321 on RNA binding*

The contribution of Asp320 and Tyr321 towards RNA binding was also evaluated. A 20 nucleotide-long Cy5-labelled U-stretch ($^{cy5}rU_{20}$) was used as the model substrate. CsA-resistance conferring mutation D320E did not alter the RNA binding of NS5A-DII (Fig. 2.3D). The Y321N mutation led to a slight increase in the K_d for binding to RNA and maximal binding remained similar to wild-type (Fig. 2.3E). The effects of non-conservative (Lys), hydrophobic (Leu) and polar (Asn) substitutions of D320 were minimal on the RNA binding property of NS5A-DII. An approximately 2-fold increase in the K_d was observed. We conclude that Asp320 is not a major contributor for RNA binding. On the other hand, both an alanine and phenylalanine substitution of Tyr321 had deleterious effects on the RNA binding capacity of NS5A-DII (Fig. 2.3E). Both substitutions reduced maximal RNA binding to less than 25% of the wild type protein.

2.4.5. Effects of CypA inhibitors on protein-protein binding

We next evaluated the potential effect of CsA and CsA derivatives on the FRET signal between NS5A-DII and CypA at a single time point. Briefly, equimolar concentrations (0.5 μM) of both proteins were preincubated in the presence and absence of CsA. Maximum FRET was observed after 15 mins, in the absence of inhibitor. We observed an increase in the Cy3 signal and a concomitant decrease in the Cy5 signal, with increasing concentration of CsA (Fig. 2.4A). The dose-dependent decrease and ultimate loss in FRET is indicative of dissociation of the ^{cy3}NS5A-DII complex with ^{cy5}CypA. The half-maximal inhibitory concentration (IC_{50}) required for CsA was 2.48 ± 0.1 μM. This experiment was also conducted with the CsA analogues alisporivir and NIM811, and the NS5A inhibitor daclatasvir. Both alisporivir and NIM811 led to a similar

decrease in FRET (Fig. 2.4B). Each of the three CypA inhibitor compounds caused an inhibitory effect to a similar extent. The addition of daclatasvir, an NS5A inhibitor that likely binds to domain I of NS5A, did not affect the FRET value.

Under similar conditions, we also compared the effect of CsA on the CypA complex with CsA^{res} NS5A-DII as compared to wild-type. A decrease in FRET was also observed after pre-incubation of CsA with the CsA^{res} NS5A-DII complex with CypA, implying that the mutant proteins remained sensitive to CsA (Fig. 2.4C).

2.4.6. Dissociation profiles of complexes with wild-type NS5A-DII and mutant proteins

Previously employed technologies have been unsuccessful in identifying the mechanism through which mutations D320E and Y321N mediate resistance to CypA inhibitors. Studies to evaluate the binding between NS5A-DII and CypA are commonly conducted at equilibrium between association and dissociation events. In attempts to focus on complex dissociation, we next studied the real-time effect of the addition of ligands to a preformed ^{cy3}NS5A-DII and ^{cy5}CypA complex. The FRET signal remained constant in the absence of inhibitor. A time-dependent decrease in FRET was evident at CsA concentrations as low as 1 μM (Fig. 2.5A). Under these conditions, the wild-type protein NS5A-DII^{WT} shows the strongest decline in FRET when compared with NS5A-DII^{D320E}, NS5A-DII^{Y321N} and NS5A-$DII^{D320E\text{-}Y321N}$. At 3 μM of CsA, a maximum decrease in FRET was seen 500s following the addition of CsA. Under these conditions, the dissociation profile recorded was the same for NS5A-DII^{WT}, NS5A-DII^{D320E}, and NS5A-DII^{Y321N} (Fig. 2.5A). However, complete dissociation of NS5A-$DII^{D320E\text{-}Y321N}$ progressed at a much slower rate. Further measurements were conducted with varying CsA concentrations to calculate the half-life of dissociation of CypA from a complex with NS5A-DII proteins expressing

either single or double CsA-resistance conferring mutations. At relatively low concentrations of 0.75 μM and 1 μM CsA, both single mutants led to a longer half-life of dissociation as compared to wild-type, and the D320E-Y321N (DEYN) double mutant showed the strongest effect in this regard (Fig. 2.5B).

Dissociation kinetics of the NS5A-DII and CypA complex were also carried out with NS5B. We and others have shown previously that NS5B and CypA share a common binding site on NS5A-DII [167, 170]. NS5B was able to effectively displace CypA from its complex with NS5A-DII. Here, different concentrations of NS5B were added to the pre-formed complex of NS5A-DII and CypA. The FRET signal and half-life of dissociation were monitored over time as described above. When NS5A-DII^{WT} was substituted with CsA^{res} NS5A-DII, the rate of dissociation remained similar to wild-type (Fig. 2.5C). At all tested concentrations of NS5B, the half-life of dissociation of the complex of NS5A-DII^{WT} with CypA was not significantly different from the CsA^{res} NS5A-DII complex. NS5B effectively disrupts the binding of CypA to NS5A-DII, irrespective of the presence of either CsA resistance-conferring mutation in NS5A-DII. We therefore conclude that the difference observed in the dissociation profile between wild-type and CsA resistant NS5A-DII and CypA complexes is specific to CsA.

2.4.7. CsA-resistance conferring mutations do not affect association of NS5A-DII with CypA

The observed difference in the rate of dissociation of the NS5A-DII and CypA complex can also be attributed to a slower association of CsA ^{res} NS5A-DII with CypA. To determine whether this was the case, ^{cy5}CypA was added to ^{cy3}NS5A-DII, and the binding of wild-type and CsA ^{res} NS5A-DII with CypA was monitored through FRET over time. An increase in FRET was observed, and this increase could be fitted to a one-phase exponential association curve (Fig.

2.6A). Increase in FRET was attributed to binding between ^{cy3}NS5A-DII and ^{cy5}CypA. Saturation in binding as measured with FRET was reached 3 minutes after injection of ^{cy5}CypA. A direct comparison of the association rate constants shows no difference in the association of either wild-type or CsA^{res} NS5A-DII with CypA (Fig. 2.6B). Therefore, we conclude that the differential dissociation rate observed for the wild-type and CsA^{res} NS5A-DII complexes with CypA is independent of the rate of association of NS5A-DII with CypA.

2.5. Discussion

In this study we demonstrate that the interaction between NS5A-DII and CypA can be directly monitored by a FRET-based assay. Purified NS5A-DII and CypA are here labeled with donor (Cy3) and acceptor (Cy5) dyes. The assay was validated by introducing the P314A mutation in NS5A-DII and the H126Q mutation in CypA. Both amino acid substitutions have been shown to affect the interaction between NS5A-DII and CypA in other assays [342, 347, 353]. Direct measurement of the binding of NS5A-DII with CypA enabled the generation of saturation binding curves from which the K_d could be extrapolated. The addition of CypA inhibitors led to reductions in the FRET signal, which is indicative for complex dissociation.

Our data corroborate previous findings in that CsA resistance-conferring mutations D320E and Y321N do not affect binding of NS5A-DII to CypA at equilibrium, nor do they affect the sensitivity of the complex to CsA [353, 356]. However, the observation that Y321A caused reductions in the FRET signal, suggests that Y321 plays an important role in maintaining the structural integrity of the protein-protein complex. Previous NMR studies identified a highly conserved motif corresponding to ^{314}PIWARP^{319} as a major CypA interaction site [357, 358]. Importantly, *in vitro* cell culture studies show that the higher CsA-resistance conferring Y321 site adjacent to this motif has

been associated with fitness deficit, while the D320 residue has been shown to be substitutable without any significant effects on HCV replication [359]. We therefore conclude that the 321 residue, and not 320, is a major contributor towards CypA binding.

Emergence of resistance conferring mutations within NS5A-DII differs from canonical modes of resistance as these mutations do not appear within the inhibitor target protein, CypA. This phenomenon makes it difficult to elucidate the mechanism through which resistance is conferred. While these mutations are able to rescue HCV replication in the presence of inhibitor, a possible mechanism of resistance has yet to be defined. In attempts to reconcile these findings, we preformed the protein-protein complex and measured its dissociation kinetics in the presence of CypA inhibitors or NS5B. We show that the rates of dissociation in the presence of CsA are reduced when the mutations D320E and Y321N are present. Changes in dissociation kinetics correlate with the level of resistance. *In vitro* susceptibility measurements in cell-based replicon assays have shown that the level of resistance to CsA and analogues follows the order Y321N/ D320E > Y321N > D320E [356]. The reductions in dissociation kinetics observed in our study follow the same order. Similar to CsA, NS5B competitively inhibits the binding of CypA to NS5A-DII. Importantly, our kinetic data show that unlike CsA, NS5B leads to a similar dissociation of wild-type and CsA^{res} NS5A-DII complexes with CypA. Therefore, our data suggests that the DEYN mutations specifically limit the inhibitory effect of CsA on the complex of NS5A-DII with CypA.

This combined data suggest that the mutations must indeed affect the structure of the protein-protein interface. NS5A-DII has been established to be essentially disordered with a mixture of turn-like and extended conformations at the CypA binding site [203]. In addition, distinct

conformational changes within NS5A-DII are induced by the presence of the D320E and Y321N mutations, which alter this structural equilibrium and favour specifically the extended conformation [203, 357]. Thus, while these structural changes do not inhibit the interaction with CypA, our data points to an increase in the longevity or sustained binding on the protein interaction with CypA.

A prolonged half-life of the protein-protein complex (which is tantamount to reduced dissociation), diminishes inhibitor binding. This prolonged half-life correlates directly with the level of resistance conferred by each mutation. Cell culture studies monitoring HCV replication have shown that the Y321N mutation confers a higher level of resistance than D320E, but lower than the double DEYN mutant [356]. CsA caused the slowest dissociation of the pre-formed NS5A-DII^{DEYN} and CypA complex. The structural changes induced by the presence of the DEYN mutations appear to have a direct effect of the accessibility of CsA and CsA analogues to the CypA hydrophobic active site pocket, an interaction that is evident through crystal structures of CypA in complex with CsA [360, 361]. Therefore, by prolonging the half-life of the protein-protein complex, these mutations effectively limit the binding of the inhibitor and decrease the inhibitory effect on the CypA complex with CsA^{res} NS5A-DII.

Hence, this data help to explain why the resistant NS5A is less dependent on CypA. DEYN mutations elicit conformational changes that reduce the need of CypA-mediated isomerization of peptide bonds. This is also supported by cell-based *in vitro* studies that show the adaptation of CsA^{res} HCV replicons in CypA knockdown cells. CsA^{res} HCV replicons are able to replicate under lower levels of CypA, but not completely independent of CypA [181]. The complex of CypA with

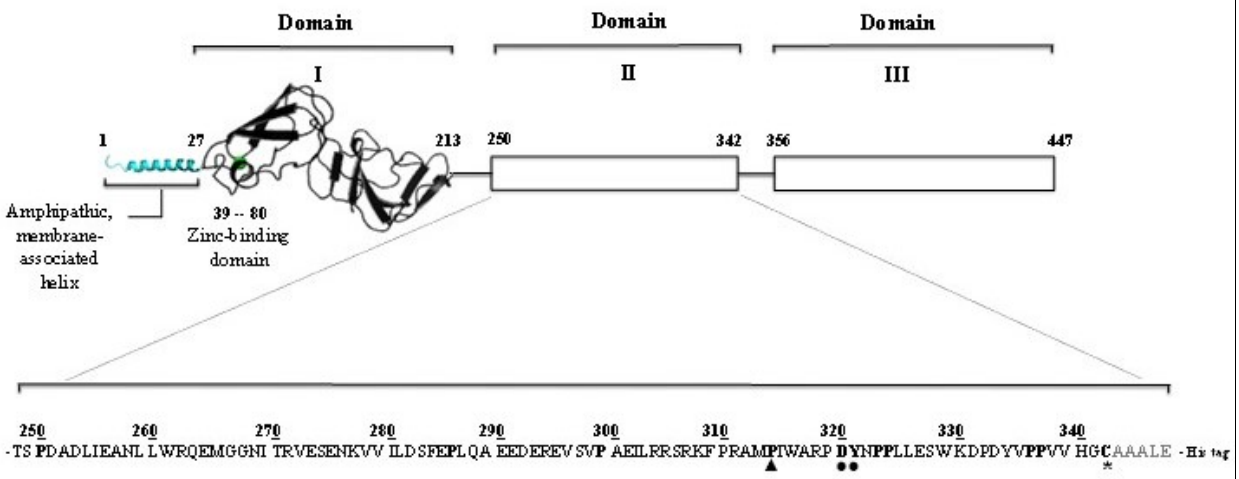
CsA^{res} NS5A becomes more efficient when compared with the wild-type protein. We therefore infer from our data that the conformational changes induced in the presence of these mutations served to reduce the sensitivity of the NS5A-DII and CypA complex to CsA inhibition.

Based on our data, we propose a model for the mechanism of resistance to CsA and derivatives (Fig. 2.7). In this model, NS5A-DII interactions with the viral RNA, CypA and NS5B are modulated by the presence of CsA-resistance conferring mutations, DEYN. Binding to CypA results in conformational changes within NS5A-DII and an increase in its RNA binding properties [200]. Subsequently, NS5B can out-compete CypA and bind to the RNA-associated NS5A protein, bringing NS5B in close proximity to the viral RNA template. In the presence of CsA, CypA is rapidly dissociated from its complex with wild-type NS5A-DII, hindering the optimal recruitment of NS5B. Presence of the DEYN mutations induce structural changes that prolong the binding to CypA by limiting the accessibility of CsA to the CypA active site. In this way, optimal recruitment of NS5B to the viral replication complex can be restored. NS5B has been shown to lack specificity for the viral RNA genome, therefore maintaining this interaction could play a role in the regulation of viral replication. One point to note is that while the dissociation kinetics observed here with CsA change with respect to each mutant, the kinetics with NS5B remain the same. Further studies into the interaction between NS5A-DII and CypA in the presence of NS5B and RNA will be essential in order to understand the precise role of CypA in regulating HCV replication.

The present analysis of CsA-resistance conferring mutations in NS5A provides novel insights into the interactions of NS5A with CypA. In addition, we have developed a robust FRET-based assay for the detection of the direct physical interaction of NS5A-DII with CypA and model

RNA substrates. This solution-based assay can be extended into a high-throughput system to screen for more specific inhibitors of the interaction of NS5A-DII with CypA. Therefore, the highly sensitive FRET-based assay provides a powerful approach that can expedite the discovery of more potent CypA inhibitors.

A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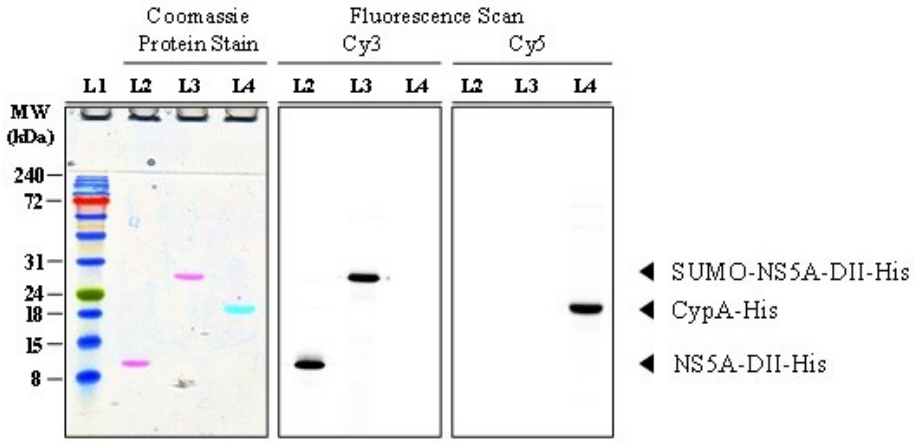
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MW (kDa)
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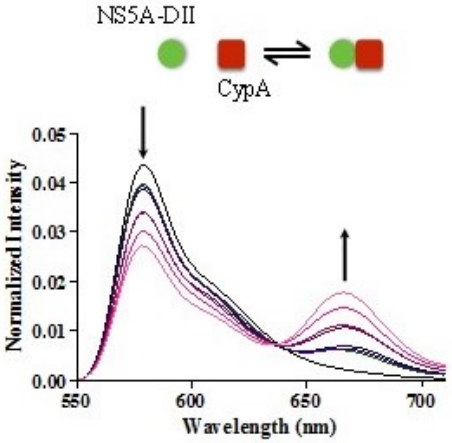
72
31
24
18
15
8
SUMO-NS5A-DII-His
CypA-His
NS5A-DII-His

Figure 2. 1: Purification of NS5A and CypA proteins.

(*A*) Domain organization of HCV non-structural 5A protein (NS5A). The NS5A protein is subdivided into 3 domains, interconnected by flexible linker regions. Domain I is highly structured and contains a zinc-finger domain, while domains II and III are largely unstructured. The sequence of NS5A-DII from Con 1b used in this study is shown. NS5A-DII is a proline-rich sequence (as indicated in bold) and binds to CypA. (▲) denotes the residue P314, a major proline involved in CypA interaction. Residues D320 and Y321 (●) are the major residues involved in conferring resistance to CypA inhibitors. (*) indicates the lone cysteine residue that is conjugated to Cy3 maleimide dye for FRET assays. **(*B*)** NS5A-DII and CypA were expressed in *E. coli*, purified, and labelled with Cy3 and Cy5 fluorescent dyes, respectively. Proteins were visualized on a 12% SDS-PAGE through Coomassie staining and fluorescently-labelled protein imaging through fluorescence scanning. *Lane 1(L1),* protein marker; *L2*, NS5A-DII with an N-terminal SUMO-tag; *L3*, CypA; *L4*, NS5A-DII with the SUMO-tag cleaved off using the SUMO protease.

A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CypA
Normalized Intensity
Wavelength (nm)

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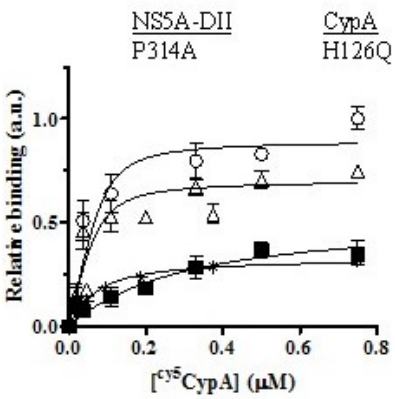
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P314A
CypA
H126Q
Relative binding (a.u.)
[cy5CypA] (μM)

C

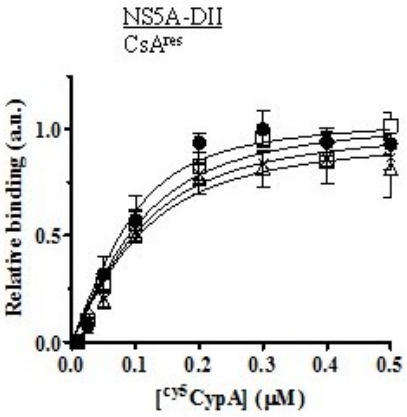
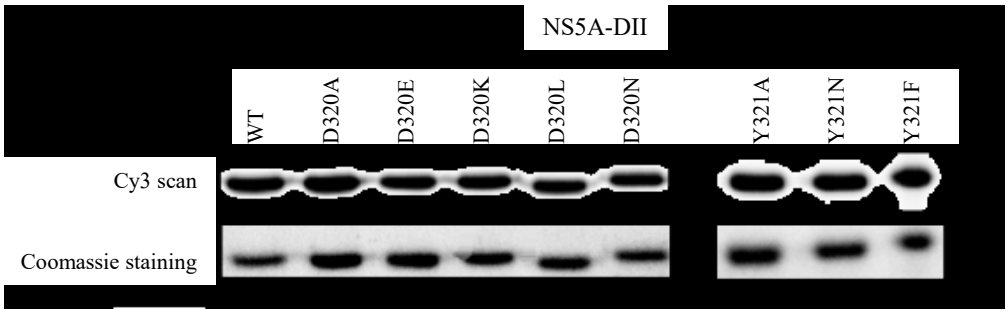

NS5A-DII
CsAres
Relative binding (a.u.)
[cy5CypA] (μM)

Figure 2. 2: Residue specificity for NS5A-DII binding to CypA.

(*A*) Steady-state fluorescence spectra for binding of 0.2 μM ^{cy3}NS5A-DII^{WT} with increasing concentrations of ^{cy5}CypA^{WT}. A diagram of the experimental setup is shown above. Note the increase in FRET with increasing CypA concentration. **(*B*)** FRET values from **(*A*)** were calculated and plotted as shown, for NS5A-DII^{WT} (Δ). A Hill slope of 2.1 ± 0.1 was determined for CypA binding. NS5A-DII^{WT} with the SUMO tag cleaved-off was used to assess the effect of the SUMO tag on binding (o). Other proteins tested were CypA mutant, H126Q (*), and NS5A-DII mutant, P314A (■) binding to NS5A-DII^{WT} and CypA^{WT} proteins, respectively. Values were normalized to the highest FRET values for each data set. **(*C*)** Comparison of CypA interaction with NS5A-DII^{WT} (●), NS5A-DII^{D320E} (o), NS5A-DII^{Y321N} (**x**) and NS5A-DII$^{D320E-Y321N}$ (Δ) mutants. (*D*) The same comparative analysis of binding was assessed with NS5A-DII^{WT} (●), NS5A-DII^{D320A} (□) and NS5A-DII^{Y321A} (Δ) mutants.


NS5A-DII
WT
D320A
D320E
D320K
D320L
D320N
Y321A
Y321N
Y321F
Cy3 scan
Coomassie staining

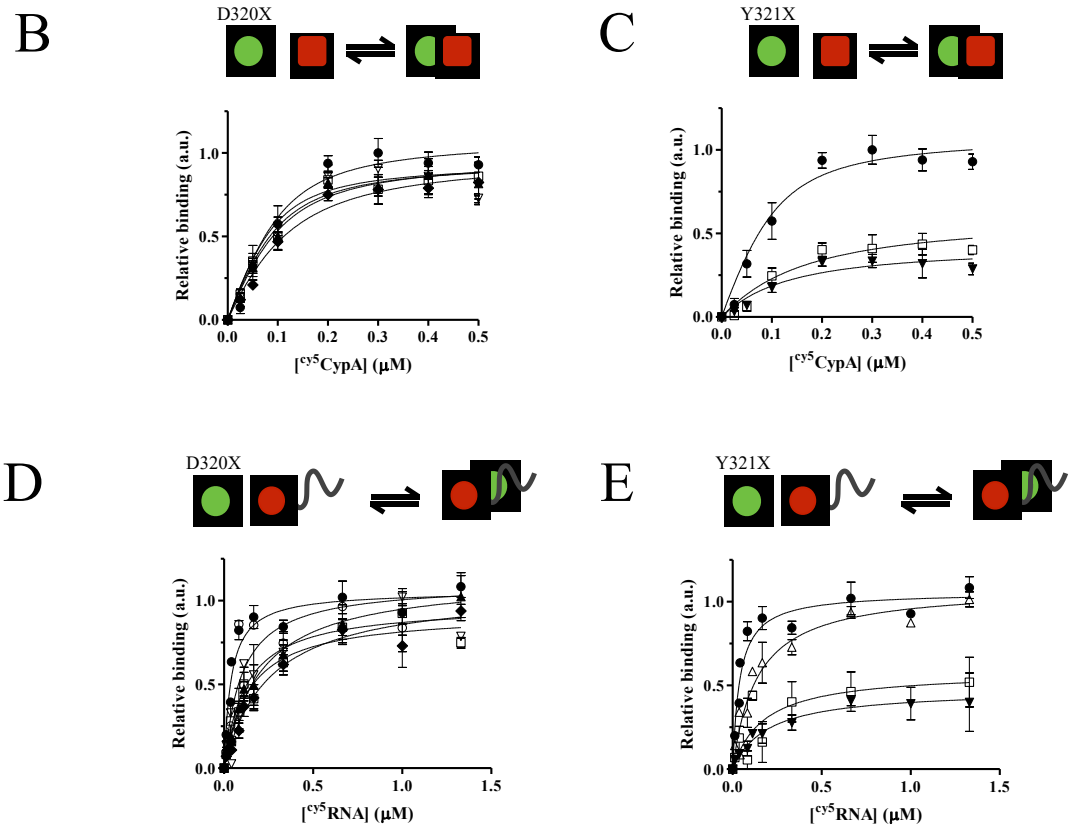

B
D320X
Relative binding (a.u.)
[cy5CypA] (μM)
C
Y321X
Relative binding (a.u.)
[cy5CypA] (μM)
D
D320X
Relative binding (a.u.)
[cy5RNA] (μM)
E
Y321X
Relative binding (a.u.)
[cy5RNA] (μM)

Figure 2. 3: FRET binding assay to assess NS5A-DII binding properties to CypA and RNA. ***(A)*** NS5A-DII mutants expressing a single mutation at D320 or Y321 were labeled with Cy3 fluorescent dye and visualized on an SDS-PAGE. Both fluorescence and protein imaging were performed to confirm Cy3 labelling of NS5A-DII. A structure-function analysis was performed to determine the contribution of each residue towards binding to ^{cy5}CypA and a model RNA substrate, $^{cy5}rU_{20}$. ***(B)*** A graphical representation is shown for the FRET assessment for the binding of ^{cy5}CypA to ^{cy3}NS5A-DII mutants D320A (□), D320K (▲), D320L (▽), D320N (◆), and ***(C)*** Y321A (□) and Y321F (▼), relative to WT (●). ***(D, E)*** Similar mutants were assessed for their RNA binding properties, with the addition to D320E (○) and Y321N (Δ) mutants. The experimental schematic is depicted above each graph.

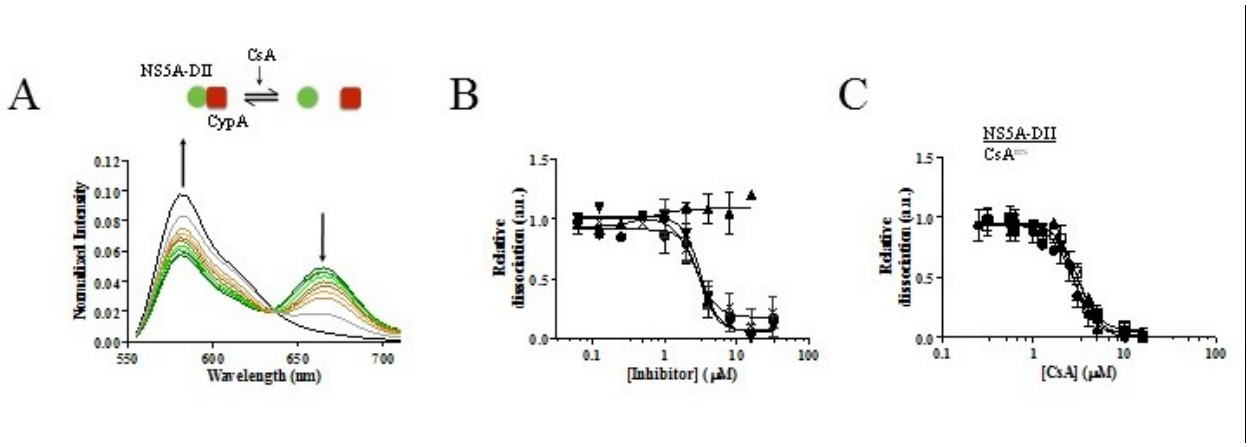
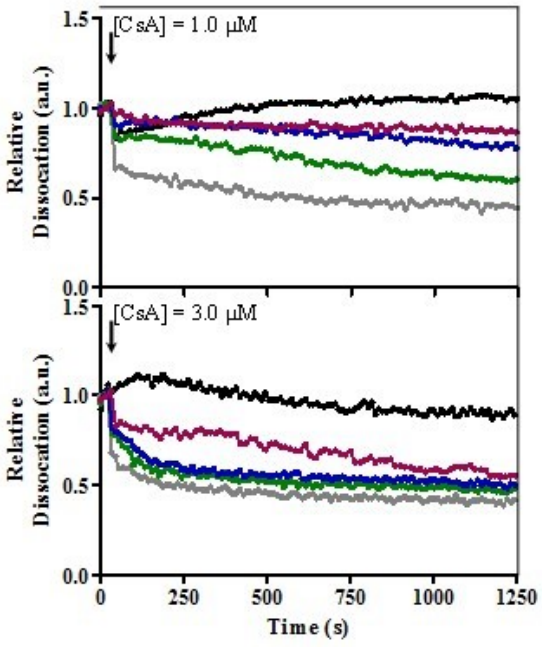


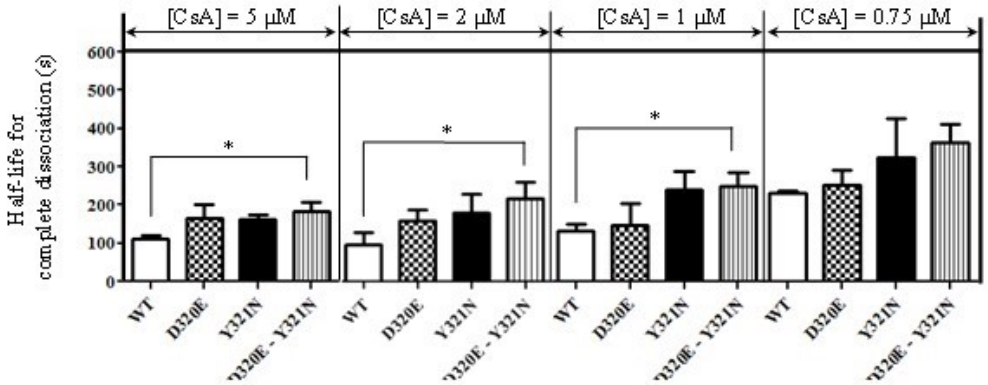
Figure 2. 4: Inhibition of the interaction between NS5A-DII and CypA.

(*A*) Steady-state fluorescence spectra of CsA inhibition of binding between NS5A-DII and CypA. A pre-formed complex of 0.5 μM ^{cy3}NS5A-DII and 0.5 μM ^{cy5}CypA was pre-incubated with increasing concentrations of CsA for 15 mins on ice, as depicted in the diagram above. Note the decrease in FRET with increasing CsA concentration. **(*B*)** FRET values from **(*A*)** were calculated and plotted, as shown. The inhibitory effect of CsA (●) was compared to alisporivir (**x**), NIM811 (▼) and daclatasvir (Δ). Values were normalized relative to the highest FRET value for each data set. **(*C*)** Comparison of CsA inhibition of CypA interaction to NS5A-DII^{WT} (●), NS5A-DII^{D320E} (□), NS5A-DII^{Y321N} (▲) and NS5A-DII$^{D320E\text{-}Y321N}$ (∇) mutants.

A


[CsA] = 1.0 µM
[CsA] = 3.0 µM
Relative Dissocation (a.u.)
Time (s)
0.0
0.5
1.0
1.5
0
250
500
750
1000
1250

B


[CsA] = 5 µM
[CsA] = 2 µM
[CsA] = 1 µM
[CsA] = 0.75 µM
Half-life for complete dissociation (s)
WT
D320E
Y321N
D320E - Y321N

C

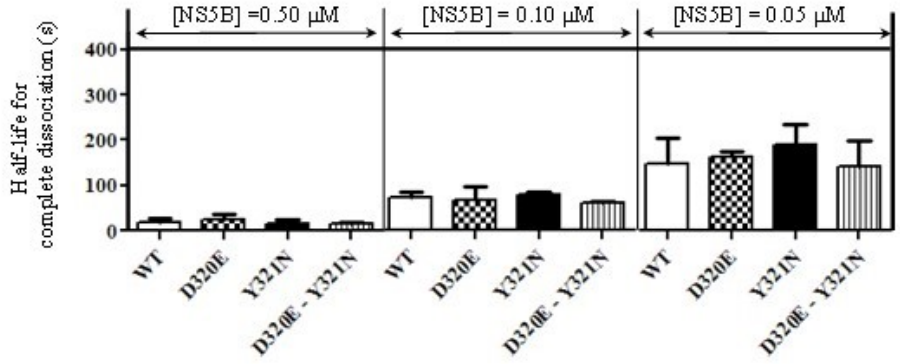

[NS5B] =0.50 µM
[NS5B] = 0.10 µM
[NS5B] = 0.05 µM
Half-life for complete dissociation (s)
WT
D320E
Y321N
D320E - Y321N

Figure 2. 5: Dissociation kinetics of NS5A-DII complex with CypA, in the presence of CsA and NS5B.

Time course experiments were designed to monitor the effect of the addition of either CsA or NS5B to a pre-formed ^{cy3}NS5A-DII and ^{cy5}CypA complex. **(*A*)** Normalized FRET time trajectories for interaction between ^{cy5}CypA and ^{cy3}NS5A-DII variants in the presence of 1 μM and 3 μM CsA. CsA was added at 30s to the final concentration shown (indicated by the arrow). Each trajectory was normalized to its initial average FRET value, prior to addition of CsA. Trajectories are shown for relative dissociation of CypA from NS5A-DII^{WT} (**grey**), NS5A-DII^{D320E} (**green**), NS5A-DII^{Y321N} (**blue**) and NS5A-DII$^{D320E-Y321N}$ (**purple**). A control to monitor binding of CypA to NS5A-DII^{WT} in the absence of CsA inhibition was also indicated (**black**). ***(B)*** Half-life values calculated from FRET time trajectories, in the presence of 0.75 μM, 1 μM, 2 μM and 5 μM CsA concentration. An asterisk (*) indicates a p-value < 0.05 (Student T-test); bars represent mean values ±SE of three independent experiments. ***(C)*** Similar dissociation experiments were carried out in the presence of 0.05 μM, 0.1 μM, and 0.5 μM NS5B, and the half-life values were calculated and plotted, as shown.

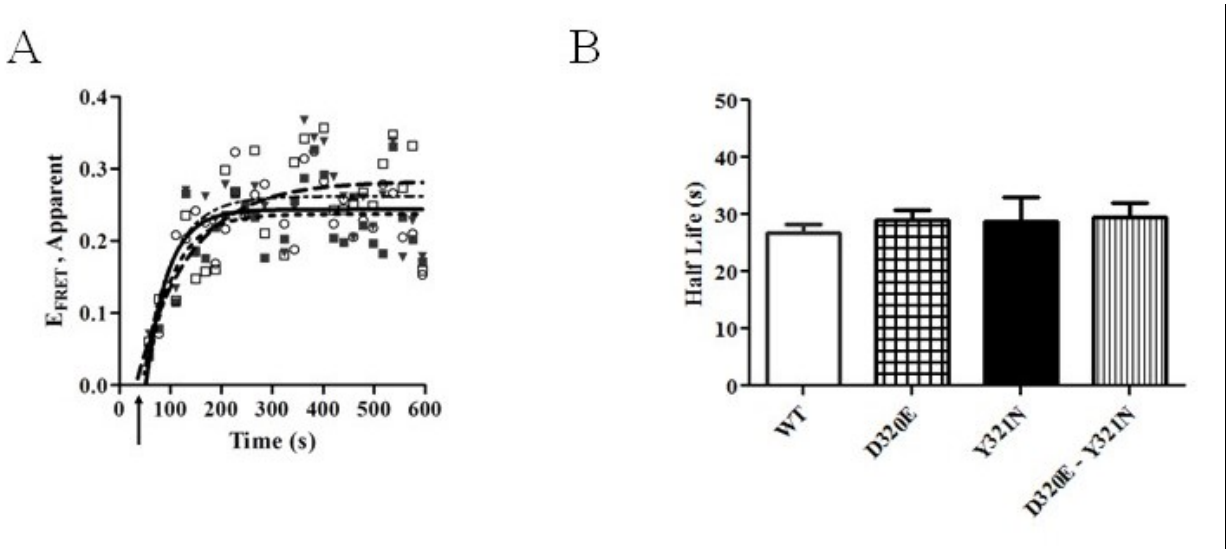


Figure 2. 6: Association kinetics of NS5A-DII complex with CypA.

(A) The rate of formation of the NS5A-DII complex with CypA was measured through FRET. 0.5 µM ^{cy5}CypA was added to a solution containing 0.5 µM ^{cy3}NS5A-DII at time = 30s (shown by the arrow). FRET observed at each time interval was calculated for the protein complex formation with CypA with NS5A-DII^{WT} (−o−), NS5A-DII^{D320E} (•■•), NS5A-DII^{Y321N} (−□−), and NS5A-DII$^{D320E\text{-}Y321N}$ (•−▼•−). ***(B)*** The association rate constant of the rate of complex formation (s^{-1}) was determined, and plotted as shown.

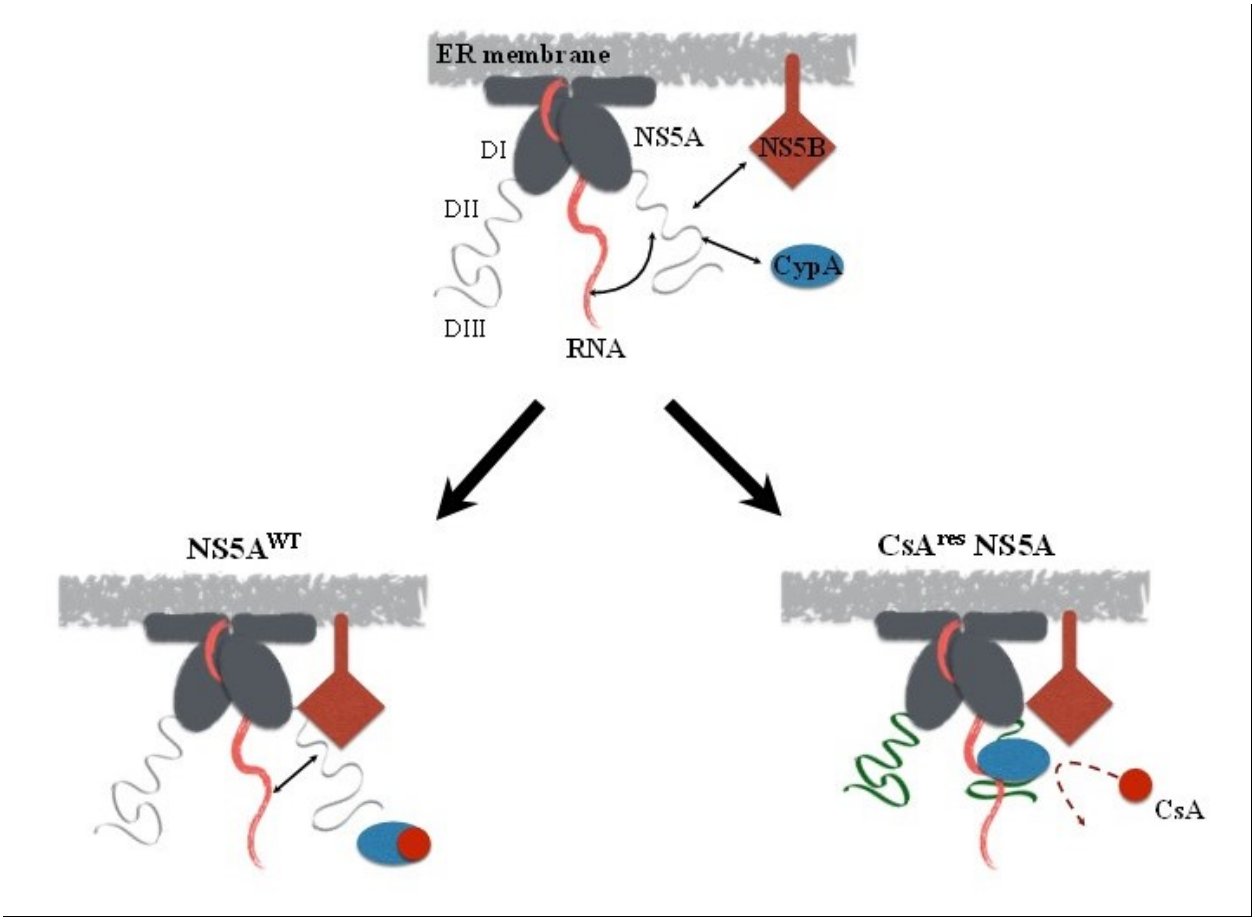


Figure 2. 7: Model for the mechanism of resistance of CsA-resistance conferring mutations, D320E and Y321N.

NS5A-DII interacts with the viral RNA, CypA and NS5B polymerase (interaction shown by short black arrows). When occupied by CypA, the RNA binding capacity of NS5A-DII is increased. NS5B can out-compete CypA and bind to NS5A, bringing NS5B in close proximity to the viral RNA for optimal RNA binding to proceed. CsA rapidly dissociates CypA from wild-type NS5A (shown in **GREY**), and optimal recruitment of NS5B is hindered. The expression of D320E and Y321N resistance-conferring mutations within CsA^{res} NS5A-DII (shown in **GREEN**) reduces the rate of dissociation of CypA (dashed red arrow), allowing for optimal recruitment of NS5B.

CHAPTER 3: INTERACTIONS OF THE DISORDERED DOMAIN II OF HEPATITIS C VIRUS NS5A WITH VIRAL RNA, NS5B AND CYPA SHOW EXTENSIVE OVERLAP

This chapter was adapted from an article authored by M. Ngure, M. Issur, N. Shkriabai, H.-W. Liu, G. Cosa, M. Kvaratskhelia, and M. Götte that appeared in the ***American Chemical Society Infectious Diseases,*** 2016, 2(11): 839-851.

3.1. Preface

Multiple binding partners have been identified that bind to domain II of NS5A (NS5A-DII)[130, 170, 200, 201]. Due to the largely unstructured nature of NS5A-DII[129, 362], a crystal structure of NS5A-DII has yet to be determined and has impeded the study into NS5A-DII functions and interactions with various binding partners. NS5A-DII possesses RNA-binding properties, and has been shown to interact with the RNA-dependent RNA viral polymerase, NS5B[170]. NS5B is responsible for the synthesis of the viral RNA genome. It interacts with NS5A-DII through a similar binding site as the cellular cofactor CypA. The specific residues involved in this interaction are unknown. In addition, the binding site of NS5A-DII on NS5B has yet to be determined. We intend to utilize high-resolution mass-spectrometry assisted protein footprinting approach to identify and characterize key residues involved in the interaction of NS5A-DII with CypA, NS5B, as well as in RNA binding. Once the protein-protein and nucleoprotein interfaces have been identified, we will validate the involvement of specific residues in these interactions using fluorescence based binding assays (FRET and anisotropy).

3.2. Abstract

Domain II of the non-structural protein 5 (NS5A) of the hepatitis C virus (HCV) is involved in intermolecular interactions with the viral RNA genome, the RNA-dependent RNA polymerase NS5B and the host factor CypA. However, domain II of NS5A (NS5A-DII) is largely disordered, which makes it difficult to characterize the protein-protein or protein-nucleic acid interfaces. Here we utilized a mass spectrometry-based protein footprinting approach in attempts to characterize regions forming contacts between NS5A-DII and its binding partners. In particular, we compared surface topologies of lysine and arginine residues in the context of free and bound NS5A-DII. These experiments have led to the identification of an RNA binding motif (305RSRKFPR311) in an arginine-rich region of NS5A-DII. Furthermore, we show that K308 is indispensable for both RNA and NS5B binding, while W316, further downstream, is essential for protein-protein interactions with CypA and NS5B. Most importantly, NS5A-DII binding to NS5B involves a region associated with RNA binding within NS5B. This interaction down-regulated RNA-synthesis by NS5B, suggesting that NS5A-DII modulates the activity of NS5B and potentially regulates HCV replication.

3.3. Introduction

Chronic infection with the hepatitis C virus (HCV) is associated with a risk for the development of severe liver disease including liver cirrhosis and hepatocellular carcinoma[363]. Drug discovery efforts have led to the development of potent direct acting antivirals (DAAs) that target crucial viral proteins. The use of combinations of DAAs is associated with high cure rates of > 90%[114, 364-366]. Different classes of approved drugs target the viral protease or non-structural protein 3 (NS3), or the RNA-dependent RNA polymerase (NS5B). Both NS3 and NS5B are logical targets with known structures and defined enzymatic activities. A rather unusual target is the non-structural protein 5A (NS5A). NS5A is a pleiotropic RNA binding protein that is involved in HCV replication and assembly[127, 132, 367-369]. Although NS5A does not possess enzymatic activities, it is a validated drug target. Daclatasvir is an approved NS5A inhibitor that shows antiviral activity in the range of picomolar concentrations[370]. Moreover, NS5A interacts with the cellular host factor CypA, and compounds that target CypA were shown to select for resistance-conferring mutations in NS5A[199, 201, 203, 205, 208, 229, 314, 371, 372]. Despite this progress, the detailed biological functions of NS5A and the mechanisms associated with antiviral effects of NS5A and CypA inhibitors remain elusive.

NS5A has been divided into 3 distinct domains[127]. Domain I (DI) possesses 80% of the RNA binding properties and provides the presumptive binding site for daclatasvir[130, 370]; DI has been crystallized in different dimer conformations and not all of these dimers seem to accommodate RNA[152, 186, 368]. Domain II (DII) and Domain III (DIII) are largely unstructured, although DII is required for viral replication[194, 373] and DIII is required for the production and release of new virus particles[172, 221, 346]. DII was also shown to exhibit RNA binding properties and interacts with CypA[130, 179]. CypA is a *cis/trans* polyprolyl isomerase that binds to a proline-rich region of NS5A-

DII (^{308}KFPRAMPIWARPDYNPP^{324} [genotype 1b]) and enhances its RNA-binding properties[200]. This proline-rich region may also provide a putative binding site for NS5B[170]. In this study, we devised a comprehensive biochemical strategy to develop a model that helps to explain how NS5A-DII binds to RNA, CypA, and NS5B. A mass spectrometry (MS)- based protein footprinting approach points to a small arginine- and proline-rich region of NS5A that seems to interact with each of the three components. Moreover, binding of NS5A-DII to NS5B seems to block the RNA entry channel of the polymerase, which in turn, affects RNA synthesis.

3.4. Experimental procedure

Chemicals and nucleic acids. Cy3 and Cy5 mono-reactive maleimide fluorescent dyes, and Cy3- *N* – hydroxysuccinimide (NHS)- ester, were purchased from GE Healthcare Life Sciences (Quebec, Canada). *N*-Hydroxysuccinimidobiotin (NHS-biotin) and *p*-hydroxyphenylglyoxal (HPG) were purchased from Thermo Scientific and freshly resuspended in solution prior to use. Cyclosporine A (CsA) was purchased from Enzo Life Sciences (New York, USA). The following nucleotide sequences were used in this study: 6mer poly-U RNA substrate (rU6ss; Dharmacon), 30mer poly-U RNA substrate (rU_{30}; IDT), 5'cy5-labeled 20mer poly-U RNA substrate ($^{cy5}rU_{20}$; IDT), 5'cy5-labeled DNA (5'-cy5-ACCTCGCGACCGTCGCCA-3') and 3' cy3-labeled RNA (5'-uggcgacggucgcgagguaacgguuucccuuuucucuc-cy3-c-3') were used to generate a dually-labeled DNA:RNA hybrid with a 3' RNA overhang (rA_{20}; single-strand substrates were purchased from Trilink), 5'-uuuuauaucuauagcgcgc-cy5-3' RNA ($^{cy5}rN_{19}$; IDT), 5′-GG-3′ (GG; Dharmacon GE Healthcare), and 5′-aaccguauccaaaacagucc-3′ RNA substrate (T20; IDT). The dinucleotide GG served as a primer for the T20 RNA template. 5′-End labeling of the GG primer was conducted with [γ-^{32}P]ATP and T4 polynucleotide kinase according to the manufacturer's recommendations

(Thermo Scientific, Inc.). Ribonucleoside triphosphates (NTPs) were purchased from Fisher Scientific. [α-^{32}P]ATP was purchased from Perkin Elmer.

Expression and purification of NS5A-DI, NS5A-DII and NS5A-DI+II. NS5A proteins constructs that span domain I (aa 33-216), domain II (aa 250-342) and domains I and II together (aa 33-342) from HCV Con1b constructs were generated. These were cloned into a modified pET-SUMO expression vector with a N-terminal SUMO tag and C-terminal peptide containing 6 consecutive histidine residues. NS5A-DII protein purification was performed as previously described in Chapter 2. The pET-SUMO expression vectors containing NS5A-DI and NS5A-DI+DII constructs were transformed into Rosetta BL21 (DE3) *E. coli* and grown at 37 ºC in Terrific Broth containing 0.5 mg/ml kanamycin and 0.034 mg/ml chloramphenicol until A_{600} of 0.6 was reached. Protein expression was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and 2% ethanol for 3hr at 22 °C. Cells were harvested by centrifugation and the pellets stored at 80 ºC. Pellets were re-suspended at 4 ºC in 10 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM BME, 5 mM imidazole and 0.1% NP-40) and the addition of lysozyme (50µg/ml). Cell lysates were sonicated to reduce viscosity and subjected to further centrifugation at 13,000 rpm for 45 mins. The supernatant was obtained and used to purify the NS5A proteins by Ni^{2+} affinity chromatography with increasing concentrations of imidazole. Fractions were collected and subjected to SDS-PAGE to determine presence of protein. Subsequent eluate with NS5A proteins were dialyzed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM DTT and 10% glycerol, and applied to a HiTrap Heparin HP resin column (GE Healthcare). Columns were washed with a phosphate buffer (10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0) and eluted with phosphate buffer containing increasing concentrations of NaCl to a final concentration

of 1M. All fractions were analyzed on an SDS-PAGE for purity and stability. Purified NS5A protein fractions were dialyzed and stored at -80°C in 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM DTT and 50% glycerol. When required, the SUMO tagged was cleaved by incubating the purified fractions with the CoolCutter SUMO protease (GeneCopoeia) at 4^{o}C for 18-20h.

Expression and purification of CypA and NS5B. Expression and purification of CypA as well as recombinant NS5BΔ21 from the HCV Con1b strain were performed as described previously in Chapter 2.

Chemical modification for Protein Footprinting. The chemical modification of solvent-accessible lysine and arginine residues were conducted as described previously[374]. Briefly, surface accessibility of lysine residues was monitored using 500 μM NHS-biotin in a 20 μL reaction in a buffer containing 50 mM HEPES (pH 7.5), 10 mM NaCl, and 2 mM DTT. NHS-Biotin reacts specifically with primary amines on proteins, resulting in covalent addition of a biotin molecule (226.30 Da) to Lys residues with the concomitant release of *N*-hydroxysuccinimide. The biotinylation reactions were carried out at 25°C for 30 min, and quenched by the addition of 100 mM Tris solution. Surface accessibility of arginine residues was monitored with 5 mM HPG modification. HPG reacts with guanidino groups on arginines, resulting in the 132-Da mass increment. The reactions were carried out at 30 °C for 1 hr in the dark and were quenched with 10 mM arginine in its free amino acid form.

Evaluating surface topology of free protein or of a nucleoprotein or protein-protein complex. In parallel experiments, NS5A-DII (10 μM) incubated with either CypA (20 μM), NS5B

(20 μM) or rU_{30} (5 μM) were compared with the free NS5A-DII protein. The SUMO tag was cleaved off of NS5A-DII prior to use in this assay. Similarly, surface topologies of free CypA and NS5B were compared with pre-formed complexes with NS5A-DII. Free and pre-bound protein complexes were subjected to chemical modification as described above. Following each modification, the proteins were separated via an SDS-PAGE and visualized by BlueFast staining. The bands of interest were excised and extensively destained in 50% methanol, 10% acetic acid. SDS was removed by washing the gel pieces with ammonium bicarbonate, dehydrated with 100% acetonitrile, and vacuum-desiccated. Samples were digested with 0.5 μg of trypsin (Roche Applied Science) in 50 mM ammonium bicarbonate overnight at room temperature. Proteolytic peptides were recovered and subjected to MS and MS/MS analysis.

MS and MS/MS Analysis. MS spectra were obtained using the Axima-CFR matrix-assisted laser desorption/ionization time of (MALDI-ToF) instrument (Shimadzu) as described previously[375]. For MS/MS analysis we employed capillary-liquid chromatography-tandem mass spectrometry (Capillary-LC/MS/MS) using a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with a microspray source (Michrom Bioresources Inc, Auburn, CA)[376]. MS data and the MASCOT automated peptide search engine (available on the World Wide Web at www.matrixscience.com) were used to identify NS5A-DII and NS5B peptide peaks from the NCBInr primary sequence data base, and the matched peaks were identified on their respective MS spectra. For accurate quantitative analysis of the modified peptide peaks, at least two unmodified proteolytic peptide peaks were used as internal controls. A protection was considered to be significant when the intensity of a modified peak formed upon treatment of the free protein

was reduced by at least 85% in the context of the nucleoprotein and protein-protein complexes. Data were reproducibly compiled and analyzed from four independent experimental groups.

Generation of mutant enzymes of NS5A-DII, NS5B and CypA. Mutant enzymes were generated through site-directed mutagenesis as previously described in Chapter 2.

Fluorescent Labeling of NS5A-DII and CypA. Conditions used to label NS5A-DII and CypA have been outlined in Chapter 2.

Fluorescent Labeling of rU6ss RNA substrate. The rU6ss RNA substrate was provided in a stable 2'-ACE® protected form. As such, the RNA was dissolved in 400 µl of 2'Deprotection buffer provided and incubated at 60^{o}C for 30 mins. Samples were then dried by speed vacuum centrifugation and ethanol precipitated to desalt the RNA oligomer stored at -20^{o}C prior to use. Subsequent pellets were stored at -20^{o}C prior to use. 100 nmols of a Cy3- NHS- ester, dissolved in 20 µL of DMSO, was used to fluorescently label 20 nmols of the rU6ss RNA oligomer in 80 µl reaction buffer of 100 mM $NaHCO_3$. Reactions were performed with constant shaking in the dark for a 6 hrs. The labeled oligo was purified by gel purification and dried. Samples were resuspended in water and stored at -80^{o}C.

Monitoring FRET. Reactions were prepared in a 96-well plate format and incubated at 4^{o}C for 30 mins in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM DTT and 5 mM $MgCl_2$. ^{Cy3}NS5A-DII (200 nM) was incubated with increasing concentration of ^{cy5}CypA. Similarly, ^{cy3}NS5A-DII (125 nM) was used to monitor nucleic acid binding with increasing concentrations of $^{cy5}rU_{20}$. Fluorescence measurements were recorded with a SpectraMax® M5

Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). The excitation wavelength was set to 520 nm, with a cutoff of 550 nm, and the emission spectra obtained from 550 nm to 710 nm. The relative binding was determined as a measure of the apparent energy transfer efficiency ($E_{FRET,\ apparent}$) between the Cy3 (donor) and Cy5 (acceptor) fluorophores. The apparent FRET efficiency denotes the observed average efficiency of the FRET, for each interacting donor in a complex. This was calculated as follows in equation 1.

$$E_{FRET,apparent} = \left(\frac{F_D - F_{DA}}{F_D}\right) \times \frac{1}{f_A} \qquad (1)$$

Where (F_D) is the fluorescence intensity of the donor recorded in the absence of the acceptor; (F_{DA}) is the fluorescence intensity of the donor recorded when the acceptor is present; (f_A) is the percent labeling efficiency of the acceptor. Where indicated, subsequent FRET values were normalized relative to maximum FRET obtained with wild-type NS5A-DII, and is presented as relative binding. All data related to protein-protein binding was fitted to equation 2.

$$y = K \times \frac{(x + [R_t] + K_d) - \sqrt{(x + [R_t] + K_d)^2 - 4[R_t]x}}{2[R_t]} \qquad (2)$$

Where K is a constant, $[R_t]$ refers to total NS5A-DII concentration used in the assay, and K_d is the equilibrium dissociation constant. The binding curves with rU_{20} RNA were fitted to a saturation-binding curve, using Graphpad Prism.

Evaluating Inhibition of NS5A-DII Binding to CypA by NS5B and CsA. FRET dissociation experiments were performed with equimolar concentrations of wild-type ^{cy3}NS5A-DII and ^{cy5}CypA proteins (0.5 μM), in the presence of a concentration gradient of either NS5B or CsA. FRET was calculated as indicated to determine the relative dissociation for each protein or

inhibitor concentration. Subsequent FRET values were normalized relative to maximum FRET obtained in the presence of NS5A-DII and CypA alone, and presented as relative binding.

Time Course for RNA Binding of NS5A-DII to a short poly RNA substrate. The binding of NS5A-DII to RNA was also monitored by anisotropy assays with a short RNA oligo. Various NS5A-DII mutants (100 nM) were preincubated at 4ºC in a half-area 96-well plate. ^{Cy3}rU6ss (15 nM) was added at time=0 and subsequence measurements were taken at 15 sec intervals, for 30 mins. Samples were excited at 490 nm and the emission was monitored at 520 nm. The change in anisotropy (Δr) was calculated as $|r-r_o|$, where r_o represents the initial anisotropy value of ^{cy3}rU6ss at time =0, and r represents the anisotropy value at each time point after the addition of NS5A-DII. As a control, the anisotropy of ^{Cy3}rU6ss in the absence of NS5A-DII was also measured. The time-dependent change in anisotropy was plotted as a function of time (s), and fitted to an allosteric sigmoidal curve, on Graphpad prism.

Binding of NS5A-DII to NS5B. The binding of NS5B to NS5A-DII was monitored by protein anisotropy assays. ^{Cy3}NS5A-DII (100 nM) was incubated with increasing concentrations of NS5B at 4ºC in a half-area 96-well plate. Measurements were performed with a SpectraMax® M5 Multi-Mode Microplate Reader. The change in anisotropy (Δr) was calculated as $|r-r_o|$, where r_o represents the initial anisotropy value of ^{cy3}NS5A-DII in the absence of NS5B, and r represents the anisotropy value at each NS5B concentration evaluated. The change in anisotropy was plotted as a function of NS5B concentration, and fitted to the equation 2 shown previously. Where indicated, subsequent anisotropy values were normalized relative to wild-type protein, and presented as relative binding.

Binding of NS5B to RNA. The binding of NS5B to RNA was monitored using the dually-labeled rA_{20} substrate, as described previously[377]. Briefly, the rA_{20} duplex DNA:RNA hybrid (200 nM) in 50 mM Tris-HCl and 20 mM NaCl at pH 7.5 was excited at 514 nm and the emission spectra was monitored in the presence of NS5B (250 nM) and a concentration gradient of NS5A-DII. FRET was calculated according to equation 1 (f_A = 1). Control experiments in the absence of any protein and in the presence of NS5A-DII alone were also carried out. For each NS5A-DII concentration, the change in FRET in the presence and absence of NS5B was calculated and plotted using GraphPad Prism.

Monitoring the effect of chemically modified NS5A-DII on binding to RNA, CypA and NS5B. 200 nM of ^{cy3}NS5A-DII was subjected to HPG-modification with varying concentrations of HPG prior to use in the described FRET binding assays with ^{cy5}CypA (500 nM) and $^{cy5}rU_{20}$ (100 nM). Labeling reactions were performed at 30ºC for 40 mins in the dark in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 2 mM DTT and 5 mM $MgCl_2$, and quenched with 10 mM arginine in its free form. ^{cy5}CypA or $^{cy5}rU_{20}$ was added to the reaction and preincubated for 30 mins. FRET measurements were taken to determine the relative binding as described previously. Similar experiments were conducted with biotinylated NS5A-DII. Labeling reactions were performed at 25ºC for 30 mins in a buffer containing 50 mM HEPES (pH 7.5), 10 mM NaCl, 2 mM DTT and 5 mM $MgCl_2$, and quenched with 100 mM Tris solution. Using protein anisotropy assays, 50 nM and 100 nM of chemically modified ^{cy3}NS5A-DII and unlabeled NS5B were used to determine the relative protein binding, respectively. Values were plotted as a function of each

HPG and NHS-biotin concentration, and fitted to a log inhibitor dose-response curve, using Graphpad Prism.

NS5B GG-primed RNA Synthesis. The reaction mixtures contained the RNA template (T20; 1 μM), purified HCV NS5B (2 μM), radiolabeled GG primer (200 nM), and an NTP mixture (10 μM). Reactions were carried out in a buffer containing 40 mM Tris-HCl (pH 8), 5 mM NaCl, 1 mM DTT, 0.2 mM $MnCl_2$, and 6 mM $MgCl_2$. The reactions were stopped at different time points by the addition of 100 mM EDTA in 100% formamide. The samples were heat denatured at 95°C for 5 min and resolved on 15% polyacrylamide-7 M urea gels. Gels were scanned and analyzed with a phosphor-imager (Molecular Imager FX; Bio-Rad). The full-length RNA product was quantified to represent the % activity of NS5B.

NS5B De Novo RNA Synthesis. The assay was run as described above with reaction mixtures containing the T20 RNA template (1 μM), HCV NS5B (2 μM), [α-^{32}P]ATP (200 nM) and an NTP mixture of ATP at 1 μM and of CTP, GTP and UTP at 100 μM each. Reactions were carried out in a buffer containing 40 mM Tris-HCl (pH 8), 5 mM NaCl, 1 mM DTT and 0.2 mM $MnCl_2$.

3.5. Results

3.5.1. Evidence for binding of NS5A-DII to RNA, CypA and NS5B

The interaction between NS5A and CypA is mediated through NS5A-DII [179, 200]. Previous reports have shown that this domain also contributes to RNA and NS5B binding[130, 131, 170, 171]. Here, we devised a comprehensive approach to characterize the binding sites for RNA, CypA and NS5B on NS5A-DII. We initially confirmed binding of RNA to NS5A-DII using a Förster Resonance Energy Transfer (FRET) assay (Fig. 3.1A). NS5A-DII proteins were site-specifically labeled by

Cy3-maleimide via maleimide-thiol coupling chemistry at residue C342, which is the only cysteine residue in NS5A-DII. In this assay, we incubated Cy3-labeled NS5A-DII (^{cy3}NS5A-DII) with increasing concentrations of a Cy5-labeled model RNA substrate ($^{cy5}rU_{20}$). A stable nucleoprotein complex was formed after 30 mins and the apparent FRET efficiency ($E_{FRET,\ apparent}$) was calculated according to equation 1 (see Materials and Methods). Increases in $E_{FRET,\ apparent}$ with increasing concentrations of RNA provide evidence for binding (Fig. 3.1A). A plateau in the $E_{FRET,\ apparent}$ indicates that the binding of ^{cy3}NS5A-DII to $^{cy5}rU_{20}$ was saturated and reached its equilibrium after the addition of 50nM RNA substrate. Maximum $E_{FRET,\ apparent}$ for RNA binding was 0.2. NS5A was previously shown using RNA filter-binding assays to preferentially bind to poly-U rich sequences in the HCV 3' UTR[130]. To confirm specificity of binding in our FRET assay, we also testing the binding of NS5A-DII to increasing concentrations of a RNA substrate with a randomized sequence, $^{cy5}rN_{19}$. We observed a 50% decrease in FRET, indicating less interaction with $^{cy5}rN_{19}$ as compared to the $^{cy5}rU_{20}$ RNA substrate (Fig. 3.1A). Total loss of FRET was not observed, and the short stretch of four consecutive uracil nucleotides within the control RNA substrate could account for the partial binding observed. Taken together, this confirmed that our FRET assay was robust and able to detect the specific interaction between NS5A-DII and our poly-U substrate. The FRET-based assay was also utilized to assess the binding of CypA to NS5A-DII. Purified CypA was subjected to labeling with Cy5 maleimide dye. CypA contains four cysteine residues. While C62, C115 and C161 are buried inside the CypA crystal structure (PDB 1CWC), C52 is solvent exposed and therefore accessible for Cy5 labelling[378]. However, simultaneous labeling of C62, C115 and C161 may not be formally excluded. In this assay, ^{cy3}NS5A-DII was incubated with increasing concentrations of Cy5-labeled CypA (^{cy5}CypA) (Fig. 3.1B). The increase in $E_{FRET,\ apparent}$ is again indicative of binding. A higher maximum $E_{FRET,\ apparent}$ of 0.4 was reached with

CypA, suggesting that NS5A-DII bound more efficiently to CypA than RNA. However, this difference may also suggest alternate modes of binding adopted by NS5A-DII for either binding partner. While the site of RNA binding is unclear, the putative binding site for CypA on NS5A-DII lays adjacent to the C342 residue bearing the Cy3 fluorophore, increasing the probability of a higher FRET efficiency. Additionally, the rU_{20} RNA substrate could potentially accommodate multiple binding sites for NS5A-DII. This would allow the NS5A-DII protein to bind farther away from the 5' Cy5-fluorophore thus resulting in a lower FRET efficient. For NS5B binding, the use of a FRET-based binding assay involving a fluorescent labelling of NS5B is not suitable because of the high number of cysteine residues present[148]. Instead, we employed a fluorescence anisotropy assay using ^{cy3}NS5A-DII to measure binding. With increasing concentrations of NS5B, a significant change in anisotropy was observed, which confirms the formation of a protein-protein complex with NS5B (Fig. 3.1C).

Both CypA and NS5B have been shown to share overlapping binding sites on NS5A-DII [170]. Hence, we investigated whether there is indeed a competition between NS5B and CypA for binding to NS5A-DII. A CypA inhibitor, Cyclosporine A (CsA), was used as a positive control, as it is known to interfere with the NS5A-DII-CypA interaction[199, 349]. When ^{cy3}NS5A-DII and ^{cy5}CypA were incubated with increasing concentrations of CsA inhibitor, a dose-dependent decrease in FRET was observed, implying that CsA inhibited the binding between NS5A-DII and CypA (Fig. 3.1D). A similar effect was observed when in the presence of NS5B, but at much lower concentrations as compared to CsA. The half-maximal inhibitory concentrations required for NS5B and CsA were 0.074 ± 0.04 μM and 2.48 ± 0.1 μM, respectively. Taken together, this shows that NS5B can effectively outcompete CypA from its complex with NS5A-DII.

3.5.2. *Presumptive binding sites for RNA, CypA, and NS5B*

In attempts to further characterize the interaction between NS5A-DII and its binding partners, we devised a MS protein footprinting approach. This technique requires chemical modification of purified NS5A-DII with *N*-Hydroxysuccinimidobiotin (NHS-biotin) and *p*-hydroxyphenylglyoxal (HPG) that react with lysines and arginines, respectively[379]. The presence of a bona fide binding partner, such as RNA or another protein, can reduce access to specific lysine or arginine residues that are involved in this interaction. As a consequence, the efficiency of chemical modification is diminished. Chemical modifications of unliganded NS5A-DII with either NHS-biotin or HPG decreased its subsequent binding to either the nucleic acid ligand (rU_{20}) or the protein co-factors (NS5B or CypA), suggesting that NS5A-DII lysines and arginines contribute to the formation of nucleoprotein and protein-protein complexes (Fig. 3.2A and B). In order to identify specific regions involved in binding, we treated both free and bound NS5A-DII with either NHS-biotin or HPG and subjected these samples to trypsin proteolytic cleavage followed by MS-based analysis (see Materials and Methods). The identified peptides covered the entire sequence of NS5A-DII (Fig. 3.3A). In the free protein, 8 out of 8 arginine residues and 1 out of the 3 lysines were readily accessible for HPG and NHS-biotin modification, respectively, which is in agreement with a largely unstructured protein. Comparative analysis of free and bound NS5A-DII next revealed several residues that were protected from modification (Table 3.1 provides a summary of the data). Representative MS data of HPG-modified and biotinylated peptides for free and complexed NS5A-DII are shown in Fig. 3.3B-D.

In the presence of the RNA substrate, residues R304, R305, R307 and R311 of NS5A-DII were protected from HPG-labeling. Only K308 was effectively labelled with NHS-Biotin in the free protein and displayed partial protection in complex with RNA (Fig. 3.3B). In the CypA-NS5A-

DII protein complex, 1 lysine and 4 arginine residues were shown to be protected from NHS-biotin and HPG-modification, respectively. Representative MS profiles showing peaks corresponding to peptides harboring R304, R305, K308, R311 and R318 are shown in Fig. 3.3C. For the NS5A-DII complex with NS5B, the MS footprinting approach gave a similar protection profile to that observed with CypA (Table 3.1). In addition, peptides with a modified R307 residue were also found to be protected in the presence of NS5B (Fig. 3.3D). R262, R271, and R294 of remained accessible for HPG-labeling in the protein complex. Together, these data provide strong evidence to show that RNA, CypA and NS5B bind to the same region on NS5A-DII. The subtle differences in protection patterns also point to specific binding events.

3.5.3. *Analysis of NS5A-DII mutant proteins*

The protections of surface lysines and arginines could arise due to direct binding of these residues to the cognate ligand, or, alternatively, indirect conformational changes induced by the ligand binding. Hence, we substituted select arginine and lysine residues to glutamic acid and evaluated the ability of the mutant proteins to form either the nucleoprotein or protein-protein complexes. Mutant NS5A-DII proteins were fluorescently labeled with Cy3 maleimide dyes, and RNA binding was then evaluated using our FRET-based assay. Subsequent FRET data obtained for RNA binding with each NS5A-DII mutant were normalized relative to wild-type, and the data represented as relative binding. The data were fitted to a saturation binding curve, and an equilibrium dissociation constant (K_d) of 15.8 ± 4.0 nM was obtained for wild-type ^{cy3}NS5A-DII. With the exception of the R304E, mutations R305E, R307E, K308E and R311E led to a 50% decrease in the FRET signal, which is indicative for reductions in RNA binding (Fig. 3.4A and B). These results suggest that R305, R307, K308 and R311 could directly contribute to binding RNA.

Interestingly, the K_d obtained for the NS5A-DII mutants was similar to wild-type, suggesting that while the nucleoprotein interaction was compromised, the mode of binding remained the same. The interaction between mutant NS5A-DII proteins and CypA was likewise studied with the FRET-based assay. The data were fitted to a quadratic equation (equation 2, see Materials and Methods) and the K_d values were extracted (Table 3.2). For the wild-type ^{cy3}NS5A-DII, a K_d of 67.5 ± 18.1 nM was obtained. However, the FRET values for each of these substitutions (R304E, R305E, R307E, K308E and R311E) did not differ significantly from wild-type (Fig. 3.4C and D). Similarly, the extrapolated mutant K_d values remained within a 2-fold range of the wild-type, suggesting that protections of these residues seen in MS-based protein footprinting of the NS5A-DII bound to CypA could arise due to protein conformational changes affecting these residues. We therefore decided to probe residues in the vicinity of the protected lysine or arginines. Residues M313, W316 and R318 are functionally conserved across all major genotypes[201, 373]. Due to the variation in charge and polarity of these residues, alanine mutations were introduced at the corresponding residues (M313A, W316A and R318A). While ^{cy3}NS5A-DII harboring the M313A or R318A mutations showed a slight shift in the binding curve for the interaction with CypA, the W316A mutation led to a significant loss in the FRET signal (Fig. 3.4E). Binding of ^{cy3}NS5A-DII expressing the W316A was severely compromised, as indicated by a calculated K_d value exceeding 1.4 μM (Table 3.2). This data suggests that W316 is directly involved in interactions with CypA. Additionally, the W316A mutation may also disrupt small localized conformations that are essential for HCV viral replication, and have been shown to favour the interaction between NS5A-DII and CypA[380]. Previously, the W316A mutation in Con1b was shown to decrease viral replication in the replicon system, and the corresponding mutation in GT 2a JFH1 (W312A)

inhibited viral replication in Huh-7 cells[373]. Moreover, W312A in GT 2a also led to a 90% loss in CypA binding[201].

Binding of NS5A-DII proteins to NS5B was again assessed using fluorescence anisotropy. Similarly, the data were fitted to a quadratic equation (equation 2) and the K_d extracted (Table 3.2). Of all the aforementioned mutant proteins tested, only K308E and W316A were found to significantly inhibit the binding of NS5A-DII to NS5B (Fig. 3.4F-H). Interestingly, the K308R change did not cause an increase in the K_d value, suggesting that a positive charge is likely required at this position (Fig. 3.4H). When K308E was combined with the W316A mutation, the double mutation synergistically reduces binding of NS5A-DII to NS5B.

3.5.4. K308 residue is major contributor to RNA binding properties of NS5A-DII

To further characterize the identified RNA binding site in NS5A-DII, we assessed the individual contribution of each arginine and lysine residue in a time-dependent fluorescence anisotropy assay. For this, measurements for each mutant protein were taken and plotted against the wild-type (Fig. 3.5). Similar to our FRET assay, R304E did not have a significant effect on the RNA binding properties of NS5A-DII. However, our time course revealed a differential effect of the selected mutations. NS5A-DII R311E mutant appeared to have a partial delay in binding to the RNA substrate while the R305E and R307E mutants required a longer time period (~17 mins) to bind. RNA binding of the K308E mutant was greatly diminished and was not observed during the 30 min time course (Fig. 3.5). Lengthy time courses up to 1hr revealed that binding was achieved with the K308E mutant after 30 mins. Taken together, this data suggests that multiple residues are important and required for the RNA association of NS5A-DII, and that the K308 residue largely contributes to these RNA properties.

3.5.5. Mapping the NS5A-DII binding site on NS5B and CypA

In the previous section, we illustrate that CypA and NS5B share a common binding site on NS5A-DII. NMR studies have shown that CypA interaction with NS5A-DII is mediated through residues within its active site[179]. Hence as a proof of concept, this method was used to map the pre-determined binding site of NS5A-DII on CypA. As expected, our data shows that NS5A-DII interaction with CypA indeed mapped to peptides located in close proximity to the binding site of Cyclophilin inhibitor, CsA (Fig. 3.6). Subsequently, the surface topology of NS5B in the absence and presence of NS5A-DII was mapped by MS-based protein footprinting. The peptides detected by MALDI-TOF analysis cover ~ 82 % of the sequence of NS5B (Fig. 3.7A). We identified 14 out of 38 arginines, and 24 out of 31 lysine residues, that were readily accessible for chemical modification with HPG and NHS-biotin, respectively (Table 3.3 and Table 3.4 provide summaries of the data). These protected (shown in red) and unprotected (shown in black) residues were found in all the three subdomains: fingers, thumb and palm (Fig. 3.7A). Mapping of the protected peptides to the NS5B protein (PDB IC2P) revealed a distinct profile of the effect of NS5A-DII on NS5B (Fig. 3.7B). Most of the peptides harboring protected amino acids (shown in RED) reside mainly within the fingers subdomain. 53% (19/36) of arginine and lysine residues within this subdomain were solvent accessible for labeling in the free NS5B protein, 68% (13/19) of which were identified within protected peptides in the protein-protein complex. 2 modified peptides from the palm subdomain were identified, containing one arginine and one lysine residue (2/11). Both remained solvent accessible in the presence of NS5A-DII. In the thumb subdomain, 29% (6/21) of arginine and lysine residues were readily accessible in the free protein, and 50% (3/6) reside in peptides shielded from chemical modification in the presence of NS5A-DII.

The consistent protection of the peptides harboring R154, R158, R277, R380 and R386 residues in the NS5B complex with NS5A-DII, imply that this may be a major site of interaction or induced conformation change. To confirm this, NS5B variants expressing glutamic acid mutations at these residues were generated and assayed for binding to NS5A-DII. R277E and R386E NS5B mutants displayed similar binding to NS5A-DII as wild-type (Fig. 3.7C). Introduction of R154E, R158E and R380E mutations led to a significant decrease in binding, with R380E having the greatest effect (Fig. 3.7D). Taken together, our data shows the NS5A-DII interaction occurs with residues within the fingers and thumb subdomains of NS5B.

3.5.6. NS5A-DII decreases the RNA binding properties of NS5B

Peptides that were found protected in the presence of NS5A-DII reside mainly in regions of the NS5B polymerase known to be associated with RNA binding[374]. R386 and R394 form part of the primer-grip that recognizes phosphates of the primer strand during RNA synthesis[150, 381]. Previously, our group also showed that K100, K106 and K270 contribute to the RNA binding properties of the NS5B polymerase[374]. We therefore adapted a fluorescence-based binding assay to monitor the RNA binding properties of NS5B in the presence of NS5A-DII. Due to difficulty of generating a fluorescently-labelled NS5B protein, we switched to the different FRET assay involving a dually-labelled DNA:RNA substrate, as previously described[377]. Briefly, ensemble FRET experiments were performed with a dually-labeled DNA:RNA duplex bearing a 20 nucleotide single stranded RNA overhang, rA_{20}. Briefly, this substrate contains a Cy3 donor dye at the 3' end of the RNA overhang, and Cy5 acceptor dye at the DNA-RNA junction. Binding of NS5B to the RNA overhang (ssRNA) reduces the flexibility of the overhang bringing closer the donor and acceptor dyes, which is characterized by a marked increase in FRET. In the absence of

any protein, the calculated FRET of 0.5 was obtained for the rA_{20} substrate. As a control, we performed similar experiments in the presence of NS5A-DII only (Fig. 3.8A). Addition of NS5A-DII alone did not result in an increase in FRET, implying that NS5A-DII does not bind to the RNA strand. Preincubation of rA_{20} substrate with 250 nM NS5B protein led to an increase in FRET to 0.7 (Fig. 3.8B). The addition of incremental amounts of the NS5A-DII protein resulted in a concomitant decrease in the calculated FRET (Fig. 3.8B and C). This suggests that the interaction of NS5A-DII with NS5B alters the affinity of NS5B for the ssRNA.

3.5.7. NS5B RNA synthesis is compromised in the presence of NS5A-DII

This led us to investigate into whether the polymerase function of NS5B was also compromised by the interaction with NS5A-DII. We assessed the *de novo* and the primer-initiated RNA synthesis by the NS5B polymerase, using the T20 RNA substrate as template (Fig. 3.9A). A solution containing 2 µM of NS5B was preincubated in the presence of NTPs with increasing concentrations of NS5A-DII. Reactions were allowed to proceed for 30 mins. The proportion of full length RNA product decreased with increasing concentration of NS5A-DII (Fig. 3.9B and C). This was observed for both *de novo* and primed RNA synthesis. Quantification of full length RNA product showed there was a slight increase in *de novo* RNA synthesis when less than 1 µM of NS5A-DII was present (Fig. 3.9D). However, for both modes of RNA synthesis, 4-fold excess NS5A-DII was required to completely inhibit NS5B RNA synthesis activity (Fig. 3.9D and E). No significant restoration of RNA synthesis activity of NS5B was observed with the addition of CypA to the NS5A-DII complex with NS5B, suggesting that NS5A-DII forms a stable complex with the NS5B polymerase (Fig. 3.10). Taken together, these results suggest that binding of NS5A-DII to

NS5B leads to diminished RNA binding of NS5B, which subsequently results in a decrease in its RNA synthesis activity.

3.5.8. Comparative effect of domains I and II of NS5A on NS5B RNA synthesis

Another NS5B binding site on NS5A also exists within domain I of NS5A (NS5A-DI)[171]. Thus, we sought to investigate into the effect of NS5A-DI on the NS5B polymerase activity, as well (Fig. 3.11A). We also assessed the NS5B RNA synthesis activity in the presence of an NS5A protein construct bearing both domains I and II (NS5A-DI+II). An inhibitory effect was observed on the NS5B RNA synthesis activity in the presence of NS5A-DI (Fig. 3.11B). Interestingly, 4-fold and 2-fold excess of NS5A-DII and NS5A-DI concentrations were required to completely inhibit RNA synthesis activity of NS5B, respectively, implying that binding of NS5A-DI to NS5B exerted a greater inhibitory effect than NS5A-DII. The NS5A-DI+II construct appeared to inhibit the NS5B RNA synthesis activity more effectively than either domains I or II alone (Fig. 3.11B). Quantification of the full-length RNA synthesis product showed a large difference in the inhibitory effect of each NS5A construct (Fig. 3.11C). The half maximal concentrations for NS5A-DI, NS5A-DII and NS5A-DI+II were 1.072 ± 0.31 μM, 2.069 ± 0.71 μM, and 0.324 ± 0.07 μM, respectively. This significant change suggests a synergistic effect when both domains are presence. Taken together, these results show that the interactions between NS5B and either domain I and II of NS5A decreases the NS5B RNA synthesis activity, and suggest that both domains I and II act synergistically to inhibit NS5B activity.

3.6. Discussion

HCV NS5A is a multi-functional protein, which has been implicated in various interactions with both viral and host factors[132]. Domain II of NS5A (NS5A-DII) directly interacts with the viral genome, the host factor CypA and the viral RNA polymerase NS5B. NS5A-DII is intrinsically disordered and this has considerably hampered structural and functional studies. In this study, we conducted MS-based protein footprinting experiments to identify key residues of NS5A-DII that are crucial in defining the nucleoprotein and protein-protein interactions. We used chemical reagents that specifically modify solvent accessible lysine and arginine residues of the free and bound protein complex. Comparative MS-based analysis allowed us to identify key residues based on their protection from modification by direct interaction or protein conformational changes in each protein complex. The importance of several amino acids that reside within these binding sites for NS5A-DII interactions was confirmed through mutational analysis.

Through MS-based analysis, key residues within NS5A-DII involved in RNA binding were identified, and this has been modelled in Fig. 3.12. These residues were found to be located within a highly positively charged region of NS5A-DII, which may favor contacts with the nucleic acid phosphate backbone. While mutations generated within this region inhibited NS5A-DII-RNA interaction, *in vitro* cell culture studies have indicated that this region may not be essential for HCV replication[373]. However, the involvement of this interaction at the level of RNA trafficking or of viral egress, which is not addressed by the replicon system, remains to be investigated. Interestingly, the identified RNA binding residues partially overlap with the highly conserved CypA binding site, from residues 308-311[179, 201]. Binding of CypA to NS5A-DII is proposed to induce local conformational changes within NS5A-DII in addition to enhancing its RNA binding

properties[200, 203]. Thus, our detailed description of the close proximity of these two binding sites suggests that the conformational changes that are induced upon CypA binding would have a favorable and positive effect on the upstream RNA binding site. In addition, our MS/MS analysis revealed overlapping binding regions for RNA, CypA and NS5B within the NS5A-DII protein. It is interesting to note that NS5A-DII peptides harboring the HPG-modified R307 residue were identified as protected in the presence of RNA and NS5B, but not when in complex with CypA. One explanation for this could be a difference in the mode in which NS5A-DII interacts to the CypA cofactor. The structural changes induced within NS5A-DII upon binding of CypA suggest an altered surface topology of NS5A-DII, rendering R307 residue solvent accessible in the NS5A-DII protein complex with CypA. This underscores the dynamic ability of NS5A-DII as an intrinsically disordered protein to adopt various conformations that facilitate the interactions with various binding partners.

NS5B and CypA have been proposed to share the same binding site on NS5A-DII [170]. Our MS analysis confirmed this observation and pointed out one key difference: K308 is a key residue in mediating the interaction with NS5B, but not with CypA. Thus, we can clearly attribute the importance of this residue to binding to NS5B and RNA. The identified binding sites reside in a highly conserved region of NS5A-DII which comprises of residues M313 and W316. Mutation of these residues leads to reduced viral replication efficiency[194, 201, 373]. Our assays indicate that the W316A mutation leads to a reduced affinity for both CypA and NS5B, which may in part explain the previously observed replication impairment. The presence of this mutation may also disrupt small structural conformations residing within this intrinsically disordered domain. Recent NMR studies have proposed the formation of a conserved functional conformation within this region,

based on the identification of a well-defined short structural motif from P314 to A317[380]. It is suggested that the side chains of P314 and W316 interact to form a Pro-Trp turn, a feature which favors CypA interaction. Introduction of the W316A mutation would therefore hinder the formation of the aforementioned structural motif. These mutations did not diminish the RNA binding properties of NS5A-DII, implying that they were not required for RNA binding (data not shown). Taken together, our data indicate that residue W316 is indispensable for protein-protein interactions and highlight a functional importance to this local conformation within NS5A-DII.

Our MS-based approach revealed a predominant site of interaction of NS5A-DII within the fingers subdomain of the viral polymerase. Several amino acids in NS5B involved in RNA binding (R154, R158, R277, R380 and R386) are also protected in the NS5A-DII complex with NS5B. This could be due to a direct interaction or induced conformational change. Figure 7 illustrates the location of these residues within the NS5B structure, co-crystallized with a short RNA fragment (PDB 4E78). R154 and R158 are located at the exit site of the NS5B RNA binding channel, while R380 forms part of a β-hairpin in the thumb subdomain[148, 157]. These residues were found to be essential for binding to NS5A-DII. Mutations generated at residues R277 (distal to the RNA binding channel) and R386 (buried within the RNA binding channel) had no effect on the NS5A-DII complex with NS5B. This suggests that the observed protection profile of R277 located further from the RNA binding channel, and R386 deep within the RNA binding cleft could be ascribed to induced protein conformational changes, rather than the direct interaction with NS5A-DII.

Mapping of the NS5A-DII binding site to a predetermined RNA binding region on NS5B suggested a functional effect on NS5B activity. Further investigation showed that NS5A-DII

binding led to decreased RNA binding and polymerase activity of NS5B. As illustrated in Fig. 3.12, this would imply that NS5A-DII must bind and block the RNA exit site, closing off the egress of nascent RNA strands. Furthermore, our data points towards the inhibition of the RNA elongation stage, rather than the initiation stage in RNA synthesis. To support this, we show that both *de novo* and primer-mediated RNA synthesis were equally inhibited. Therefore, NS5A-DII would appear to not affect the initiation phase of RNA synthesis. A slight increase in *de novo* RNA synthesis was observed, however the exact significance of this in the context of viral replication would need to be determined. The observed inhibition suggests a possible regulatory role of NS5A-DII on the NS5B polymerase activity. Several studies have alluded to the down regulation of viral replication by NS5A, and this could serve as a molecular switch in the regulatory process between transcription and translation[164, 171]. However, the exact role of domain II in this regulation still remains to be investigated.

Overall, we propose a model of the interplay of intermolecular interactions mediated through NS5A-DII within the HCV replication complex (Fig. 3.12). Here, we model the existence of an RNA binding site overlapping the binding region for the CypA host factor and NS5B polymerase. This may suggest different modes of binding with respect to a specific ligand. CypA binds through several key residues like W316 within NS5A-DII. Conformational changes induced by CypA interaction facilitate a preferred RNA binding mode through the upstream RNA binding site, and this interaction with CypA is essential for HCV replication[179]. In the absence of CypA, this conformation is lost. The K308 and W316 residues, integral to NS5A-DII protein interaction, are available to bind NS5B. Consequently, this mode of binding inhibits NS5B activity, thus

decreasing RNA replication. The differential mode of binding could have alternate positive and negative effects on HCV replication.

In summary, for the present study we used state of the art technique to map the interaction surface of NS5A-DII at the amino acid level when it is bound to different ligands. We pinpoint distinct residues that are specifically involved in interacting with either a nucleic acid or a protein ligand. Our data demonstrates that although NS5A-DII is intrinsically disordered, it does possess inherent features which enable its interaction with different ligands. In addition, our MS-based analysis of NS5B revealed a previously undefined site of interaction for NS5A-DII. The results of our study provide an in-depth characterization of the intermolecular interactions mediated through NS5A-DII, and highlight a potential functional role in the HCV life cycle. Our data sets the groundwork for the validation of newly described sites for the rational design and development of the next generation of therapeutics for the treatment of HCV infection.

Table 3. 1: NS5A-DII peptide fragments containing modified arginines and lysines identified from a complex with RNA, CypA and NS5B.

Modifying agent	Residues present in NS5A-DII	Peptide coverage	Modified Residues	NS5A-DII Peptide Protection Profile with:		
				RNA	CypA	NS5B
HPG	R262	248-271	R262	- [a]	-	-
	R271	263-277	R271	-	-	-
	R294	278-304	R294	-	-	-
	R304	295-305	R304, R305 (2) [c]	+ [b]	+	+
	R305	295-308	R304, R305, R307 (3)	+	-	+
	R307	295-311	R304 / R305 / R307 (2)	+	-	+
		305-311	R305 / R307 (1)	+	-	+
	R311	309-311	R311	+	+	+
	R318	309-330	R311, R318 (2)	-	+	+
		312-330	R318	-	+	+
NHS-Biotin	K277	N.A. [d]	N.A.	N.D. [e]	N.D.	N.D.
	K308	308-311	K308	+	+	+
		308-330	K308	-	+	+
	K330	N.A.	N.A.	N.D.	N.D.	N.D.

[a] Denotes protected peptides
[b] Denotes unprotected peptides
[c] Number of modified residues
[d] N.A. = not applicable; no peptides bearing this modified residue were detected.
[e] N.D. = not determined

Table 3. 2: Table of K_d values obtained for binding of NS5A-DII to CypA and NS5B

	Ligand			
	CypA		NS5B	
NS5A-DII	K_d (nM)	Fold Change	K_d (nM)	Fold Change
WT	67.5 ± 18.1	1.0	14.5 ± 4.8	1.0
R304E	89.1 ± 31.2	1.2	29.3 ± 9.3	2.0
R305E	87.4 ± 15.7	1.3	11.5 ± 3.6	0.8
R307E	122.2 ± 29.7	1.8	11.9 ± 2.7	0.8
R311E	143.1 ± 18.0	2.2	34.6 ± 17.4	2.4
M313A	364.4 ± 125.2	5.3	4.9 ± 1.7	0.3
W316A	> 1400	20	58.0 ± 15.1	4.0
R318A	342.3 ± 102.3	5.1	30.4 ± 9.2	2.1
K308E	100.7 ± 28.5	1.3	55.5 ± 17.1	3.8
K308R	N.D. [a]		8.9 ± 3.2	0.6
K308E_W316A	N.D.		> 500	35

[a] N.D. = not determined

Table 3. 3 NS5B peptide fragments containing modified arginines identified from a complex with NS5A-DII

Modifying agent	Arginine residues present in NS5B	Peptide coverage	Modified Residues	Peptide Protection Profile
HPG	38	49-65	R56	- [a]
		57-71	R65	+ [b]
		91-106	R98	+
		110-124	R120	+
		142-168	R154, R158 (2) [c]	+
		155-172	R158, R168 (2)	+
		271-278	R277	+
		271-280	R277 / R278 (1)	+
		281-307	R304	-
		380-394	R380, R386 (2)	+
		380-401	R380, R386, R394 (3)	+
		381-394	R386	+
		524-533	R531	-

[a] Denotes unprotected peptides
[b] Denotes protected peptides
[c] Number of modified residues

Table 3. 4: NS5B peptide fragments containing modified lysines identified from a complex with NS5A-DII

Modifying agent	Lysine residues present in NS5B	Peptide coverage	Modified Residues	NS5B Protection Profile in complex with NS5A-DII
NHS-Biotin	31	49-51	K50	- [a]
		51-56	K51	-
		70-74	K72	-
		99-109	K100, K106 (2) [c]	+ [b]
		101-109	K106	+
		110-120	K114	+
		142-158	K151 / K155 (1)	+
		152-158	K155	+
		169-172	K172	-
		201-211	K209	-
		260-277	K270	+
		491-501	K491	-
		518-523	K523	-
		532-535	K533	-

[a] Denotes unprotected peptides
[b] Denotes protected peptides
[c] Number of modified residues

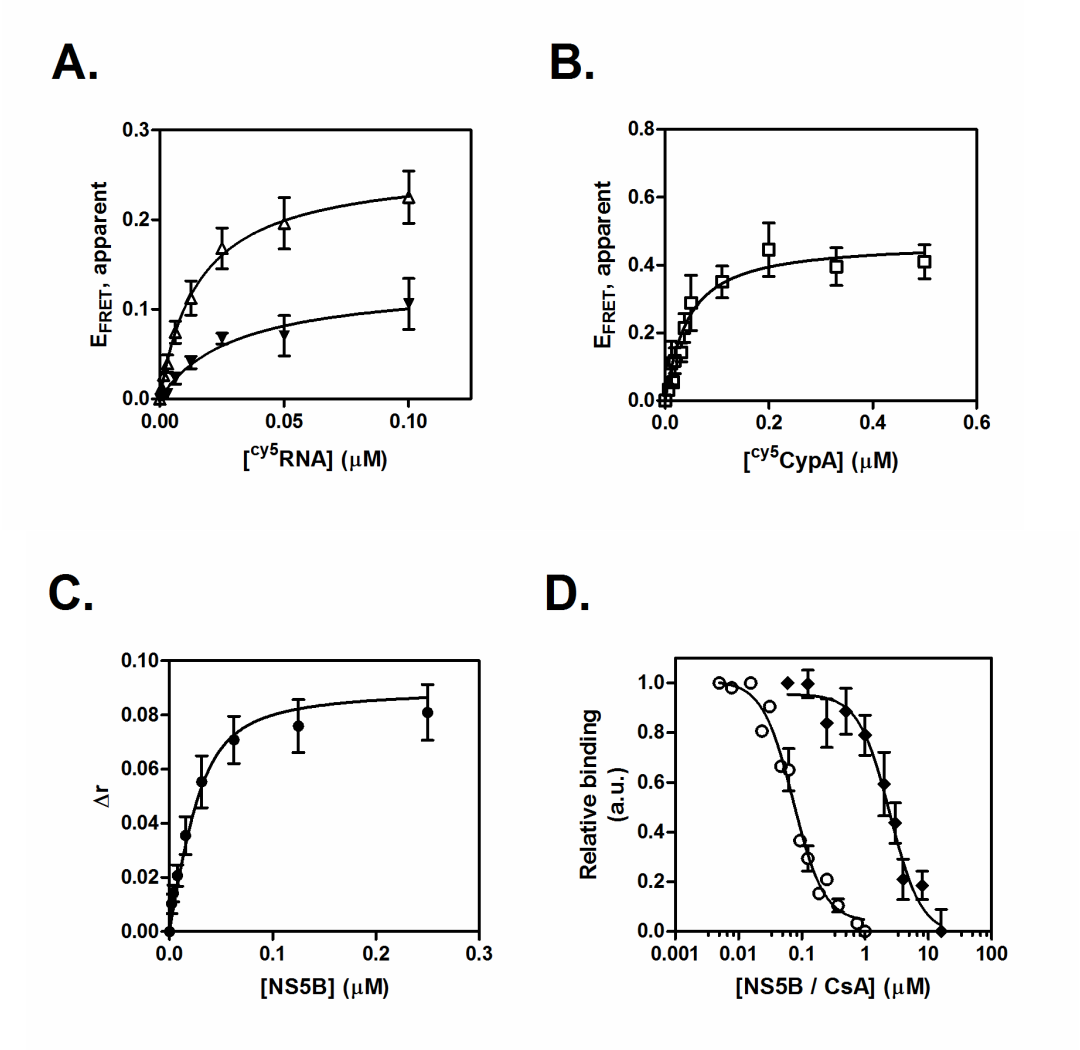


Figure 3. 1: Binding of NS5A-DII to RNA, CypA and NS5B, respectively.

(A) FRET values between ^{cy3}NS5A-DII and increasing concentrations of $^{cy5}rU_{20}$ (Δ), $^{cy5}rN_{19}$ (▼) and ***(B)*** ^{cy5}CypA. ***(C)*** Change in anisotropy (Δr) for the binding between ^{cy3}NS5A-DII with increasing concentration of NS5B. Data points represent average values of at least three independent measurements with error bars indicating standard error. ***(D)*** Relative binding data representative of competitive inhibition of binding between NS5A-DII and CypA by NS5B (○) and CsA (◆).

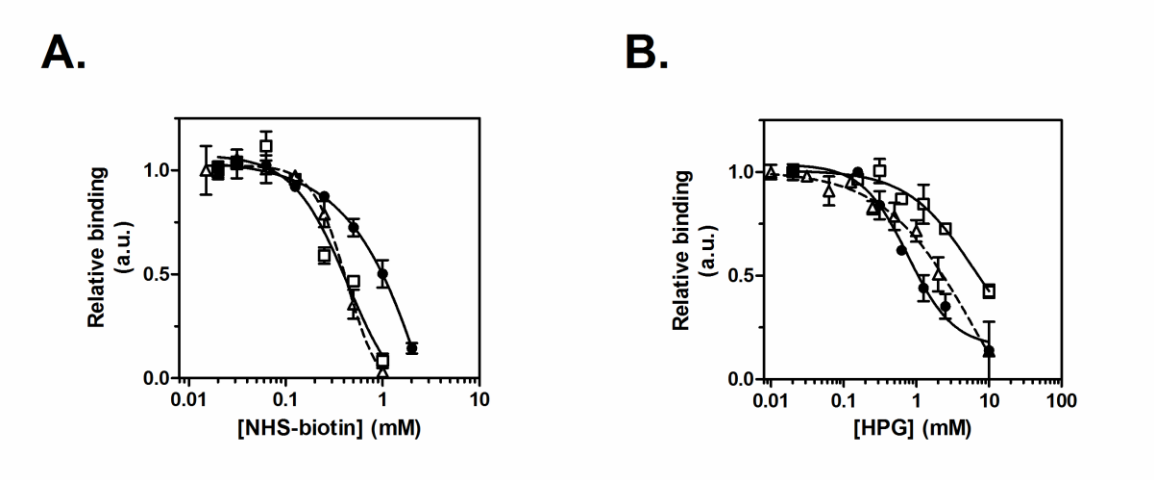
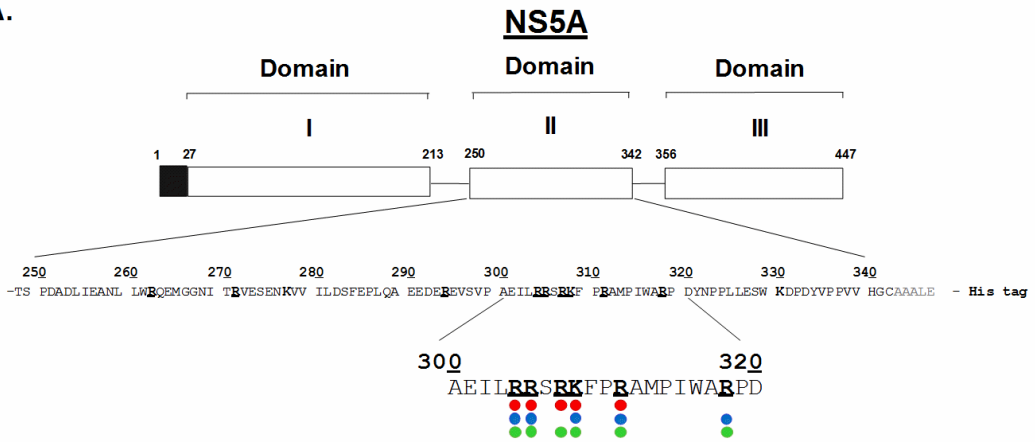


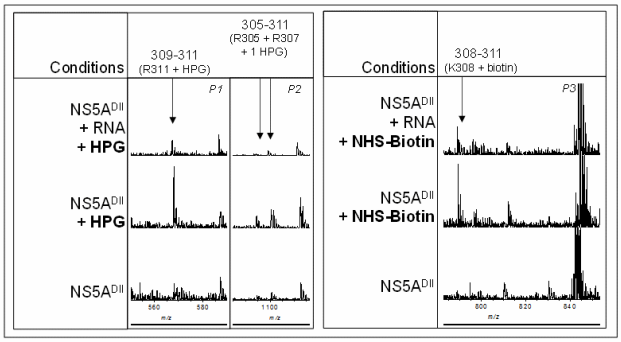
Figure 3. 2: Effect of HPG-modified and biotinylated on NS5A-DII on protein-protein and protein-RNA interactions.

(A) Relative binding of biotinylated NS5A-DII to either rU_{20} (Δ), CypA (■), or NS5B (●). ***(B)*** Similar experiments were conducted with HPG-modified NS5A-DII. Relative binding to rU_{20} and CypA is representative of actual FRET values that were normalized relative to the initial FRET value obtained for the protein-protein or protein-RNA complex, in the absence of any chemical modifier. Relative binding for NS5B represents the change in anisotropy normalized relative to the initial value of the protein-protein complex.

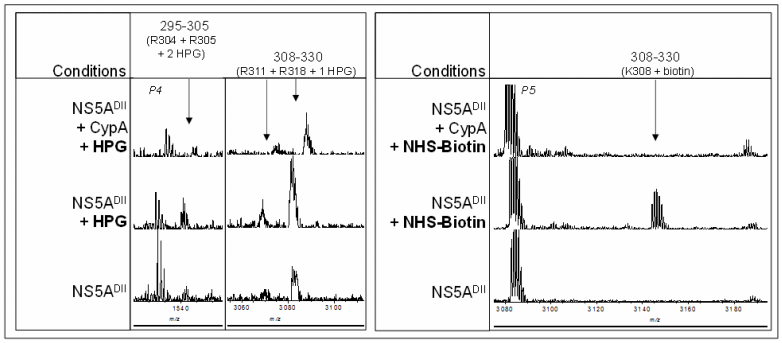
A.



B. [NS5A^{DII} : RNA]



C. [NS5A^{DII} : CypA]



D. [NS5A^{DII} : NS5B]

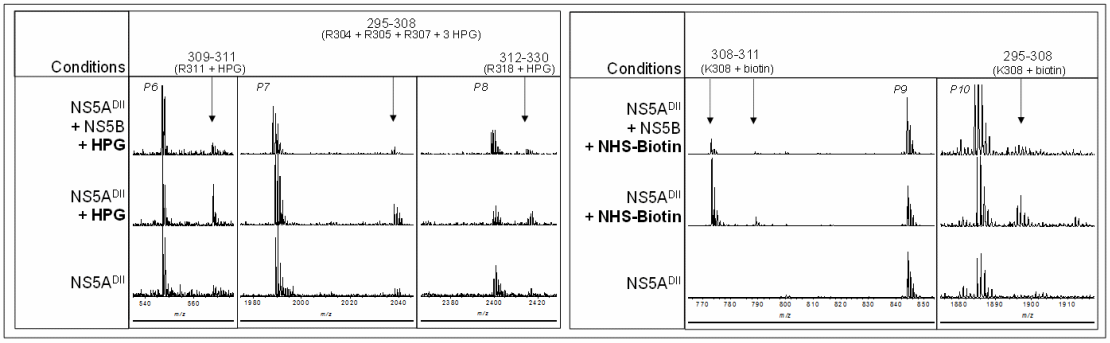


Figure 3. 3: Characterization of the RNA, CypA and NS5B binding sites on NS5A-DII.

(A) NS5A domain organization, with a C-terminal amphipathic helix (shown in black). The sequence of NS5A-DII from HCV Con1b used in this study is shown. The arginine and lysine residues are shown in **bold**. Underlined are those that were readily accessible for labeling in the free NS5A-DII protein. HPG modified arginines or NHS-biotinylated lysines residing in peptides protected in the NS5A-DII complex with RNA (●), CypA (●) and NS5B (●) are indicated. ***(B)*** A representative segment of MALDI-TOF spectrum showing tryptic fragments of NS5A-DII with HPG modifications of arginine residues in the presence of RNA. Peaks containing modified R305, R307 and R311 residues are significantly diminished in the NS5A-DII complex with RNA. Similarly, representative spectra showing tryptic fragments of chemically modified NS5A-DII in the presence of ***(C)*** CypA, and ***(D)*** NS5B. Peaks corresponding to peptides harboring residues R304, R305, R307, K308, R311 and R318 residues of NS5A-DII were labeled with either HPG or NHS-Biotin. Several of these peaks were significantly reduced in the NS5A-DII complex with CypA and NS5B. Unmodified NS5A-DII peptide peaks P1-P9 serve as internal controls.

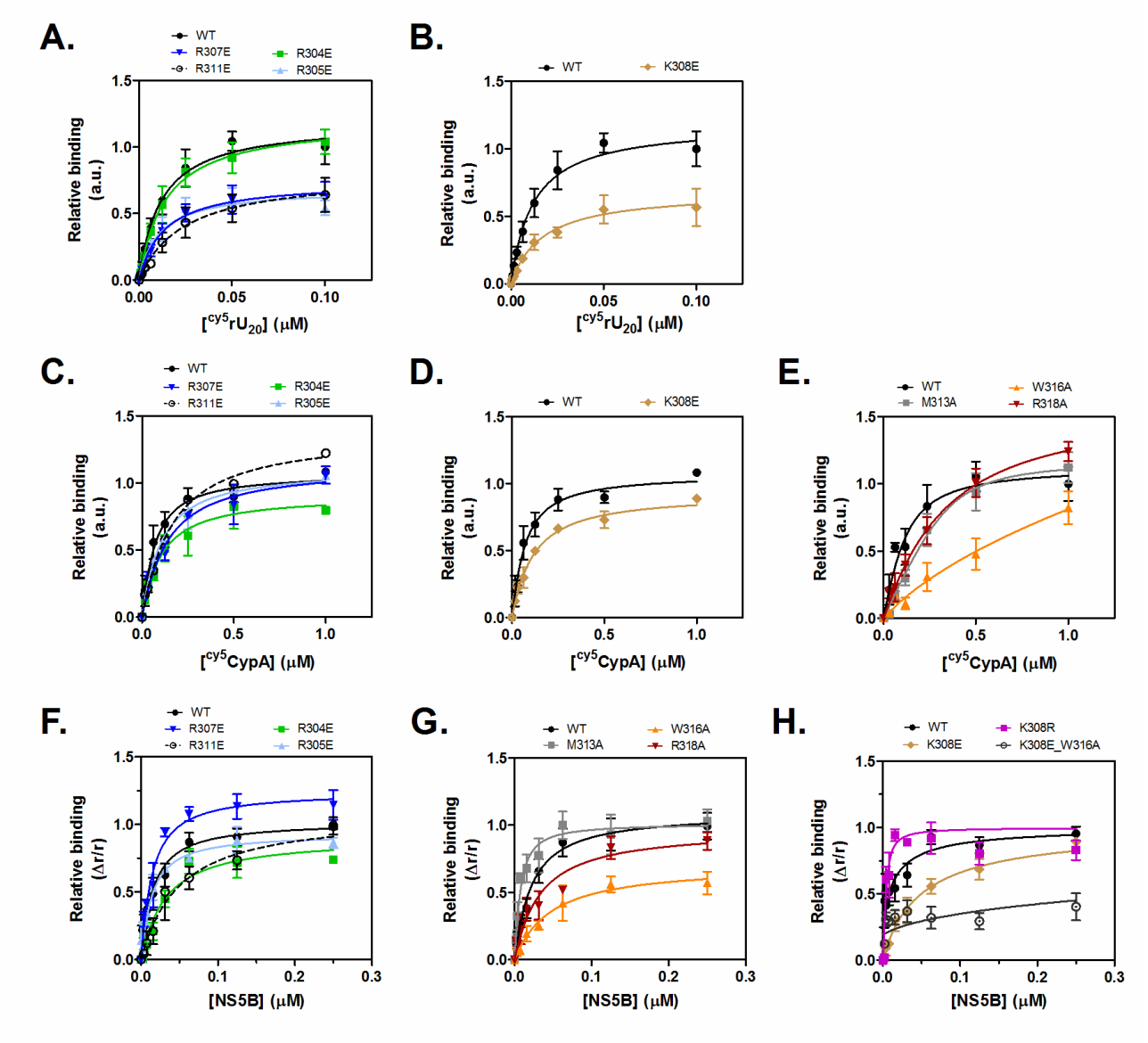

A.
WT
R307E
R311E
R304E
R305E
Relative binding (a.u.)
[cy5rU20] (μM)
B.
WT
K308E
Relative binding (a.u.)
[cy5rU20] (μM)
C.
WT
R307E
R311E
R304E
R305E
Relative binding (a.u.)
[cy5CypA] (μM)
D.
WT
K308E
Relative binding (a.u.)
[cy5CypA] (μM)
E.
WT
M313A
W316A
R318A
Relative binding (a.u.)
[cy5CypA] (μM)
F.
WT
R307E
R311E
R304E
R305E
Relative binding (Δr/r)
[NS5B] (μM)
G.
WT
M313A
W316A
R318A
Relative binding (Δr/r)
[NS5B] (μM)
H.
WT
K308E
K308R
K308E_W316A
Relative binding (Δr/r)
[NS5B] (μM)

Figure 3. 4: Mutational analysis of NS5A-DII.

^{Cy3}NS5A-DII mutants harboring single mutations (as indicated) were assessed for their RNA binding, CypA binding, and NS5B binding properties. FRET and anisotropy values for each mutant were normalised relative to wild-type ^{cy3}NS5A-DII (●), and represented as relative binding. ***(A)*** For RNA binding, ^{cy3}NS5A-DII mutants harboring the R304E (■), R305E (▲), R307E (▼), R311E (○) and ***(B)*** K308E (◆) mutations were assessed using ^{cy5}rU$_{20}$ through FRET. ***(C, D)*** Similarly, these mutants were assessed for binding to ^{cy5}CypA. **(E)** Relative binding to CypA is shown for additional ^{cy3}NS5A-DII mutants harboring M313A (■), W316A (▲) and R318A (▼) mutations. ***(F, G)*** Data representative of relative binding of ^{cy3}NS5A-DII mutants to NS5B through fluorescence anisotropy. ***(H)*** Binding of ^{cy3}NS5A-DII K308E (◆) mutant to NS5B is shown in comparison to K308R (■) and the K308E-W316A double mutant (**Θ**).

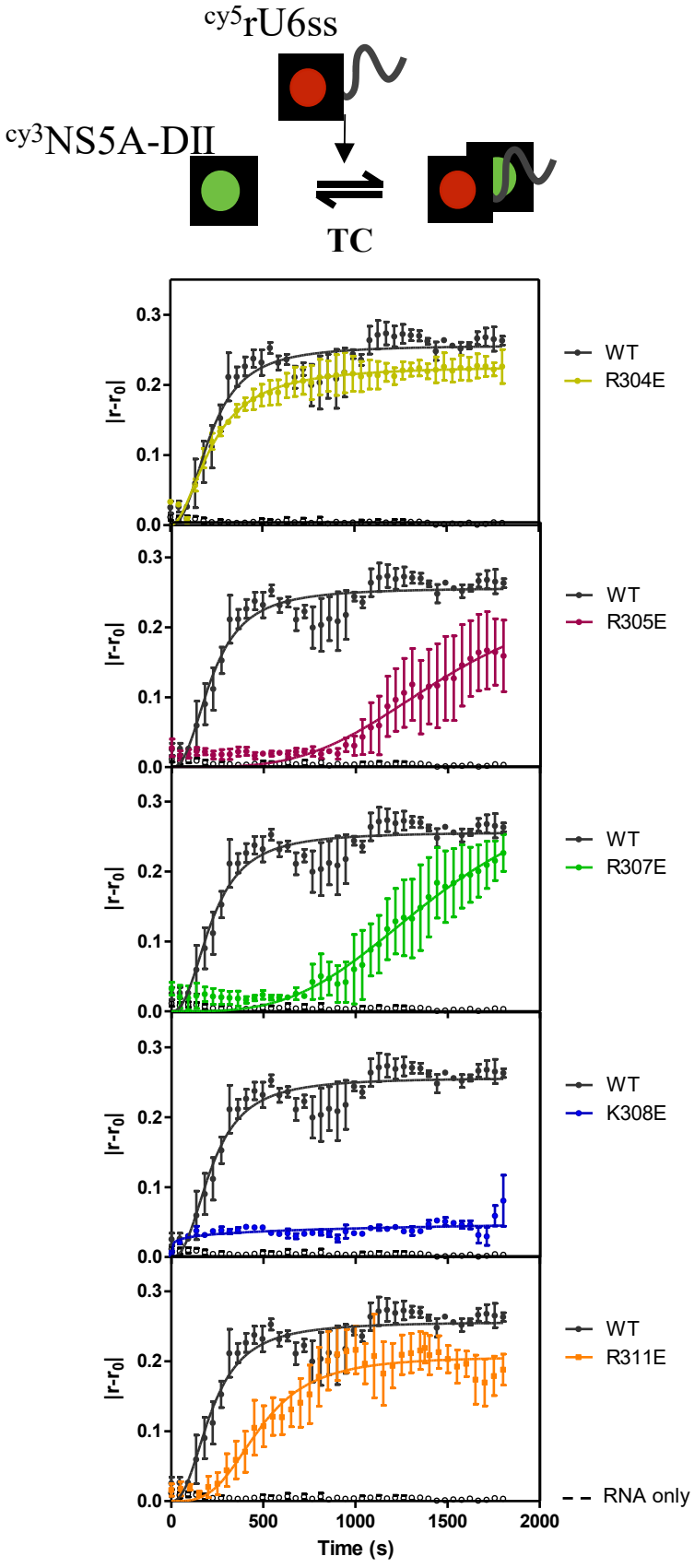

cy5rU6ss
cy3NS5A-DII
TC
|r-r0|
Time (s)
WT
R304E
WT
R305E
WT
R307E
WT
K308E
WT
R311E
RNA only

Figure 3. 5: Presence of K308E on NS5A-DII greatly diminishes RNA binding.

Experiment schematic of the time-dependent Anisotropy RNA binding of NS5A-DII. TC refers to a time course experiment. NS5A-DII mutants expressing a single mutation were used to assess the contribution of each residue towards RNA binding to a model RNA substrate, ^{cy3}rU6ss. A graphical representation is shown for the anisotropy assessment for the binding of NS5A-DII mutants R304E, R305E, R307E, R3I1E and K308E, to ^{cy3}rU6ss.

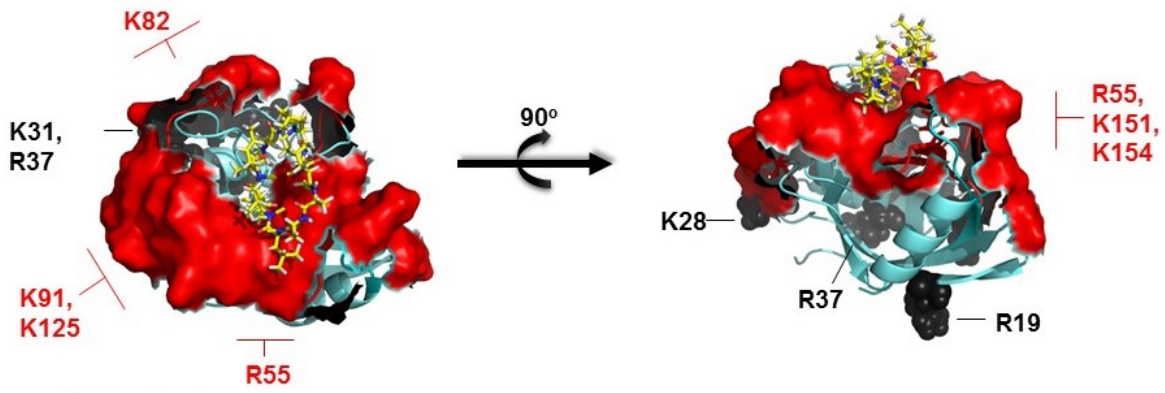


Figure 3. 6: Summary of MS-based footprinting results of the CypA interaction with NS5A-DII.

CypA crystal structure in complex with the CypA inhibitor, Cyclosporine A (PDB 3CYS). Peptides which include protected lysine or arginine residues are shown in **red**. Unprotected lysine and arginine residues are shown in **black**

.

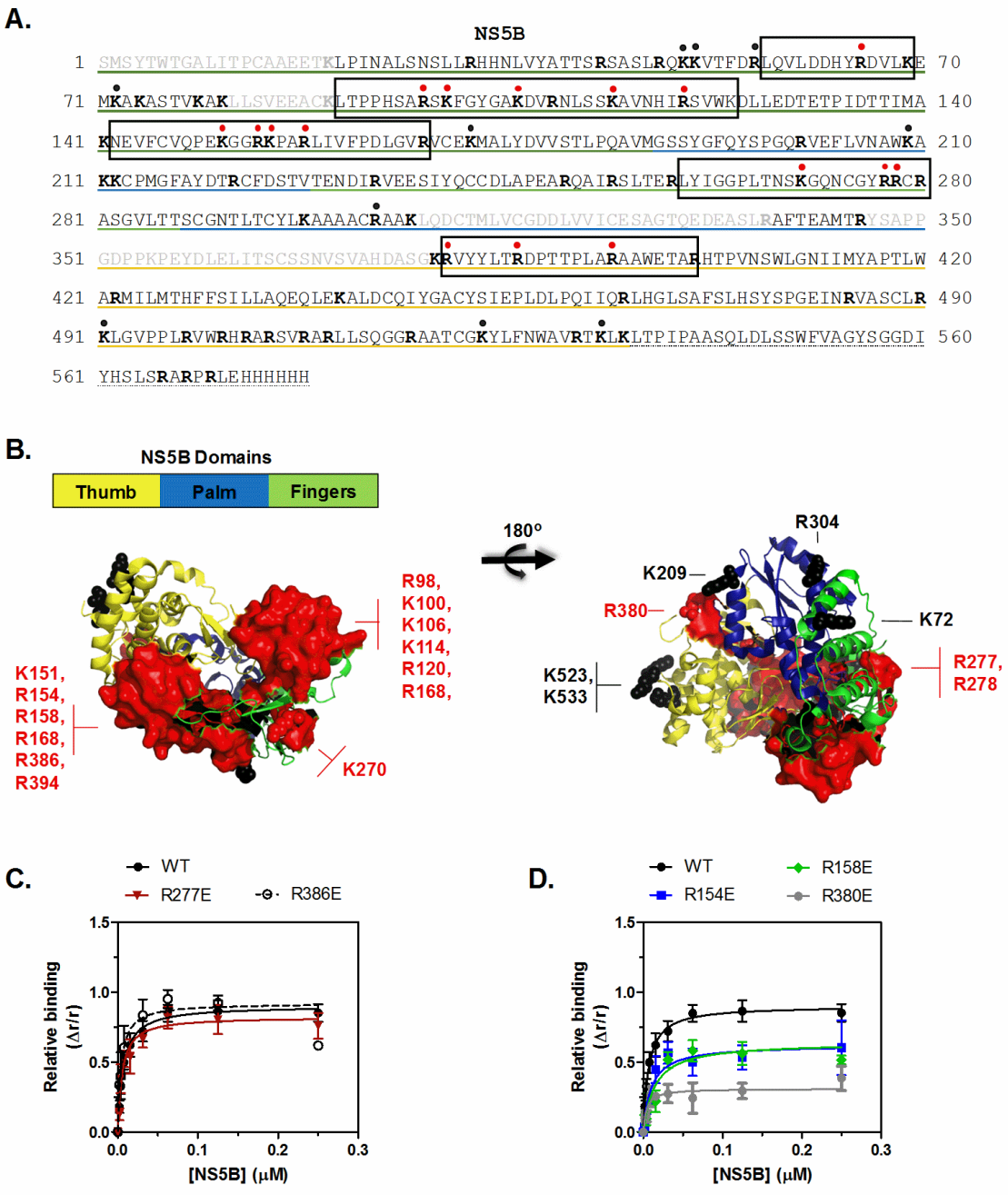

A.
NS5B
1 SMSYTWTGALITPCAAEETKLPINALSNSLLRHHNLVYATTSRSASLRQKKVTFDRLQVLDDHYRDVLKE 70
71 MKAKASTVKAKLLSVEEACKLTPPHSARSKFGYGAKDVRNLSSKAVNHIRSVWKDLLEDTETPIDTTIMA 140
141 KNEVFCVQPEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQYSPGQRVEFLVNAWKA 210
211 KKCPMGFAYDTRCFDSTVTENDIRVEESIYQCCDLAPEARQAIRSLTERLYIGGPLTNSKGQNCGYRRCR 280
281 ASGVLTTSCGNTLTCYLKAAAACRAAKLQDCTMLVCGDDLVVICESAGTQEDEASLRAFTEAMTRYSAPP 350
351 GDPPKPEYDLELITSCSSNVSVAHDASGKRVYYLTRDPTTPLARAAWETARHTPVNSWLGNIIMYAPTLW 420
421 ARMILMTHFFSILLAQEQLEKALDCQIYGACYSIEPLDLPQIIQRLHGLSAFSLHSYSPGEINRVASCLR 490
491 KLGVPPLRVWRHRARSVRARLLSQGGRAATCGKYLFNWAVRTKLKLTPIPAASQLDLSSWFVAGYSGGDI 560
561 YHSLSRARPRLEHHHHHH
B.
NS5B Domains
Thumb
Palm
Fingers
K151,
R154,
R158,
R168,
R386,
R394
K270
R98,
K100,
K106,
K114,
R120,
R168,
180°
R304
K209
R380
K72
K523,
K533
R277,
R278
C.
WT
R277E
R386E
Relative binding
(Δr/r)
[NS5B] (μM)
D.
WT
R154E
R158E
R380E
Relative binding
(Δr/r)
[NS5B] (μM)

Figure 3. 7: Summary of MS-based footprinting results of the NS5B interaction with NS5A-DII.

(A) HCV NS5B amino acid sequence mapping of the modified arginine and lysine residues. The assigned MS peptide peaks cover ~82% of the NS5B sequence. The segments that were not detected by MS are written in gray. Unprotected modified residues in the free NS5B protein are indicated with a black circle. Arginine and lysine residues that reside in peptides protected (black box) from modification in the NS5B complex with NS5A-DII are shown with red circles. Underlined sequences refer to the NS5B subdomains: fingers in green, palm in blue, thumb in orange, and C terminus with a dashed line. ***(B)*** NS5B crystal structure (PDB 1C2P). Color codes for each domain are indicated. Peptides which include protected lysine or arginine residues are shown in **red**. Unprotected lysine and arginine residues are shown in black. ***(C)*** NS5B proteins harbouring mutations R277E (▼), R386E (○), and ***(D)*** R154E (■), R158E (◆) and R380E (●) were assessed for their binding properties to ^{cy3}NS5A-DII by protein anisotropy. Relative binding is indicative of anisotropy values normalised relative to wild-type NS5B (●).

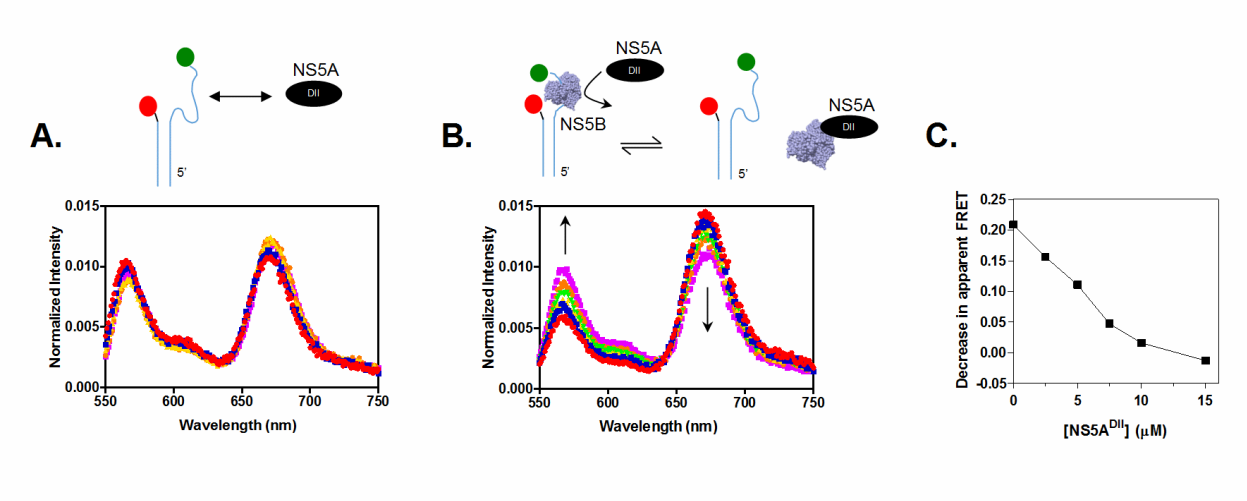


Figure 3. 8: Monitoring binding of NS5B to rA20 in the presence of NS5A-DII.

(A) The RNA substrate was incubated with increasing concentrations of NS5A-DII and either buffer alone, or ***(B)*** NS5B (250 nM). Note that in the absence of NS5B, calculated FRET for rA_{20} is at 0.5, compared to 0.7 when pre-incubated with only NS5B. ***(C)*** The decrease in apparent FRET, as calculated by the difference in FRET in the presence and absence of NS5B, is plotted as a function of NS5A-DII concentration.

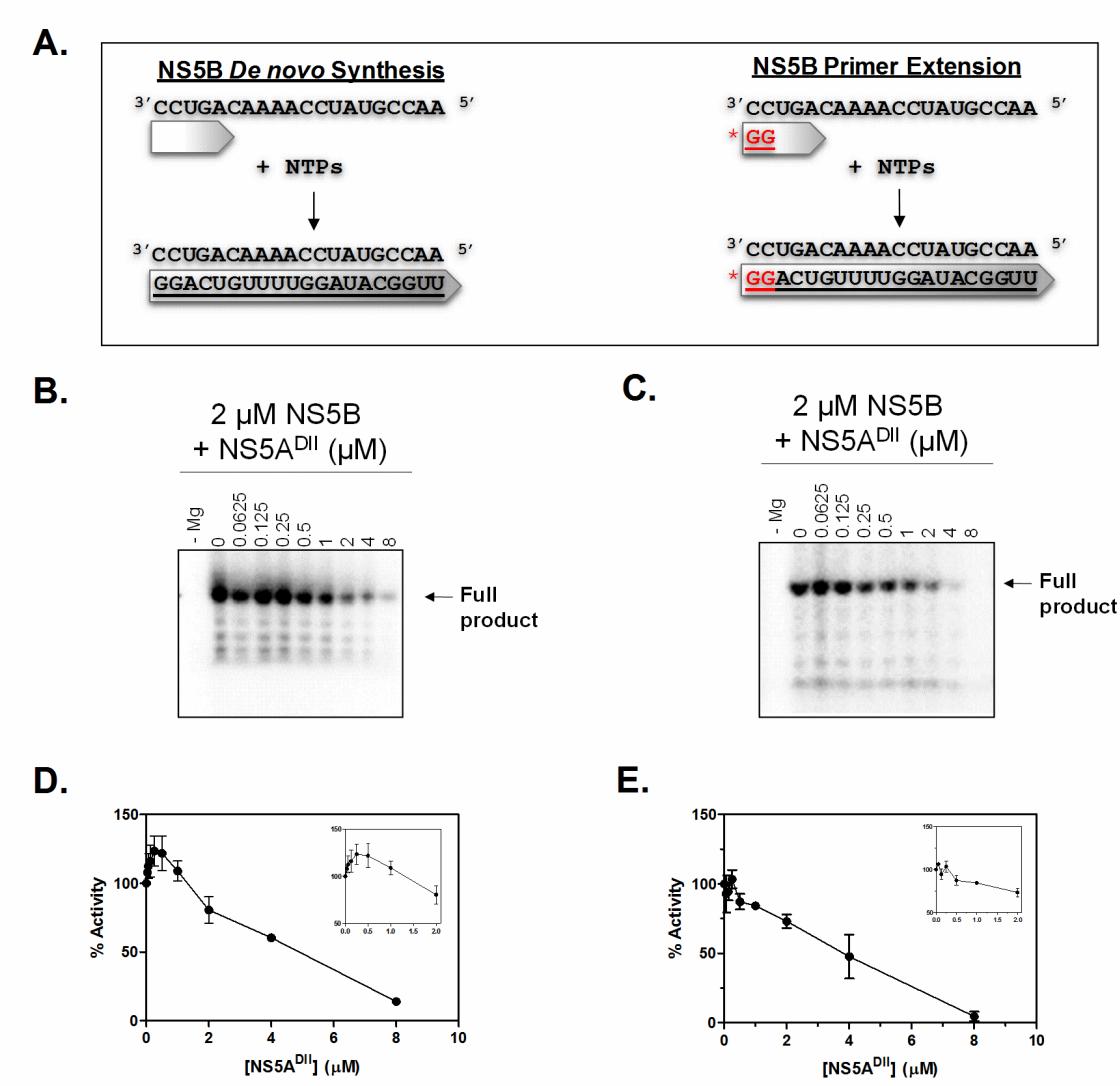

A.
NS5B De novo Synthesis
3′CCUGACAAAACCUAUGCCAA 5′
+ NTPs
3′CCUGACAAAACCUAUGCCAA 5′
GGACUGUUUUGGAUACGGUU
NS5B Primer Extension
3′CCUGACAAAACCUAUGCCAA 5′
*GG
+ NTPs
3′CCUGACAAAACCUAUGCCAA 5′
*GGACUGUUUUGGAUACGGUU
B.
2 μM NS5B
+ NS5ADII (μM)
- Mg
0
0.0625
0.125
0.25
0.5
1
2
4
8
Full product
C.
2 μM NS5B
+ NS5ADII (μM)
- Mg
0
0.0625
0.125
0.25
0.5
1
2
4
8
Full product
D.
% Activity
[NS5ADII] (μM)
E.
% Activity
[NS5ADII] (μM)

Figure 3. 9: Polymerase activity of NS5B.

(A) Schematic of NS5B RNA synthesis assays. For *de novo* and primer extension assays, new RNA products are synthesised by the NS5B polymerase from the T20 RNA template. Radiolabeled GG dinucleotide primer was used in the primer extension assay. Denaturing gel electrophoresis analysis of *de novo* and primed RNA synthesis by NS5B are shown in ***(B)*** and ***(C)***, respectively. Gels were cropped to show just the full-length RNA product. Reactions proceeded for 30 mins in the presence of a NS5A-DII concentration gradient. Increasing NS5A-DII concentration led to a decrease in RNA synthesis. ***(D, E)*** Graphical representation of the % full-length RNA product from ***(B)*** and **(C)** are shown, respectively. ***(D,** inset)* indicates the slight increase in *de novo* NS5B polymerase activity at low NS5A-DII concentrations, but not evident for primer-mediated activity *(**E**, inset)*.

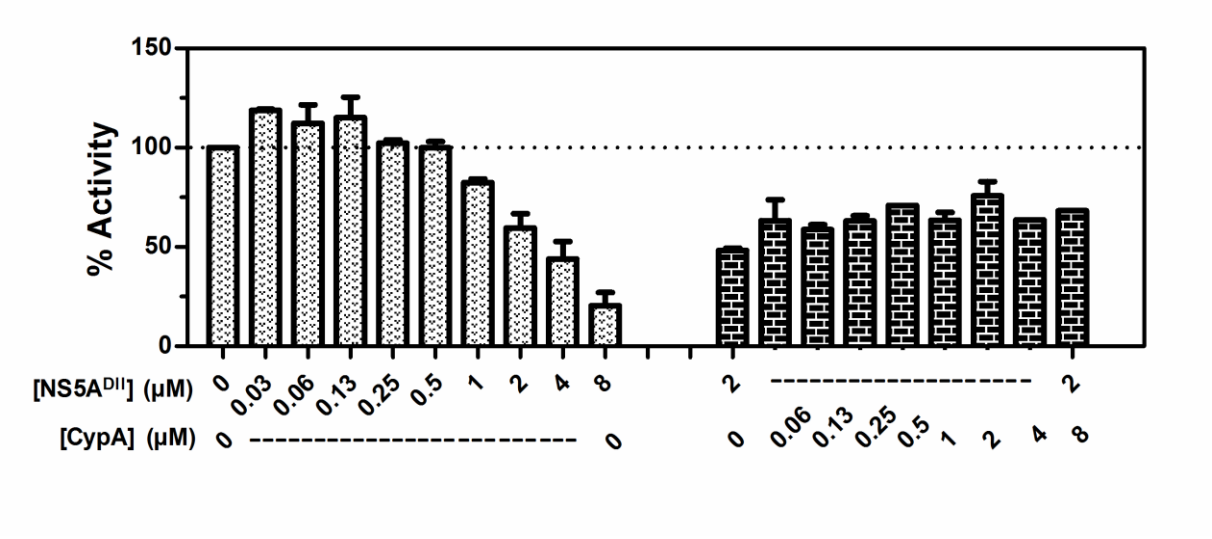
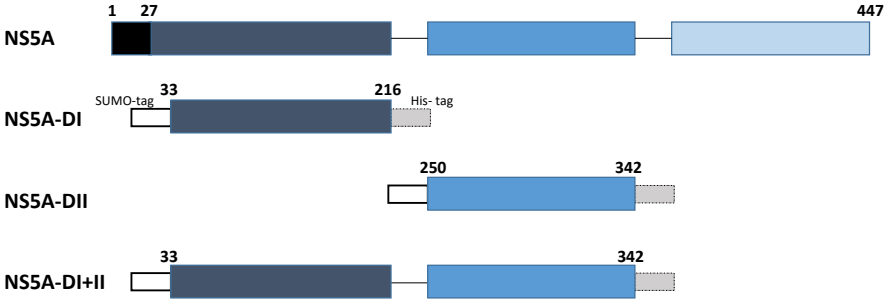


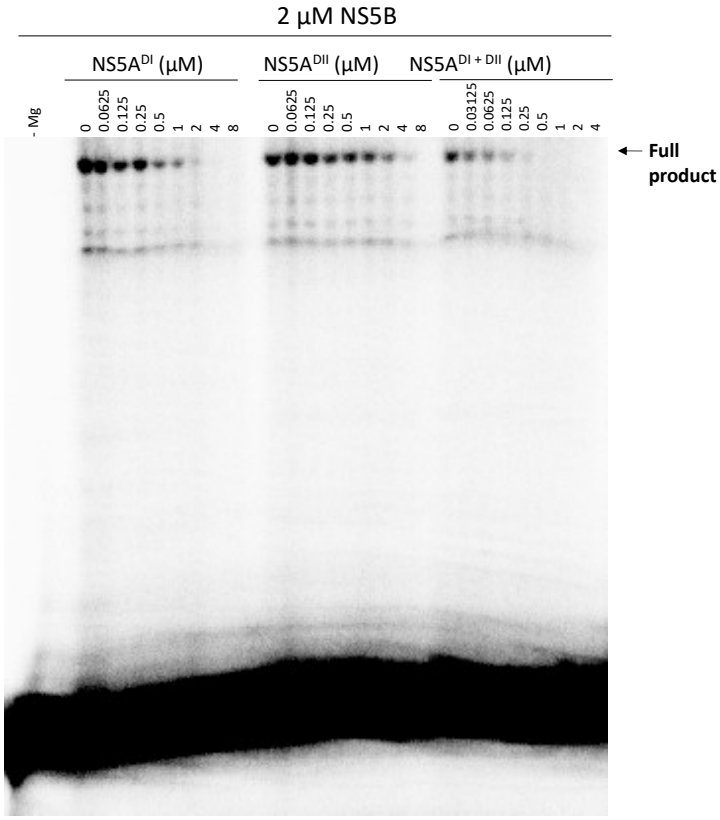
Figure 3. 10: Effect of CypA and NS5A-DII on NS5B RNA synthesis activity.

Graphical representation of NS5B primed RNA synthesis activity in the presence of increasing concentrations of NS5A-DII and in the absence or presence of CypA. Primer extension assays were performed with the T20 RNA template and radiolabeled GG dinucleotide primer. Reactions were resolved on a polyacrylamide gel, and NS5B % activity is indicative of full length RNA product.

A.


NS5A
1
27
447
NS5A-DI
SUMO-tag
33
216
His- tag
NS5A-DII
250
342
NS5A-DI+II
33
342

B.


2 µM NS5B
NS5A^DI (µM)
NS5A^DII (µM)
NS5A^DI + DII (µM)
- Mg
0
0.0625
0.125
0.25
0.5
1
2
4
8
0.03125
Full product

C.

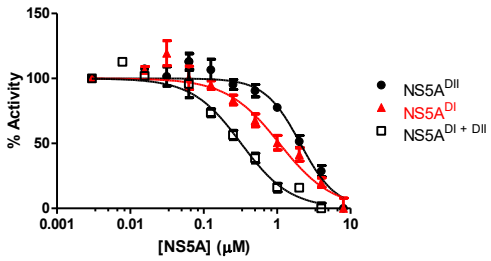

% Activity
150
100
50
0
0.001
0.01
0.1
1
10
[NS5A] (µM)
NS5A^DII
NS5A^DI
NS5A^DI + DII

Figure 3. 11: Comparative effect of domain I and II of NS5A on the RNA synthesis activity of NS5B.

(A) Constructs of NS5A proteins used spanning NS5A domain I and II individually and combined. ***(B)*** NS5B RNA synthesis assay using a radio-labelled GG primer on a heteropolymeric RNA template. NS5A RNA synthesis was assessed in the presence of increasing concentrations of NS5A-DI, DII, and DI+II constructs. Reactions were quenched with formamide, and resolved on a 20% acrylamide gel, as shown. ***(C)*** Graphical representation of the % full-length RNA product of NS5B from ***(B)*** in the presence of NS5A domain I (●), domain II (▲), and domain I+II (□).

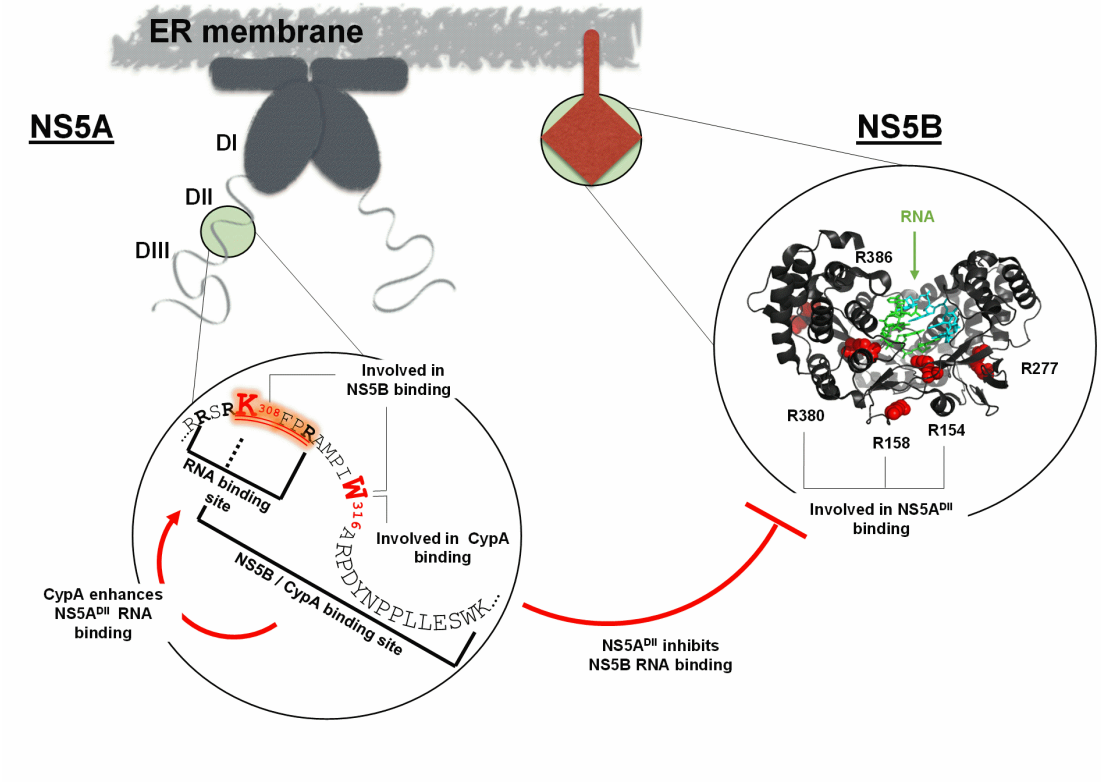

ER membrane
NS5A
DI
DII
DIII
NS5B
RNA
R386
R277
R380
R158
R154
Involved in NS5B binding
RNA binding site
Involved in CypA binding
NS5B / CypA binding site
CypA enhances NS5A^DII RNA binding
Involved in NS5A^DII binding
NS5A^DII inhibits NS5B RNA binding

Figure 3. 12: Model of the complex intermolecular interaction between NS5A-DII, NS5B, CypA and RNA.

The RNA binding site resides in an arginine rich region of NS5A-DII. This site overlaps the binding region for the CypA and NS5B by 4 amino acids, 308-311 (highlighted in **orange**). CypA host factor binds through several key residues including W316 within NS5A-DII. When CypA is bound, conformational changes are induced that enhance the RNA binding properties of NS5A-DII, potentially through the upstream RNA binding site. Alternatively, in the absence of CypA, the K308 and W316 residues, integral to NS5A-DII protein interaction with NS5B, are available to bind NS5B. This interaction occurs through NS5B residues residing in the RNA exit channel (R154, R158 shown in **red**). Possible conformational changes are also indicated in other regions of NS5B involved in RNA binding (R270, R386 in **red**). This mode of binding inhibits the RNA binding properties of NS5B, and RNA synthesis is decreased.

CHAPTER 4: DISCUSSION

4.1. Understanding the HCV mechanism of resistance to CypA inhibitors

It is a great achievement that over the past decade, major research advances into understanding the Hepatitis C life cycle have led to >90% cure rates for the majority of the patient population. These effects were heralded with the development of DAAs that target viral proteins and blocked their function, abrogating virus replication. However, pitfalls were identified that were plagued with the variability in treatment outcome for each HCV GT, as well as the emergence of resistance in a subset of patients. The direction in drug development shifted to identified drug targets that offered a high barrier to the emergence of resistance, and drug candidates featuring a pangenotypic profile. This was rapidly followed by the development of HTAs that were highly effective across all HCV GTs and offered alternate treatment options where treatment failure had occurred. CypA was identified as an essential host factor for HCV replication, and several HTAs directed against CypA have been evaluated in preclinical or clinical trials[284, 307, 317, 382]. Alisporivir, a non-immunosuppressive analogue of CsA and the most advanced CypA inhibitor in clinical development, has shown high cure rates in monotherapy in phase II and III clinical trials[320]. The mechanism of action of CsA and analogues is unclear, partly due to the fact that the exact function of CypA in the HCV life cycle is not fully understand. It is clear, however, that the polyprolyl *cis-trans* isomerase active is absolutely required to sustain HCV replication[180, 202, 306]. Binding of catalytically active CypA to HCV NS5A-DII induces a conformation change in the proline-rich binding site. Due to the high barrier to the emergence of resistance, mutations that confer resistance to CypA inhibitors take 3-6 months to be selected, and have been associated with relatively low, <10 fold, reduction in drug susceptibility[320, 357, 372]. Interestingly, *in vitro* biochemical studies revealed that the presence of the D320E and Y321N resistance-conferring mutations in GT 1b did not alter the binding of NS5A-DII to CypA, but were shown to alter the conformation of NS5A-

DII that binds to CypA[203]. Therefore, our goal was to evaluate whether differences in the protein complex formation of WT and mutant NS5A-DII with CypA could alter the binding kinetics of CypA inhibitors, implying a mechanism of resistance. We observed that CsA caused a slower rate of dissociation of the CypA complex with NS5A-DII mutants conferring a higher level of resistance. How does this apply and translate in the context of the HCV life cycle? Let's consider that the D320E/Y321N resistance phenotype was detected in *in vitro* cell culture studies under selection pressure. One scenario could be that the prolonged CypA interaction results in a prolonged enhancement of the RNA binding properties of NS5A-containing replicase. This would be highly favourable for subsequent steps in the HCV life cycle. Additional mutations associated with lesser resistance towards CypA inhibitors have been identified within the NS3 and NS5B proteins, and resistance mutations that emerge within NS5B have been associated with increased RNA binding of the viral polymerase[229, 372]. Taken together, this implies that the mechanism of resistance allows for prolonged binding to the viral RNA and stable formation of the replication complex, in the presence of CypA inhibitors. An additional scenario applies to the suggested role of CypA as a chaperone protein in aiding in trafficking of viral proteins from the ER site of translation to sites of replication in the membranous web[372]. NIM811 was shown to inhibit VLDL trafficking pathways used to traffic host factors essential for formation of the HCV replication complex, and colocalization of NS5A with LDs.[383]. Therefore an enhanced binding phenotype of mutant NS5A proteins to the CypA would facilitate this process. The emergence of the resistance-conferring mutations at residues D320 (GT 3a) and the corresponding D316 (GT 2a, 2b, 3b) within some patients in a recent clinical trial using Alisporivir were not correlated with the viral breakthrough observed[320, 324]. Lengthier studies would be needed to determine whether emergence of multiple clinically relevant mutations over time would lead to a decrease in susceptibility to this

class of inhibitors. Lastly, it has been shown that catalytically-active CypA is required for the proper formation of double membrane vesicles (DMV) that are essential for HCV replication[384]. CypA inhibitors inhibited DMV formation, suggesting that CypA-induced conformational changes within NS5A are functionally relevant and vital for this process[206, 384]. CypA inhibitors did not inhibit DMV formation by a T7 promoter-driven JFH-1 NS3-NS5B DNA plasmid that expressed D316E and Y321N resistance conferring mutations[206, 384]. Indeed, our observed sustained NS5A-DII complex with CypA could explain this phenomenon, and this adds another layer to the added advantage/ resistance to CypA inhibitors in the context of HCV viral replication.

4.2. The future of HTAs for the treatment of Hepatitis C infections

4.2.1. Cyclosporine analogues and other CypA inhibitor treatment options

HTAs targeting CypA have shown a great deal of promise in providing a successful, complementary treatment option for difficult-to-treat populations, including previous treatment failures and non-responders to prior therapy, as well as patients with cirrhosis[317]. There has been some debate as to the association of Alisporivir treatment with some adverse effects like acute pancreatitis and hyperbilirubinemia. This has forced the scientific community to re-evaluate the clinical safety profile of the use of CsA analogues in HCV drug therapy. CsA analogues have been shown to have off-target effects on cellular transporter proteins involved in bilirubin transport, and the structural similarity of CsA-based inhibitors may confer broad specificity to Cyp isoforms, undermining their safety profile[385]. One group showed that targeted delivery of nanoparticles encapsulating CsA to the mouse liver had a potent antiviral effect in a HCV mouse model[386]. This type of targeted drug delivery also drastically decreased CsA-associated cellular toxicity and immunosuppression. Other efforts are underway to generate optimized CypA inhibitors with

increased specificity for CypA. Combinatory application of bioengineering and semi-synthesis of SFA led to the development of sangamides that possess enhanced binding to CypA[387]. These second-generation CypA inhibitors have improved specificity for CypA, and likewise, enhanced drug potency as compared to the starting parent molecule. Similarly, using a structure-based screening approach coupled with computational modeling techniques, a class of bis-amide natural products were predicted to possess high binding affinity and specificity for the CypA active site[385]. In particular, the bis-amide **25** was identified as a lead CypA inhibitor by virtue of its low cytotoxicity (CC_{50} > 100 μM), potent anti-HCV effect (EC_{50} = 5.2 ± 0.2 μM), and effectively low nanomolar inhibition of CypA isomerase activity (IC_{50} = 5.5 nM). The authors endeavoured to show the specificity of their lead compound **25** for CypA. Surface plasmon resonance (SPR) experiments showed that **25** possessed a high binding affinity for CypA (K_D = 570 ± 20 nM) over its closely related isoform CypB (K_D = 2.1 ± 4 μM), implying its selective and highly specific binding to CypA[385]. Taken together, their data suggests that **25** is a potent and selective CypA inhibitor. These results support the clinical efficacy of HTAs and the potential for their therapeutic application.

4.2.2. Potential treatment options for diverse cancer therapy

A subset of HCV infected patients with unmet medical needs, such as the immunocompromised and those with advanced liver disease and/or HCV-induced hepatocellular carcinoma, often have limited therapeutic options. Although the clinical implications of CypA in cancer progression are still unclear, it is proposed to be a biomarker for hepatocellular carcinoma, and other cancer subtypes[388]. Studies into carcinogenesis have indicated up to 5-fold increase in CypA expression in several carcinomas, as well as a potential correlation with malignant

transformation and metastasis[308, 388]. Thus, these specific HTAs are highly attractive for cancer therapy. CypA inhibitors have the potential to provide therapeutic alternatives that interfere with host factors required for pathogenesis, and boost the host immune system at the same time. Han et. al. showed that the combination therapy of Cisplatin, a commonly used DNA-damaging cytotoxic anticancer drug, with either CsA or SFA had a synergistic apoptotic effect on glioblastoma cells resistance to treatment[389]. This serves as a proof-of-concept, and future studies using clinically relevant non-immunosuppressive CypA inhibitors may prove promising as effective treatment options for clinical use in cancer therapy.

4.2.3. Anti-liver inflammation and fibrosis effects of CypA inhibitors

Chronic HCV infection is often associated in liver inflammation and fibrosis. As an inflammatory response, several cyclophilins including CypA are often secreted from the cell[390]. Extracellular CypA can interact with cell surface receptors CD147 on leukocytes to trigger production of inflammatory cytokines, such as TNF-α, and activate pro-inflammatory processes[308, 390, 391]. Non-immunosuppressive cyclophilin inhibitors, like NIM811, have been shown to decrease expression of hepatic TNF- α and IL-1β expression in an *in vivo* animal model, as well as illicit a strong anti-inflammatory effect via inhibition of cyclophilin interaction with CD147[391, 392]. As such, CypA inhibitors have the potential to serve a dual function by inhibiting HCV replication as well as reducing liver injury due to inflammation. TNF-α and other pro-inflammatory cytokines also induce the production of fibrogenic factors, contributing to fibrosis in chronic HCV infection[308]. Liver fibrosis occurs due to dysfunction in the synthesis and degradation of extracellular matrix components, primarily in hepatic stellate cells (HSCs)[393-395]. HSCs are nonparenchymal liver cells that adopt an activated myofibroblastic phenotype upon liver injury.

They proliferate and produce extracellular components, like collagen, while also releasing matrix metalloproteases which contribute to extracellular matrix degradation[396, 397]. CsA and NIM811 have been shown to induce a dose-dependent suppression of cell proliferation and collagen production in HSCs *in vitro*[396]. SCY-635, a more potent CypA inhibitor, was also shown to decrease collagen production in HSCs[398]. In this study, stimulation of the production of the matrix metalloprotease-1 (MMP-1) was also observed, while production of the tissue inhibitor of metalloprotease-1 (TIMP-1) was decreased, enhancing MMP-1 activity in collagen matrix degradation. Other results from a randomized clinical study also indicated a lower frequency of liver fibrosis progression when liver-transplant patients were administered a steroid-free CsA treatment regimen, implying an anti-fibrotic effect of cyclophilin inhibition[399]. These results underscore the potential antifibrogenic effect of CypA inhibitors. Liver fibrosis is a compounding factor in the survival rate of HCV-infected liver transplant recipients, and immunosuppressive therapy with CsA was shown to decrease chances of graft cirrhosis[400-402]. Further *in vivo* studies would be required to confirm the clinical application of CypA inhibitors in the treatment of hepatic fibrosis.

4.2.4. CypA inhibitor applications for other clinically relevant infections?

As mentioned previously, CypA host factor is implicated in a myriad of other human viral pathogens from various virus families, including HIV (*Retroviridae*), Hepatitis B (*Hepadnaviridae*), and Influenza A (*Orthomyxoviridae*), just to name a few. Although Alisporivir is generally regarded as a potent HCV antiviral drug, it was initially developed for the treatment of HIV[403]. The interaction between CypA and a unique exposed loop of the HIV capsid protein (CA) is essential for replication, and facilitates the incorporation of CypA into virus particles

during assembly. Alisporivir and other CsA analogues were shown to effectively inhibit interaction, impairing HIV-1 replication in clinical isolates[403-405]. Unlike HCV, naturally occurring major resistance conferring mutation H87Q and minor variants (V86P/I91V/M96I) were identified within the capsid loop derived from HIV-1 Group M and O strains, rendering HIV-1 replicatively active and independent of CypA[403, 406, 407]. The underlying mechanism of resistance is thought to relate to modulation of the CA loop conformation that does not necessitate CypA binding or isomerase activity[408]. In support of this, HIV-2 CA proteins do not bind to CypA, and this strain does not require CypA for viral replication, rendering CypA inhibitors ineffective[403]. Taken together, HIV variants associated with resistance to CypA inhibitors have developed a mechanism of resistance that facilitate viral replication in human cells independently from CypA.

CsA and analogues have proven to be effective against currently circulating influenza A and B viruses, including resistant variants to clinically approved classes of Influenza antivirals[309]. Interestingly, there is mounting evidence that CsA antiviral effect against Influenza virus replication is not mediated in a CypA-dependent manner[309, 409, 410]. Its inhibitory effect was exerted at the viral replication intermediary stage. Contrary to the essential role of CypA in enhancing HIV and HCV replication, CypA is thought to restrict Influenza by binding to the M1 protein and inducing its proteosomal degradation[410]. CsA analogs that were engineered to possess diminished binding to CypA were shown to potently inhibit Influenza A and B viral replication as effectively as CsA, and serial viral passages of clinical viral isolates proved that indeed CypA inhibitors do exhibit a high barrier to the emergence of resistance[309]. Thus, the rationale drug design of non-immunosuppressive CsA analogs, additively lacking affinity for CypA, would be suitable for the development of a promising HTA drug candidate for Influenza treatment.

4.3. Redefining the functional role of NS5A-NS5B interactions

HCV NS5A is a multifunctional protein, with several interactions mediated through its largely disordered domain II as outlined in Chapter 1's introduction. Following our characterization of the residues involved in conferring resistance to CypA inhibitors, Chapter 3 focuses on expanding our understanding of NS5A-DII function through characterization of key residues that mediate its protein interactions with CypA, NS5B and RNA. NS5A was previously shown to interact with the NS5B RdRp via two independent regions[171]. By introducing internal deletions within a GT 1b isolate, both binding sites shown to mediate interactions with NS5B were also deemed crucial to sustain viral replication[164]. One site of interaction exists within the intrinsically disordered NS5A-DII protein, and overlaps with the CypA binding site. Our data is the first indication of the specific regulatory role of NS5A-DII in modulating the activity of the NS5B polymerase. This begs the question: what does the overlapping CypA binding and NS5B interaction with NS5A-DII mean in the context of HCV replication? And what is the biological relevance in the context of full-length NS5A? Both stimulatory and inhibitory effects of the NS5A on viral replication, as well as the interaction with NS5B have been reported, making it difficult to discern a definite functional role[156, 164, 166, 168, 169, 171].

CypA binding to NS5A-DII is known to increase its RNA binding properties. The identified RNA binding region was not shown to be essential to HCV replication, however optimal binding to the RNA genome may play a role in recruitment of host factors and viral proteins, like NS5B, to sites of replication. The partial stimulation in NS5B RNA synthesis we and others have observed at sub-stoichiometric concentrations of domain II-containing NS5A implies an early co-

stimulatory role of NS5A on NS5B RNA replication[168, 169, 171]. The mode and significance of this are unclear. One study suggests that during elongation, NS5A may bind and induce conformational changes in the NS5B β-hairpin loop found in the thumb subdomain[168, 169]. Ultimately, this could favour selective binding to the 3'UTR and enhancing RNA synthesis. Crystal structures illustrate the protrusion of β-hairpin into the NS5B active site in the closed conformation, and as such hinders egress of newly synthesized RNA substrates[148, 150, 157]. In chapter 3, we confirmed that residues located within the NS5B β-hairpin and other RNA binding regions were crucial for interaction with NS5A-DII. Another hypothesis is that binding of NS5A may facilitate effective interaction and transcription from the 3' UTR, as NS5B is shown to poorly synthesize RNA derived from the 3' UTR[411]. Therefore, future NMR studies would help to determine whether the NS5A-NS5B interaction does indeed result in these or any other suggested conformational changes in the polymerase.

Domain II of NS5A, as well as domain I, were found to synergistically inhibit NS5B RNA synthesis activity, in a dose dependent manner. It is unclear what is the functional relevance; however, we speculate that this may signal a modulatory switch between transcription and translation. Using a HCV RNA translation reporter system, Hoffman et.al. identified a region within NS5A-DII important for the modulation of HCV 1b translation[196]. The presence of an alanine mutation at residue K312 within this region was sufficient to abrogate this modulation. It is important to note that the corresponding residue already exists as an alanine residue in our Con 1b-derived NS5A-DII (A312), GT 2a JFH1 (A308), and several other genotypes[179, 201]. Thus, characterization studies into NS5A-DII involvement in HCV translation modulation would be needed in the context of other HCV genotypes. Lastly, we observed a synergistic inhibitory effect

with our NS5A-DI+II construct. Using a HCV RNA translation reporter system, further studies into the effect of NS5B interaction with full-length NS5A at the translational level would be relevant in understanding how both domains modulate HCV translation in the viral life cycle.

4.4. Conclusion

The in-depth study into HCV mechanisms of resistance towards CypA inhibitors, as well as the functional role of the intrinsically unstructured HCV NS5A-DII outlined in this thesis defines new concepts into the key factors necessary for an optimal viral replication complex.

While no HCV-specific vaccine exists, the discovery and continued development of highly potent DAAs and HTAs continue to improve the success in disease treatment. In Chapter 2, we designed fluorescence-based biochemical assays to explore the mechanism of emergent resistance mutations within HCV NS5A-DII to inhibitors of CypA host factor. We demonstrate that the level of resistance correlates inversely with the rate of dissociation of the NS5A-CypA complex in the presence of CsA (NS5A WT > D320E > Y321N). This correlation was also found to be independent of the rate of association of the complex. This data provides a possible biochemical mechanism of resistance to CypA inhibitors. Understanding the mechanism of resistance of resistance-conferring mutations can allow for better rationale design of pangenotypic host-targeting inhibitors. In addition, HTAs can subsequently be employed in clinical combination therapy for treatment of HCV, as well as other clinically relevant infections.

NS5A-DII interactions extend from host factors like CypA, to viral proteins like NS5B as well as the viral genome. The intrinsically disordered nature of this domain has been an obstacle in determining a crystal structure, impeding structural and functional studies. Thus, the specific

residues that mediate these protein-protein and nucleoprotein interactions are unknown. In Chapter 3, we utilized high-resolution protein footprinting techniques to identify and characterize previously unidentified key residues within NS5A-DII and NS5B involved in each interaction. Our data demonstrates the involvement of conserved residues in NS5A-DII specific to NS5B and CypA interactions, which implies that these interactions mediate a conserved role across multiple HCV genotypes. By mapping of a novel NS5A-DII binding site on NS5B, we propose a possible regulator role of NS5A in the transition between transcription and translation of the viral genome.

Taken together, with these initial insights we can start to decipher the complex interplay of the intermolecular interactions mediated by various viral and cellular factors within the HCV replication complex. This paves the way to understanding the functional relevance of each component that constitutes a replication-competent HCV replicase.

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