Quantifying the Relative Contributions of the Three Roles of miR-122 in the HCV Life Cycle

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ABSTRACT

Hepatitis C virus (HCV) recruits two molecules of the human liver-specific microRNA, miR-122, to two sites (site 1 and site 2) at the 5′ end of its genome, and these interactions promote viral RNA accumulation. Studies conducted over the last decade have led to the identification of three specific roles for miR-122 in the HCV life cycle: 1) it acts as an RNA chaperone, or "riboswitch" to promote the conformational change from an alternative structure called stem-loop II alternative ($SLII^{alt}$) to the functional internal ribosomal entry site (IRES) structure, consisting of SLII-IV; 2) it stabilizes the viral genome by protecting the 5′ terminus from pyrophosphatase activity and subsequent exoribonuclease-mediated decay; and 3) it promotes translation via an interaction between the Argonaute (Ago) protein at site 2 and the viral IRES. Thus far, most studies have considered some or all of these roles in combination, but the contribution of each of the individual roles to the overall impact of miR-122 on the HCV life cycle remains unclear. We hypothesized that each of the three roles attributed to miR-122 in the HCV life cycle has a distinct contribution to the overall impact of miR-122 on viral RNA accumulation.

To evaluate the contribution of each of the three roles, we used a combination of viral mutants or resistance-associated variants (RAVs) that compensate for one or more of miR-122's roles in the HCV life cycle, and independently manipulated miR-122 binding to the viral genome, to isolate each role in the context of full-length luciferase reporter viral RNAs. We show that the overall contribution of each of miR-122's roles in HCV RNA accumulation differs during the establishment phase (before genome replication is initiated) compared with the maintenance phase of the infection (during active RNA replication). Specifically, during the establishment phase, our results suggest that the riboswitch effect has a minimal contribution to

HCV RNA accumulation, while genome stabilization and translational promotion contribute to a similar extent. However, in the maintenance phase, after several rounds of RNA replication, the impact of the genome stabilization lessens, and translational enhancement becomes the dominant role.

Importantly, because we observed a minimal contribution of the riboswitch effect, we wondered why the SLII^{alt} structure, and the need to riboswitch to form the functional IRES, was maintained in some viral genotypes. To this end, we used point mutations to stabilize the SLII structure and measured viral RNA accumulation and infectious particle production using the HCV cell culture (HCVcc) system. We observed that successive stabilization of SLII over SLII^{alt} resulted in a reduction in infectious particle production, suggesting that SLII^{alt} is important for efficient virion assembly. This implies that in addition to its riboswitch, genome stability and translational promotion activities, miR-122 may also regulate progeny RNA engagement in translation or virion assembly activities. This may also provide insight into the success of HCVcc systems as well as have implications for the outcome of HCV infection.

Taken together, this study provides important insight into an unusual host-virus interaction and the mechanics of a non-canonical miRNA-target RNA interaction. A growing body of evidence suggests that several diverse RNA viruses similarly use cellular or virally derived small RNAs to regulate their life cycles. Notably, miR-122 inhibitors have shown promising results for the treatment of HCV in the clinic. Thus, furthering our understanding of the unusual interaction between miR-122 and HCV RNA may provide important insight for the design of RNA-based therapeutic approaches, and could be applicable to other important human and veterinary pathogens.

RÉSUMÉ

Le virus de l'hépatite C (VHC) recrute deux molécules du microARN humain spécifique au foie, miR-122, à deux sites (site 1 et site 2) à l'extrémité 5′ de son génome, et ces interactions favorisent l'accumulation de l'ARN viral. Des études complétées au cours de la dernière décennie ont mené à l'identification de trois rôles spécifiques joués par miR-122 dans le cycle de vie du VHC : 1) il agit comme un ARN chaperon, ou « riboswitch » pour promouvoir le changement de configuration d'une structure alternative appelée $SLII^{alt}$ (tige-boucle alternative) vers la structure fonctionnelle de l'IRES (*internal ribosomal entry site*), qui consiste des tige-boucles SLII à IV; 2) il stabilise le génome viral en protégeant son extrémité 5′ de l'activité des pyrophosphatases et de la dégradation subséquente par les exoribonucléases; et 3) il favorise la traduction via une interaction entre la protéine Argonaute (Ago) au site 2 et l'IRES viral. Jusqu'à présent, la plupart des études ont considéré plusieurs ou tous ces rôles combinés, mais la contribution de chacun de ces rôles à l'impact global de miR-122 sur le cycle de vie du VHC demeure incertaine. Nous avons émis l'hypothèse que chacun des trois rôles de miR-122 contribue de manière distincte à l'impact global de miR-122 sur le cycle de vie du VHC.

Afin d'évaluer la contribution de chacun de ces trois rôles, nous avons utilisé une combinaison de virus mutants et de mutations associées à la résistance (RAVs, *resistance-associated variants*) qui compensent pour un ou plusieurs des rôles de miR-122 dans le cycle de vie du VHC, et nous avons indépendamment manipulé l'association de miR-122 avec le génome viral pour isoler chaque rôle dans le contexte de reporters de luciférase basés sur l'ARN viral complet. Nous démontrons que la contribution de chacun des rôles de miR-122 à l'accumulation de l'ARN du VHC diffère durant la phase initiale (avant que la réplication du génome soit débutée), comparée à la phase de maintenance de l'infection (durant la réplication active de

l'ARN viral). Plus précisément, durant la phase initiale, nos résultats suggèrent que l'effet riboswitch a une contribution minimale à l'accumulation de l'ARN du VHC, tandis que la stabilisation du génome et la promotion de la traduction contribuent de manière similaire. Cependant, durant la phase de maintenance, après plusieurs cycles de réplication de l'ARN, l'impact de l'effet de stabilisation diminue, alors que la promotion de la traduction devient le rôle principal.

Notamment, puisque nous avons observé une contribution minimale de la part de l'effet riboswitch, nous nous sommes demandé pourquoi la structure SLII^{alt}, et le prérequis de procéder au riboswitch pour former l'IRES fonctionnel, étaient maintenus dans certains génotypes viraux. Afin de répondre à cette question, nous avons utilisé des mutations ponctuelles qui stabilisent la structure SLII et avons mesuré l'accumulation d'ARN viral et la production de particules infectieuses en utilisant le système de culture cellulaire du VHC. Nous avons observé que la stabilisation successive de SLII au lieu de SLII^{alt} a donné lieu à une réduction de la quantité de particules infectieuses produites, ce qui porte à croire que SLII^{alt} est une structure importante pour l'assemblage des virions. Cela implique qu'en plus des activités de riboswitch, de stabilisation du génome et de promotion de la traduction, miR-122 pourrait également réguler la distribution des génomes nouvellement synthétisés entre les activités de traduction et de production de virions.

Le tout considéré, cette étude fournit des notions importantes relatives à une interaction hôte-virus inusuelle et par rapport aux mécanismes d'une interaction non canonique entre un microARN et son ARN cible. Un ensemble grandissant de preuves suggère que de nombreux différents virus à ARN utilisent de manière similaire des petits ARN cellulaires ou dérivés du génome viral afin de réguler leur cycle réplicatif. Notamment, des inhibiteurs de miR-122 ont

démontré des résultats prometteurs dans le traitement de l'hépatite C en clinique. Par conséquent, l'amélioration de notre compréhension de l'interaction particulière entre miR-122 et l'ARN du VHC pourrait fournir des informations pertinentes dans le développement de thérapies basées sur l'ARN, et qui pourraient être transférables à l'étude d'autres pathogènes humains et animaux importants.

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PREFACE

This thesis was written according to McGill University's "Guidelines for Thesis Preparation". The candidate has chosen to present in a "Manuscript-based thesis" format by following these guidelines:

"As an alternative to the traditional format, a thesis may be presented as a collection of scholarly papers of which the student is the first author or co-first author. […] A manuscript-based Master's thesis must include the text of one or more manuscripts. […]

Manuscripts for publication in journals are frequently very concise documents. A thesis, however, is expected to consist of more detailed, scholarly work. A manuscript-based thesis will be evaluated by the examiners as a unified, logically coherent document in the same way a traditional thesis is evaluated."

The information of the manuscript in preparation is listed below. The contributions of all authors are detailed in the "Contribution of Authors" section.

Manuscript presented in this thesis:

Marylin Rheault, Sophie E. Cousineau, Danielle R. Fox, Quinn H. Abram, and Selena M. Sagan. Elucidating the distinct contributions of the three roles of miR-122 in the HCV life cycle reveals insights into virion assembly. *Manuscript in preparation.*

CONTRIBUTION OF AUTHORS

Chapter 2:

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M.R. and S.M.S. designed the study. M.R. performed and analyzed the J6/JFH-1 electroporation experiments. S.E.C. performed and analyzed the JFH-1_T infection experiments. D.R.F. optimized and performed the 5′ RACE protocol. Q.H.A. performed the SHAPE analysis. M.R. and S.M.S. drafted the manuscript and all authors contributed to editing the manuscript.

LIST OF ABBREVIATIONS

ΔG	Change in Gibb's free energy
λN	Bacteriophage Lambda N peptide
A cap	G(5′)ppp(5′)A cap
Ago	Argonaute
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhea virus
CCR4-NOT	Carbon catabolite repression 4-negative on TATA-less
CD81	Cluster of differentiation 81
cDNA	Complementary DNA
C.I.	Confidence interval
C-terminal	Carboxy-terminal
Ctrl	Control
CypA	Cyclophilin A
D1	Domain 1
D2	Domain 2
DAAs	Direct-acting antivirals
DCP1	Decapping protein 1
DCP2	Decapping protein 2
DENV	Dengue virus
DGCR8	DiGeorge chromosomal region 8
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DOM3Z/DXO	Dom-3 homolog Z/Decapping and exoribonuclease protein
dsRNA	Double-stranded RNA
DUSP11	Dual specificity phosphatase 11
E1	Envelope protein 1
E2	Envelope protein 2
ECL	Enhanced chemiluminescence
EHcV	Equine hepacivirus
eIF3	Eukaryotic initiation factor 3
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
EV-71	Enterovirus 71
GBV-B	GB virus B
FFU	Focus-forming unit
FLuc	Firefly luciferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GW182	Glycine-tryptophan repeat containing protein of 182 kDa
GW-repeats	Glycine-tryptophan repeats
FXR1	Fragile-X-mental-retardation-related protein 1
h	Hours
HCC	Hepatocellular carcinoma
HC-J6	HCV clone J6

HCV	Hepatitis C Virus
HCVcc	Cell culture-derived HCV
HEK	Human embryonic kidney
HITS-CLIP	High throughput sequencing of RNA isolated by crosslinking immunoprecipitation
HRP	Horseradish peroxidase
HTAs	Host-targeted antivirals
Huh-7	Human hepatoma 7
HuR	Human antigen R
IFN	Interferon
IRES	Internal ribosomal entry site
ITAF	IRES *trans*-acting factor
JFH-1	Japanese Fulminant Hepatitis-1
KO	Knockout
LD	Lipid droplet
LDL	Low-density lipoprotein
LDLR	LDL-receptor
LLPS	Liquid-liquid phase separation
MAVS	Mitochondrial antiviral signaling protein
min	Minutes
miR-122	microRNA-122
miRISC	microRNA induced silencing complex
miRNA	microRNA

mRNA	Messenger RNA
neo	Neomycin phosphotransferase gene
NPHV	Non-primate hepacivirus
NrHV	Norway rat hepacivirus
NS	Nonstructural
N-terminal	Amino-terminal
NTP	Nucleoside triphosphate
NTPase	Nucleoside triphosphatase
nt	Nucleotide
OH	Hydroxyl
PAN2	PABP-dependent poly(A)-nuclease 2
PAN3	PABP-dependent poly(A)-nuclease 3
PAZ	Piwi-Argonaute-Zwille
P-body	Processing body
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIWI	P-element induced wimpy testis
Pri-miRNA	Primary miRNA
P-site	Peptidyl site
RAV	Resistance-associated variant
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid inducible gene I
RLuc	*Renilla* luciferase

RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse-transcription PCR
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scr	Scrambled
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SHAPE	Selective 2′-hydroxyl acylation analyzed by primer extension
SL	Stem-loop
$SLII^{alt}$	Stem-loop II alternative
SVR	Sustained virological response
TBS-T	Tris buffered saline with Tween 20
TNRC6	Trinucleotide repeat-containing gene 6
TRBP	Transactivation response element RNA-binding protein
TRIF	Toll-like receptor 3 adaptor TIR-domain-containing-adapter-inducing-interferon-β
Trp	Tryptophan
UTR	Untranslated region
V	Voltz
vsRNA	Viral small RNA
Xrn1	5′-3′ exoribonuclease 1
Xrn2	5′-3′ exoribonuclease 2
ZIKV	Zika virus

CHAPTER 1: INTRODUCTION

1.1 Hepatitis C Virus

Discovery of Hepatitis C Virus

During the 1970s, several studies indicated that the majority of transfusion associated hepatitis cases were not attributable to Hepatitis A or Hepatitis B viruses (1-8). Rather, the cause was an as-yet unidentified infectious agent which was chloroform-sensitive and smaller than 80 nm in diameter, consistent with the characteristics of a small, enveloped virus (9-11). In 1989, a complementary DNA (cDNA) library generated using infectious material from experimentally infected chimpanzees enabled the identification of an approximately 10,000 nucleotide (nt)-long single-stranded positive-sense RNA virus, subsequently named Hepatitis C virus (HCV) (12). The development of an HCV-specific antibody assay was an instrumental step in lowering the risk of post-transfusion acquired HCV (7, 13). Two decades later in 2020, the Nobel Prize in Physiology or Medicine was awarded to Harvey J. Alter, Michael Houghton, and Charles M. Rice for the discovery of HCV (14, 15).

Genotypes and distribution

HCV sequences are represented by 7 genotypes which differ by 30-35% in their nucleotide identity and over one hundred subtypes with less than 15% sequence variability (16-19). Different genotypes/subtypes have distinct overall prevalence, with genotypes 1 and 3 being the most prevalent (16). Each genotype also has a distinct geographic distribution (**Figure 1.1**). Genotypes 1 and 2 are mostly found in West Africa, genotype 3 in South Asia, genotype 4 in Central Africa and the Middle East, genotype 5 in Southern Africa, and genotype 6 in South East Asia (16). Genotype 7 was only recently identified and is thought to originate from Central

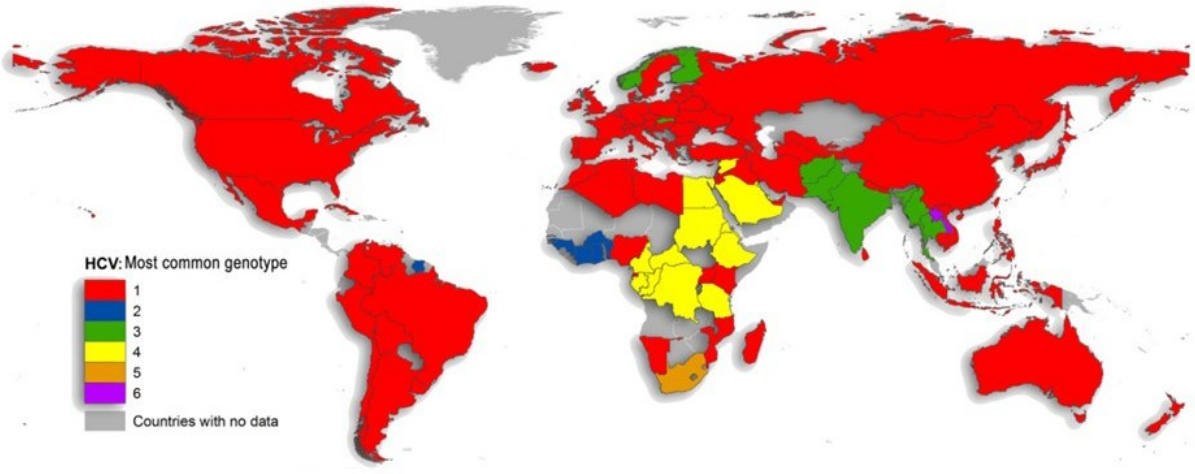

Figure 1.1. Geographic distribution of HCV genotypes. Figure reproduced from Messina *et al*. 2015 (16).

Africa (16, 20, 21).

Modes of transmission and pathogenesis

HCV is transmitted parenterally via contact with blood products, mainly by transfusion and injection drug use (22, 23). In rarer occasions, it may be acquired following high-risk sexual activity or through vertical transmission from mother to child (22). Transfusion-associated transmission of HCV greatly decreased after the implementation of HCV antibody screening of blood products in most countries in 1991 (24). As such, in developed countries, the primary mode of transmission today is injection drug use (25, 26).

HCV infection manifests asymptomatically in up to 50% of acutely infected individuals, and the absence of symptoms can be maintained into chronic infection until severe liver complications develop (24, 27). On average 75-85% of HCV-infected individuals progress to chronic infection, which is defined as the presence of HCV RNA in the serum for >6 months beyond the initial infection (27-30). Chronic HCV can lead to steatosis (fatty liver), fibrosis, and can progress to cirrhosis (27). Other complications include end-stage liver disease and hepatocellular carcinoma (HCC), which make chronic HCV infection one of the leading causes of liver transplant worldwide (24, 27, 28, 31, 32).

1.2 HCV Genome Organization and Life Cycle

HCV Genome

The HCV genome is a 9.6 kb single-stranded RNA molecule of positive polarity. It consists of a single open-reading frame (ORF) encoding a ~3000 amino acid-long polyprotein, flanked by highly structured 5′ and 3′ untranslated regions (UTRs) (33). The UTRs contain functional *cis*-acting elements which aid in translation and replication of the viral genome. The 5′ UTR

contains four stem-loop (SL) structures, termed SLI through IV, and the latter three make up the viral internal ribosomal entry site (IRES) (34-36).

Internal ribosome entry site (IRES)

Translation of the viral polyprotein is initiated in the absence of a 5′ cap via the viral IRES (SLII-IV), a complex structural element at the 5′ UTR of the viral genome. The IRES is composed of nucleotides 40 through 372 of the viral genome, which includes the initiator AUG (positions 342) and the beginning of the ORF (5′ end of the core protein coding region). (34, 37-41). It is divided intro three structural domains, namely domains II, III and IV, and it is the RNA secondary and tertiary structure, rather than the nucleotide identity, which gives function to the IRES (34, 36, 37, 39, 42-44).

The IRES sequentially recruits ribosomal subunits to ensure cap-independent translation. The 40S ribosomal subunit is recruited first and positioned such that the AUG start codon is placed in (or near) the peptidyl site (P-site). This is followed by recruitment of eukaryotic initiation factor 3 (eIF3) and the other components required for formation of the 48S and the 80S complexes (**Figure 1.2**) (34). The HCV IRES also employs a number of host IRES *trans*-acting factors (ITAFs) which aid in ribosome recruitment.

3′ UTR

The 3′ UTR is separated into three main parts: the variable region, the polyU/UC-tract, and the highly conserved 3′ X-tail (**Figure 1.2**) (45-47). The variable region consists of about 40 nucleotides with a poorly conserved sequence (46, 47). It is not essential for genome replication,

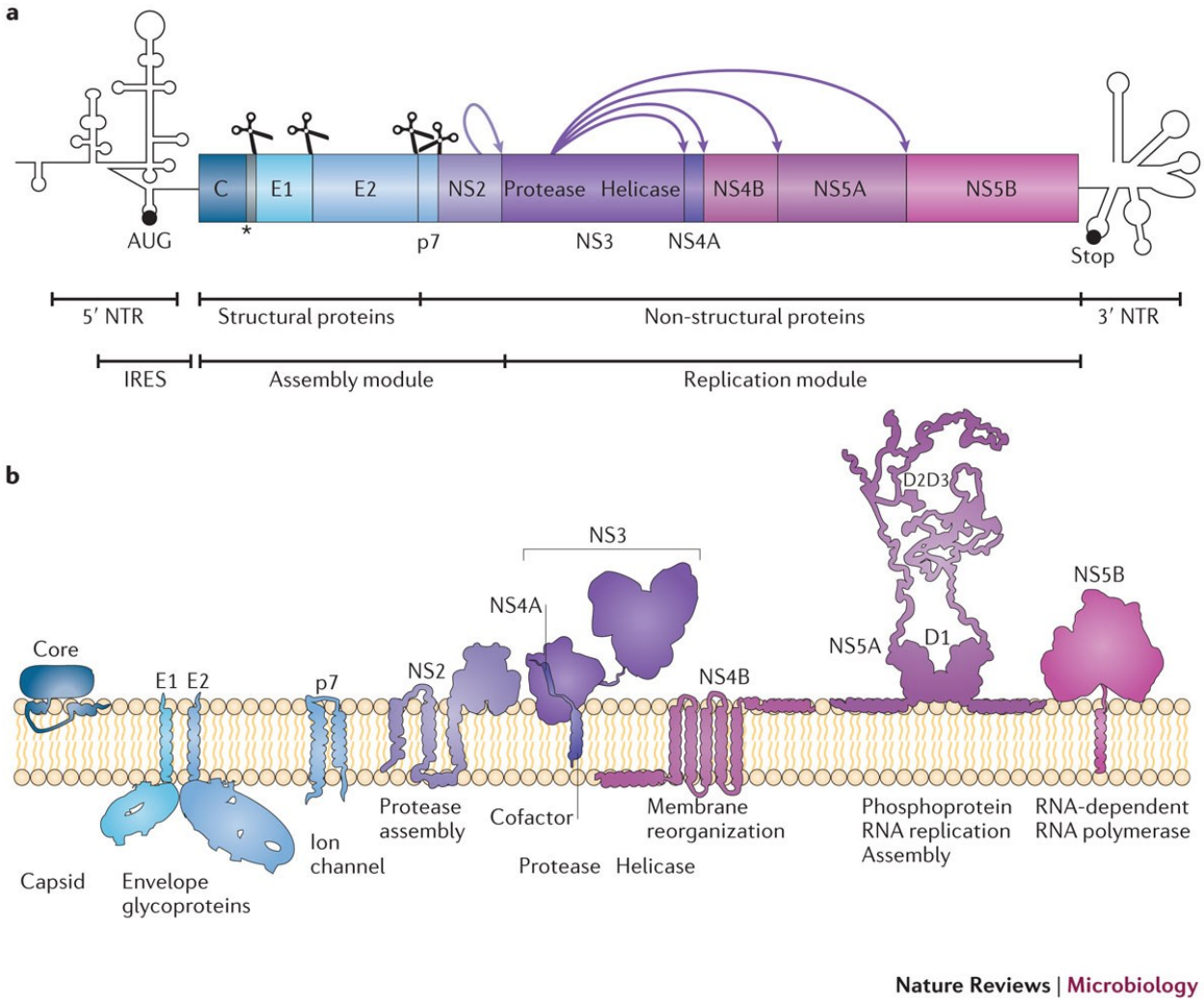

Figure 1.2. The HCV genome and viral proteins. A) The HCV genome contains highly structured 5′ and 3′ UTRs, depicted in black. SLII-IV in the 5′ UTR form the IRES. The single ORF encodes a polyprotein which is processed co- and post-translationally into four structural and six nonstructural proteins. Host protease-mediated cleavage events are indicated by scissors while viral protease-mediated cleavage events are indicated by arrows from the relevant protease to each site. **B)** Topology and structure representation of the HCV viral proteins and their respective functions. Figure reproduced from Bartenschlager *et al*. 2013 (48).

but nonetheless required for the completion of the viral life cycle in cell culture (49, 50). Its secondary structure is predicted to consist of two stem-loop structures, the first of which contains the stop codon (46, 47, 51). The polyU/UC-tract varies in length from 30 to 80 nucleotides depending on the HCV isolate, and consists of consecutive uridine residues interspersed by single cytosine residues (52). A minimum of 26-33 consecutive uridines are required for replication in cell culture, but the position of this polyU sequence within the polyU/UC-tract varies (49, 53). This tract is proposed to serve as a recruitment platform for viral and host proteins, and the viral nonstructural (NS) proteins NS3, NS5A and NS5B all have a preference for polyU sequences *in vitro* (54-56). Finally, the 3′ X-tail is 98 nucleotides long and forms three stem-loop structures (SL1, 2 and 3), and both its sequence and its structure are essential for viral replication (49, 50).

HCV Viral Proteins

The single HCV ORF is translated by cellular ribosomes via the IRES into a ~3000 amino acid-long polyprotein, which is cleaved co- and post-translationally into the 10 mature viral proteins, by both cellular and viral proteases (**Figure 1.2**) (33, 57, 58). The three structural proteins, core and the envelope glycoproteins E1 and E2, make up the viral particle (33, 57, 58). The nonstructural proteins include the small hydrophobic protein p7, followed by NS2, NS3, NS4A, NS4B, NS5A and NS5B (33, 57, 58).

The core protein is released from the viral polyprotein by two host protease-mediated cleavage events after which it localizes to the surface of lipid droplets (LDs) (59, 60). Core forms the viral nucleocapsid, while E1 and E2 compose the viral envelope. P7 is thought to form ion channels (viroporins) which are necessary for HCV virion assembly and release (57, 58, 61, 62).

The NS2 protein, together with NS3, forms a dimeric cysteine protease responsible for cleavage of the NS2/3 boundary (63-65). Following cleavage, processed NS2 also plays a role in virion assembly (66). NS3 contains RNA helicase, serine-type protease, and nucleoside-triphosphatase (NTPase) activities. In addition to cooperating with NS2, its serine protease activity, enhanced by the NS4A cofactor, is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B sites (54, 67-70). The NS3 helicase activity is also required for viral RNA replication and packaging (71). Additionally, NS3-4A inhibits the host antiviral response through cleavage of toll-like receptor 3 adaptor TIR-domain-containing-adapter-inducing-interferon-β (TRIF) and mitochondrial antiviral signaling protein (MAVS) (72-74). The NS4B protein is an integral membrane protein which provides membrane curvature for the formation of the membranous web and replication organelle biogenesis (see the subsection *HCV Life Cycle*) (75-77). NS5A is an RNA-binding phosphoprotein important for replication organelle biogenesis as well as virion assembly. Finally, the NS5B protein is the RNA-dependent RNA polymerase (RdRp), responsible for replication of the viral genome via a negative-sense replicative intermediate, which is used to generate progeny positive-sense genomic RNAs (58).

HCV Life Cycle

HCV selectively infects humans and chimpanzees, mainly targeting hepatocytes, but occasionally other cell types, including B cells and dendritic cells (58). Viral particles circulate in association with low-density lipoproteins (LDL) and first come in contact with the cell surface by interacting with heparin sulfate and the LDL-receptor (LDLR) (33, 78-81). Following initial attachment, the viral particle sequentially engages with scavenger receptor B class 1 (SR-B1), Cluster of differentiation 81 (CD81), claudin-1 and occludin-1, before entering the cell via clathrin-mediated endocytosis (33, 81-86). The viral membrane then fuses with the endosomal

membrane, releasing the viral genome into the cell, although the precise steps of the uncoating process remain poorly characterized (33, 87, 88). The HCV IRES allows for cap-independent translation of the viral polyprotein containing the 10 viral proteins. Host and viral proteases cleave the junctions separating individual proteins (detailed in the *HCV Viral Proteins* subsection) (33). The nonstructural proteins NS4B and NS5A then induce the formation of endoplasmic reticulum (ER) membrane-derived invaginations termed the membranous web, which consists of single-, double- and multi-membrane vesicles as well as LDs, and can contain membranes derived from other cellular compartments including mitochondria and endosomes (89). This membranous web is the replication organelle, i.e., the site of genome replication. During viral replication, a full-length negative-sense replicative intermediate is generated and is subsequently used to synthesize an excess of positive-sense genomic RNA molecules, which can be used as translation templates or packaged into progeny virions. Viral assembly occurs in close proximity with LDs: HCV infection promotes the generation of new LDs in infected cells, and both viral proteins and host factors are found to localize to these structures during infection (90). During packaging, the core protein associates with the viral RNA to form nucleocapsids which bud into the ER membrane, acquiring the viral envelope. Nascent HCV particles are trafficked via the secretory pathway during which time they mature and acquire the envelope glycoproteins E1 and E2 as well as host apolipoproteins, before being exported out of the cell via exocytosis (33, 91, 92).

1.3 HCV cell culture systems

From its discovery up until the early 2000s, HCV could not be grown in cell culture, and the only available method to study this virus *in vitro* were replicon systems. These systems are useful for

studying RNA replication, but they do not recapitulate the entire viral life cycle, and are unable to produce infectious viral particles (58). The discovery of a fully infectious HCV genotype 2a clone which could propagate in cell culture and undergo the complete viral life cycle was a turning point in the study of HCV (93).

Replicon systems

The first functional HCV sub-genomic replicon system was developed in 1999 (94). It relies on a bicistronic construct (i.e. a construct containing two separate ORFs) encoding the neomycin phosphotransferase gene (*neo*), which confers resistance to the antibiotic G418, or a luciferase reporter gene, followed by the nonstructural proteins required for viral RNA replication (NS2- or NS3-NS5B) (94). In this system, translation of *neo* or luciferase is directed by the HCV IRES, while translation of the viral nonstructural proteins is directed by the encephalomyocarditis virus (EMCV) IRES. This system could be used to select cells which stably replicate the sub-genomic replicon RNA based on their survival in G418 containing media (94). These sub-genomic replicons could replicate in human hepatoma 7 (Huh-7) cells, allowing for the study of HCV replication in cell culture (94). Subsequently, sub-genomic and full-length replicons have been generated for genotypes 1 through 6 as well as chimeric genomes (58, 95).

Cell lines

To date, the only cell line which can support robust HCV replication (without the introduction of modifications) are Huh-7 cells and its derivatives (96). A subline of Huh-7 cells, termed Huh-7.5, was developed by curing Huh-7 cells of HCV using interferon alpha (IFN-α) treatment. It was found to be highly permissive to replication of HCV sub-genomic replicons thanks to an

inactivating mutation in retinoic acid inducible gene I (RIG-I). This enabled the study of unmodified HCV genomes whose translation is directed by the HCV IRES rather than the EMCV IRES (96, 97).

Cell culture-derived HCV (HCVcc)

In 2005, a breakthrough study described a genotype 2a HCV isolate named Japanese Fulminant Hepatitis-1 (JFH-1), which could recapitulate the entire infectious life cycle in cell culture (93). Around the same time, a cell culture competent chimeric genotype 2a HCV clone was described (98). This isolate encodes the core-NS2 region of the HCV clone J6 (HC-J6) and the remainder (NS3-NS5B) is from the JFH-1 isolate (98, 99). Through passaging of JFH-1 in Huh-7.5 cells, a derived strain, termed JFH-1_T was generated, which contains five cell culture adaptive mutations leading to higher titers of infectious virus than the parental JFH-1 strain (100). Subsequently, cell culture competent clones were established for strains of genotypes 1, 2, 3, 4 and 6, and chimeras were developed which further expand the range of cell culture derived HCV (HCVcc) models (101). The availability of these fully infectious clones that can undergo the complete replication cycle in cell culture is a valuable tool to the study of HCV. However, limitations arise from the difficulty in recapitulating the inherent genetic variability of HCV by only being able to study select strains in cell culture.

1.4 HCV therapy

Initial therapies and direct-acting antivirals (DAAs)

HCV infectious status is routinely measured by quantifying the viral RNA present in serum, while liver sampling, although more invasive, may provide a more representative quantification

of the viral load. When treating a patient, the term sustained virological response (SVR) refers to the absence of detectable HCV RNA by the end of the treatment which is maintained for 6 months after. Rebound after this period is possible but rare, and thus a SVR is tantamount to a cure (102). Historically, IFN and ribavirin were used to treat HCV and were of limited efficacy and associated with significant side effects and contraindications (102, 103). More recently approved treatments include highly effective direct-acting antivirals (DAAs) which target viral proteins, and host-targeted antivirals (HTAs, described in subsection *Host-targeted antivirals HTAs*). Approved DAAs include protease inhibitors, nucleoside and nucleotide analogs, and NS5A inhibitors. Current treatment regimens consist of all-oral combinations of DAAs and achieve SVR in >95% of cases (104, 105).

Host-targeted antivirals (HTAs)

Recently developed antiviral compounds directed against host factors have the advantage of providing a high barrier to resistance due to the low mutational rate of eukaryotic genes, while being effective against a broad range of HCV genotypes (106). Examples of HTAs include inhibitors of Cyclophilin A (CypA) and microRNA-122 (miR-122) inhibitors, among others. CypA is bound by HCV NS5A, and the neutralization of this interaction using CypA inhibitors has antiviral effects which seem to result from a defect in membranous web formation (107, 108). The liver-specific microRNA (miRNA), miR-122, is known to be co-opted by HCV and is an important host factor for optimal viral RNA accumulation (109-111). Consequentially, inhibitors of miR-122 have been investigated as HCV treatments and two molecules, Miravirsen™ and RG-101, have demonstrated significant efficacy and tolerability in clinical

trials (discussed in further detail in the subsection titled *Interactions Between miR-122 and the HCV RNA Genome*) (112, 113).

1.5 MicroRNAs

miRNAs are short noncoding RNAs measuring about 19-25 nt that are encoded within the genome of multicellular organisms and generally serve cytoplasmic regulatory functions (114). The first miRNA was discovered in *C. elegans* in 1993 (115). The abnormal cell lineage 4 (*lin-4*) gene, which was known to negatively regulate the lin-14 protein, was demonstrated to produce short transcripts with complementarity to the 3′ UTR of the *lin-14* messenger RNA (mRNA), which were proposed to downregulate translation from this mRNA via RNA-RNA interactions (115). Since then, the miRNA field has grown substantially and there are now over 30,000 miRNAs from more than 200 species in the miRBase database (http://mirbase.org/) (116).

Biogenesis of miRNAs

miRNAs are transcribed from the genome and undergo a series of nuclear and cytoplasmic processing events before they can fulfill their regulatory functions. Highly structured primary miRNA (pri-miRNA) transcripts are typically transcribed in the nucleus by RNA Polymerase II and then processed by the RNase III family enzyme Drosha, in cooperation with DiGeorge chromosomal region 8 (DGCR8)/Pasha, its double-stranded RNA binding cofactor (117, 118). The product of this processing step is a hairpin-shaped precursor miRNA (pre-miRNA) of about 60-75 nt (117). The pre-miRNA is then exported into the cytoplasm via the Exportin-5 receptor and further processed in the cytoplasm by another RNase III enzyme, Dicer, as well as transactivation response element RNA-binding proteins (TRBPs), which yields the mature (~21-

25 nt) miRNA duplex, containing a 5′ monophosphate and 2 nucleotide, 3′ hydroxyl (OH) overhangs (114, 117, 119). The miRNA duplex is then taken up into the miRNA-induced silencing complex (miRISC), which contains an Argonaute (Ago) protein. Ago proteins are composed of four domains arranged in two lobes: one lobe contains the middle (MID) as well as the P-element induced wimpy testis (PIWI) domains, while the other consists of the N-terminal and the Piwi-Argonaute-Zwille (PAZ) domains (120). Ago is an essential component of the miRISC, and the human paralogs have been shown to repress translation of mRNAs when artificially tethered to their 3′ end, in the absence of a miRNA (121). When loaded with a miRNA duplex, Ago unwinds the duplex, and uses one strand, termed the "guide strand" to target complementary mRNAs, while the other strand, the "passenger strand", is discarded (117, 122). Several features of the miRNA duplex are involved in strand selection: in human cells, Ago2 has a strong preference for Uracil at the 5′ terminus of the guide strand, and the monophosphate is also required for loading. Moreover, the strand with the lowest relative thermostability is generally selected as guide strand (123). Out of the four mammalian Ago proteins (Ago1-4), only Ago2 possesses slicing activity that enables it to cleave the passenger strand during strand selection (122, 124).

miRISC-mediated gene silencing

miRNAs are primarily known to base-pair with the 3′ UTR of partially complementary target mRNAs to repress their translation, and this interaction may lead to mRNA decay (114, 125). Each miRNA can have multiple target mRNAs, just as there can be multiple miRNA binding sites on a single transcript, both allowing for downregulation of transcript expression (126). After engagement of an Ago:miRNA complex with a complementary target mRNA, the RNA

silencing effector protein, glycine-tryptophan repeat containing protein of 182 kDa (GW182), known as trinucleotide repeat-containing gene 6 protein (TNRC6) in mammals, is recruited to the target mRNA, which leads to translational repression, decapping and deadenylation of target mRNAs (**Figure 1.3**) (127-129). Silencing of transcripts is achieved on one hand by repressing cap-dependent translation, and on the other hand via the recruitment of deadenylase enzymes which remove the poly(A) tail and promote the decay of the target mRNA (114). mRNAs targeted by miRNAs localize with the miRISC to cytoplasmic foci termed processing bodies (P-bodies), which also contain components of other mRNA silencing pathways (130). While deadenylation and decapping are known to be effected by TNRC6-mediated recruitment of the carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) and decapping protein 1 (DCP1)-DCP2 complexes respectively, the mechanism(s) of translational repression has been the subject of much debate and remains incomplete (114, 124).

1.7 Interaction between miR-122 and the HCV genome

miR-122 is primarily expressed in the liver of all vertebrates, where it accounts for approximately 70% of all miRNAs, with an estimated 135,000 copies per human hepatocyte (131-133). Its endogenous functions include the regulation of cholesterol and fatty acid metabolism, as well as systemic iron homeostasis (134-138). In addition to its endogenous functions in maintaining the integrity of liver cells, miR-122 was found to be a critical host factor required for efficient HCV RNA accumulation. As mentioned above, miRNAs generally target the 3′ end of mRNAs and downregulate protein expression (114, 124). In contrast to this canonical pathway, miR-122 interacts with two sites in the 5′ UTR of the HCV genome and

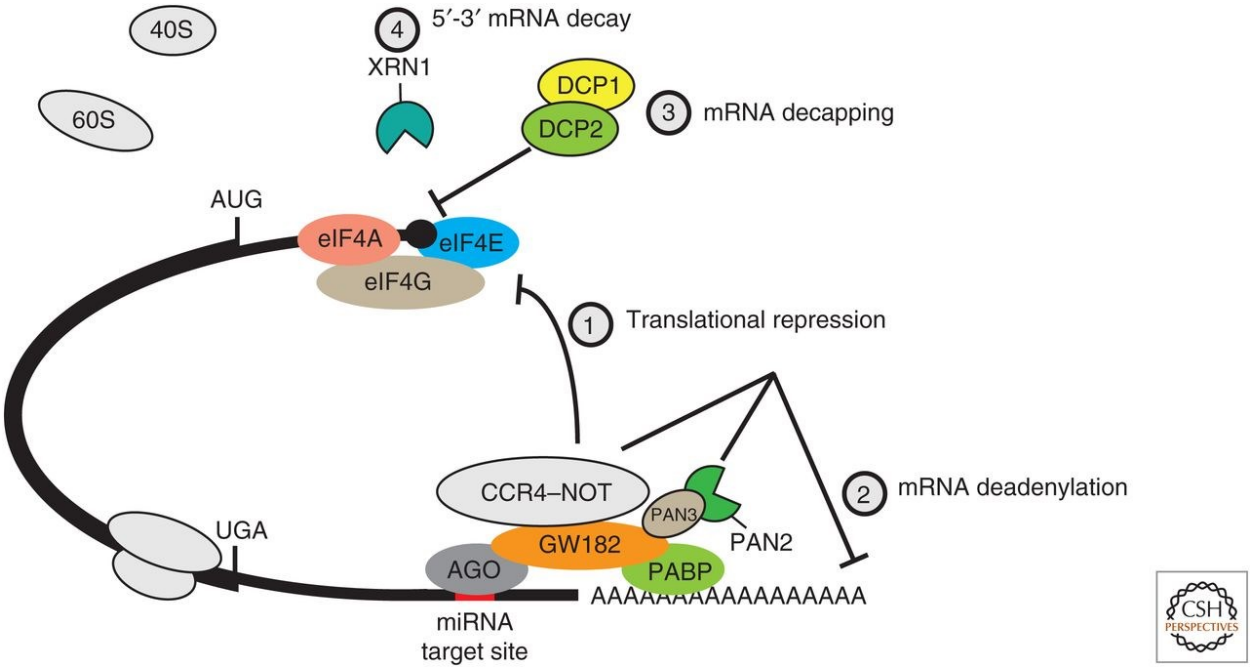

Figure 1.3. Model for miRNA-mediated gene silencing. A miRNA loaded Ago is recruited to a target mRNA by base-pairing between the miRNA seed sequence and the mRNA 3′ UTR. GW182/TNRC6 binds to Ago and recruits silencing factors. 1) The CCR4-NOT complex mediates translational repression prior to deadenylation; 2) the CCR4-NOT and the PABP-dependent poly(A)-nuclease (PAN)2-PAN3 complexes deadenylate the target mRNA; and 3) the DCP1-DCP2 complex mediates mRNA decapping, thereby promoting mRNA decay (4). Figure reproduced from Duchaine & Fabian 2019 © Cold Spring Harbor Laboratory Press (114).

promotes viral RNA accumulation (109-111). Sequestration of miR-122 leads to a dramatic decrease in HCV RNA accumulation *in vitro* and *in vivo* (109, 112, 113, 139). It is clear that this effect is due to a direct interaction with the HCV genome, as mutations in the HCV 5′ UTR which ablate endogenous miR-122:HCV RNA interactions can only be rescued by exogenous supplementation with miR-122 molecules containing compensatory mutations (109, 111). Additionally, at least one study has demonstrated that the miR-122 sites can be replaced with miR-15 sites, to generate a miR-15-dependent viral RNA (140). Consequently, due to its reliance on miR-122, optimal HCV RNA accumulation relies on the microRNA biogenesis machinery – including Dicer, Drosha, DGCR8, TRBP and the Ago proteins (111, 141-144).

Interestingly, interactions between miR-122 and the HCV genome are also implicated in HCV pathogenesis and the virus effectively "sponges" miR-122 in cell culture and in the livers of infected individuals, such that miR-122's normal cellular targets are de-repressed during infection (140, 145). Since miR-122 is a tumour suppressor, this de-repression is associated with cancer progression and may contribute to the onset and/or progression of HCC in patients with HCV (140, 146-148).

Requirements and roles of miR-122 in the HCV life cycle

miR-122 interacts with two sites in the 5′ UTR of the HCV RNA genome, referred to as site 1 and site 2, respectively (**Figure 1.4**). Each site base pairs with nucleotides 2-8 of miR-122, termed the "seed sequence", and also makes contact through 3′ accessory base pairing interactions (109, 111). The two miR-122 seed sequences are separated by 15 nt (from the beginning of site 1 to the beginning of site 2), which is an optimal distance for cooperative action of miRNAs in canonical RNA silencing (149, 150). There are a total of six predicted binding

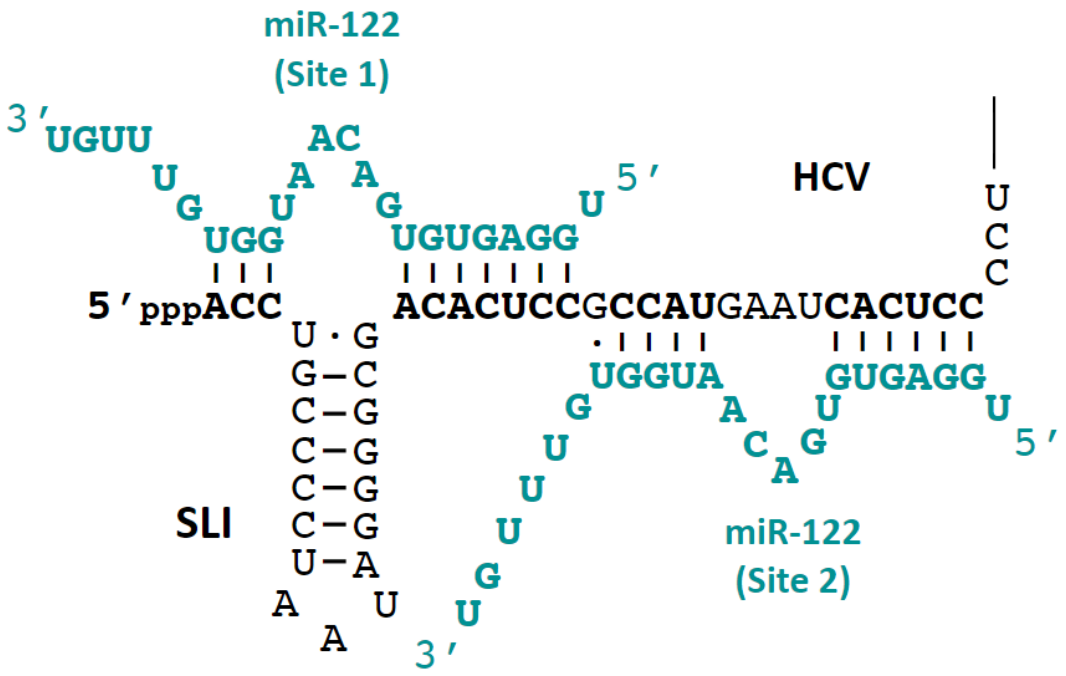

Figure 1.4. miR-122 binding sites on the HCV genome. Nucleotides 1-42 of the HCV genotype 2a isolate JFH-1 are shown in black, with miR-122 binding sites 1 and 2 in bold. Two copies of miR-122 (blue) interact with the HCV genome at site 1 (positions 1-3 and 21-27) and at site 2 (positions 28-32 and 37-42), respectively.

sites for miR-122 on the HCV genome, but although Ago high throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) analyses suggest that they can all be bound by Ago:miR-122 complexes in HCV-infected cells, only the two sites in the 5′ UTR are required for efficient viral RNA accumulation (140, 151). Early studies aimed at elucidating the function of miR-122 in the HCV life cycle suggested a moderate role in promotion of IRES-mediated translation and little to no impact on viral RNA replication, at least in the elongation phase of viral RNA synthesis (152-154). However, the impact of miR-122 on viral translation was not sufficient to explain the dramatic decrease in viral RNA accumulation observed upon miR-122 sequestration, indicating that miR-122 likely had additional roles in the HCV life cycle (152, 153). Recent studies have shown that miR-122 plays at least three roles in the HCV life cycle: 1) it acts as an RNA chaperone or "riboswitch" to promote the formation of the functional IRES structure; 2) it protects the 5′ terminus of the genome from pyrophosphatase activity and subsequent 5′-to-3′ exoribonuclease-mediated decay; and 3) it promotes translation through interactions between the Ago protein at site 2 and the viral IRES (155, 156).

miR-122 acts as a riboswitch to promote the formation of the functional HCV IRES

Analysis of the HCV 5′ UTR by *in vitro* selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) suggests that when the viral RNA is present alone, the most energetically favorable structure for the 5′ UTR is a non-canonical configuration in which SLII is replaced by an alternative RNA structure, termed stem-loop II alternative ($SLII^{alt}$) (156) (**Figure 1.5**). In this configuration, miR-122 binding site 2 is accessible, but site 1 is partially base paired. Thus, assuming that the viral genome is present in this energetically favorable conformation when it enters the cell, miR-122 is likely recruited first to site 2 (156). Binding of miR-122 to site 2 is

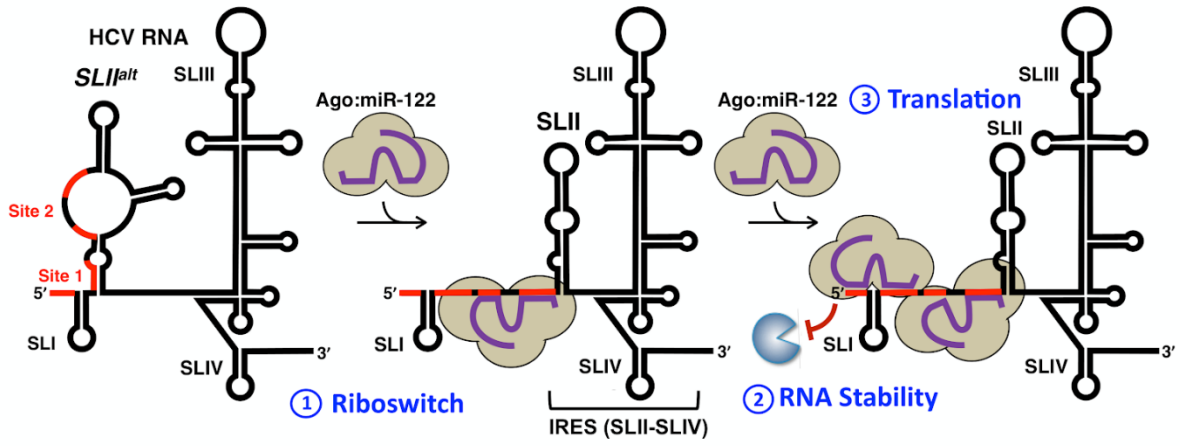

Figure 1.5. Model for the mechanism of Ago:miR-122-mediated promotion of HCV replication. Binding of Ago:miR-122 to site 1 and site 2 in the HCV 5′ UTR is thought to serve at least three purposes to promote the viral life cycle: it 1) acts as an RNA chaperone to promote the riboswitch mechanism allowing the formation of the functional IRES; 2) stabilizes the 5′ terminus by masking the triphosphate moiety from pyrophosphatase activity and subsequent exoribonuclease-mediated decay, and 3) enhances viral translation via interactions between Ago and the IRES.

predicted to alter the conformation of the HCV genome and promote formation of the canonical IRES (SLII-IV) structure. This model is supported by SHAPE as well as computational predictions and mutational analyses (156-158). As such, miR-122 binding to site 2 of the HCV 5′ UTR promotes a conformational change from the non-canonical SLII^{alt} structure to the SLII structure, allowing the viral IRES to form.

miR-122 stabilizes the viral genome by protecting the 5′ triphosphate

Mutational analyses indicated that miR-122 interacts with the 5′ terminus of the HCV genome, suggesting that the Ago:miR-122 complex may shield the 5′ terminus from cellular sensors of RNA or protect it from 5′-to-3′ decay (111) (**Figures 1.5 and 1.6**). Several groups proceeded to investigate the impact of miR-122 binding on stability of the viral RNA and its detection by innate immune sensors of RNA. Collectively, these studies found that miR-122 could shield the viral RNA 5′ terminus from degradation mediated by 5′-3′ exoribonuclease (Xrn)1/2 (159-163). However, Xrn1/2 have a preference for a 5′ monophosphate while the HCV genome contains a 5′ triphosphate (164, 165). Subsequent studies indicated that miR-122 could protect the viral 5′ terminus from two cellular pyrophosphatases, Dom-3 homolog Z/Decapping and exoribonuclease protein (DOM3Z/DXO) and/or Dual specificity phosphatase 11 (DUSP11) (155). Thus, miR-122 binding to the 5′ terminus protects the HCV genome from cellular pyrophosphatase activity and subsequent 5′-to-3′ exoribonuclease-mediated decay.

Ago:miR-122 binding enhances IRES-mediated translation

Early studies of miR-122-mediated promotion of HCV RNA accumulation focused on translational enhancement mediated by miR-122 (110, 152). Indeed, one study suggested that miR-122 could promote ribosome association with the HCV IRES; however, the stimulation was

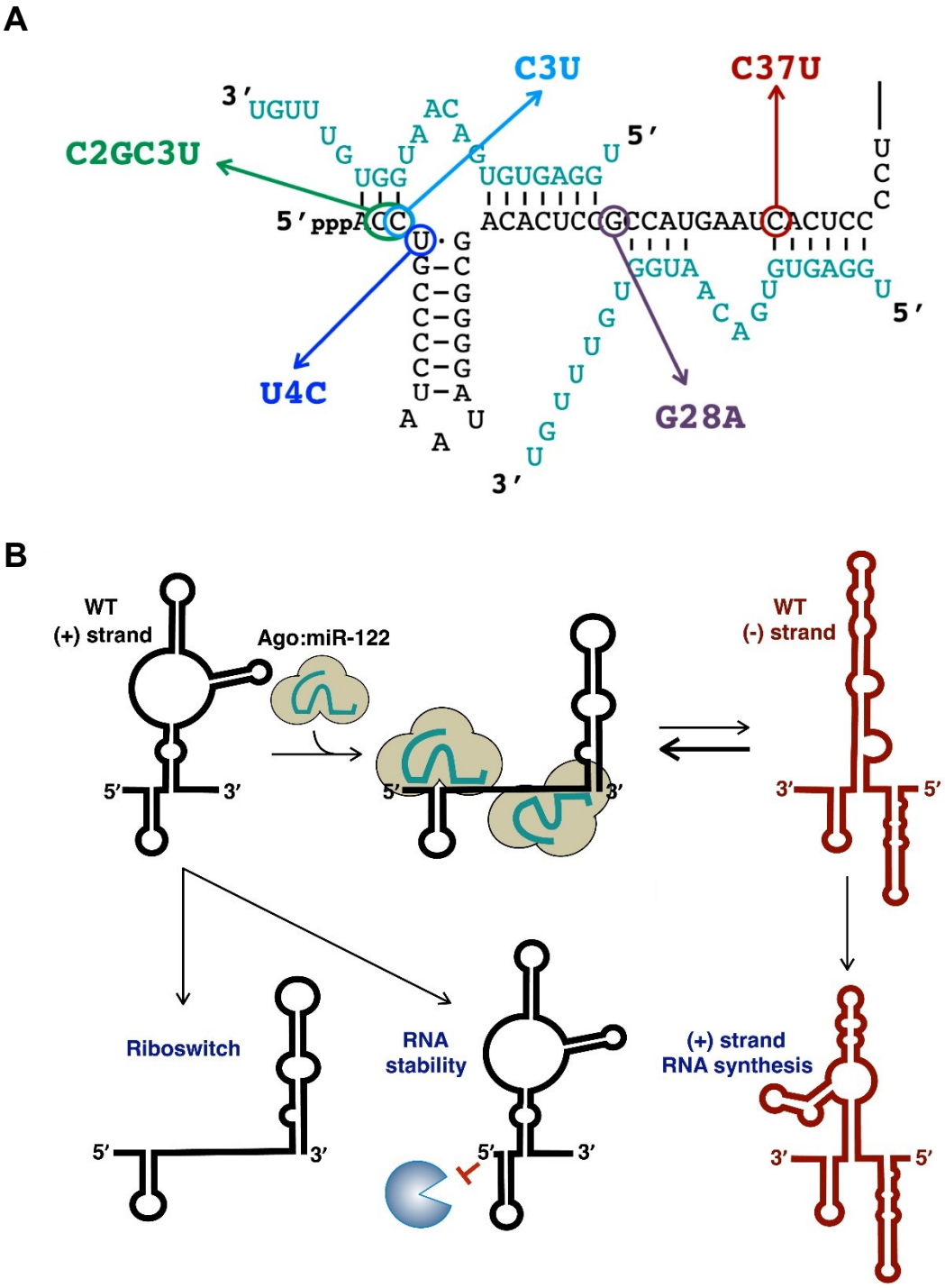

Figure 1.6. Resistance-associated variants and their mechanism of action. A) Indication of the RAVs on the HV 5′ end sequence. **B)** Model for the mechanism of action of the RAVs. WT HCV requires miR-122 binding to two sites in its 5′ UTR to form the functional IRES structure, protect its 5′ end from degradation, and enhance IRES-mediated translation. The C2GC3U, U4C and G28A RAVs are able to form the functional SLII structure preferentially in the absence of miR-122. The C2GC3U, C3U and U4C RAVs have a shortened single-stranded 5′ end which confers protection against pyrophosphatase recognition and subsequent exoribonuclease-mediated decay. C37U has an altered negative-sense intermediate 3′ end structure which is a better promoter for genomic viral RNA synthesis. Both panels reproduced from Chahal *et al.* 2021 (166).

relatively modest, at approximately 1.4-fold (152). More recent studies suggest that translational enhancement might be partially mediated by the riboswitch effect, which allows the HCV IRES to form, and then subsequently through further stabilization provided by contacts between the Ago protein at site 2 and the HCV IRES (**Figure 1.5**). Through *in vitro* SHAPE analysis and computational modeling, the Ago protein at site 2 was demonstrated to make direct contact with the HCV IRES, both at SLIIa (the apical stem of SLII) and in the region between SLII and SLIII of the HCV IRES (156). Specifically, residues 719-728 of Ago2's PIWI domain appear to make direct contact with SLIIa, which may further stabilize this structure and thereby enhance IRES activity (156). Interestingly, these residues are highly conserved across human Ago1-3, suggesting that any of these proteins are likely able to provide translational promotion in this context (156). Thus, in addition to the riboswitch effect, Ago:miR-122 binding to site 2 on the HCV genome may further promote translation by stabilizing the IRES structure, through direct contact between Ago and the HCV IRES.

miR-122 as a therapeutic target

Due to the importance of miR-122 in the HCV life cycle, miR-122 inhibitors have been developed for the treatment of chronic HCV infection. Two such molecules, Miravirsen™ and RG-101, have made it to clinical trials: they were well tolerated and led to sustained viral load reductions and even SVR in some patients (112, 113). Thus, targeting of miR-122 is a promising novel approach to HCV treatment, which may benefit from further investigation in the event of resistance to DAA therapies.

Resistance-associated variants compensate for distinct miR-122 roles

While Miravirsen™ and RG-101 were highly efficacious in clinical trials, in a few cases, when miR-122 levels rebounded after cessation of treatment, HCV RNA also rebounded (167). In these clinical trials, together with cell culture-based studies, a group of resistance-associated variants (RAVs) have been identified in the 5′ UTR of the HCV genome (113, 145, 168) (**Figure 1.6**). A recent study has provided evidence that these RAVs compensate for some of miR-122's roles, thereby reducing their need for the miRNA. More specifically, C2GC3U, U4C and G28A, are "riboswitched" and adopt the functional SLII structure even in the absence of miR-122 (156, 166, 169). C2GC3U, C3U and U4C, have additional base-pairing interactions at the 5′ terminus which decreases the number of single-stranded nucleotides at the 5′ end of the genome, thereby stabilizing the RNA in the absence of site 1-bound miR-122 (166, 169). Finally, C37U changes the conformation of the 3′ end of the negative-strand replicative intermediate (the positive-strand promoter), which enhances viral RNA synthesis (166, 169).

1.8 Hypothesis and Specific Aims

As previously discussed, the liver-specific miRNA, miR-122, binds to two sites in the 5′ UTR of the HCV genome and promotes translation and viral RNA accumulation. Studies conducted over the last decade have led to the identification of three specific roles for miR-122 in the HCV life cycle: 1) it acts as an RNA chaperone, or "riboswitch" to promote the conformational change from an alternative structure called $SLII^{alt}$ to the functional IRES structure, consisting of SLII-IV; 2) it stabilizes the viral genome by protecting the 5′ terminus from pyrophosphatase activity and subsequent exoribonuclease-mediated decay; and 3) it promotes translation via an interaction between the Ago protein at site 2 and the viral IRES. However, the relative contribution of each

of these three roles to the overall impact of miR-122 on the HCV life cycle, and whether additional roles have yet to be identified, remains unclear. Thus, in ***Chapter 2***, we explore the importance of each of these three roles in the HCV life cycle. More specifically, we hypothesized that we could quantify the relative contribution of each of the three known roles of miR-122 using a combination of reporter viral RNAs, point mutations, and miR-122 complementation, in live cell assays. To test this hypothesis, we performed the following specific aims: ***aim 1***, to quantify the maximum effect of miR-122 on HCV RNA accumulation, and ***aim 2***, to quantify the relative contributions of each of the three roles attributed to miR-122 in the HCV life cycle. To measure the maximum effect (***aim 1***), we electroporated wild-type (WT) HCV into miR-122 knockout (KO) cells and compared luciferase activity and viral RNA levels in the presence or absence of miR-122. To quantify the relative contributions of each of the three roles attributed to miR-122 in the HCV life cycle, namely riboswitch, genome stabilization, and translational promotion (***aim 2***), we used a series of viral point mutations and complementation strategies to isolate each role.

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CHAPTER 2: Elucidating the distinct contributions of miR-122 in the HCV life cycle reveals insights into virion assembly

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Keywords

Hepatitis C Virus, miR-122, riboswitch, genome stability, translational promotion

2.1 Preface to Chapter 2

As discussed in ***Chapter 1***, at least three roles have been attributed to miR-122 in the HCV life cycle: 1) riboswitch; 2) genome stabilization; and 3) translational promotion (1-7). However, it remains unclear what the relative contribution is of each of these roles to the overall impact of miR-122 on the HCV life cycle, and whether more functions remain to be identified. We hypothesized that the relative contributions of each of the three roles attributed to miR-122 in the HCV life cycle could be isolated using a combination of point mutations, miR-122 complementation, and live cell assays. Using a full-length HCV genomic RNA encoding a luciferase reporter gene, we used point mutations and mutant miRNAs to study each of the three roles of miR-122 in isolation. Our results provide an estimate of the relative contributions of each of the three roles to the establishment and the maintenance of HCV infection, and also reveal new insights into the regulation of virion assembly.

This chapter was adapted from the following manuscript in preparation: "Elucidating the distinct contributions of miR-122 in the HCV life cycle reveals insights into virion assembly" by **Marylin Rheault**, Sophie E. Cousineau, Danielle R. Fox, Quinn H. Abram, and Selena M. Sagan.

2.2 Abstract

During infection, the Hepatitis C virus (HCV) genome interacts with two molecules of the human liver-specific microRNA, miR-122, and this unusual interaction promotes viral RNA accumulation. Three distinct roles have been attributed to miR-122 in the HCV life cycle: an RNA chaperone, or "riboswitch" activity that allows the formation of the viral internal ribosomal entry site (IRES), a genome stabilization function which protects the 5′ terminus of the viral RNA from exoribonuclease-mediated decay, and a translational promotion activity involving the Argonaute (Ago) protein in complex with miR-122. However, the relative importance of each of these three roles in the HCV life cycle remains unclear. To evaluate this, we used a combination of point mutations and mutant miRNAs in the context of an HCV *Renilla* luciferase (RLuc) reporter RNA system to study each of the three roles in isolation and evaluate their contribution to the overall impact of miR-122 in the HCV life cycle. Our results suggest that the riboswitch effect has a minimal contribution in isolation, while the stabilization and translational promotion roles have similar contributions in the establishment phase of infection. However, in the maintenance phase, after several rounds of replication, translational enhancement becomes the dominant role. Additionally, by using point mutations that stabilize the SLII conformation over $SLII^{alt}$, we found that the $SLII^{alt}$ structure was important for efficient virion assembly. Taken together, our results clarify the importance of each of the established roles for miR-122 in the HCV life cycle and provide new insights into the factors regulating the balance of viral RNAs in the translating/replicating pool and those engaged in virion assembly.

2.3 Introduction

Hepatitis C virus (HCV) affects approximately 71 million people worldwide and typically results in a chronic infection that can lead to steatosis, cirrhosis, and hepatocellular carcinoma (8-10). HCV is a positive-sense RNA virus of the *Flaviviridae* family. Its ~9.6 kb genome encodes a single open-reading frame flanked by highly structured 5′ and 3′ untranslated regions (UTRs) (11). The 5′ and 3′ UTRs contain several *cis*-acting RNA elements which play important roles in the viral life cycle. Specifically, the 5′ UTR contains the internal ribosomal entry site (IRES), composed of stem-loops (SL) II-IV, which directs cap-independent translation of the viral polyprotein (12). Additionally, SLI-II in the 5′ UTR as well as the 3′ UTR (including the variable region, polyU/UC-tract, and 98-nt X-tail) are required for viral replication (13-17). The 5′ terminus of the HCV genome also contains two conserved binding sites for the human liver-specific microRNA (miRNA), miR-122 (5-7).

miR-122 is highly conserved across the vertebrate lineage and specifically expressed in the liver, with ~135,000 copies per hepatocyte (6, 18). While the canonical function of miR-122 is in regulation of cholesterol synthesis and fatty acid metabolism, miR-122 interacts with two sites (site 1 and site 2) in the 5′ terminus of the HCV genome, and these interactions promote viral RNA accumulation (4-7). Several recent studies have revealed a new model for miR-122:HCV RNA interactions that suggests that miR-122 has at least three roles in the HCV life cycle (**Figure 2.1A**) (1-3, 19, 20). Firstly, upon entry into the cell, the viral 5′ UTR is thought to adopt the most energetically favorable conformation (termed $SLII^{alt}$), in which only the second miR-122 binding site (site 2) is accessible. As such, an Argonaute (Ago):miR-122 complex is recruited to site 2 of the HCV genome. This results in an RNA chaperone-like switch in conformation, akin to a riboswitch, converting $SLII^{alt}$ to the SLII structure, thereby allowing the

viral IRES (SLII-IV) to form (2, 3, 20). This conformational change then reveals site 1, allowing recruitment of a second Ago:miR-122 complex, which promotes genome stability by base pairing with the 5′ terminus, protecting the viral RNA from cellular pyrophosphatase activity and subsequent exoribonuclease-mediated decay (1, 7, 21-24). Finally, the Ago protein bound to site 2 makes direct contact with SLII, further stabilizing the IRES and promoting viral translation (**Figure 2.1A**) (1-3, 21-26).

Herein, we explored the importance of each of the three roles attributed to miR-122, namely the riboswitch, genome stabilization, and translational promotion activities, in the overall impact of miR-122 in the HCV life cycle. To do so, we used a combination of point mutations and luciferase reporter viral RNAs, combined with compensatory miR-122 molecules, which allowed us to study each of the three roles in isolation. We found that the relative contributions of each of the three roles differed during the establishment and maintenance phases of the viral life cycle. Specifically, during the establishment phase of the viral life cycle (i.e. before viral replication is established), our results suggest that the riboswitch activity alone does not play a significant role, while genome stabilization and translational enhancement contribute similarly to translation and viral RNA accumulation. However, during the maintenance phase (i.e. after several rounds of viral replication), genome stabilization becomes less important and translational promotion contributes predominantly to viral RNA accumulation. Interestingly, since we observed that the riboswitch activity did not have a significant effect on viral RNA accumulation using luciferase reporter RNAs, we decided to explore the importance of the riboswitch activity using the fully packaging-competent cell culture-derived HCV (HCVcc) system. By using point mutations that destabilized the $SLII^{alt}$ conformation and stabilized the SLII conformation, we observed a progressive decrease in infectious particle production,

suggesting that SLII^{alt} is required for efficient viral packaging. Thus, our results provide a new model whereby SLII^{alt} and miR-122 interactions with the viral 5′ UTR regulate the balance of viral RNAs in the translating/replicating pool and those engaged in virion assembly.

2.4 Results

Overall contribution of miR-122 to HCV translation and viral RNA accumulation.

To determine the overall contribution of miR-122 to HCV translation and viral RNA accumulation, we co-electroporated full-length wild-type (WT) and GNN (replication-defective) HCV *Renilla* luciferase (RLuc) reporter RNAs and miR-122 or control miRNA duplexes into miR-122 knockout (KO) cells and monitored luciferase activity over time (**Figure 2.1B-E**). Luciferase activity was used as a proxy for viral RNA accumulation and/or decay over time. As shown previously, and because miR-122 is required for efficient viral RNA accumulation, introduction of HCV reporter RNAs into miR-122 KO cells in the absence of exogenously provided miR-122 resulted in transient translation of the input RNA from 2-8 h post-electroporation, but the signal quickly decayed to background by the 24 h time point, in both the replication-defective (GNN) and WT conditions (**Figure 2.1B-E**) (7, 19). In contrast, the exogenous addition of miR-122, which gets loaded into an Ago protein and interacts with the HCV genome at both site 1 and site 2, results in an approximately 3.6-fold increase in luciferase activity in the establishment phase of the viral life cycle, quantified using the GNN condition at the 6 h time point, which we considered the initial impact on translation prior to viral RNA replication (**Figure 2.1B-C**). In the maintenance phase of the viral life cycle, exogenous addition of miR-122 results in an approximately 1863.4-fold increase in luciferase activity, quantified using the WT condition at the 72 h time point, after several rounds of viral RNA replication

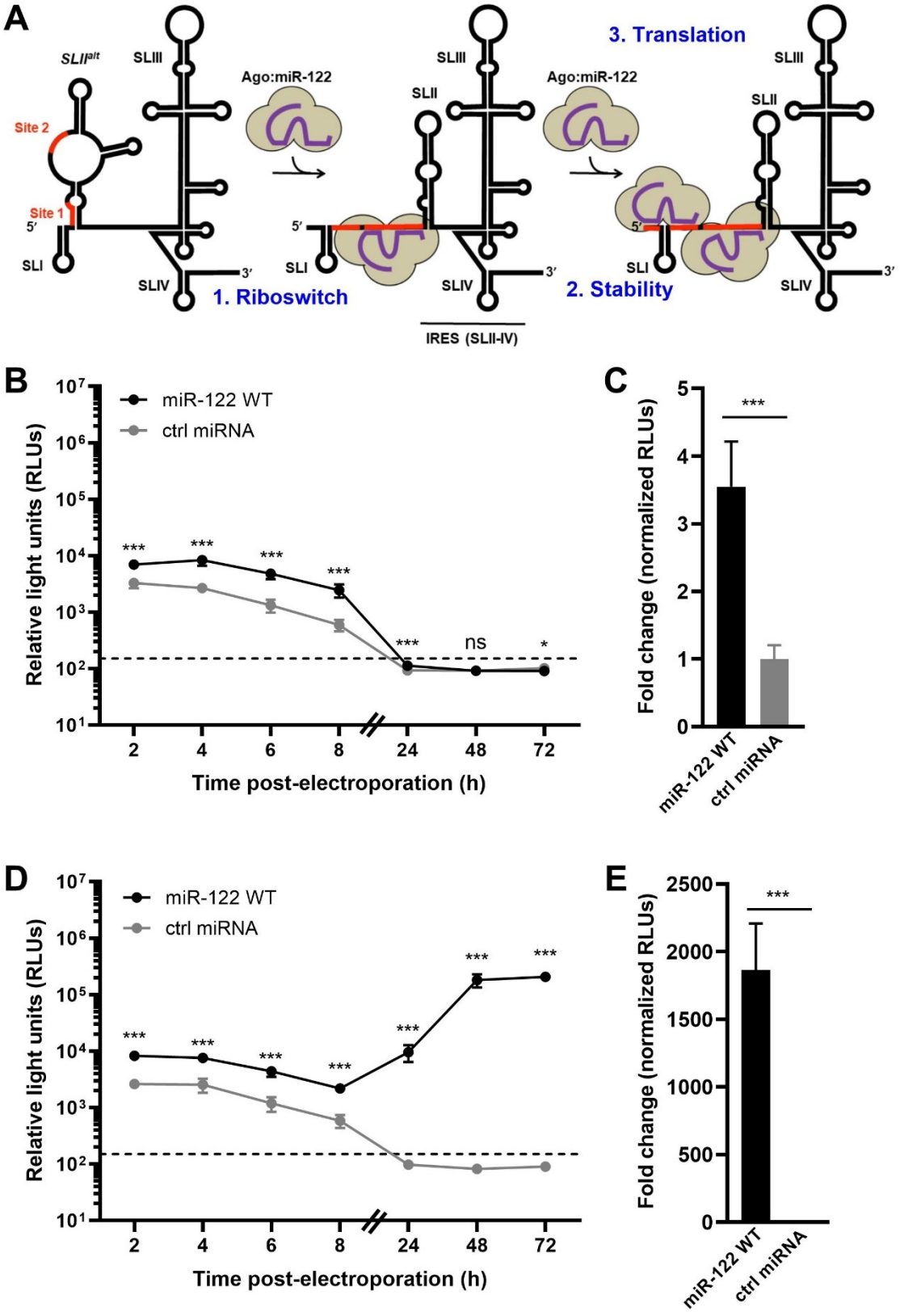

Figure 2.1. Overall contributions of miR-122's riboswitch, genome stability and translational promotion activities. (A) Schematic representation of the three roles attributed…*continued on next page*.

(**Figure 2.1D-E**). To further confirm that luciferase activity was an appropriate proxy for HCV reporter RNA levels, we also performed strand-specific RT-qPCR to quantify viral RNA levels in the WT experiment (**Figure 2.1D and Supplementary Figure S2.1**). In contrast to our luciferase activity results, we observed similar viral RNA levels in the miR-122 and control miRNA conditions at early time points (2-8 h) post-electroporation, while we observed significant differences in viral RNA accumulation at later time points (24-72 h) post-electroporation. The fold-change in viral RNA accumulation at the 72 h time point based on RT-qPCR analysis was approximately 410.6-fold (**Supplementary Figure S2.1**). These results are not surprising given that miR-122 promotes translation and the luciferase assay serves as a proxy for the proportion of viral RNAs engaged in translation, while RT-qPCR is reporting on total viral RNA levels. Moreover, HCV RNA containing cells are also likely diluted out at the later time points post-electroporation, since HCV naïve cells have a growth advantage, and thus are likely to contribute disproportionately to the total RNA. Nonetheless, the results are largely concordant and allow us to estimate the overall contribution(s) of miR-122 complementation at both the establishment and maintenance phases of the viral life cycle. Taken together, our results

Figure 2.1. Overall contributions of miR-122's riboswitch, genome stability and translational promotion activities. *continued from previous page…*to miR-122 in the HCV life cycle. In the absence of miR-122, WT HCV RNA favors the $SLII^{alt}$ conformation, and recruitment of miR-122 to site 2 promotes RNA chaperone or riboswitch activity, allowing the viral internal ribosomal entry site (IRES) to form (including SLII-IV). Subsequent recruitment of a second miR-122 molecule to site 1 provides genome stability (protecting the 5′ triphosphate from pyrophosphatases and exoribonuclease-mediated decay), and translational promotion (through interactions between the Argonaute (Ago) protein at site 2 and the viral IRES. (**B-E)** Full-length RLuc HCV RNAs were co-electroporated into miR-122 knockout (KO) cells with wild-type (WT) miR-122 or control (ctrl) miRNA, and a capped Firefly luciferase (FLuc) reporter RNA. Half the number of cells were plated for the 24-72 h time points and the 48-72 h lysates were diluted 2-fold to ensure values were within the range of the luciferase assay. Rluc activities for **(B-C)** GNN and **(D-E)** WT viral RNAs were monitored over time. In **(C)** and **(E)**, RLuc activity for GNN or WT HCV RNAs at 6 h or 72 h, respectively, normalized to the FLuc (transfection efficiency) control at 2 h, were used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. Numbers are displayed as the mean of three biological replicates, and error bars represent the standard deviation (SD) of the mean. Statistical significance was determined by multiple Student's *t* test, $***p \leq 0.0001$; $*p \leq 0.01$; ns, not significant ($p \geq 0.01$).

suggest that full complementation of miR-122 at both site 1 and site 2 on the HCV genome provides an approximately 3.6- and 1863.4-fold increase in translation and viral RNA accumulation as measured by luciferase activity during the establishment and maintenance phases of the viral life cycle, respectively.

miR-122's riboswitch activity has a negligible impact on HCV translation and viral RNA accumulation.

Next, we wanted to specifically dissect the precise contributions of each of the three roles attributed to miR-122 in the HCV life cycle, namely the: 1) riboswitch; 2) genome stability; and 3) translational promotion activities. To do so, we used a combination of point mutations and manipulated miR-122 binding to site 1 and site 2 on the HCV genome.

Firstly, miR-122's riboswitch activity is fulfilled when an Ago:miR-122 complex binds to site 2 on the HCV genome, converting the $SLII^{alt}$ conformation to the SLII conformation (**Figure 2.1A**). However, miR-122 binding to site 2 also provides translational promotion via interactions between the Ago protein and SLII of the HCV IRES, which could conflate these two roles. As such, we decided to make use of the U4C mutant, which we previously demonstrated is "riboswitched", even in the absence of miR-122 (**Figure 2.2A**) (19). We therefore compared WT and U4C HCV reporter RNA translation and viral RNA levels via luciferase assay in miR-122 KO cells. However, since these viral RNAs do not replicate in the absence of miR-122 (**Figure 2.1B-E**), we only performed this experiment in the GNN (replication-defective) context (**Figure 2.2B-C**) (19). Interestingly, we did not observe a significant difference in luciferase activity between WT and U4C at any time point post-electroporation, suggesting that the riboswitch effect has a minimal impact on HCV translation and viral RNA accumulation. To

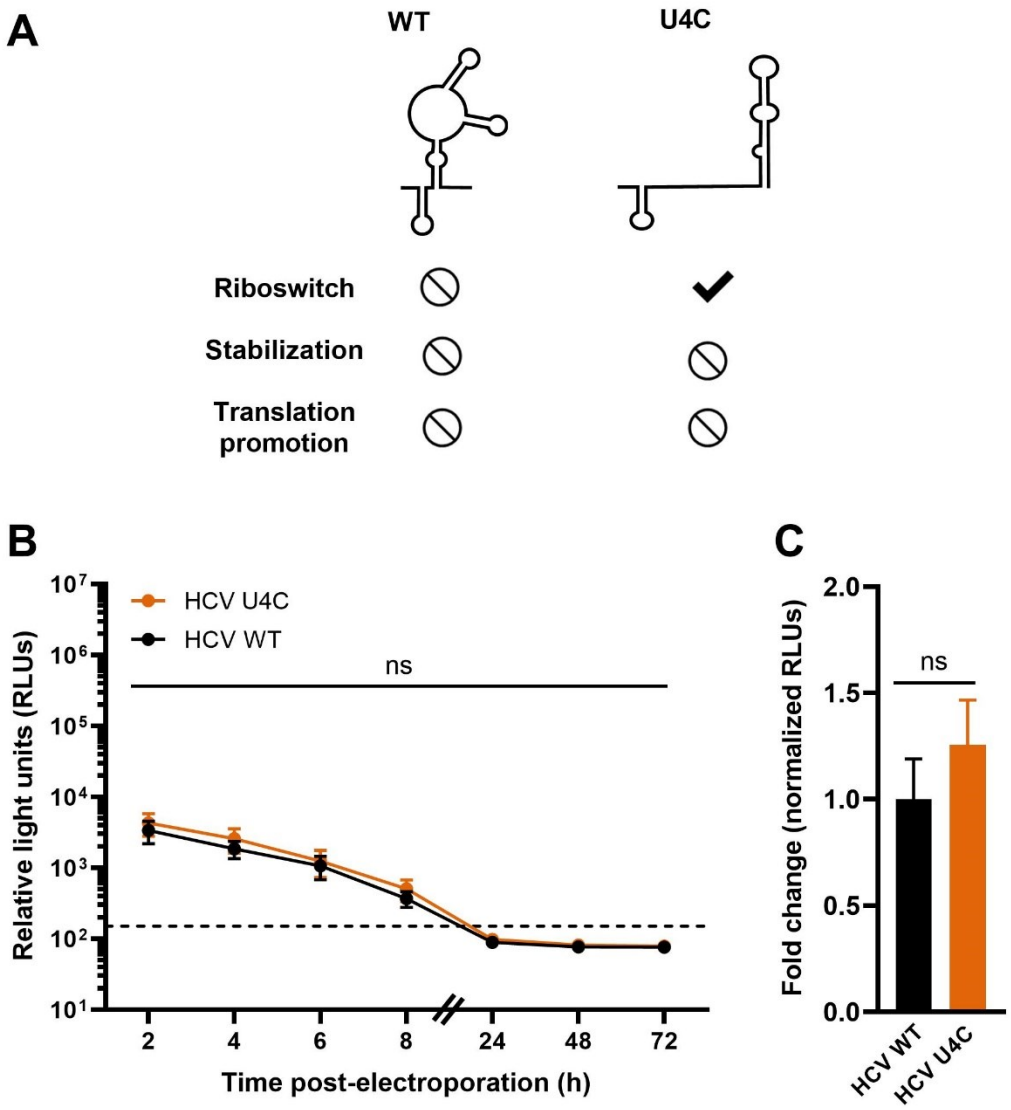

Figure 2.2. The riboswitch activity on its own has a negligible impact on viral translation. (A) Schematic representation of the experimental set-up for the riboswitch activity assay. In the absence of miR-122, WT HCV RNA favors the SLII^{alt} conformation, while the U4C mutant favors the riboswitched (SLII) conformation. In the absence of miR-122, the stabilization and translational enhancement roles of miR-122 are not fulfilled in either condition, allowing for the isolation of the riboswitch effect. **(B)** Full-length RLuc GNN HCV (WT or U4C) RNAs were co-electroporated into miR-122 KO cells with a capped FLuc reporter RNA. RLuc activity was monitored over time as described in *Figure 2.1*. **(C)** RLuc activity for HCV GNN at 6 h normalized to FLuc (transfection efficiency) control at 2 h, was used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. RLuc is displayed as the mean of three biological replicates, and error bars represent the SD of the mean. Statistical significance was determined by multiple Student's *t* test. ns, not significant ($p \geq 0.01$).

confirm these results, we also generated an additional mutant, G20A, which we demonstrated via *in vitro* selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) analysis is similarly "riboswitched" in the absence of miR-122 (**Supplementary Figure S2.3**). In agreement with the U4C results, we did not observe any significant differences in luciferase activity between WT and G20A GNN RNAs when electroporated in miR-122 KO cells. Given that the WT GNN reporter viral RNA is able to produce luciferase even in the absence of miR-122 (**Figures 2.1B-C and 2.2B-C**), the viral 5′ UTR must be able to spontaneously "riboswitch", even in the absence of miR-122. This is not surprising, given that the Gibb's free energies (ΔG) of the $SLII^{alt}$ and SLII conformations are -39.7 and -37.8, respectively (**Supplementary Figure S2.7**). As such, the viral 5′ UTR likely exists in an equilibrium between these conformations within the host cell. Thus, although the riboswitch activity is required for viral translation as it allows the HCV IRES to form, this is likely to be able to occur even in the absence of miR-122. Taken together, our results suggest that miR-122's riboswitch activity has a negligible contribution to HCV translation and viral RNA accumulation.

miR-122-mediated genome stabilization plays a more important role in the establishment phase than in the maintenance phase of the viral life cycle.

Next, we focused on miR-122's role in genome stabilization, which is primarily mediated by the Ago:miR-122 interactions with site 1 through base pairing with the 5′ terminus of the viral RNA (**Figure 2.1A**) (1-3, 19, 22-24). As such, we made a point mutation in site 1 (C26A) in the HCV RNA, termed S1:p3, which prevents wild-type miR-122 from binding, but maintains the secondary structure of the viral 5′ UTR. Ago:miR-122 interactions with site 1 can then be rescued by exogenous complementation of miR-122 p3U molecules, which contain a compensatory mutation at position 3 of the miRNA (**Figure 2.3A**). To account for the riboswitch

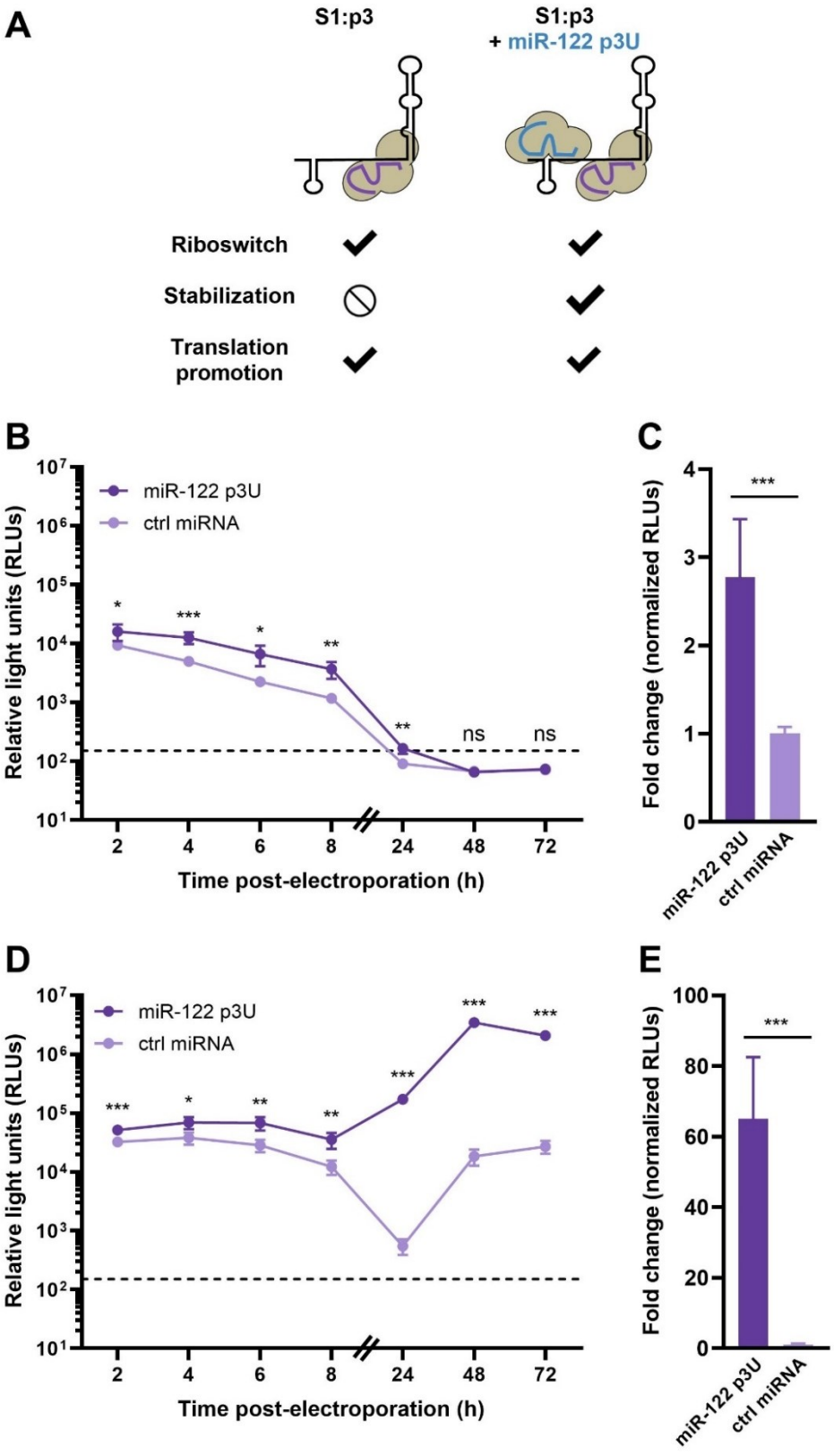

Figure 2.3. miR-122's effect on genome stability has a greater impact in the establishment phase than in the maintenance phase of the HCV life cycle. miR-122-mediated genome *…continued on next page*.

and translational promotion activities, we complemented with miR-122 p3U or a control miRNA in Huh-7.5 cells, where endogenous wild-type miR-122 is available to interact with site 2 on the HCV genome. GNN or WT S1:p3 HCV reporter RNAs were co-electroporated with miR-122 p3U or a control miRNA into Huh-7.5 cells and luciferase activity was monitored over time (**Figure 2.3B-E**). In the establishment phase, we observed an approximately 2.8-fold increase in luciferase activity upon complementation with miR-122 p3U (**Figure 2.3B-C**). However, in the maintenance phase, we observed an approximately 65.0-fold increase in luciferase activity upon miR-122 p3U complementation (**Figure 2.3D-E**). Again, we saw no significant difference in HCV RNA copies as measured by RT-qPCR during the establishment phase, and the half-lives of the GNN viral RNAs were 1.8 h [95% confidence interval (C.I.) 1.2 – 2.7 h] and 3.5 h (95% C.I. 1.9 – 7.6 h) for the miR-122 p3U and control miRNA conditions, respectively (**Supplementary Figure S2.4A-B**). These results suggest that genome stabilization accounts for a significant proportion of miR-122's effects during the establishment phase, but accounts a lower proportion of the effect during the maintenance phase of infection.

Figure 2.3. miR-122's effect on genome stability has a greater impact in the establishment phase than in the maintenance phase of the HCV life cycle. *Continued from previous page…*stabilization has an impact during both the establishment and maintenance phases of viral replication. **(A)** Schematic representation of the experimental set-up for genome stability assays. Endogenous WT miR-122 binds to site 2 only on HCV S1:p3 RNAs, thereby fulfilling the riboswitch and translational enhancement roles, while addition of miR-122 p3U allows binding to site 1 and measurement of the stabilization effect in isolation. **(B-E)** Full-length RLuc HCV S1:p3 RNAs were co-electroporated in Huh-7.5 cells with miR-122 p3U or ctrl miRNA, as well as a capped FLuc reporter RNA. Rluc activities for **(B-C)** GNN or **(D-E)** WT S1:p3 viral RNAs were monitored over time as in *Figure 2.1*. In **(C)** and **(E)**, RLuc activities for GNN or WT S1:p3 HCV RNAs at 6 h or 72 h, respectively, normalized to the FLuc (transfection efficiency) control at 2 h, were used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. RLuc is displayed as the mean of three biological replicates, and error bars represent the SD of the mean. Statistical significance was determined by multiple Student's *t* test, ***$p \leq 0.0001$; **$p \leq 0.001$; *$p \leq 0.01$; ns, not significant ($p \geq 0.01$).

miR-122-mediated translational promotion is the predominant role in the maintenance phase of the viral life cycle.

Finally, we focused on miR-122's translational promotion effect, which is primarily mediated by contacts between the Ago protein bound to site 2 and the HCV IRES at SLII (**Figure 2.1A**). To measure the translational promotion effect in isolation, we introduced a point mutation at site 2 (C41A) in the HCV RNA, termed S2:p3, which prevents wild-type miR-122 from binding, but maintains the secondary structure of the viral 5′ UTR. Like the S1:p3 mutation, Ago:miR-122 binding to S2:p3 can be rescued by exogenous complementation with miR-122 p3U molecules, which contain a compensatory mutation at position 3 of the miRNA (**Figure 2.4A**). To account for the riboswitch and genome stabilization activities, we also made use of the U4C point mutation ("riboswitched" *a priori*) and performed these experiments in Huh-7.5 cells, such that endogenous miR-122 can interact with site 1, providing the stabilization effect. We therefore co-electroporated WT or GNN U4C S2:p3 HCV reporter RNAs with miR-122 p3U or a control miRNA into Huh-7.5 cells and monitored luciferase activity over time (**Figure 2.4B-E**). In the establishment phase, we observed an approximately 3.6-fold increase in luciferase activity upon addition of miR-122 p3U (**Figure 2.4B-C**). Interestingly, in the maintenance phase, we observed an approximately 2450.1-fold increase in luciferase activity upon addition of miR-122 p3U (**Figure 2.4D-E**). Notably, we also observed a similar magnitude effect when this experiment was performed using G20A S2:p3 viral RNAs (**Supplementary Figure S2.5**). Taken together, these results suggest that the translational promotion and genome stabilization effects have a similar overall contribution in the establishment phase of the viral life cycle, while translational promotion is the predominant role in the maintenance phase.

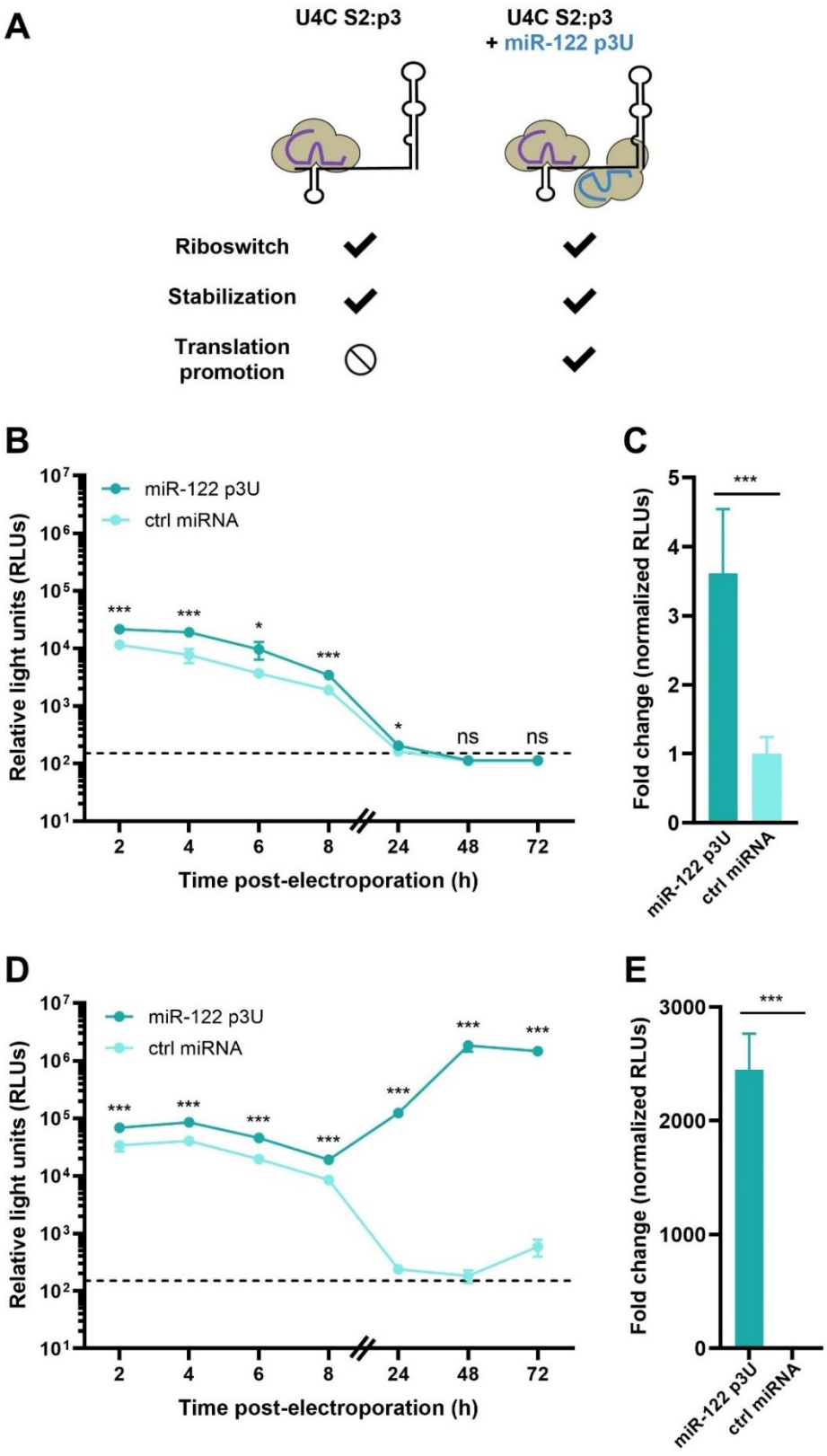

Figure 2.4. miR-122-mediated translational promotion is the dominant role during the maintenance phase of viral replication. (A) Schematic representation of the experimental*...continued on next page.*

Ago-mediated translational promotion is context-specific.

Given that the Ago protein was first identified as a ribosome-associated protein and that it has also been reported to promote mRNA translation of AU-rich element-containing transcripts during cell stress, we wondered whether recruitment of Ago to the 5′ UTR of an unrelated transcript would lead to translational promotion (27-29). To investigate this, we used the Bacteriophage λN-BoxB system to directly tether human Ago2 to the 5′ end of a luciferase reporter mRNA (**Supplementary Figure S2.6**) (30). We transcribed the mRNAs using an "A cap" [i.e. G(5′)ppp(5′)A] to stabilize the transcript, but to prevent cap-dependent translation, since the eukaryotic initiation factor 4E (eIF4E) is not recruited to an A cap (31). As positive- and negative-controls, we used λN-eIF4G and λN-LacZ, respectively (**Supplementary Figure S2.6A-C**). When we expressed λN-Ago2, we did not observe any increase in luciferase activity beyond the negative control, suggesting that Ago2 does not universally promote translation when tethered to the 5′ end of a reporter mRNA (**Supplementary Figure S2.6B**). However, it is possible that Ago2's subsequent recruitment of the miRNA silencing effector protein, trinucleotide repeat-containing gene 6 (TNRC6), could affect Ago2's translational promotion activity. As such, we also engineered point mutations which disrupt the Tryptophan (Trp)-binding pockets in Ago2 that mediate TNRC6 recruitment. Of the three TNRC6 Trp-binding

Figure 2.4. miR-122-mediated translational promotion is the dominant role during the maintenance phase of viral replication. *Continued from previous page…* set-up for translational promotion assays. HCV U4C S2:p3 is riboswitched *a priori*, and endogenous wild-type miR-122 can bind to site 1, thereby fulfilling the stabilization effect. Addition of miR-122 p3U allows binding to site 2 and measurement of the translational enhancement effect in isolation. **(B-E)** Full-length RLuc HCV U4C S2:p3 RNAs were co-electroporated into Huh-7.5 cells with miR-122 p3U or ctrl miRNA, as well as a capped FLuc reporter RNA. RLuc activities for **(B-C)** GNN or **(D-E)** WT U4C S2:p3 viral RNAs were monitored over time as described in *Figure 2.1*. In **(C)** and **(E),** RLuc activities for GNN or WT U4C S2:p3 HCV RNAs at 6 h or 72 h, respectively, normalized to FLuc (transfection efficiency) control at 2 h, were used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. RLuc is displayed as the mean of three biological replicates, and error bars represent the SD of the mean. Statistical significance was determined by multiple Student's *t* test, ***$p \leq 0.0001$; **$p \leq 0.001$; *$p \leq 0.01$; ns, not significant ($p \geq 0.01$).

pockets (termed 1, 2 and 3) present on Ago2, we generated all single and double mutants and tested them in the same tethering assay. We did not include a triple Trp-binding pocket mutant, as this mutant is unstable (Ian MacRae, personal communication) (32). In all cases, we did not observe translational promotion by any of the Ago2 Trp-binding pocket mutants, suggesting that even when TNRC6 recruitment is impaired, Ago2 is not able to promote cap-independent translation when directly tethered to the 5′ end of a target mRNA (**Supplementary Figure S2.6C**). Thus, Ago-mediated translational promotion is context-dependent.

The SLII^{alt} conformation is maintained as it promotes efficient virion assembly.

In our efforts to understand the contribution of miR-122's riboswitch, genome stabilization, and translational promotion activities, we were somewhat surprised by the negligible contribution of the riboswitch effect. While it is clear that the viral RNA can spontaneously riboswitch even in the absence of miR-122, we questioned why HCV would retain the need to riboswitch at all (i.e. why conserve the ability to form SLII^{alt} over SLII). To test this, we made use of point mutations in the 5′ UTR that stabilize the SLII conformation, specifically G20A, U4C, and G28A (**Figure 2.5A and Supplementary Figure S2.7**) (3, 19). The U4C and G20A mutations change the U-G wobble base pair at the base of SLI into a C-G (U4C) or U-A (G20A) Watson-Crick base pair, which results in similar energetic stabilities (ΔG) for the SLII^{alt} and SLII conformations. Meanwhile, the G28A mutation destabilizes SLII^{alt}, rendering the SLII conformation comparatively more stable (2, 3, 19, 20). Since we did not observe a significant effect of the riboswitch activity in the establishment phase of the viral life cycle, and because the large luciferase reporter insertion impairs virion production, we decided to test these mutants in the context of the JFH-1$_T$ HCVcc system (33-36). Thus, we introduced each of the point mutations

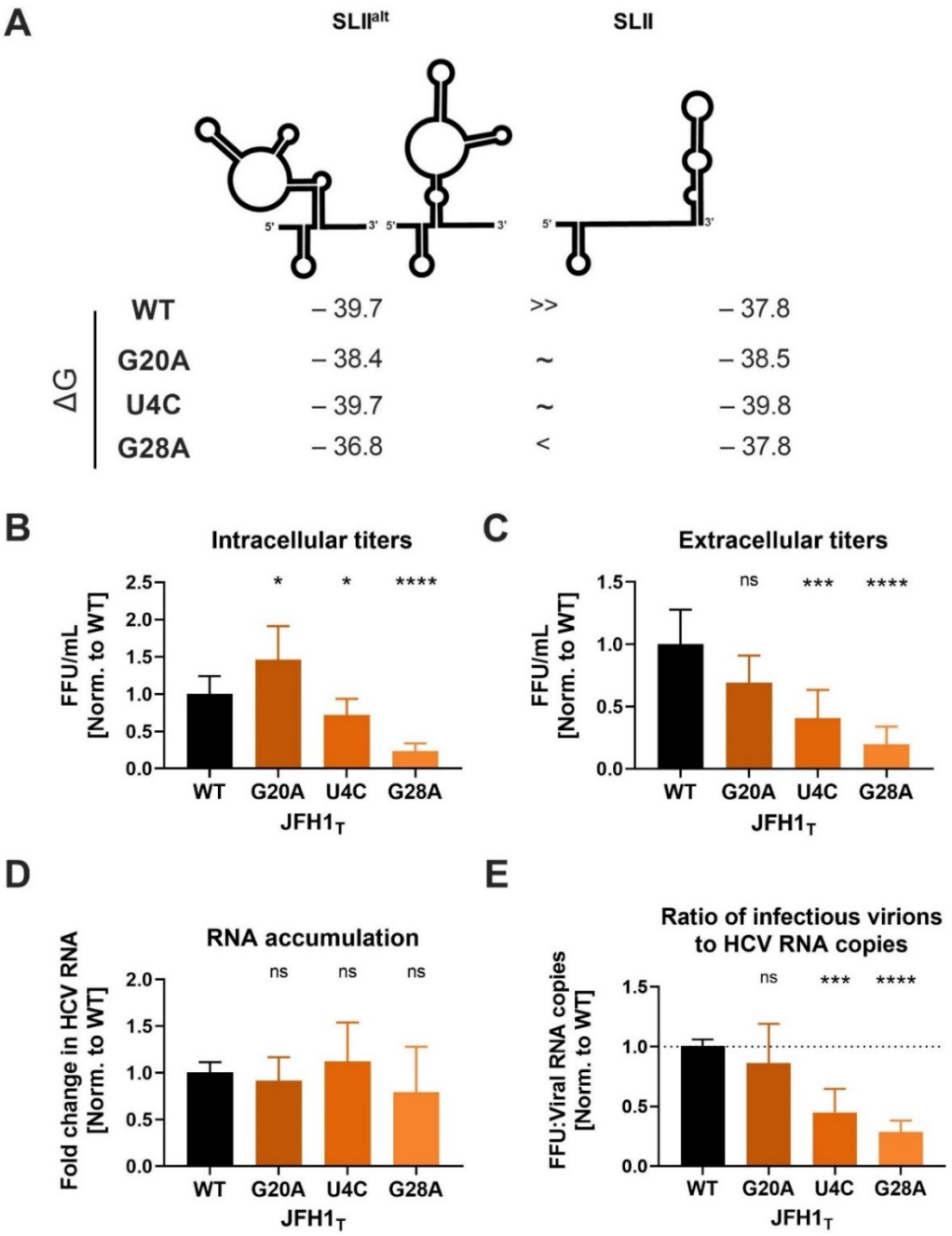

Figure 2.5. Successive stabilization of the SLII conformation leads to a reduction in viral particle production. A) RNA conformations of the first 117 nucleotides of the HCV 5′ UTR, showing the Gibb's free energies (ΔG) of SLII^{alt} and SLII for WT, G20A, U4C and G28A RNAs. Three days post-electroporation of JFH-1$_T$ RNA with the indicated mutations into Huh-7.5 cells, **(B)** intracellular viral titers and **(C)** extracellular viral titers were assessed by focus-forming unit (FFU) assay, and **(D)** viral RNA accumulation was assessed by RT-qPCR. **E)** The proportion of packaged RNAs was ascertained by calculating the ratio of total FFUs **(A-B)** to viral genome copies **(C)**. The relative titers, RNA levels, and percentages of packaged virions **(A-D)** were all normalized to the WT condition. Error bars represent the mean of at least three independent replicate and error bars represent the standard deviation of the mean. Statistical significance was determined by multiple Student's *t* test, ****$p < 0.0001$, ***$p < 0.001$; ** < 0.01; ns, not significant ($p \geq 0.01$).

into the JFH-1_T genome, and electroporated these viral RNAs into Huh-7.5 cells (which are replete with endogenous miR-122), then monitored viral RNA accumulation, as well as intracellular and extracellular viral titers (**Figure 2.5B-E**). Fascinatingly, despite similar amounts of viral RNA accumulation, we noticed an inverse correlation between the relative stability of SLII and intracellular and extracellular viral titers at day 3 post-electroporation. Notably, the U4C and G28A viral RNAs consistently packaged a smaller proportion of viral genomes than the WT or G20A RNAs (**Figure 2.5E**). This suggests that the $SLII^{alt}$ conformation is likely retained as it is required for efficient packaging of the genomic RNA into virions.

2.5 Discussion

Herein, we investigated the overall contribution of each of the three roles attributed to miR-122, (namely riboswitch activity, genome stabilization, and translational promotion) in the HCV life cycle. In general, we found that the relative contributions of the three roles differed during the establishment and maintenance phases of the viral life cycle (**Figure 2.6**). Firstly, we used replication-defective (GNN) viral RNAs to estimate the contribution of each of the three roles to miR-122's activity in the establishment phase of the HCV life cycle (i.e. prior to the initiation of replication). While we observed that the riboswitch effect played a negligible role in the establishment phase of the viral life cycle, we know that the genome must be "riboswitched" for translation, as the SLII conformation forms part of the HCV IRES (SLII-IV). Thus, while it is likely that WT HCV can spontaneously switch conformations due to the similar thermodynamic stabilities between the $SLII^{alt}$ and SLII conformations (**Supplementary Figure S2.7**), it is reasonable to assume that the effect of stabilizing the SLII conformation is below the limit of detection in our assay. As such, in our summary, we have attributed <5% of miR-122's overall

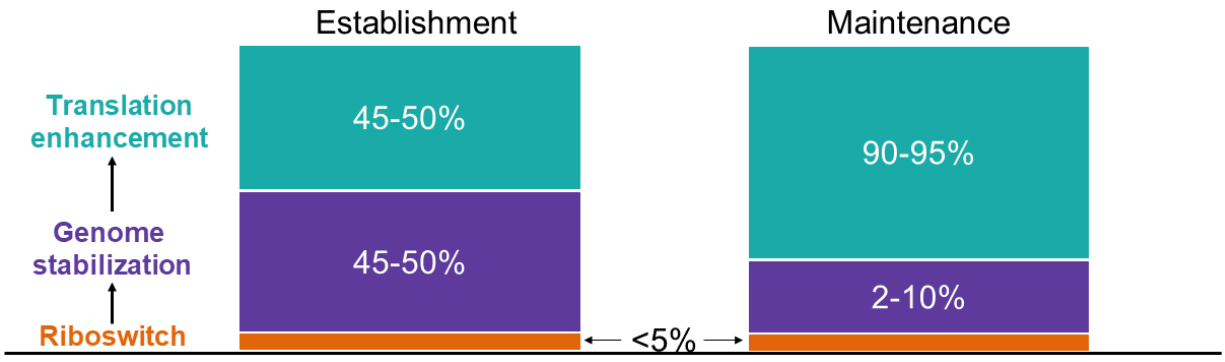

Figure 2.6. Estimated contribution of riboswitch, genome stabilization and translational promotion to the overall impact of miR-122 on the HCV life cycle. The relative contributions of each role are estimated based on the results of the luciferase assays where each role was isolated, compared to the overall effect of miR-122 on viral RNA accumulation (*Figures 2.1-2.4*). During the establishment phase (before significant genome replication takes place), the riboswitch effect has a minimal contribution, which is estimated to represent less than 5% of the overall effect, while genome stabilization (45-50%) and translational promotion (45-50%) have similar contributions. In contrast, during the maintenance phase of the infection (after several rounds of replication), stability becomes less important (2-10%) while translational promotion is the predominant effect, accounting for ~90-95% of miR-122's overall effect.

activity in the establishment phase of the viral life cycle to the riboswitch effect (**Figure 2.6**). Additionally, we found that during the establishment phase, the contributions of the genome stability and translational promotion effects were similar in magnitude, each accounting for ~45-50% of the overall effect of miR-122. Notably, at the time point (6 h) used for quantification, we observed that both the replication-defective (GNN) and WT luciferase values were similar in magnitude and fold-change suggesting that no significant viral replication has occurred at this time point (**Figures 2.1-2.4**).

In contrast, using replication competent (WT) viral RNAs, we were able to estimate the contribution of each of the three roles to miR-122's activity in the maintenance phase of the HCV life cycle (i.e. after several rounds of viral replication). Given that the riboswitch is needed for viral translation, we again attributed <5% of miR-122's activity in the maintenance phase to the riboswitch effect (**Figure 2.6**). However, in contrast to the establishment phase, where the genome stability and translational promotion effects had similar magnitudes, we found that translational promotion was the predominant role accounting for ~90-95% of miR-122's activity during active viral replication (**Figure 2.6**). These results align with the current understanding of the HCV life cycle, given that in the early phases of infection, prior to the establishment of viral replication, each HCV RNA molecule must be stable long enough and produce sufficient levels of viral proteins to establish a replication organelle. However, once a replication organelle is established, the negative-strand replicative intermediate can produce many positive-strand progeny RNA molecules. As such, viral RNA stability becomes less important, while sufficient levels of viral proteins to establish subsequent replication organelles becomes a more limiting factor. This is consistent with previous observations that suggest that miR-122-independent replication is possible, albeit highly inefficient, when HCV nonstructural protein translation is

driven by the more efficient encephalomyocarditis virus (EMCV) IRES (22). Our findings are also in agreement with a recent study that similarly explored the contributions of the riboswitch, genome stability, and translational promotion activities in the HCV life cycle (37).

Interestingly, while the negligible contribution of the riboswitch effect can be explained by the similar thermodynamic stabilities of the SLII^{alt} and SLII conformations in the 5′ UTR of the HCV genome, our experiments using the HCVcc system revealed that the SLII^{alt} conformation is important for efficient virion assembly (**Figure 2.7**). This conclusion is based on our observation that mutations that stabilized SLII over SLII^{alt} reduced viral packaging efficiency in cell culture. More specifically, without significantly altering intracellular viral RNA accumulation, we found that the U4C and G28A mutations significantly reduced both intracellular and extracellular viral titers. The latter is consistent with a prior study, which reported that a G28A virus produced significantly lower infectious viral progeny than WT HCVcc, even in miR-122-replete conditions (38). Herein, we also found that U4C, which stabilizes SLII without destabilizing SLII^{alt}, reduced virion production, but to a lesser extent than G28A (**Figure 2.5**). Interestingly, the G20A mutation did not have any detectable impact on virion production, even though its secondary structure closely matches that of U4C (**Figure 2.5 and Supplementary Figure S2.2**). Notably, both U4C and G20A stabilize the SLII conformation by replacing a G-U wobble with a canonical Watson-Crick base pair at the base of SLI: G20A generates an U-A pair, which has a similar stability to the G-U wobble, while U4C creates a C-G pair, which is thermodynamically more stable (39). Thus, it may be that although U4C or G20A are similarly likely to form the SLII^{alt} or SLII conformations, the structure of the G20A RNA provides a similar thermodynamic stability to WT and may simply be more dynamic. Nonetheless, our results suggest a model whereby the SLII^{alt} conformation promotes

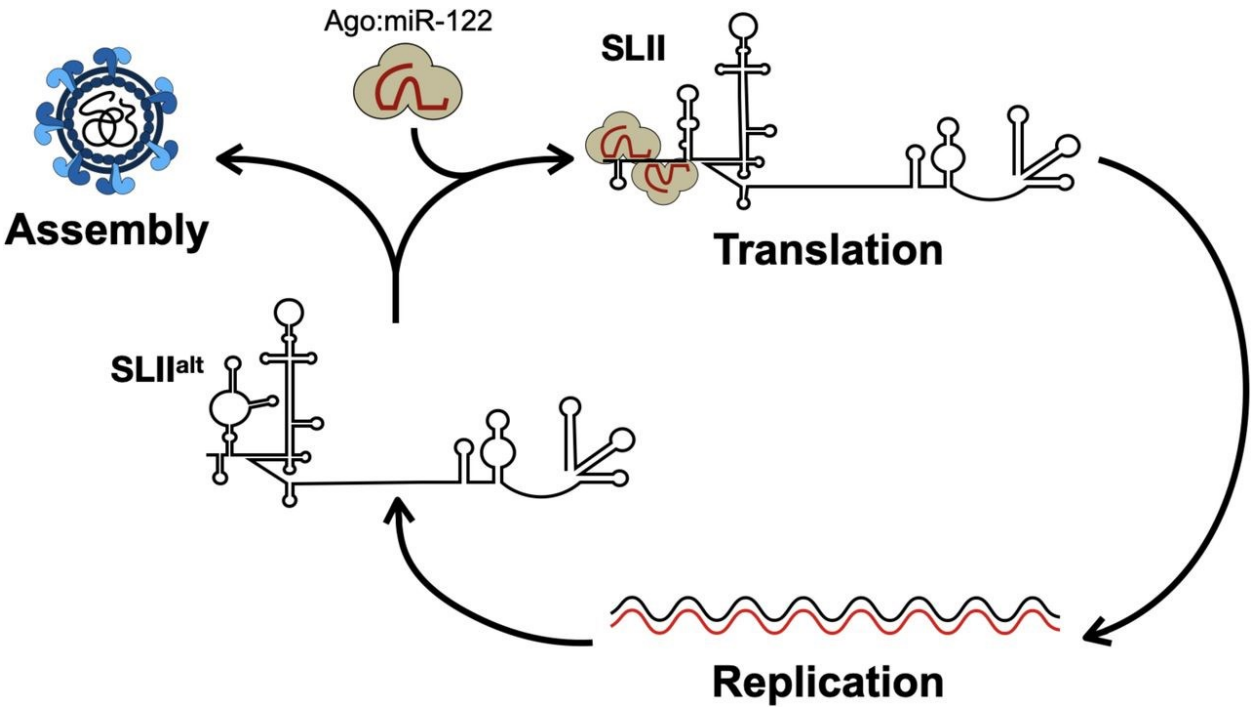

Figure 2.7. Model for the functional role of SLII^{alt} in the HCV life cycle. Newly synthesized viral genomes are at a junction point in the HCV life cycle: they can either engage in virion assembly and be packaged into new virions that exit the cell, or they can engage in a new cycle of translation and RNA replication. Our model suggests that newly synthesized RNAs that take on the most energetically stable conformation, SLII^{alt}, can engage in packaging. However, if they spontaneously or through binding to Ago:miR-122 convert to the functional SLII conformation, they form the viral IRES and recruit ribosomes, committing to translation.

virion assembly, while the SLII conformation promotes viral translation (**Figure 2.7**). Interestingly, our results may help explain the ability of the JFH-1 strain to produce infectious particles in cell culture (**Figure 2.8**). The JFH-1 strain was the first infectious HCV isolate to recapitulate the entire HCV life cycle in cell culture without adaptive mutations, including the production of infectious viral particles (40-43). Subsequently, chimeric viruses that combine the JFH-1 (genotype 2a) genome with core-NS2 or 5′ UTR-NS2 sequences derived from genotypes 1-6 were developed and shown to produce infectious viral particles in cell culture (reviewed in (44)). While the ability to produce infectious virions is certainly due at least in part to contributions from the JFH-1 nonstructural genes, our findings herein suggest that there may also be a contribution from the G residue at position 28 (G28) in the 5′ UTR. Notably, ~80% of all HCV isolates contain an A at this position (A28) (45). In JFH-1, the presence of G28 provides thermodynamic stability to $SLII^{alt}$, and as we demonstrate herein, helps to prioritize virion assembly (**Figure 2.8**) (46-48). In contrast, in other HCV genotypes, the A28 residue stabilizes the SLII conformation, which promotes viral translation to the detriment of virion assembly (**Figure 2.8**). Moreover, the A28 residue also increases the affinity of the viral RNA for Ago:miR-122 at both miR-122 binding sites, which likely further reinforces a commitment to translation (19, 45, 49, 50). Taken together, this suggests that while JFH-1 can prioritize virion assembly over translation, the other genotypes end up lost in translation.

Finally, it is interesting to consider whether our findings may also help explain HCV infection outcomes. Notably, JFH-1 was isolated from a patient with fulminant (acute) hepatitis, and several case reports have suggested an association between genotype 2 and fulminant hepatitis or severe recurrence post-transplant (41, 51, 52). However, this association may be related to higher incidence of genotype 2 in these patient populations, as fulminant hepatitis

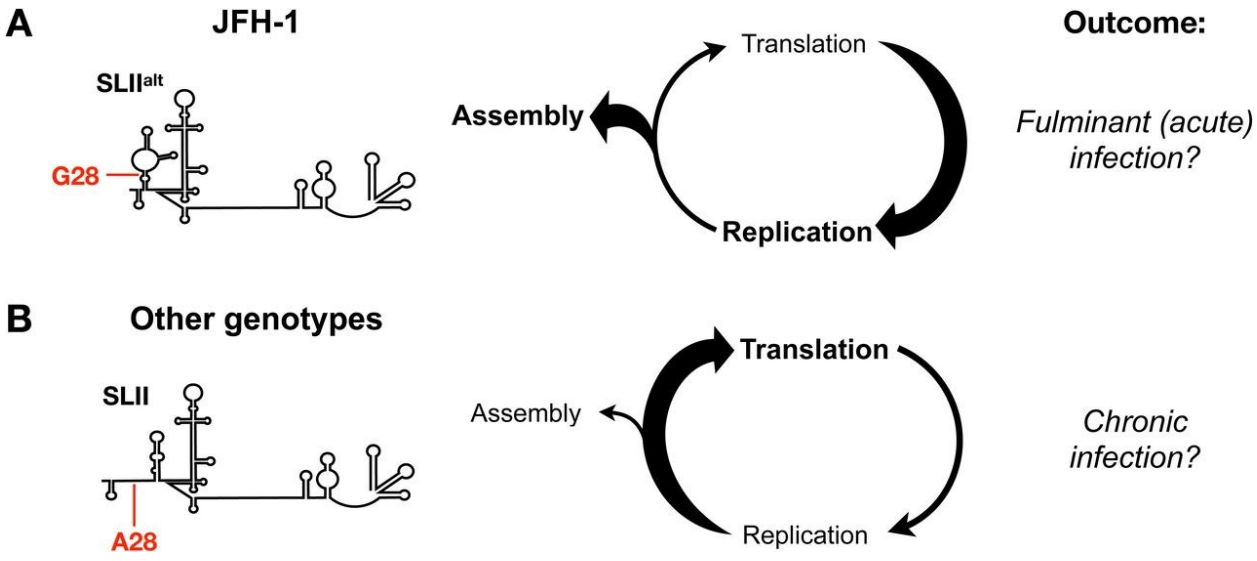

Figure 2.8. The formation of SLII^{alt} may explain why JFH-1 is able to produce virions in cell culture and may have implications for the outcome of HCV infections. (A) Previous studies indicate that JFH-1 nonstructural proteins provide it with a replicative advantage over other HCV isolates. Additionally, the G28 residue in the 5′ UTR allows JFH-1 (and likely other genotype 2 isolates) to energetically favor the SLII^{alt} conformation, which we demonstrate promotes virion assembly. This prioritization of virion assembly over translation may contribute to JFH-1 pathogenesis, considering it was isolated from a patient with fulminant (acute) hepatitis and genotype 2 has been associated with increased incidence of fulminant hepatitis. **(B)** In contrast, other HCV genotypes (~80% of all HCV isolates) have an A at position 28 (A28), which energetically favors the SLII conformation rather than SLII^{alt}. As a result, these other genotypes favor translation over virion assembly. Moreover, A28 increases the affinity of the 5′ UTR for Ago:miR-122 complexes, further reinforcing translation. This bias may allow the virus to better evade intracellular and systemic immune responses, thereby resulting in chronic HCV infection.

cases have been reported with other genotypes (53-55). Moreover, despite its worldwide distribution, genotype 2 (G28) only accounts for an estimated 9.1% of HCV cases globally, and is highly responsive to interferon-free direct-acting antiviral regimens (55-57). It is unclear if the lower prevalence of genotype 2 is related to a higher rate of spontaneous viral clearance or to other factors as genotype 2 has been sporadically reported to be less likely to progress to chronic infection — with estimates between 22-33% for genotype 2 versus 57-92% for genotype 1 (57, 58). However, spontaneous clearance rates between genotypes are unreliable, as they largely rely on the assessment of outbreak studies of single genotypes in distinct patient populations and are thus subject to cohort bias (56-58). Nonetheless, it is possible that more efficient replication and infectious particle production generates a more robust intracellular and systemic immune response that can effectively control HCV infection at the acute stage. In contrast, prioritizing translation to the detriment of virion assembly may allow HCV to better evade intracellular antiviral responses and avoid induction of a systemic immune response, thereby favoring the establishment of a chronic infection. Consistent with this idea, treatment-naïve chronic genotype 2 patients have been demonstrated to have greater HCV-specific T cell responses at baseline, which could be reflective of early viral infection kinetics (59). However, more research is needed to provide further insight into whether G28 status influences disease pathogenesis.

In conclusion, our results help clarify the overall contributions of the three roles attributed to miR-122 in the HCV life cycle, namely the riboswitch, genome stability and translational promotion activities. During the establishment phase of the viral life cycle, miR-122's stabilization and translational promotion activities each contribute to a similar extent to the overall impact on viral translation and RNA accumulation, suggesting that a balance between these two functions is important for optimal establishment of an infection. In the maintenance

phase, once replication complexes have been established, the contribution of the stabilization effect represents a minor fraction of the overall impact, and the translation promotion effect becomes the dominant role. Moreover, we found that while the riboswitch effect had a negligible impact on viral translation and RNA accumulation, the ability of the viral genomic RNA to form the SLII^{alt} conformation was important for efficient virion assembly. Our data suggest that the ability to form SLII^{alt} may partially explain infectious particle production in HCVcc models and may offer insight into the outcome of HCV infection. Overall, our results provide insight into the importance of miR-122 as well as SLII^{alt} in modulating the balance of viral RNAs in the translating/replicating pool and those engaged in virion assembly.

2.6 Materials and Methods

Cell culture

Wild-type (WT) and miR-122 knockout (KO) Huh-7.5 human hepatoma cells were provided by Drs. Charlie Rice and Matthew Evans, respectively (38, 60). All cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1X MEM nonessential amino acids, and 2 mM L-glutamine. All cells were maintained at 37°C/5% CO_2 and were routinely screened for mycoplasma contamination.

Viral RNAs

The pJ6/JFH1 full-length (FL) *Renilla* luciferase (RLuc) WT and GNN plasmids bear full-length viral sequences derived from the J6 (structural genes) and Japanese Fulminant Hepatitis-1 (JFH-1, nonstructural genes) genotype 2 isolates of HCV, with a RLuc reporter inserted between the p7 and NS2-coding regions (61). The pJ6/JFH1 FL RLuc GNN plasmid also bears inactivating mutations (GDD → GNN) within the NS5B RNA polymerase active site (61). The S1:p3

(C26A), S2:p3 (C41A) and additional point mutations (U4C, G20A, and G28A) were generated by overlapping PCR and were subcloned into the J6/JFH-1 plasmid using the *EcoRI* and *KpnI* restriction sites (19). The pJFH-1_T plasmid encodes a cell culture-adapted JFH-1 with three adaptive mutations that increase viral titers in cell culture (36). The JFH-1_T U4C, G20A, and G28A mutations were similarly generated by overlapping PCR and were subcloned into the JFH-1_T plasmid using *EcoRI* and *AgeI* restriction sites.

MicroRNAs

miR-122: 5′ – UGG AGU GUG ACA AUG GUG UUU GU – 3′, miR-122*: 5′ – AAA CGC CAU UAU CAC ACU AAA UA – 3′, miR-122p3U: 5′ – UGU AGU GUG ACA AUG GUG UUU GU – 3′, ctrl miRNA: 5′ – UAA UCA CAG ACA AUG GUG UUU GU – 3′, and ctrl miRNA*: 5′ – AAA CGC CAU UAU CUG UGA GGA UA – 3′ were all synthesized by Integrated DNA Technologies (7). MicroRNA duplexes were diluted to a final concentration of 20 µM in RNA annealing buffer (150 mM HEPES pH 7.4, 500 mM potassium acetate, 10 mM magnesium acetate). After a denaturation step of 1 min at 95 °C, miRNAs were annealed at 37 °C for 1 h, and stored at -20 or -80 °C until use. The same passenger strand (miR-122*) was annealed with both miR-122 and miR-122 p3U guide strands.

In vitro transcription

To make full-length viral RNAs, all templates were linearized with *XbaI* and *in vitro* transcribed using T7 RNA polymerase (NEB). Briefly, 1 µg of linearized template DNA was incubated at 30°C for 1 h 10 min with 1 mM each ATP, UTP and CTP, 1.2 mM GTP, 0.8U/µL RiboLock RNase inhibitor (ThermoFisher Scientific) and 200 U T7 RNA polymerase in a final volume of

50 µL, followed by a 20 min DNase I (NEB) digestion at 37°C. Capped Firefly luciferase (FLuc) mRNAs were generated from the pT7Luc plasmid (Promega) linearized with *XmnI* and were *in vitro* transcribed using the mMESSAGE mMACHINE T7 Transcription Kit (Invitrogen) according to the manufacturer's instructions. *In vitro* transcribed RNAs were precipitated in 0.1 volume of 3M NaOAc pH 5.2 and 2.5 volumes 95-100% ethanol with 1 µL GlycoBlue co-precipitant and stored at -80°C until use.

Electroporations

Electroporations were carried out as previously described (1, 62). Briefly, 4×10^6 cells resuspended in 400 µL cold phosphate-buffered saline (PBS; Wisent) were mixed with 10 µg WT or GNN J6/JFH-1 FL RLuc RNAs, 1 µg FLuc capped mRNA, and in some cases 60 pmol duplexed miR-122 molecules (WT, ctrl or p3U) and electroporated using 4 mm cuvettes at 270 V, 950 µF and infinite resistance, optimized for the Bio-Rad Gene Pulser XCell (Bio-Rad). Electroporated cells were resuspended in 7.5 mL media and 1 mL per time point were plated in 12-well plates for luciferase assays. For replication competent (WT) assays, only 0.5 mL was plated for the 24 –72 h time points.

For simultaneous luciferase assays with RT-qPCR, 8×10^6 cells were used in electroporations and 3 cuvettes were pooled in 7 mL media. Subsequently, 200 µL per time point was plated in 12-well plates for luciferase assays (100 µL for 24 – 72 h time point in replication-competent assays), and 1 mL (2 – 8 h) or 0.5 mL (24 – 72 h) per time point were plated in 6-well plates for RT-qPCR analysis.

Luciferase assays

For luciferase assays, cells were washed in PBS and harvested in 200 µL (400 µL for 48 – 72 h time points in replication-competent assays) of 1X passive lysis buffer (Promega). Luciferase data was analyzed using the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions, with the modification that 25 µL of reagent was used per 10 µL sample. Luciferase assays were performed with a 20/20 luminometer with an integration time of 10 seconds and each sample was read in duplicate.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analyses

For strand-specific RT-qPCR analysis, electroporated Huh-7.5 or miR-122 KO cells plated in 6-well plates were lysed in 500 µLTRIzol reagent (ThermoFisher Scientific) and total RNA was extracted according to the manufacturer's instructions. Reverse transcription was performed using the HCV-specific primer: Tag4-NS5B-REV: 5′ – GAA GCT GAC TTG ACA TGT TGC CGC GTC AAG CCC GTG TAA CC – 3′ as well as the human GAPDH-specific primer: hsGAPDH RT: 5′ – GCT CCT GGA AGA TGG TGA TGG GAT TTC C – 3′ (63). Briefly, 500 ng total RNA was incubated with 100 nM primers and dNTP mix (50 µM each) at 95 °C for 5 min to denature the sample. The mixture was then cooled to 55 °C prior to addition of reverse transcriptase buffer, 40 U RiboLock (ThermoFisher Scientific) and 100 U of Maxima H Minus reverse transcriptase. The reaction was incubated at 55°C for 30 min for cDNA synthesis, and heat inactivated at 85°C for 15 min. The cDNA was subsequently purified using the DNA clean and concentrator kit (Zymo) according to manufacturer's instructions, with the inclusion of the optional RNA hydrolysis step, and samples were eluted in 10 µL of water. Subsequently, 3 µL was used for qPCR using the iTaq Universal Probes Supermix (Bio-Rad) according to

manufacturer's instructions using 400 nM each Tag4 (5′ – GAA GCT GAC TTG ACA TGT TGC C – 3′) and NS5B-FOR (5′ – AGA CAC TCC CCT ATC AAT TCA TGG C – 3′) and 200 nM of the NS5B-FAM probe (5′ – ATG GGT TCG CAT GGT CCT AAT GAC ACA C – 3′). GAPDH was simultaneously detected using 150 nM each hsGAPDH-FOR (5′ – GGA AGG TGA AGG TCG GAG TCA ACG G – 3′), hsGAPDH-REV (5′ – GCT CCT GGA AGA TGG TGA TGG GAT TTC C – 3′) and GAPDH-HEX probe (5′ – AGC TTC CCG TTC TCA GCC TTG AC – 3′).

For analysis of JFH-1_T WT and RAV accumulation, the iTaq Universal Probes One-Step kit (Bio-Rad) was used to perform duplex assays probing for the HCV genome with primers NS5B-FOR (above) and NS5B-REV (5′ – GCG TCA AGC CCG TGT AAC C – 3′) and NS5B-FAM probe (5′ – ATG GGT TCG CAT GGT CCT AAT GAC ACA C – 3′) as well as a GAPDH loading control (PrimePCR Probe assay with HEX probe, Bio-Rad) (33). Each 20 µL reaction contained 500 ng of total RNA, 1.5 µL of the HCV primers and probe, and 0.5 µL of the GAPDH primers and probe. All RT-qPCR reactions were conducted in a CFX96 Touch Deep Well Real-Time PCR system (Bio-Rad). Genome copies were calculated against a genomic RNA standard curve, and fold-differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (64).

RNA structure prediction

To predict the secondary structures and Gibb's free energy (ΔG) of the first 117 nucleotides of the JFH-1 sequence, the RNAstructure 6.4 secondary structure prediction software was accessed from the Matthews lab server https://rna.urmc.rochester.edu/RNAstructureWeb/ (65). To avoid spurious interactions that artificially decreased the calculated ΔG, the first three nucleotides of

the sequence were constrained to be single-stranded. The results were saved as dot bracket files and used to generate the predicted structures with the RNA2Drawer browser app https://rna2drawer.app/ (66).

Focus-forming unit (FFU) assays

One day prior to infection, 8-well chamber slides (Lab-Tek) were seeded with 4×10^5 Huh-7.5 cells/well. Infections were performed with 10-fold serial dilutions of viral samples in 100 µL for 4 h, after which the supernatant was replaced with fresh media. Three days post-infection, slides were fixed in 100% acetone and stained with anti-HCV core antibody (1:100, clone B2, Anogen), and subsequently with the AlexaFluor-488-conjugated anti-mouse antibody (1:200, ThermoFisher Scientific) for immunofluorescence analysis. Viral titers are expressed as the number of focus-forming units (FFU) per mL. Extracellular virus titers were determined directly from cell supernatants.

Data analysis and availability

Statistical analyses were performed using GraphPad Prism v9 (GraphPad, USA). Multiple unpaired Student's *t* test was performed between the two relevant data sets for each time point, with the assumptions of Gaussian distribution and same standard deviation for samples at the same time point.

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2.9 Author contributions

M.R. and S.M.S. designed the study; M.R., S.E.C., D.R.F. and Q.H.A. performed the experiments and analyzed the data, and M.R. and S.M.S. wrote and edited the manuscript.

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2.11 Supplementary Materials

Supplementary Methods

Cell culture

HEK 293T human embryonic kidney cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1X MEM nonessential amino acids, and 2 mM L-glutamine. Cells were maintained at 37°C/5% CO_2 and were routinely screened for mycoplasma contamination.

Plasmids

The plasmid pMSCV-LN-eIF4G encoding a FLAG-tagged Bacteriophage Lambda N peptide (λN) fused eIF4G protein as well as the plasmids containing the 6X BoxB and 6X scrambled (scr) sequences were provided by Dr. Jerry Pelletier (McGill University). The pcDNA3.1+Lambda-N-HA-Peptide (Addgene plasmid # 92005; http://n2t.net/addgene:92005 ; RRID:Addgene_92005) and pcDNA3.1+_FH-AGO2-WT (Addgene plasmid #92006 ; http://n2t.net/addgene:92006 ; RRID:Addgene_92006) were gifts from Dr. Joshua T. Mendell (67). The plasmid pCDNA3.1+LN-FLAG-HA-LacZ encodes a FLAG- and HA-tagged λN fused LacZ. The plasmid pcDNA3.1+LN-FLAG-HA-Ago2 encoding a FLAG-tagged λN fused human Ago2, was generated using the In-Fusion cloning kit (Takara Bio) by PCR amplification of the Ago2 gene from the pcDNA3.1+_FH-AGO2-WT plasmid and insertion into the *XhoI* digested pcDNA3.1+Lambda-N-HA-Peptide plasmid by Gibson assembly according to the manufacturer's instructions. The Trp-binding pocket mutations (K660S, P590G and R688S) were introduced into the pcDNA3.1+LN-FLAG-HA-Ago2 by site-directed mutagenesis using

the QuikChange XL II kit (Agilent) according to manufacturer's instructions. All plasmid sequences were verified by Sanger sequencing (Génome Québec).

In vitro transcription

To make A capped 6X BoxB and 6X scrambled (scr) mRNAs, the plasmids were linearized with *BamHI* and *in vitro* transcribed using T7 RNA polymerase (NEB). Briefly, 1 µg of template DNA was incubated at 37 °C for 1 h with 500 µM each G(5′)ppp(5′)A cap analog (NEB), ATP, CTP and UTP, 100 µM GTP, 0.2 U/µL RiboLock RNase inhibitor and 250 U T7 RNA polymerase in a final volume of 100 µL, followed by a 20 min DNase I (NEB) digestion at 37°C. Capped RLuc mRNAs were generated from the pRL-TK plasmid (Promega) linearized with *BglII* and were *in vitro* transcribed using the mMESSAGE mMACHINE T7 Transcription Kit (Invitrogen) according to the manufacturer's instructions. *In vitro* transcribed RNAs were precipitated in 0.1 volume of 3M NaOAc pH 5.2 and 2.5 volumes 95-100% ethanol with 1 µL GlycoBlue co-precipitant and stored at -80°C until use.

Tethering assays

For hAgo2 tethering assays, 1×10^6 HEK 293T cells were seeded in each well of three 6-well plates approximately 16 h prior to transfection. Subsequently, 3 µg of pMSCV-LN-eIF4G or pcDNA3.1+LN-FLAG-HA-Ago2 (WT as well as single and double Trp-binding pocket mutants), or 1 µg pCDNA3.1+LN-FLAG-HA-LacZ plasmid were transfected using 2.7 µL Lipofectamine 2000 (ThermoFisher Scientific) per well in 1 mL of Opti-MEM reduced serum media (ThermoFisher Scientific) in duplicate. Transfection complexes were replaced with complete media at 3.5 to 4 h post-transfection. At 24 h post-transfection, the cells were split into

12-well plates and 5-6 h later were transfected with 400 ng each A capped [A(5′)ppp(5′)G] 6X BoxB or 6X scr mRNAs and RLuc control mRNA using 2 µL DMRIE-C reagent (ThermoFisher Scientific) in 400 µL Opti-MEM per well. Transfection complexes were left on the cells overnight, and cells were harvested in 100 µL 1X PLB 16 h post-transfection.

Western blot analysis

Whole cell lysates were prepared in 1X passive lysis buffer (Promega) and stored at -80 °C until use. Lysates were cleared by centrifugation at 16,000 × g and supernatant protein concentration was assessed by Bradford assay using the Pierce Coomassie assay kit (ThermoFisher Scientific) according to manufacturer's instructions, with the modification that 1-2 µL of supernatant and 5 µL of standard was used with 200 µL of Coomassie protein assay reagent. Ten micrograms of protein was loaded on 10% SDS-PAGE gels and run at 80V for 20 min, followed by 100V. Samples were transferred onto Immobilon-P PVDF membranes (Millipore), blocked in 5% skim milk for 1 h and incubated overnight with the following primary antibodies diluted in 5% bovine serum albumin (BSA): mouse anti-FLAG M2-horse radish peroxidase (HRP) (F8592, Sigma, 1:20,000 or 1:200,000) rabbit anti-actin (A2066, Sigma, 1:20,000) When probing for actin, blots were washed in Tris buffered saline with Tween 20 (TBS-T) and incubated for 1 h with HRP-conjugated goat anti-rabbit (111-035-144, Jackson ImmunoResearch Laboratories, 1:50,000). After washing, blots were visualized using enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, Fisher Scientific).

In vitro selective 2´ hydroxyl acylation analyzed by primer extension (SHAPE) analysis

In vitro SHAPE analysis was performed as previously described (19). Briefly, 5 pmol of HCV 5´ UTR RNA was re-folded and incubated in SHAPE buffer (333 mM HEPES, pH 8.0; 20 mM $MgCl_2$; 333 mM NaCl) for 1 h at 37ºC. RNA was then exposed to 0.01 M NAI-N_3 or dimethyl sulfoxide (DMSO) (treatment control) for 5 min at 37ºC, and then extracted using TRIzol reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Extracted labelled RNA was precipitated and stored at -80ºC. Labelled RNA was used for SHAPE analysis by capillary electrophoresis as previously described (3).

Supplementary Figures

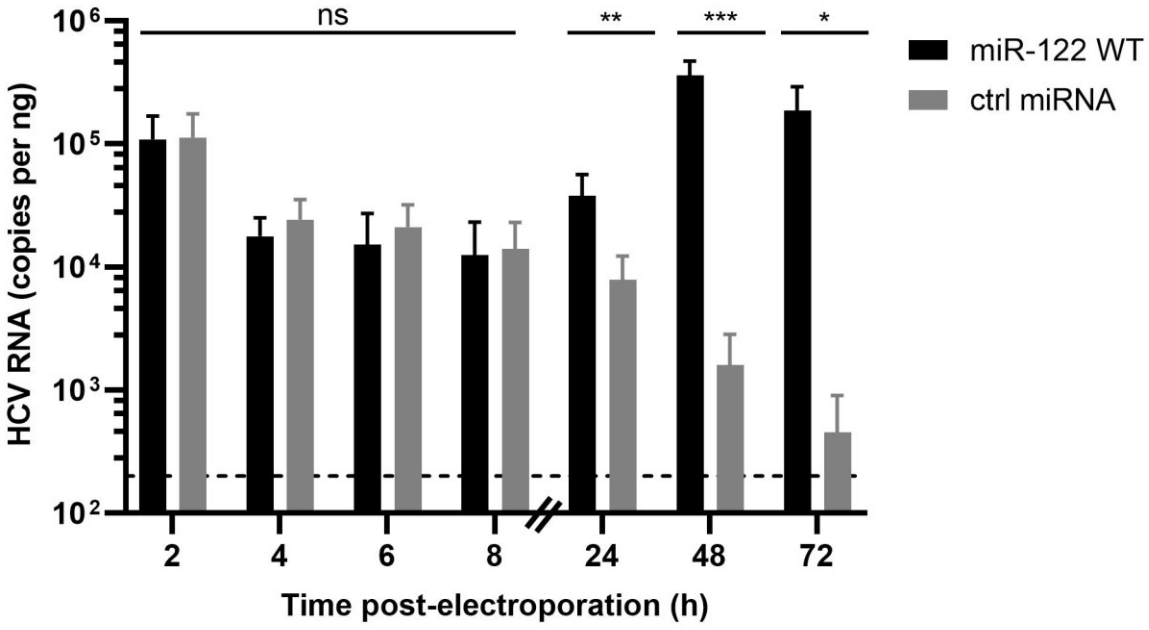

Figure S2.1. RT-qPCR analysis of viral RNA accumulation in miR-122 KO cells supplemented with miR-122 or control miRNA. WT HCV was co-electroporated into miR-122 KO cells with miR-122 or ctrl miRNA duplexes, and luciferase activity and viral RNA levels were monitored over time as described in *Figure 2.1*. HCV copy numbers were quantified by RT-qPCR and normalized to GAPDH. HCV RNA is displayed as the mean copy number per ng of total RNA, with error bars representing the SD of the mean. The lower limit of quantitation is indicated by a dashed line. Statistical significance was determined by multiple Student's *t* test, ***$p \leq 0.0001$; **$p \leq 0.001$; *$p \leq 0.01$; ns, not significant ($p \geq 0.01$).

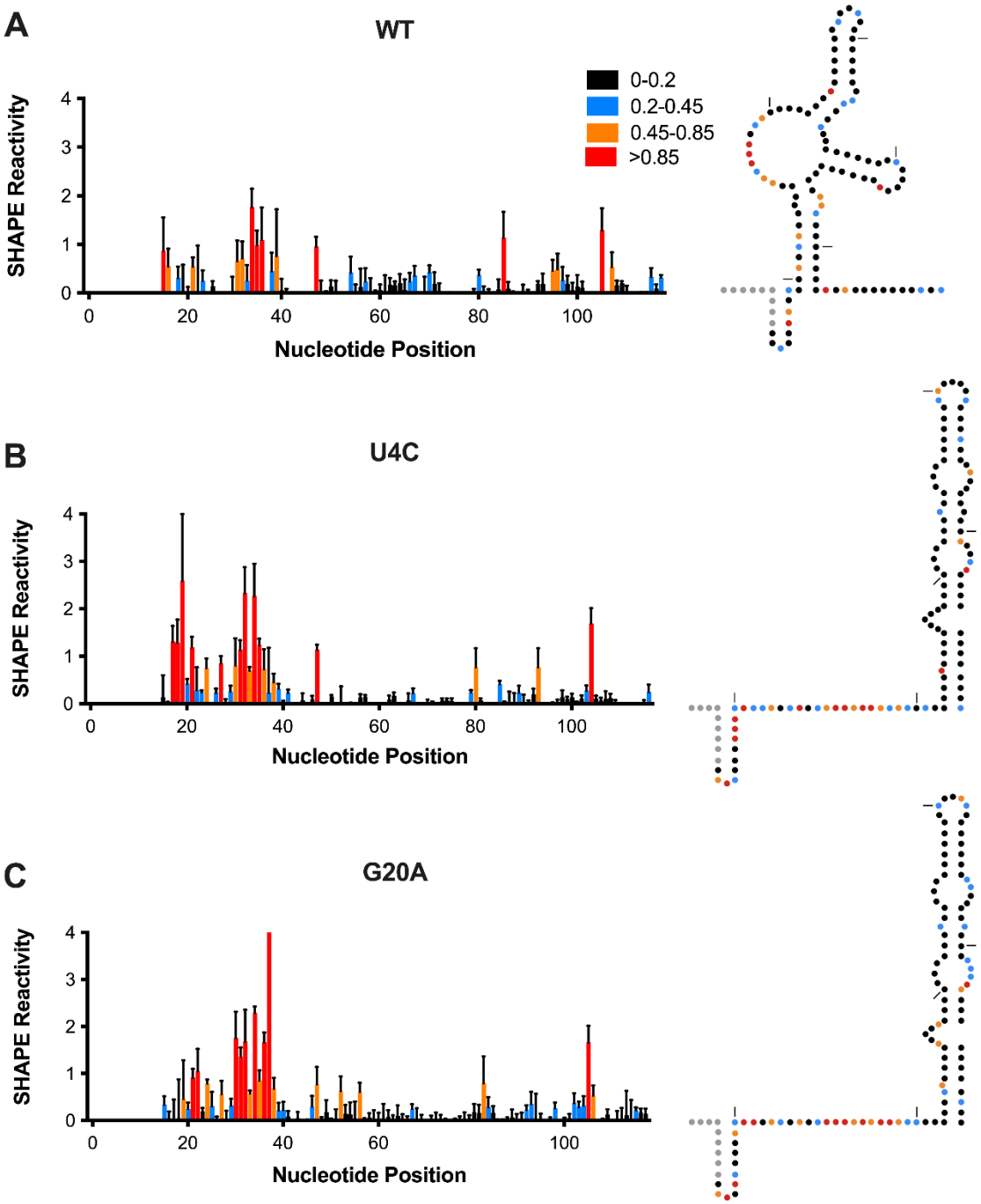

Figure S2.2. *In vitro* SHAPE analysis of the 5′ terminus of WT, U4C and G20A HCV RNAs. Normalized SHAPE reactivities of nucleotides 1-117 of **(A)** WT, **(B)** U4C, and **(C)** G20A HCV RNAs (*Left*). Nucleotides with high (≥ 0.85, red), intermediate (0.4 - 0.85, orange), low (0.2 - 0.4, blue) and very low (≤ 0.2) SHAPE reactivity are indicated. Nucleotides 1-9 were omitted due to high background reactivity. Data is shown as mean normalized SHAPE reactivity + standard error of the mean and is representative of four independent replicates. Prediction of the lowest free energy structure formed by the first 117 nt of the HCV genome for each variant constrained by SHAPE reactivity is depicted via dot plot (*Right)* with relative SHAPE reactivity superimposed. Tick marks represent 20 nucleotide intervals.

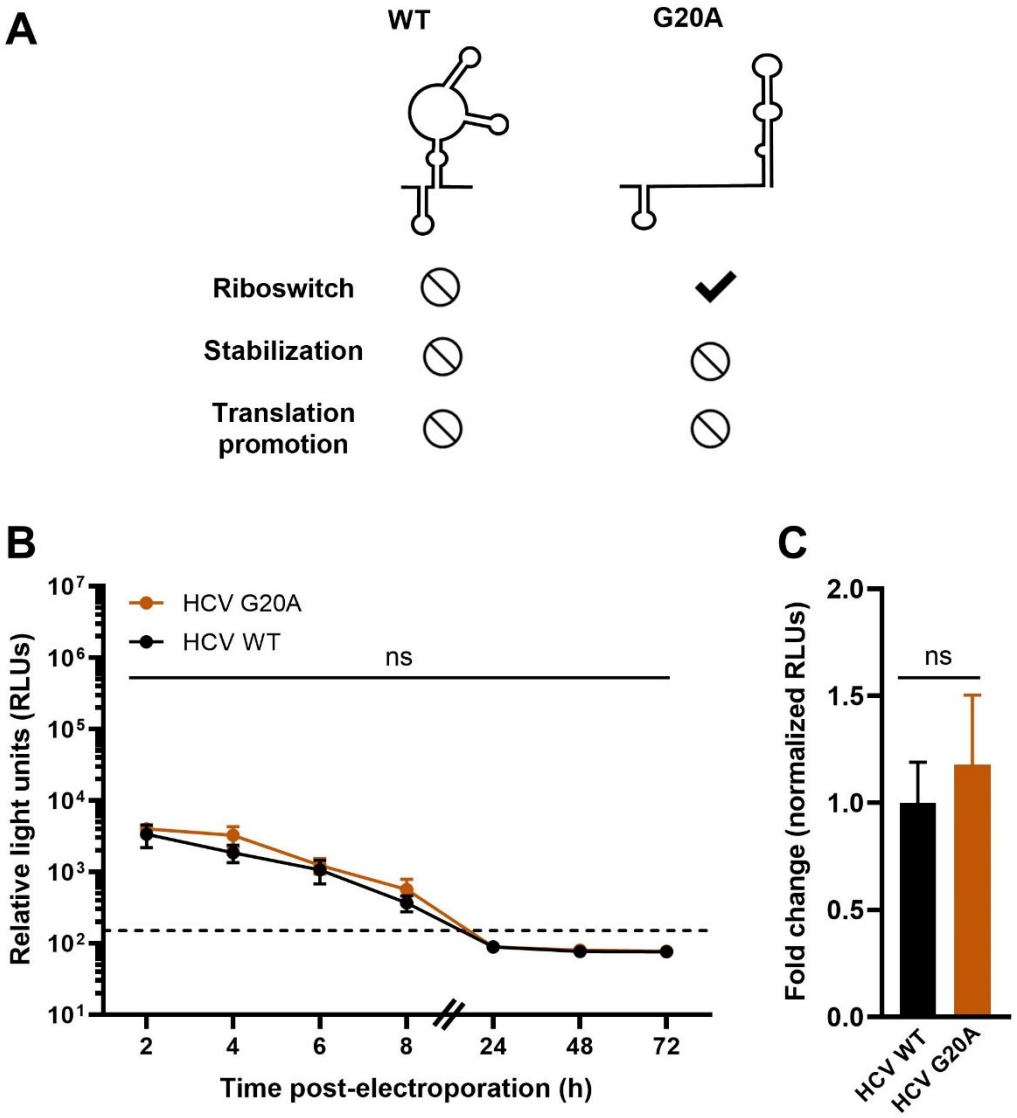

Figure S2.3. Alternative quantification of the riboswitch effect using the G20A mutant. (A) Schematic representation of the experimental set-up for riboswitch activity assay using G20A. In the absence of miR-122, WT HCV RNA favors the SLII^{alt} conformation, while the G20A mutant favors the riboswitched (SLII) conformation. In the absence of miR-122, the stabilization and translational enhancement roles of miR-122 are not fulfilled in either condition, allowing for the isolation of the riboswitch effect. **(B)** Full-length RLuc GNN HCV (WT or G20A) RNAs were co-electroporated into miR-122 KO cells with a capped FLuc reporter RNA. Luciferase activity was monitored over time as described in *Figure 2.1*. **(C)** RLuc activity for HCV GNN at 6 h normalized to FLuc (transfection efficiency) control at 2 h, was used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. RLuc is displayed as the mean of three biological replicates, and error bars represent the SD of the mean. Statistical significance was determined by multiple Student's *t* test, ns, not significant ($p \geq 0.01$).

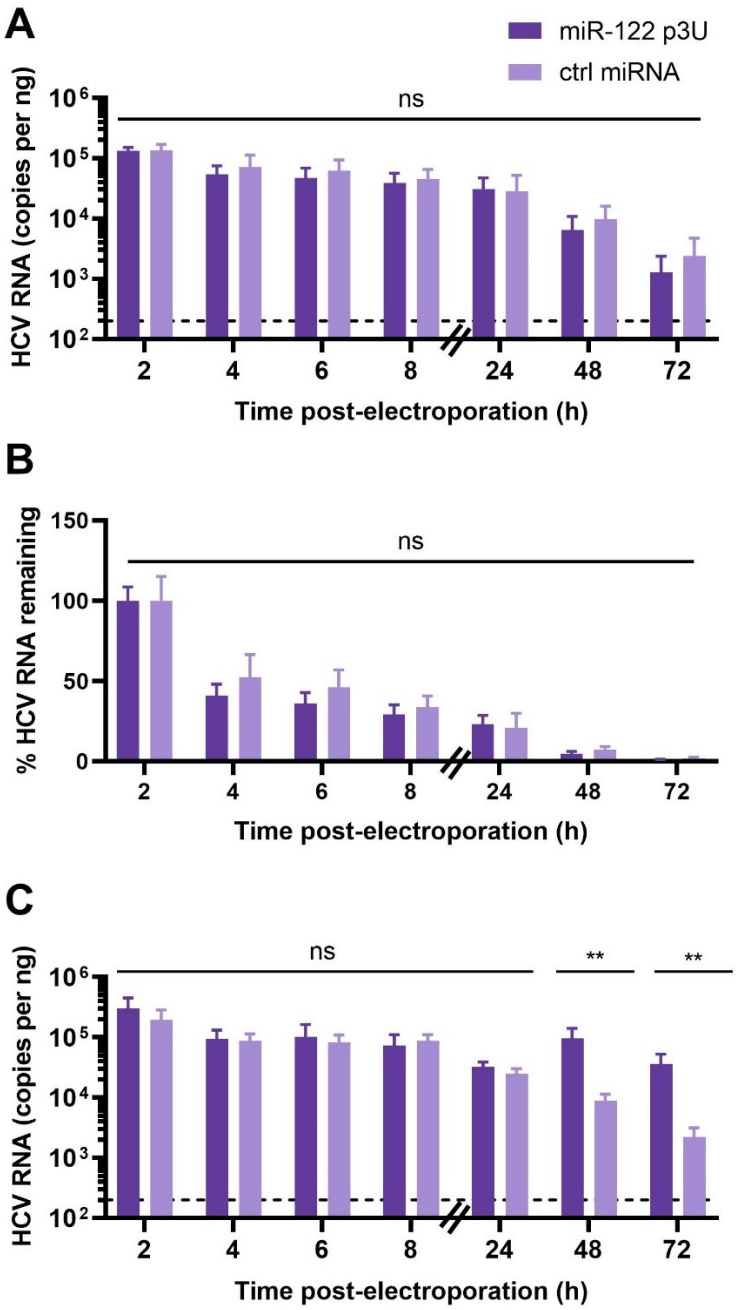

Figure S2.4. RT-qPCR analysis of viral RNA accumulation and decay from the stability experiment. GNN **(A-B)** or WT **(C)** HCV S1:p3 was co-electroporated in Huh-7.5 cells with miR-122p3U duplexes or a control miRNA as well as a capped FLuc reporter RNA, and luciferase signal and viral RNA levels were monitored over time as described in *Figure 2.1*. **(A,C)** HCV copy numbers were quantified by RT-qPCR and normalized to GAPDH. HCV RNA is displayed as the mean copy numbers per ng total RNA, and error bars represent the SD of the mean. The lower limit of quantitation is indicated by the dashed line. **(B)** The percentage of remaining RNA at each time point was calculated by setting the copy number at the 2 h time point to 100%. This decay curve was fit to a one phase decay function to calculate the half-life of HCV RNA for each condition. Statistical significance was determined by multiple Student's *t* test, ***$p \leq 0.0001$; **$p \leq 0.001$; *$p \leq 0.01$; ns, not significant ($p \geq 0.01$).

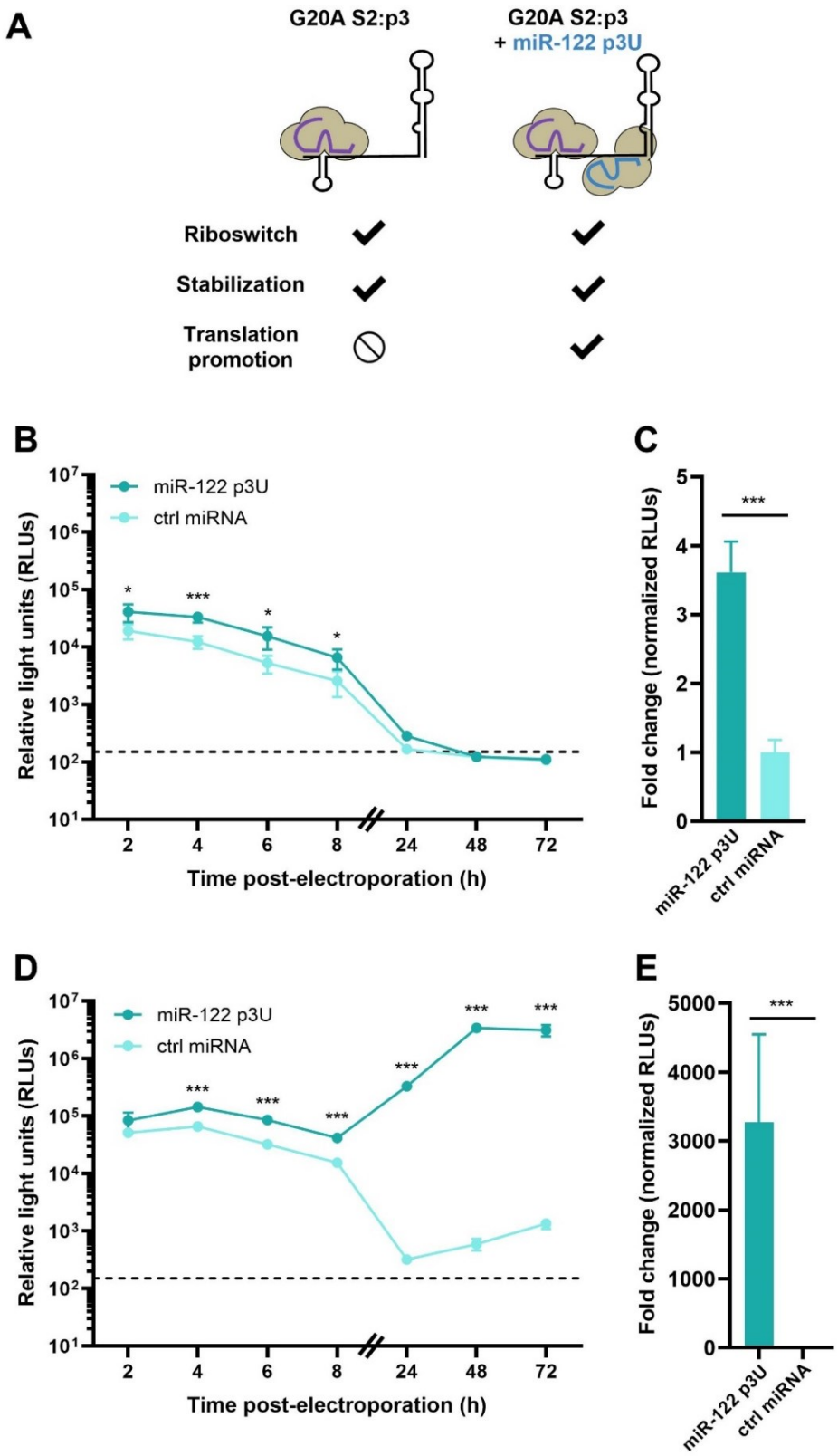

Figure S2.5. Alternative quantification of the translational promotion effect using the G20A S2:p3 mutant. (A) Schematic representation of the experimental set-up for translational…*continued on next page*.

Figure S2.5. Alternative quantification of the translational promotion effect using the G20A S2:p3 mutant. *Continued from previous page…*promotion assays. HCV G20A S2:p3 is riboswitch *a priori*, and endogenous WT miR-122 can bind to site 1, thereby fulfilling the stabilization effect. Addition of miR-122 p3U allows binding to site 2 and measurement of the translational promotion effect in isolation. **(B-E)** Full-length RLuc HCV G20A S2:p3 RNAs were co-electroporated into Huh-7.5 cells with miR-122 p3U or ctrl miRNA, as well as a capped FLuc reporter RNA. RLuc activities for **(B-C)** GNN or **(D-E)** WT G20A S2:p3 viral RNAs were monitored over time as described in *Figure 2.1*. In **(C)** and **(E)**, RLuc activities for GNN or WT G20A S2:p3 HCV RNAs at 6 h or 72 h, respectively, normalized to FLuc (transfection efficiency) control at 2 h, were used to calculate the fold change (with the control condition set to 1). The limit of detection is indicated by the dashed line. RLuc is displayed as the mean of three biological replicates, and error bars represent the SD of the mean. Statistical significance was determined by multiple Student's t test, ***$p \leq 0.0001$; **$p \leq 0.001$; *$p \leq 0.01$; ns, not significant ($p \geq 0.01$).

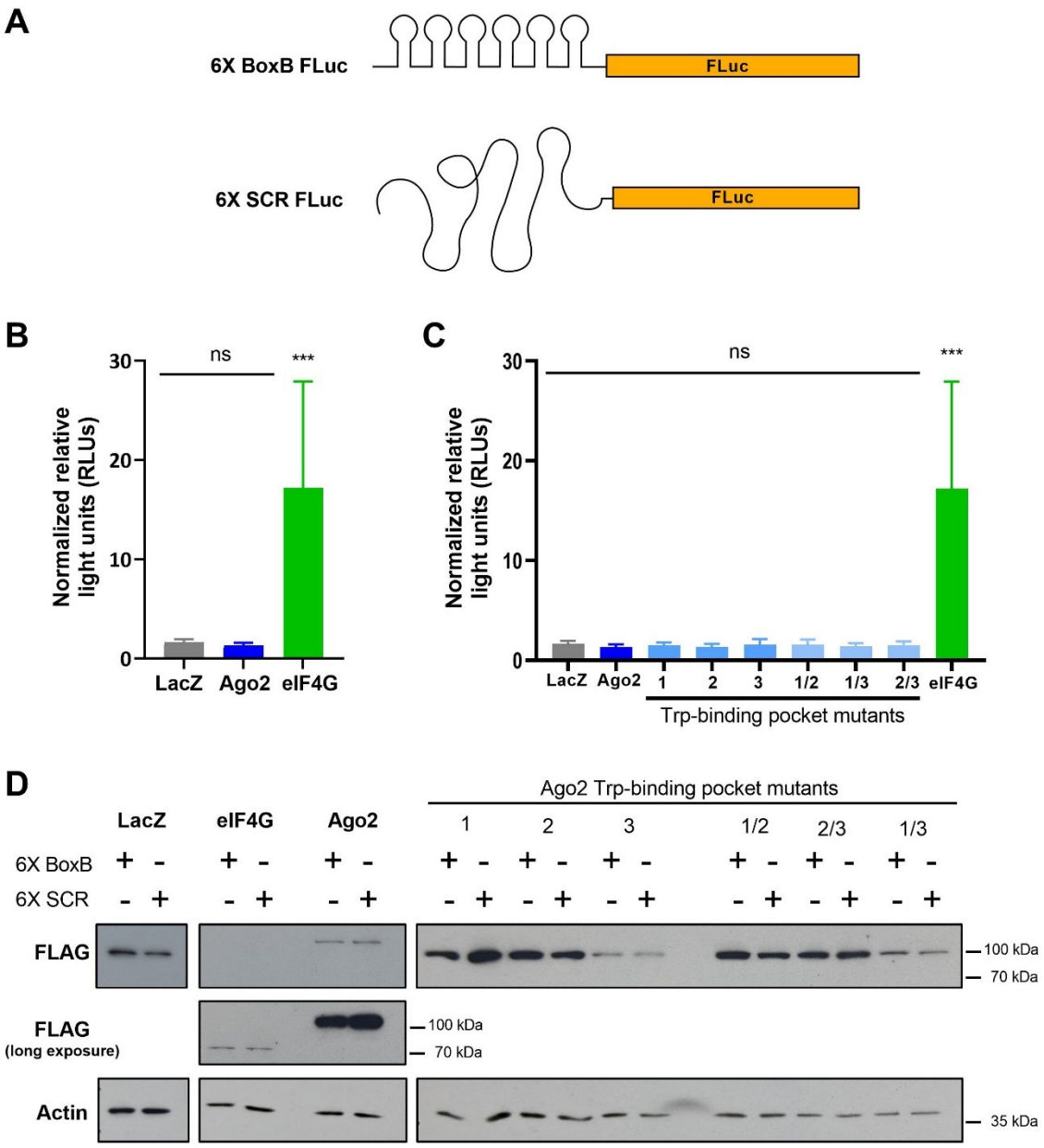

Figure S2.6. Tethering Ago2 to the 5′ UTR of a reporter RNA does not promote translation. (A) Schematic representation of 6X BoxB and 6X scrambled (SCR) reporter mRNAs. **(B-D)** Individual λN-fusion protein encoding plasmids were co-transfected in HEK 293T cells with a control RLuc reporter encoding plasmid. One day post-transfection, cells were transfected with 6X BoxB or 6X scr FLuc reporter RNAs and harvested for western blot and luciferase assay at 16 h post-transfection. **(B)** Luciferase activity of WT λN-Ago2, λN-LacZ and λN-eIF4G conditions. **(C)** Luciferase activity for the WT and mutant λN-Ago2 constructs. Luciferase activity is presented as FLuc normalized to the RLuc control, and all conditions were further normalized to the LacZ scr control condition. Normalized luciferase signal is reported as the mean of three independent biological replicates, and error bars represent to the SD of the mean. Statistical significance was determined by multiple Student's *t* test, ***$p \leq 0.0001$; ns, not significant ($p \geq 0.01$). **(D)** Expression of WT and mutant λN-Ago2, λN-LacZ and λN-eIF4G was verified by Western blot.

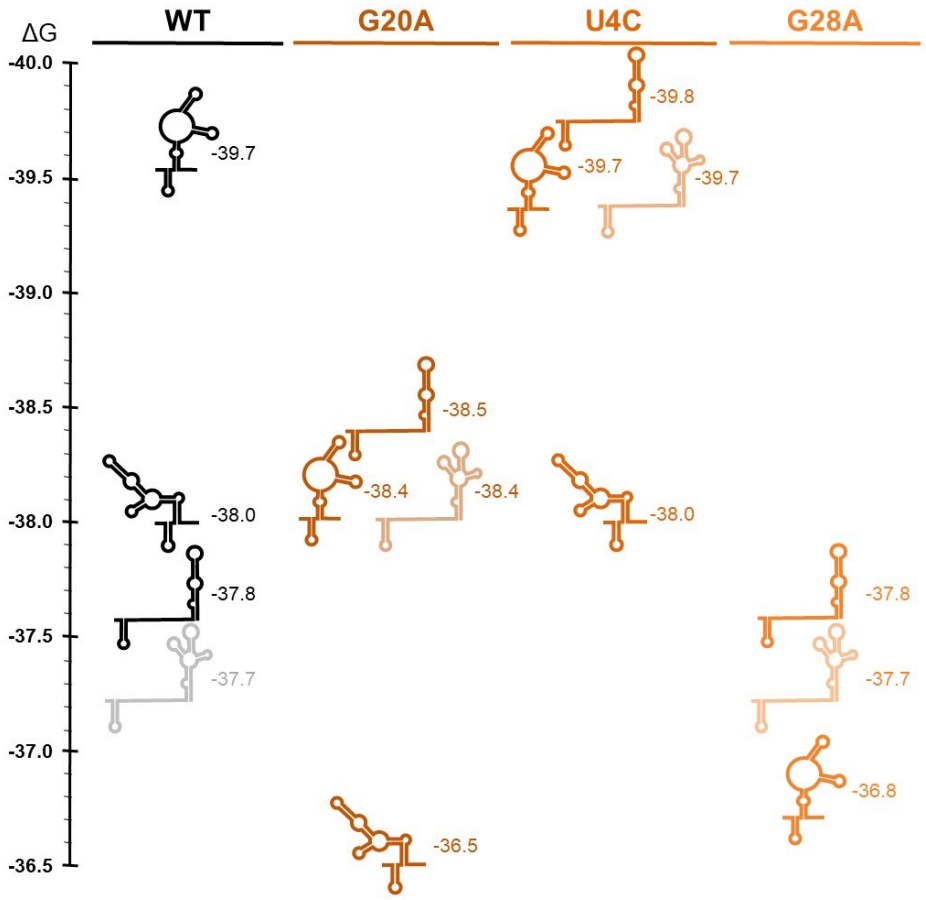

Figure S2.7. RNA structure predictions for the first 117 nucleotides of the WT, G20A, U4C and G28A JFH-1 genomes. Predicted RNA secondary structures and their associated Gibb's free energy (ΔG) predictions were calculated using RNAStructure, with the first 3 nucleotides constrained as single-stranded.

CHAPTER 3: DISCUSSION

3.1 Summary

As discussed herein, the liver-specific miRNA, miR-122, engages in non-canonical interactions with the 5′ UTR of the HCV genome. Unlike typical miRNA-target interactions, this results in promotion of viral translation and RNA accumulation (1-6). Recent studies have attributed at least three roles to miR-122 in the HCV life cycle: 1) RNA-chaperone or "riboswitch" activity, whereby Ago:miR-122 binding to site 2 on the HCV genome promotes the formation of the functional SLII structure (and the viral IRES, SLII-IV); 2) genome stabilization, as miR-122 binding to site 1 protects the 5′ terminus of the HCV genome from pyrophosphatase activity and subsequent exoribonuclease-mediated decay; and 3) translational promotion, fulfilled by interactions between the Ago protein bound to site 2 and SLII-III of the viral IRES (1-7). Herein, we show that the riboswitch effect contributes minimally to miR-122-mediated promotion of HCV translation and viral RNA accumulation, while the genome stability and translational promotion effects have similar contributions to the establishment of an infection. In contrast, we demonstrate that during the maintenance of an on-going infection, translational promotion becomes the dominant role. Finally, we identified a previously unknown function for the SLII^{alt} structure in viral particle production.

3.2 Estimation of the three roles of miR-122 in the HCV life cycle.

In this study, we used different approaches to isolate each of the three roles attributed to miR-122 and estimate their contribution to the overall impact of miR-122 on viral RNA accumulation. However, some limitations were associated with these experiments. Firstly, to estimate the riboswitch effect, we made use of point mutations which were predicted to decrease the Gibb's

free energy of the functional SLII structure, to approximate the "riboswitched" conformation. In this experiment, the riboswitch effect could not be assessed in the context of replication due to the inability of these constructs to replicate in the absence of miR-122. Unlike our subsequent experiments where we could subtract the role of interest from the combination of the three roles, here neither of the other two roles of miR-122 are fulfilled since we predict that binding at either site would result in formation and/or stabilization of the SLII structure, thereby also fulfilling the riboswitch effect. Moreover, it is important to note that these mutated genomes are also able to form SLII^{alt} structures and therefore are not likely to represent a uniformly "riboswitched" state, but rather, like WT HCV RNA, they are likely to sample multiple conformations due to their similar energetic stabilities (**Supplementary Figure S2.7**). An alternative approach to measure the riboswitch effect could have been to measure the combined effects of translation and riboswitch by comparing HCV WT S2:p3 +/- miR-122 p3U (or S1:p3 +/- WT miR-122) in miR-122 KO cells, and to subtract the translation effect (measured using U4C S2:p3 as described in ***Chapter 2***). However, limitations also arise in this scenario because miR-122 binding to site 2 would likely provide some degree of stability to HCV which may be different in the U4C experiment where site 1 is bound at baseline. As such, we chose to use the SLII stabilizing point mutations in the context of GNN virus and in the absence of miR-122, as the most straightforward approach to estimate the riboswitch effect.

When measuring the stability effect, we isolated the effect of miR-122 binding to site 1; however, there is some evidence to suggest that Ago:miR-122 at site 2 also provides a modest stabilization effect (8, 9). We hypothesize that this may be due to increased association of the RNA with the ribosome; suggesting that the stability and translational promotion effects may also be linked. However, in our experiments, we chose to focus on stability provided by

Ago:miR-122 interactions at site 1, which result in protection of the 5′ terminus from cellular pyrophosphatases and exoribonucleases, as we felt that this likely accounted for the vast majority of the stability effect (1, 3, 8).

When measuring translational promotion, we observed a similar impact as the stabilization effect in the establishment phase of the viral life cycle, and our results are in accordance with the estimated impact of miR-122 on translation in another study that used non-replicative reporter RNAs (7). Importantly, our study shows that in the context of active viral replication, miR-122-mediated translational promotion has the largest impact of the three roles. This is not surprising since during the establishment stage of infection, where the genome can only be translated or decay, it is important for the virus to extend the viral RNA half-life in order to translate sufficient levels of viral proteins to allow for the formation of a replication organelle. However, once replication has been initiated, an excess of new positive-sense genomes is produced from every negative-sense intermediate generated (10, 11). Moreover, the vesicular replication organelle can shield the dsRNA replicative intermediate and newly synthesized viral RNAs from cellular sensors of RNA (12). Additionally, the replication organelle has also been shown to protect the viral RNA from nucleases, and we hypothesize it may also protect against cellular pyrophosphatases (10, 11, 13, 14). Additionally, since replication results in the production of many progeny positive-strand RNAs, stability may be less important in the maintenance phase and rather generating enough viral proteins to seed additional replication organelles and for the production of new virions, becomes greater in importance. Thus, consistent with our findings herein, it is likely that stability is more important in the establishment phase, but its importance is outweighed by translational promotion in the maintenance phase of HCV infection.

Early studies of miR-122 and HCV were contradictory with regards to the exact mechanism of miR-122-mediated promotion of viral RNA accumulation, some pointing to enhancement of replication with no impact on translation or stability, while others demonstrated modulation of translation rather than viral RNA replication (5, 15, 16). An important confounding factor resided in the fact that most of these studies manipulated miR-122 binding using mutations which are predicted to alter the secondary structure of the viral 5′ UTR (i.e. p3G, p3,4 and p6 mutations), which may negatively impact viral kinetics independently of miR-122 binding (5, 6, 15, 16). Additionally, several studies observed no HCV RNA accumulation when a single miR-122 binding site was mutated (4, 6, 16). This may be explained by alterations to the secondary structure since we now know that HCV can accumulate, albeit to a low level, when miR-122 is bound to only one site on the HCV 5′ UTR (*see* ***Chapter 2***) (17). Additionally, without the knowledge that miR-122 binding provides these three roles (riboswitch, genome stability and translational promotion), previous studies have overestimated the importance of site 1 given that interactions with site 1 only would inevitably provide both genome stability and the riboswitch effect (2, 3). Thus, in contrast to previous studies which may have conflated these roles, by focusing on point mutations that do not alter the secondary structure of the viral RNA and carefully isolating each role, we believe that we have provided the most accurate estimation to date of these three roles in the HCV life cycle (1, 5, 6, 16-18).

3.3 Confirming that viral RNA accumulation is site-mutant dependent rather than due to revertant mutations.

When quantifying the stabilization effect, we observed significant amounts of replication in the negative control condition of the replication competent (WT) experiment. We also observed

modest replication in the absence of site 2 binding in the translation promotion experiment. As such, one outstanding question is whether the viral replication we observed in the absence of miR-122 at one of the two sites was due to viral reversion or was really in the absence of miR-122 interactions. To answer this question, we have devised a modified 5′ Rapid Amplification of cDNA Ends (5′ RACE) protocol which will enable us to sequence the 5′ terminus of HCV genomes recovered at the endpoints of these experiments (**Figure 3.1**). Briefly, the experimental design involves reverse transcribing and ligating the HCV 5′ UTR to produce a circular cDNA product, which is then amplified by PCR, ligated into a vector, and transformed into competent cells to screen and sequence transformants. Thus far, our preliminary results have been promising, and we have recovered the expected S1:p3 sequences from the experimental endpoint of the stability experiments performed in ***Chapter 2*** (data not shown). Interestingly, the 5′ RACE analysis also provided us with new insights into this experiment as we observed that a significant proportion of viral genomes isolated (and more so in the negative control condition) had 5′ truncations, suggestive of active 5′ to 3′ decay. Interestingly, many of the truncations recovered occur within the viral IRES, suggesting that these viral RNAs are no longer able to engage in translation. In future, our 5′ RACE protocol will enable us to confirm our quantification of the three roles of miR-122 by verifying the integrity of the mutations engineered in the HCV 5′ UTR at the 72 h experimental endpoint.

3.4 miR-122 binding sites are conserved across hepaciviruses

It is interesting to note that miR-122 binding sites are conserved across all the members of the hepacivirus genus for which 5′ UTR sequences are available (19-26). Only a single binding site has been identified for some of them, while others have two, but it remains unclear whether

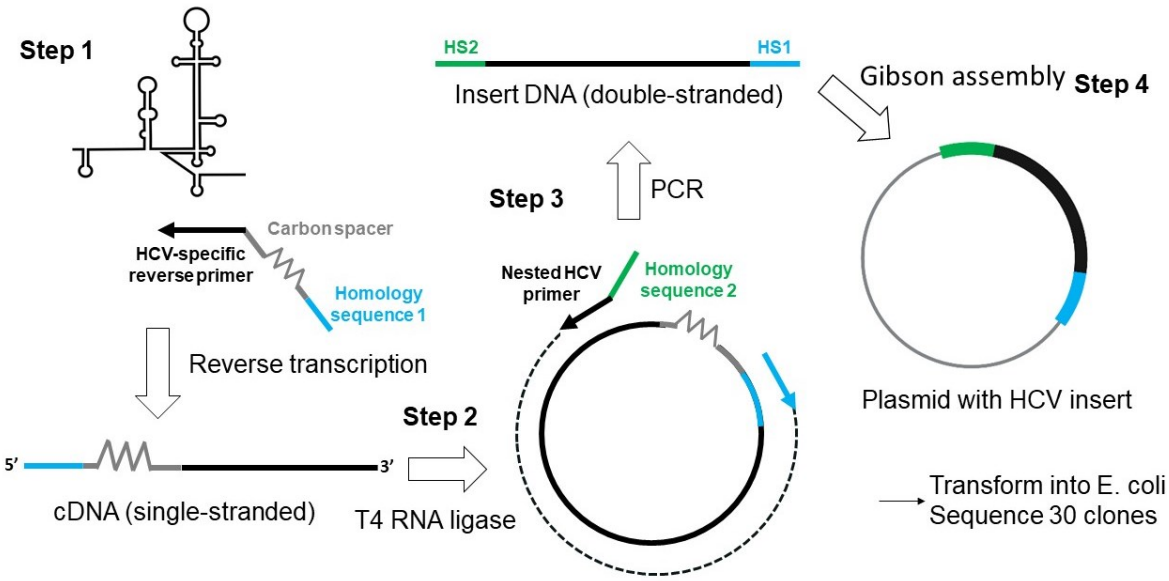

Figure 3.1. Schematic of HCV 5′ Rapid Amplification of cDNA Ends (5′ RACE) experimental design. Schematic representation of HCV 5′ RACE protocol. Briefly, in Step 1, the total RNA from HCV-infected cells is subjected to reverse transcription using an HCV-specific primer containing carbon spacer and homology sequence 1 (HS1, a sequence with homology to the sub-cloning vector for Gibson assembly). In Step 2, the cDNA product is circularized using T4 RNA ligase. In Step 3, PCR is performed on the circular single-stranded DNA product using a nested HCV reverse primer containing a homology sequence 2 (HS2) matching the other end of the sub-cloning vector, and a forward primer annealing to HS1. Finally, in Step 4, the linear PCR product is ligated into a PCR-amplified linear sub-cloning vector using Gibson assembly. The ligation product is transformed into *E. coli* and transformants are isolated and sequenced via Sanger sequencing.

miR-122 similarly promotes viral RNA accumulation for all these viruses. Notably, GB virus B (GBV-B), equine hepacivirus (EHcV), Norway rat hepacivirus (NrHV) and bovine hepacivirus (BovHepV) were all shown to be dependent on miR-122 to some degree either by assessing IRES activity in reporter assays or infectivity in cell culture or *in vivo* (19, 27-33). These conserved miR-122 binding sites across the hepaciviruses infecting a variety of species suggest the presence of a common hepatotropic ancestor (34). The ability of some non-primate hepaciviruses (NPHVs), like EcHV, to readily become independent from miR-122 in cell culture also suggests that there is some evolutionary advantage to maintaining the interaction(s) with miR-122 (28). This may be due to their liver tropism or miR-122 may aid in the development of chronicity (or long enough infectivity to spread to more hosts) (28). As discussed herein, in the case of HCV, at least some mutations which render the virus less dependent on miR-122 come at a fitness cost as less viral particles are produced (*see* ***Chapter 2***). However, the lack of cell culture systems in which to study fully infectious authentic NPHVs limits our ability to assay similar aspects in these viral life cycles (26). Once those systems become available, it will be interesting to study the entire life cycle of miR-122 independent variants to determine whether they also display reduced virion production or defects in other aspects of their life cycle. It will also be interesting to find out whether miR-122's mechanism of action is entirely conserved between HCV and related hepaciviruses or if new mechanisms of miRNA-mediated modulation of viral RNA accumulation are at play (35).

3.5 Other viruses are regulated by miRNAs

In addition to HCV, other viruses have been shown to interact with host or virally derived small RNAs to modulate their life cycles. For example, human miR-21 was reported to be pro-viral for

two flaviviruses, Zika virus (ZIKV) and Dengue virus serotype 2 (DENV2) (36, 37). In the case of ZIKV, the interaction takes place in the 5′ cyclization sequence in the 5′ UTR, which is reminiscent of miR-122 and HCV interactions (36). Bovine viral diarrhea virus (BVDV), a member of the pestivirus genus, also in the family *Flaviviridae*, interacts with two host miRNAs, miR-17 and let-7, via binding sites in the viral 3′ UTR (29, 38). These interactions also promote viral RNA accumulation, suggesting that non-canonical miRISC interactions similar to miR-122 and HCV can also take place at the 3′ end of transcripts and promote viral RNA accumulation (29, 38). It would be interesting to identify the other factors which are recruited to the miRISC complex and determine whether the positioning of the binding sites to either end of the viral genome results in differential recruitment of specific factors.

On the other hand, some viruses antagonize host miRNAs to modulate cell metabolism, such as *Herpesvirus saimiri* (a gamma herpesvirus that infects New World primates), which expresses non-coding RNAs that bind to and promote the decay of host miR-27, resulting in dysregulation of endogenous miR-27 targets (39). In contrast, other viruses encode their own small RNAs, which may influence host protein expression and immune responses, or play roles in the viral life cycle (40-43). Herpesviruses and polyomaviruses have been known for several years to encode viral miRNAs, and although their function is not always clear, in many cases these viral small RNAs play roles in immune evasion by downregulating the host immune response (40-43). More recently, RNA virus encoded small RNAs have been identified (44-47). For example, the Influenza virus H5N1 encodes a miRNA-like small viral RNA which contributes to cytokine production during infection by modulating the host innate antiviral response (47). Moreover, enterovirus 71 (EV-71, *Picornaviridae* family) infection leads to the production of several viral small RNAs (vsRNAs), one of which, vsRNA-1, was suggested to

bind to the viral IRES and modulate its activity (discussed in more detail in section **3.6**) (44-46). Given how much remains to be elucidated regarding cellular miRNA-mediated gene silencing, while numerous atypical functions of miRNAs and small RNAs have already been identified both in cellular and viral systems, it seems that we are only beginning to scratch the surface of the endless functions that can be fulfilled by these non-coding RNAs. Importantly, antisense inhibitors can be used to target miRNAs *in vivo*, as exemplified by miR-122 inhibitors which successfully reduced HCV viral loads in clinical trials (48-50). Therefore, identifying pro-viral miRNAs, both host- and virally encoded, could provide novel therapeutic targets. As RNA based therapeutics gain in popularity and delivery systems continue to improve, miRNA inhibitors may thus prove important tools in the control of important human and veterinary pathogens (51).

3.6 The controversial role of Ago in translation promotion

As discussed in ***Chapter 2***, the Ago protein was initially identified as a ribosome-associated factor (52, 53). Additionally, some reports suggest that in conditions of cellular stress, Ago is involved in the promotion of translation of AU-rich element containing transcripts (54, 55). There are also other examples of miRNAs acting to promote translation in specific cellular contexts, but the role of Ago in these processes remains unclear (56, 57). Interestingly, miRISC-mediated translational promotion is not unique to HCV infection, and recent studies suggest that this may also be the case in EV-71 infection (44). Briefly, EV-71 was shown to bind to a small RNA generated by Dicer-mediated processing of its own 5′ UTR, called vsRNA-1 (44). Experiments using vsRNA-1 mimics suggested promotion of viral translation; however, the authors' use of single-stranded vsRNA-1 mimics, as opposed to vsRNA-1 duplexes which are competent for loading into Ago, confound these results (58). Nonetheless, Ago2 was also found

to interact with the EV-71 5′ UTR and to be a positive regulator of EV-71 translation, but whether or not this is mediated via vsRNA-1 still remains unclear (46). Overall, there is anecdotal evidence which suggests that the versatility of Ago in translation regulation remains underappreciated, but more studies are needed to more definitively identify the determining factors of Ago-mediated translational promotion. In the context of EV-71, human antigen R (HuR) was suggested to positively regulate IRES activity, whereas in the case of Ago-mediated translation upregulation during cell stress, fragile-X-mental-retardation-related protein 1 (FXR1) was identified as a co-factor (46, 54). Thus, in future it will be interesting to perform proximity biotinylation and mass spectrometry analysis to identify the host and viral factors which are enriched at these sites of miRNA-mediated translational promotion, and compare them with the factors associated with the canonical miRISC pathway.

3.7 How does HCV escape canonical silencing?

As discussed in ***Chapters 1 and 2***, Ago:miR-122 binding to the HCV genome, unlike typical miRNA interactions, promotes translation and viral RNA accumulation. Despite a good understanding of how this occurs, an important question remains unanswered: how does HCV escape canonical miRISC-mediated silencing? In the canonical miRNA pathway, a miRNA-loaded Ago protein binds to its mRNA target and triggers a series of events which includes the recruitment of the TNRC6 silencing effector protein. TNRC6 is known to promote translational inhibition, and recruit the deadenylation and decapping machinery, promoting decay of the targeted mRNA (58, 59). Given that HCV doesn't have a 5′ cap or a poly(A) tail, the decapping and deadenylation machinery are unlikely to have any impact on the viral genomic RNA. While the mechanism of TNRC6-mediated translational inhibition has been controversial, a recent

study has proposed a new model of translational inhibition, as Ago and TNRC6 interactions were shown to promote liquid-liquid phase separation (LLPS) *in vitro* and in cells (60). LLPS is a process by which a select set of macromolecules crowd together to form a dense phase, distinct from the surrounding, dilute phase (61). This process is driven by multivalent interactions between the GW-repeats in TNRC6 and the Trp-binding pockets on Ago, and leads to accelerated deadenylation of target mRNAs. LLPS has also been shown to serve as a barrier to large molecular complexes, such as the ribosome (62-64). Thus, LLPS helps explain TNRC6-mediated translational inhibition (60). Moreover, at least one HCV protein, the NS5A protein, also displays the features of a protein which may undergo phase separation. More specifically, NS5A is a phosphoprotein implicated in replication organelle biogenesis, inhibition of the antiviral response, and virion assembly (65-71). NS5A is a homodimer (creating multivalency), an RNA-binding protein (with high affinity for the polyU/UC-tract in the viral 3′ UTR), it contains large intrinsically disordered regions (IDRs), and it is known to be regulated by phosphorylation; these properties are all commonly found in proteins that undergo LLPS (61, 65-69).

Given this information, we propose a new model for miR-122-mediated viral RNA accumulation. Firstly, as described herein, when the HCV genome enters the cytoplasm, it is likely present in the most energetically favorable conformation, $SLII^{alt}$. Recruitment of Ago:miR-122 to the HCV 5′ UTR results in riboswitch activity, genome stabilization, and translational promotion. This leads to the accumulation of the viral proteins, including the NS3 helicase, the NS5A phosphoprotein, and the NS5B RdRp. These proteins all have affinity for polyU sequences and therefore accumulate on the polyU/UC-tract in the 3′ UTR of the viral RNA; and, due to the membrane association of these proteins, they tether the viral RNA to the

endoplasmic reticulum (ER) membrane. As NS5A is a phase separation protein, it can then begin to condense the viral RNA into a replication organelle. However, this is hampered by ongoing translation. As such, we believe that subsequent recruitment of TNRC6 to the Ago:miR-122 complexes at the 5′ terminus of the HCV genome results in translational inhibition, effectively excluding ribosomes and allowing the viral RNA to be fully condensed into a replication organelle. As such, Ago:miR-122 complexes both serve to switch on translation, and to switch it back off for viral replication organelle biogenesis. If this model is correct, then HCV does not "escape" canonical silencing after all; instead, it makes use of the canonical silencing pathway to mediate the switch from translation to viral RNA replication. Future studies will therefore be focused on providing more insight into this model for miR-122-mediated viral RNA accumulation.

It is well recognized that phase separation underlies the formation of the replication organelles used by negative-sense RNA viruses, which are membrane-less cytoplasmic compartments referred to as inclusion bodies (64, 72-74). However, this has been largely unexplored in positive-sense RNA viruses which have vesicular replication organelles (75, 76). However, this does not preclude their formation by phase separation, and accumulating evidence implicates phase separation in replication organelle biogenesis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (77-81). Thus, it is likely that in the years to come, we will begin to recognize the importance of phase separation in the life cycles of both positive-sense and negative-sense RNA viruses alike (76).

3.8 Expansion of the roles of miR-122 in the HCV life cycle

In this study, we quantify the three previously identified roles of miR-122 in the HCV life cycle, but we also provide evidence for additional role(s) for miR-122. Firstly, in the regulation of the balance between translation/replication and virion production, since miR-122 interactions with the HCV genome promote the conformational change to the translation-competent 5′ UTR structure, to the detriment of viral particle assembly. Secondly, we suggest a potential role for miR-122 in the switch from translation to replication, aiding in the establishment of replication organelles. As such, our results and new models predict at least 5 roles for miR-122 in the HCV life cycle: 1) riboswitch activity; 2) genome stabilization; 3) translational promotion; 4) the switch from translation to replication; and 5) the decision point between virion assembly and translation. While these putative additional roles require more study, they could explain the challenges in dissecting the importance of miR-122 in the HCV life cycle as by attempting to quantify the three identified roles herein, we may have only scratched the surface with respect to miR-122's effects in the HCV life cycle.

3.9 Concluding remarks

In conclusion, the study presented here provides a deeper understanding of the importance of each of the three roles previously attributed to miR-122 in the HCV life cycle. Our results show that riboswitch has the least impact on the establishment of HCV infection, while genome stabilization and translational promotion contribute similarly. After several rounds of replication, translational promotion becomes the dominant role in the maintenance of viral RNA accumulation. Excitingly, we identified a previously unknown function for the non-canonical $SLII^{alt}$ structure in virion production, and our findings herein have led us to propose a new model

whereby miR-122 also plays a role in the balance between translation/RNA replication and virion production, as well as mediates the switch between translation and replication in the HCV life cycle. Given that many viruses interact with miRNAs, the mechanisms elucidated herein may not be unique to HCV:miR-122 interactions and future work will help to reveal new insights into these miRNA:viral RNA interactions. As such, we are hopeful that further research into these interactions will provide new targets for the development of antivirals, but may also uncover new mechanisms of miRNA-mediated gene regulation in both health and disease.

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4.1 APPENDIX

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