The poly(rC)-binding proteins 1 and 2 regulate hepatitis C virus infectious particle assembly

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ABSTRACT

The hepatitis C virus (HCV) is a positive-sense, single-stranded RNA virus; as such, its genome directly participates in multiple mutually exclusive steps of the viral life cycle, namely translation, RNA replication, and virion assembly. To complete its viral life cycle, HCV co-opts many cellular elements — including proteins, lipids, and microRNAs. The cellular poly(rC)-binding proteins (PCBPs), specifically PCBP1 and PCBP2, were previously reported to bind the HCV genome, yet their specific importance in the viral life cycle had remained unclear. We hypothesized that these interactions were biologically significant, and that PCBPs played a role in the viral life cycle. Specifically, we aimed to identify the precise step(s) of the viral life cycle affected by PCBP1 and PCBP2.

Firstly, we found that knocking down endogenous PCBP1 decreased HCV RNA accumulation, yet increased secreted viral titers. By systematically assessing each step of the viral life cycle, we were able to rule out a role for PCBP1 in viral entry, translation, genome stability, or RNA replication in the absence of efficient virion assembly. We found that PCBP1 knockdown increased infectious particle assembly as well as the rate of infectious particle secretion. Thus, we propose that endogenous PCBP1 normally limits virion assembly and egress, thereby indirectly enhancing viral RNA accumulation in infected cells.

Secondly, we found that knocking down endogenous PCBP2 reduced both viral RNA accumulation and infectious particle production. We ruled out a role for PCBP2 in viral entry, translation, genome stability, or RNA replication. We found that PCBP2 knockdown only affected viral RNAs that could engage in the early steps of packaging into viral particles, or that could form a stable RNA conformation termed $SLII^{alt}$. We also found that PCBP2 knockdown

accelerated virion assembly without affecting the rate of virion secretion. In all, these results suggest that PCBP2 normally delays the early steps of virion assembly, thereby increasing the number of viral genomes engaged in translation and RNA replication.

Based on this research, we propose that PCBP1 and PCBP2 both indirectly promote HCV translation and RNA replication by preventing viral genome packaging into virions, and that PCBP1 plays an additional role to limit virion secretion. Beyond identifying the roles played by PCBPs in the HCV life cycle, this research furthers our understanding of how cellular RNA-binding proteins influence how HCV uses its genomic RNA over the course of its viral life cycle.

RÉSUMÉ

Le virus de l'hépatite C (VHC) est un virus dont le génome — un ARN à simple brin de polarité positive — participe directement à plusieurs étapes mutuellement exclusives du cycle viral, c'est-à-dire la traduction, la réplication du génome, et l'assemblage des virions. Pour compléter son cycle viral, le VHC s'empare de nombreuses composantes cellulaires, dont des protéines, des lipides, et des microARNs. Les protéines qui se lient aux séquences poly(rC) (poly(rC)-binding proteins, PCBP), spécifiquement PCBP1 et PCBP2, ont déjà été décrites comme étant des protéines humaines qui se lient au génome du VHC, mais leur rôle exact dans le cycle viral est encore inconnu. À partir de l'hypothèse que leurs interactions avaient un impact biologique, donc que les PCBP jouaient un rôle dans le cycle viral, le but de cette recherche était d'identifier les étapes précises du cycle viral qui étaient affectées par PCBP1 et PCBP2.

En premier lieu, nous avons constaté que réduire le niveau de PCBP1 endogène lors d'une infection du VHC diminuait la quantité d'ARN viral accumulé, mais augmentait sa production de particules infectieuses. En évaluant de manière systématique chaque étape du cycle viral, nous avons réussi à exclure un rôle pour PCBP1 dans l'entrée du virus, la traduction ou stabilité de son ARN, ou dans la réplication de son génome. Nous avons aussi constaté que réduire les niveaux de PCBP1 augmentait à la fois l'assemblage de particules infectieuses ainsi que leur taux de sécrétion. Nous proposons donc que PCBP1 limite normalement l'assemblage et la sécrétion des virions, et augmente donc d'une manière indirecte l'accumulation d'ARN viral dans les cellules infectées.

En deuxième lieu, nous avons constaté que diminuer la quantité de PCBP2 endogène réduisait à la fois l'accumulation d'ARN viral et la production de particules infectieuses. Nous

avons confirmé que PCBP2 n'était pas nécessaire pour l'entrée du VHC, la traduction ou la stabilité de son ARN, ou la réplication de son génome. Nous avons remarqué que seuls les ARN viraux qui pouvaient participer aux étapes initiales de l'assemblage, ou qui pouvaient former une conformation d'ARN stable appelée $SLII^{alt}$, étaient affectés par l'inhibition de l'expression de PCBP2. Nous avons également constaté que réduire les niveaux de PCBP2 accélérait l'assemblage des virions sans affecter leur taux de sécrétion. En tout, ces résultats suggèrent que PCBP2 cause normalement un délai des premières étapes de l'assemblage, et augmente ainsi le nombre de génomes viraux disponibles pour la traduction et la réplication d'ARN.

Nous proposons donc que PCBP1 et PCBP2 favorisent la traduction et réplication de l'ARN du VHC d'une manière indirecte, en empêchant les génomes viraux d'être empaquetés dans des virions, et que le PCBP1 joue un rôle supplémentaire pour limiter la sécrétion des virions. En plus d'identifier les rôles que les PCBP jouent dans le cycle de vie du VHC, cette recherche nous a permis de mieux comprendre comment des protéines cellulaires qui se lient à l'ARN peuvent influencer la manière dont le VHC régule les fonctions de son génome pendant son cycle viral.

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its challenges, and to meet inspiring researchers and leaders in the field.

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PREFACE

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation." The format of this thesis conforms to the "Manuscript-based thesis" option, which states that:

"As an alternative to the traditional thesis format, 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 doctoral thesis must include the test of a minimum of two manuscripts published, submitted or to be submitted for publication. 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 with regard to the submitted and published articles, such as journal name and date of publication are listed below. The contributions of all authors to each article are detailed in the "Contributions of authors" section.

Manuscripts presented in this thesis:

1. **Sophie E. Cousineau** and Selena M. Sagan. KH domain proteins and viruses: adaptable tools for molecular parasites. *Manuscript In Preparation*
2. **Sophie E. Cousineau** and Selena M. Sagan. Poly(rC)-binding protein 1 limits hepatitis C virus virion assembly and egress. *bioRxiv* [preprint] 2021 Mar 1. doi: 10.1101/2021.02.28.433252
3. **Sophie E. Cousineau** and Selena M. Sagan. The poly(rC)-binding protein 2 prevents the early steps of hepatitis C virus assembly. *Manuscript In Preparation*

CONTRIBUTIONS OF AUTHORS

Chapter 1:

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Part of this review manuscript was used in the sections "Poly(rC)-binding proteins" and "Interactions with viruses." S.E.C drafted the manuscript; S.E.C. and S.M.S revised and edited the final manuscript

Chapter 2:

Sophie E. Cousineau and Selena M. Sagan. Poly(rC)-binding protein 1 limits hepatitis C virus virion assembly and egress. *bioRxiv* [preprint] 2021 Mar 1. doi: 10.1101/2021.02.28.433252

S.E.C. and S.M.S. designed the study; S.E.C. performed the experiments and analyzed the data, and S.E.C and S.M.S. wrote and edited the manuscript.

Chapter 3:

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S.E.C. and S.M.S. designed the study; S.E.C. performed the experiments and analyzed the data, and S.E.C and S.M.S. wrote and edited the manuscript.

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2´CMA	2´C-methyladenosine
Ago	Argonaute
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
CDK1N1A	Cyclin-dependent kinase inhibitor 1A
CLDN1	Claudin-1
DAA	Direct-acting antiviral
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
eEF	Eukaryotic translation elongation factor
eIF	Eukaryotic translation initiation factor
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FFU	Focus-forming unit
FLuc	Firefly luciferase
g2a	Genotype 2a
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCVpp	HCV pseudoparticles
HPV	Human papillomavirus
hnRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
iCLIP	Cross-linking immunoprecipitation

IFN	Interferon
IGF2BP	Insulin growth factor 2 binding protein
IRES	Internal ribosomal entry site
ITAF	IRES *trans*-acting factor
IVT	*In vitro* transcribed RNA
JFH-1	Japanese Fulminant Hepatitis-1
kb	Kilobase
kDa	Kilodalton
KH	HnRNP K homologous domain
KO	Knockout
LCS	Low complexity sequence
LD	Lipid droplet
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
m^6A	*N*6-methyladenosine
MAVS	Mitochondrial antiviral signalling
METTL	*N*6-adenosine-methyltransferase
miRNA	MicroRNA
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NANBH	Non-A, non-B hepatitis
NPC1L1	Niemann-Pick C1-Like 1 receptor
NS	Nonstructural
NSAP1	NS1-associated protein 1
nt	Nucleotide
OAS	2′-5′-oligoadenylate synthetase
OCLN	Occludin
ORF	Open reading frame

PABP	Poly(A)-binding protein
PCBP	Poly(rC)-binding protein
PKR	Protein kinase R
PV	Poliovirus
RIG-I	Retinoic acid inducible gene I
RISC	RNA interference silencing complex
RLuc	Renilla luciferase
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription & quantitative polymerase chain reaction
SARS-CoV	Severe acute respiratory syndrome coronavirus
siRNA	Short interfering RNA
SL	Stem-loop
SR-B1	Scavenger receptor class B type I
STAT1/2	Signal transducer and activator of transcription 1/2
TRIF	Toll like receptor adaptor inducing interferon-β factor
UTR	Untranslated region
VSVpp	Vesicular stomatitis virus pseudoparticles
VLDL	Very low density lipoprotein
WT	Wild-type
YBX-1	Y box binding protein 1
YTHDF	YTH domain family protein

CHAPTER 1: Introduction

1.1 Hepatitis C virus

Discovery of the hepatitis C virus

The 2020 Nobel prize in Medicine or Physiology was awarded to Harvey J. Alter, Michael Houghton, and Charles M. Rice for their discovery of the hepatitis C virus (1). During the 1970s, Alter and colleagues observed that nearly 10% of patients who received blood transfusions developed hepatitis that could not be attributed to hepatitis A or B viruses (2, 3). This non-A, non-B hepatitis (NANBH) was then shown to be caused by a filterable agent, likely an enveloped virus, that could infect both humans and chimpanzees (4-6). By 1989, during their search for the causative agent of NANBH, Houghton and colleagues at Chiron corporation isolated a nearly 10,000 nucleotide (nt) long flavivirus-like RNA from infected chimpanzee blood, that encoded an antigen recognized by antibodies exclusively found in NANBH patients; this isolated virus was termed hepatitis C (HCV) (7, 8). Isolation of this HCV RNA was instrumental to develop serological tests (for anti-HCV antibodies), and helped functionally eliminate transfusion-associated HCV transmission (9). In 1997, eight years after the HCV genome was first published, the Rice laboratory confirmed that the full-length viral RNA was, by itself, sufficient to cause hepatitis when injected directly into the chimpanzee liver (10).

Epidemiology

Since HCV is transmitted through blood products, intravenous drug use is currently the most important risk factor for HCV infection in developed countries, and accounts for roughly 60% of

HCV cases in Canada (11). Still, healthcare procedures can still pose a risk of exposure if infection prevention practices are improperly followed (12-14). Recent estimates of HCV prevalence predict that about 71 million people worldwide live with a chronic HCV infection, including 268,000 Canadians (15).

HCV has a high degree of genetic diversity, and viral isolates are classified into eight distinct genotypes and over 90 subtypes, which vary in their pathogenesis, susceptibility to treatment, and geographic distribution (16). Genotypes 1, 2 and 3 have a worldwide distribution, and account for roughly 60%, 15%, and 22% of HCV infections in Canada, respectively (11). The remaining genotypes have a more restricted geographic distribution: genotype 4 is mainly found in Africa and the Middle East; genotype 5 is confined to Southern Africa; genotype 6 is limited to Southeast Asia; genotype 7 was found in the Democratic Republic of Congo; and genotype 8, which was only recently identified, is limited to India (17-19).

Pathology and treatment

Although 15 to 30% of patients can spontaneously clear HCV during the acute phase of infection, most patients will go on to develop a persistent viral infection. This chronic infection generally presents few or very mild symptoms, yet can lead to the development of liver fibrosis, cirrhosis, hepatocellular carcinoma and end-stage liver disease over the course of decades (**Figure 1.1**) (reviewed in (20)). Successfully curing a patient of their HCV infection generally halts liver fibrosis progression, reduces by 70% their likelihood of developing liver cancer, and decreases by more than 10-fold their odds of dying from liver-related causes over the next 10 years (21-24).

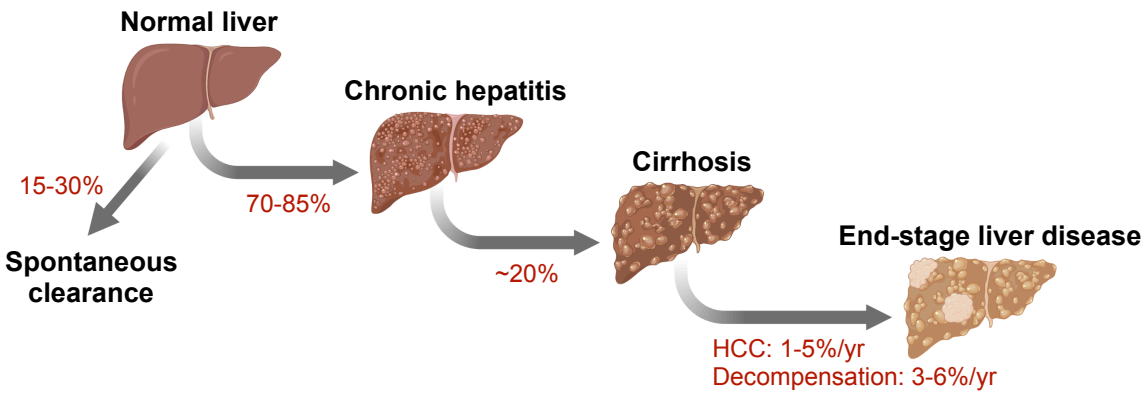

Figure 1.1. Natural history of HCV infection. About 15-30% of HCV infections are spontaneously cleared without treatment; the remaining 70-85% establish a persistent infection. Roughly 20% of patients with chronic HCV will develop liver cirrhosis, which gives patients a 1-5% annual risk of hepatocellular carcinoma (HCC) and a 3-6% annual risk of liver decompensation. This figure was generated in BioRender with data reported by Westbrook and Dusheiko, *J Hepatol*, 2014 (20).

Historically, chronic HCV infection was treated with a nearly year-long regimen of interferon (IFN) and ribavirin injections, which caused numerous adverse effects and were only partially effective (25-27). More recently, direct-acting antiviral (DAA) drugs have been developed to block viral replication by specifically targeting viral proteins (e.g. viral protease and polymerase inhibitors). These small molecules have revolutionized HCV treatment, as a 12-week course of combination DAA therapy can now cure over 94% of chronic HCV infections (28). These highly effective pan-genotypic treatments have led the World Health Organization to set the goal of global elimination of HCV by 2030 (29).

1.2 The molecular biology of HCV

1.2.1 HCV genome

Like all viruses, HCV is a molecular parasite that co-opts the molecular machinery of its host cell to reproduce itself. The HCV genome is, by itself, sufficient to establish a productive viral

infection in a permissive cell (30). This genome consists of a ~9.6 kb long uncapped, non polyadenylated single-stranded RNA that encodes a single open reading frame flanked by highly structured 5′ and 3′ untranslated regions (UTRs) (**Figure 1.2A**). Although secondary RNA structures form throughout the viral genome, the importance of the secondary structures in the viral 5′ and 3′ UTRs are the most well-characterized (reviewed in (31)) (32, 33). Briefly, the 5′ UTR contains four stem-loop (SL) structures termed SLI through SLIV (34). SLI, which is most proximal to the 5′ terminus, is dispensable for translation but is necessary for viral RNA replication, along with SLII; interestingly, it is SLI's hairpin structure, rather than its precise nucleotide sequence, that is required for genome replication (34-36). SLII through SLIV, along with the first 30 nucleotides of the core-coding sequence, make up the viral internal ribosomal entry site (IRES) (34, 37-40). In the viral 3′ UTR, the polyprotein stop codon is located in a structured hypervariable region (poorly conserved between HCV isolates), which is followed by a poly(U/UC) tract (of variable length) and a highly conserved 96-nt X-tail, consisting of three stem-loop structures (41, 42).

The viral 5′ and 3′ UTRs flank a single open reading frame that encodes a ~3,000 amino acid-long polyprotein, which is co- and post-translationally processed into ten mature viral proteins (**Figure 1.2B**). Like other members of the *Flaviviridae* family, the structural proteins (core, E1 and E2) are processed from the N-terminal end of the polyprotein, and the viral RNA-dependent RNA polymerase is the C-terminal nonstructural (NS) protein (which consist of p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (43, 44).

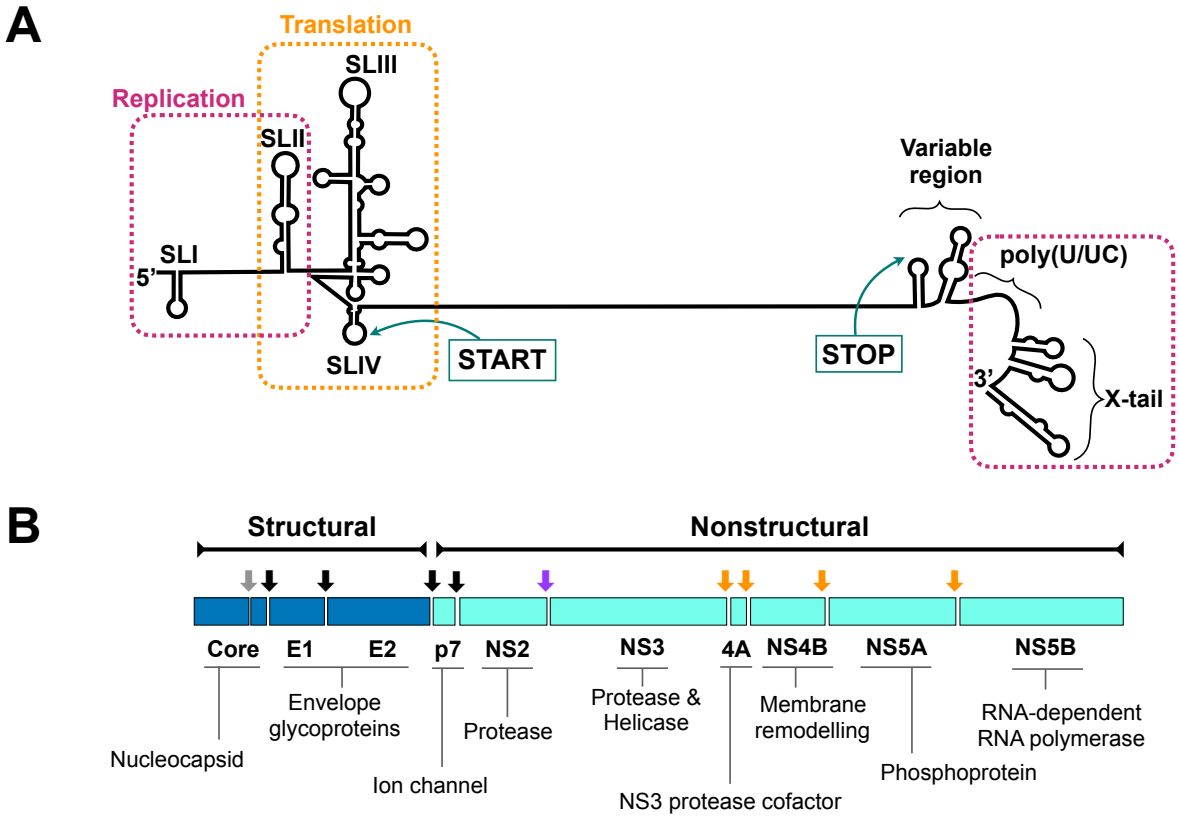

Figure 1.2. HCV genome structure and organization. (**A**) Structure of the 5′ and 3′ untranslated regions of the genome. RNA structures that play important roles in translation and RNA replication are indicated with boxes. The location of the polyprotein start and stop codons are also indicated. (**B**) Organization of the viral polyprotein, indicating the function of each of the mature viral proteins. Arrows indicate protein junctions processed by signal peptidase (black), signal peptide peptidase (grey) the NS2/NS3 protease (violet) and the NS3/4A protease (orange).

Structural proteins

HCV infectious particles are comprised of a nucleocapsid surrounded by an envelope, a lipid bilayer derived from the host ER membrane. This envelope is decorated with heterodimers of the viral glycoproteins (E1 and E2) which mediate the attachment, entry and fusion steps of the viral life cycle (45, 46). The nucleocapsid is composed of a viral genomic RNA packaged into a protein shell made of the core protein, a 21 kDa protein that directly associates with the viral RNA (47). The core protein contains three domains: a N-terminal hydrophilic domain (residues 1-117), a hydrophobic domain (residues 118-174), and a C-terminal signal peptide that is cleaved from the mature protein, but is required for orienting the E1 protein into the ER lumen during protein synthesis (48-50). In the mature core protein, the N-terminal domain mediates RNA-binding and homo-oligomerization, while the hydrophobic domain anchors core to the surface of lipid droplets (48-51) (**Figure 1.3**). In addition to its role as the capsid protein, core has been proposed to contribute to HCV pathology, since transgenic mice engineered to express core develop fatty liver disease and hepatocellular carcinoma, and exogenous core protein expression can alter cellular signalling pathways involved in lipid metabolism, cell proliferation, apoptosis, and inflammation (reviewed in (52)) (53-57).

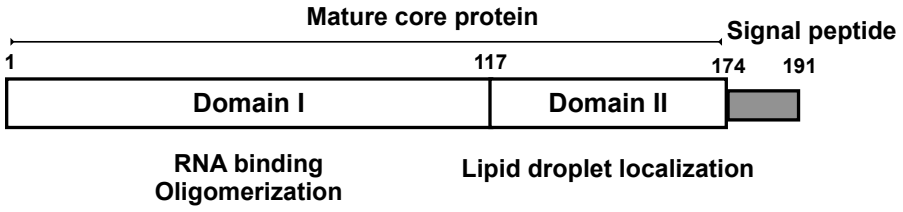

Figure 1.3. Domain organization of the core protein. After its C-terminal signal peptide (dark grey) is cleaved off by the host signal peptide peptidase, the mature core protein contains a N-terminal hydrophilic domain and a C-terminal hydrophobic domain; their associated functions are indicated below each domain.

Nonstructural proteins

The HCV nonstructural proteins — p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B — mediate the polyprotein processing, RNA replication, immune evasion, and virion assembly steps necessary for a productive HCV infection. The NS3-5B proteins form the viral replicase, responsible for viral RNA replication; while p7 and NS2 are not strictly required for RNA replication, they perform key roles in virion assembly and polyprotein processing (58, 59). Briefly, p7 is a small hydrophobic protein that participates in virion assembly and forms a viroporin ion channel to protect newly assembled virions from acidic cellular compartments (60-62). NS2 is a cysteine protease that cleaves the NS2-NS3 junction of the polyprotein and also coordinates virion assembly (63-65). NS3 has both serine protease and RNA helicase activities, and is tethered to the ER membrane through its association with its cofactor NS4A (66, 67). Through its serine protease activity, NS3 is responsible for polyprotein processing at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions and has also been implicated in the inhibition of antiviral signalling, primarily through cleavage of the cellular MAVS and TRIF proteins (68, 69). Through its RNA helicase activity, NS3 is an integral component of the HCV replication complex, where it unwinds secondary structures within the viral RNA to enable its replication (70). NS4A is a short transmembrane protein; in addition to its role as an NS3 cofactor, it has also been recently implicated in virion assembly (71). NS4B is a transmembrane protein that induces extensive ER membrane rearrangements required for replication complex formation (72). NS5A is a multifunctional, highly phosphorylated RNA-binding protein with a preference for poly(U) sequences (73). NS5A is composed of three domains connected by low complexity sequences (LCS) that are highly conserved between viral isolates: Domain I (residues 1-213)

contains an N-terminal amphipathic helix unnecessary for RNA binding, but crucial to localize NS5A to lipid membranes during the viral life cycle (73-75); meanwhile, domains II (residues 250-342) and III (residues 356-447) are intrinsically disordered and are implicated in viral replication and assembly, respectively (76, 77) (**Figure 1.4**). While no enzymatic activity has been attributed to NS5A, domains I and II are crucial for viral RNA replication and have been proposed to downregulate viral translation in a poly(U/UC) tract-dependent manner (74, 78); these domains have also been reported to interfere with cellular antiviral responses through protein-protein interactions with cellular sensors of double-stranded RNA, such as protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS) (79-81). Finally, NS5B is the viral RNA-dependent RNA polymerase, responsible for viral RNA synthesis (82).

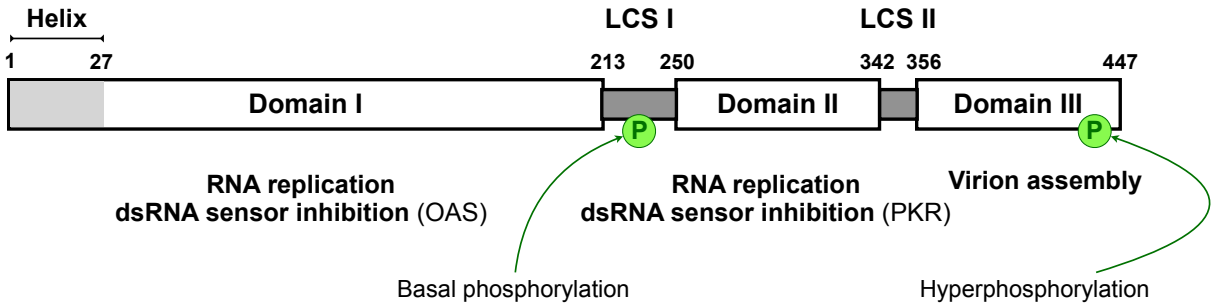

Figure 1.4. Domain organization of the NS5A protein. NS5A contains three domains, separated by low-complexity sequences (LCS); functions associated with each region of the protein are indicated below each domain. The N-terminal amphipathic helix is indicated with grey shading in domain I. The approximate locations of phosphorylation sites in LCS I and in domain III have been indicated with green P symbols and arrows. Adapted from Tellinghuisen *et al. J Biol Chem*, 2004 (73)

1.2.2 HCV life cycle

Viral entry

To complete its viral life cycle, HCV must first enter a permissive liver cell. This multi-step process begins with the adsorption of the viral particle to the cell surface, followed by receptor engagement by the viral glycoproteins, internalization by clathrin-mediated endocytosis, and the eventual release of the viral genome into the cytosol (**Figure 1.5**). In the bloodstream of infected individuals, HCV particles circulate in close association with low density and very low density lipoproteins (LDL and VLDL) to form buoyant lipo-viro-particles (**Figure 1.6**) (84). These associations facilitate the initial interactions between HCV particles and host cells, as the apolipoproteins incorporated in these lipo-viro-particles interact with the scavenger receptor class B type I (SR-B1) high density lipoprotein receptor, the low density lipoprotein receptor (LDLR), and heparan sulfate proteoglycans on the cell surface (85-89). These interactions enable the viral E2 glycoprotein to interact with SR-B1, which facilitates further E2 interactions with CD81, the major cellular receptor for HCV (86, 90, 91). CD81 recruits the viral particle to tight junctions, where further interactions with multiple entry factors — including claudin-1 (CLDN1), occludin (OCLN), and Niemann-Pick C1-Like 1 (NPC1L1) — ultimately lead to clathrin-mediated endocytosis of the viral particle (92-97). In the last step of viral entry, the viral genome is released into the cytoplasm after a decrease in endosomal pH causes conformational changes in E1 and E2 that promote fusion of the host and viral membranes (98, 99). That HCV entry requires the concurrent expression of numerous host proteins partially explains this virus' specific tropism for human liver cells (reviewed in (100)).

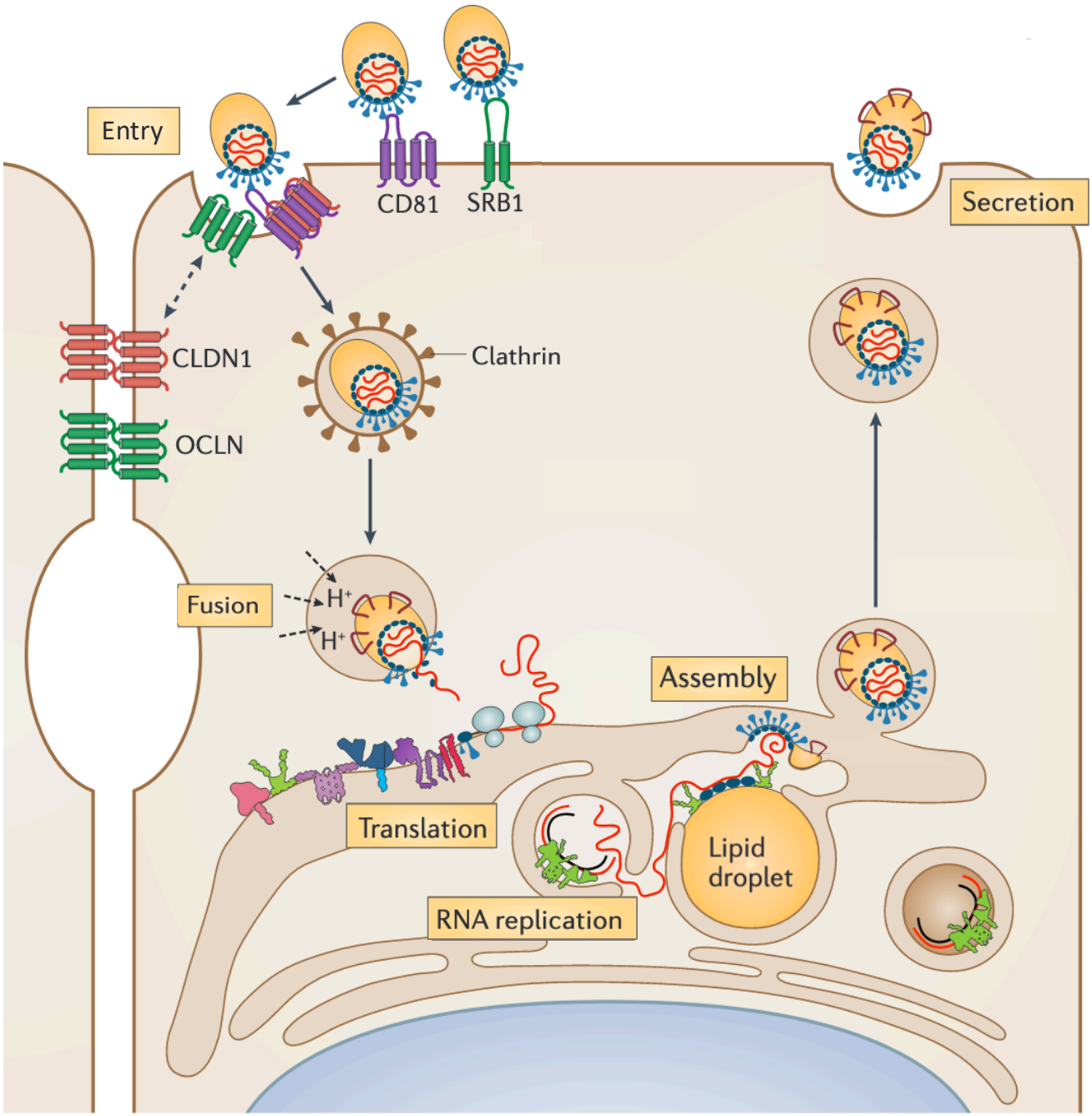
Entry
CD81
SRB1
Secretion
CLDN1
Clathrin
OCLN
Fusion
H+
H+
Assembly
Translation
Lipid
droplet
RNA replication

Figure 1.5. **The HCV life cycle**. To generate a viral progeny, HCV (1) engages with cellular receptors (which include SRB1, CD81, CLDN1 and OCLN) and enters the cell by clathrin-mediated endocytosis, after which (2) membrane fusion and uncoating releases the viral genome into the cytoplasm. (3) The viral RNA then undergoes IRES-mediated translation on ER-associated ribosomes, resulting in the production of a viral polyprotein that is co- and post-translationally processed into ten mature viral proteins. (4) The positive-sense genomic RNA is then used as a template by the replicase complex to synthesize a complementary antigenome replicative intermediate, which in turn is used to synthesize new positive-strand genomic RNAs. (5) Nascent viral genomes either engage in new rounds of translation, or are shuttled to the core protein at the surface of lipid droplets to undergo packaging and assembly into new viral particles. (6) Nucleocapsids are assembled and simultaneously enveloped by budding through the ER membrane. Finally, (7) mature virions egress through the Golgi network and are secreted from the cell. Adapted from Neufeldt CJ *et al*., *Nature Reviews Microbiology,* 2018 (83).

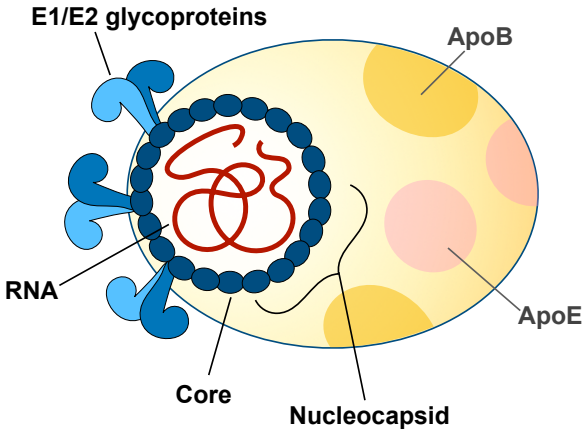

Figure 1.6. Structure of HCV lipo-viro-particles. The viral genome is packaged in a nucleocapsid composed of the core protein and the viral RNA. The nucleocapsid is enveloped by an ER-derived lipid layer, decorated with E1/E2 glycoproteins and is tightly associated with host lipids and lipoproteins, including ApoA and ApoE. Adapted from Piver *et al. Gut,* 2017 (101).

Translation and polyprotein processing

Once the viral genome is released into the cytoplasm, it can be directly translated by ER-associated ribosomes, and the synthesized viral polyprotein is co- and post-translationally processed into the ten mature viral proteins. HCV IRES-mediated translation initiation is a relatively simple process, compared to canonical cap-mediated translation. Briefly, while cap-mediated translation requires numerous translation initiation factors (eIFs) and scanning of the 40S ribosomal subunit to locate the initiation codon before assembling the 80S ribosome, the HCV IRES requires very few translation initiation factors (i.e. eIF3 and eIF2 are sufficient), and it assembles the ribosomal subunits directly over the initiation codon, such that no scanning is necessary for translation initiation (reviewed in (102)). The HCV IRES directly recruits these components: SLIII binds to eIF3 and the 40S ribosomal subunit, while SLII correctly orients the 40S subunit to unfold SLIV (revealing the initiation codon), which subsequently recruits the 60S ribosomal subunit (reviewed in (103)). While these are the minimal requirements for viral

translation, HCV IRES activity can be enhanced by IRES *trans*-acting factors (ITAFs), which are proteins that do not participate in canonical translation initiation yet can enhance viral translation, such as the lupus autoantigen (La), the NS1-associated protein 1 (NSAP1, also known as hnRNP Q), and the insulin growth factor 2 binding protein 1 (IGF2BP1, also called IMP-1), among others (103-106). While the precise mechanism of action of most ITAFs has not yet been fully elucidated, they have been proposed to promote translation by helping melt SLIV to reveal the initiation codon, by correctly orienting the ribosomal 40S and 60S subunits, or by facilitating ribosome recycling over multiple rounds of translation by circularizing the viral genome (107-109).

Finally, the viral polyprotein is co- and post-translationally processed into the ten mature viral proteins, as host signal peptidase and signal peptide peptidase cleave the boundaries between the structural proteins, and the viral NS2 and NS3 proteases cleave the junctions between the nonstructural proteins (**Figure 1.2B**) (63, 66, 110-113). Being processed from a common precursor facilitates interactions between proteins encoded by neighbouring genes and, by extension, increases the efficiency of viral processes choreographed by viral protein complexes (such as viral RNA replication and virion assembly) (64).

RNA replication

To generate new copies of itself, the viral genome must serve as a template to synthesize a negative-sense replication intermediate that is, in turn, used to generate positive-sense genomic progeny viral RNAs (**Figure 1.5**). This process takes place in replication organelles, double-membrane vesicles derived from the ER membrane by the NS4B and NS5A proteins (72, 114).

These double-membrane vesicles help shield the viral proteins and RNAs from cellular proteases, nucleases, and sensors of double-stranded RNA (reviewed in (83)) (115, 116). Viral replicase complexes, which are formed of the NS3 through NS5B proteins, accumulate within these replication organelles (58, 117). Within the replicase complex, the NS3 helicase unwinds secondary structures in the viral genome, NS5A binds the viral genome's poly(U/UC) tract and fine-tunes NS5B's affinity for RNA templates, and NS5B synthesizes an RNA complementary to its RNA template (70, 82, 118-121). Thus, from a positive-sense genome template, the replicase produces a full-length, negative-sense (antigenome) replication intermediate that is, in turn, used as a template to synthesize positive-sense genomic RNAs, a process that yields 5 to 10 genomic RNAs for every negative-sense replication intermediate (116). Since NS5B is an error-prone polymerase that introduces an error every 10^3-10^6 nucleotides and lacks proofreading activity, the vast majority of nascent viral genomic RNAs will contain one substitution compared to the original genomic template RNA (122, 123).

As HCV is a single-stranded positive-sense RNA virus, the viral genome itself must serve as a template for translation, replication and packaging. Since these processes are mutually exclusive (i.e. ribosomes travel over the RNA in a 5′ to 3′ direction, while NS5B polymerization occurs in the 3′ to 5′ direction), a mechanism must exist to switch off translation so that a given RNA can be used as a template for replication. However, this switch from translation to replication during HCV infection remains unclear. Nonetheless, there is some evidence that this is at least partly mediated by NS5A, as its binding to the poly(U/UC) tract is already known to be the main driver of viral replication complex assembly, and interactions between NS5A and the poly(U/UC) tract have been demonstrated to be important for 3′ UTR-mediated translational inhibition (74, 78).

Virion assembly and egress

Once progeny viral genomic RNAs have been synthesized, they can either re-engage with the translation machinery to complete more rounds of translation and RNA replication, or they may be packaged into new virions. Viral genome packaging and assembly takes place on the surface the ER membrane in close association with lipid droplets (LDs, **Figure 1.5**). Assembly is essentially a two-step process that consists of a transfer of the viral RNA to the core protein followed by the simultaneous formation and envelopment of the nucleocapsid. Briefly, during viral infection, the core protein causes cellular LDs to localize in close proximity to viral replication complexes (124, 125). These LDs serve as storage facilities, where core protein accumulates until it becomes associated with a viral genome to initiate the packaging process (65). Viral RNA delivery to core is mediated by the NS5A protein and is dependent on the phosphorylation of specific residues in domain III of NS5A (126-128). Once core proteins are associated with a viral genome, they are displaced by the NS2 and p7 proteins from the LD to the sites of virion assembly on the adjacent ER membrane (65, 124). There, the NS2 and NS4A proteins interact with core, NS3, and the E1/E2 glycoproteins to coordinate core oligomerization and nucleocapsid formation, as well as acquisition of the envelope as the nucleocapsid buds into the ER lumen (64, 71, 129, 130). It is not yet entirely clear how HCV regulates whether a given viral genomic RNA will be engaged in translation/replication or be destined for packaging into a new virion. Still, it has been proposed that this process may be influenced by NS5A hyperphosphorylation, by chemical modifications of the viral RNA (e.g. adenosine methylation into m^6A), and/or by select host proteins, which will be discussed in more detail in Chapter 4 of this thesis (65, 131-134).

Once virions bud into the ER lumen, they are fully infectious — but they still need to leave the infected cell and return to the bloodstream. To do so, HCV virions follow the existing very low and low density lipoprotein (VLDL and LVL) secretion pathways through the ER and Golgi; during this egress, the virions incorporate cellular apolipoproteins, including ApoB and ApoE (reviewed in (135)) (85, 101, 136, 137). Accordingly, the HCV particles that emerge from cells are relatively buoyant lipo-viro-particles, with a lipid composition that closely resembles (V)LDL (**Figure 1.6**) (84).

Superinfection exclusion

To establish an infection, a newly secreted virion must find a naïve cell to infect since cells that harbour a pre-existing HCV infection are refractory to subsequent HCV infection, a phenomenon known as superinfection exclusion. This block has been proposed to occur at two different steps of the viral life cycle: HCV infection leads to the down-regulation of its entry factors CLDN1, OCLN and NPC1L1, and replicating viral RNAs have also been reported to further block a post-entry step of a secondary virus' life cycle, at the level of viral translation or RNA replication (95, 138-141). This superinfection exclusion helps explain why recombinant HCV genomes are rare, as their generation would require viruses from two different strains of HCV to infect the same cell at the same time — an unlikely combination of events (142)

1.2.3 HCV cell culture systems

The details of the viral life cycle described in the preceding sections is the result of years of fundamental research. This was no small feat, as HCV is notoriously difficult to study in the laboratory. Clinical isolates do not generally replicate in cell culture, and most liver-derived cell

lines do not support cell culture-adapted HCV replication. The exception is Huh-7 cells (and derivatives thereof), a human hepatocellular carcinoma cell line initially isolated from a 57-year-old Japanese man (30, 143, 144). The more permissive and commonly used Huh-7.5 cell line was derived from Huh-7 cells that had acquired an inactivating mutation in the retinoic acid inducible gene I (RIG-I) cytosolic sensor of viral RNA (145, 146). These cells have been key to develop HCV cell culture models, including those used in this thesis to study viral RNA replication, entry, and the full viral life cycle.

In 1999, ten years after HCV was first cloned, Lohmann *et al.* developed the first reliable and efficient viral RNA replication system in cell culture: a subgenomic replicon (58). Instead of the full-length viral genome, this replicon consisted of a bicistronic RNA flanked by the HCV 5′ and 3′ UTRs, where the HCV IRES drove the expression of a reporter or selection gene (such as a luciferase or antibiotic resistance gene), while an internal encephalomyocarditis virus (EMCV) IRES drove the expression of the HCV NS3 through NS5B genes (**Figure 1.7A**). When introduced into permissive cells by transfection or electroporation, this replicon RNA can undergo translation and RNA replication, but is unable to form infectious particles due to its lack of structural proteins. This system has been crucial to develop direct-acting antivirals that inhibit HCV RNA replication (reviewed in (147)). However, the replicon model has some significant limitations. Firstly, the EMCV IRES does not have the same translation efficiency as the HCV IRES; this has motivated the development of monocistronic HCV replicons, where translation of the viral NS proteins is mediated by the HCV IRES (148-150). Furthermore, while subgenomic replicons can undergo the translation and RNA replication steps of the viral life cycle, they are not suitable for studies of viral entry or assembly. These limitations led to the development of

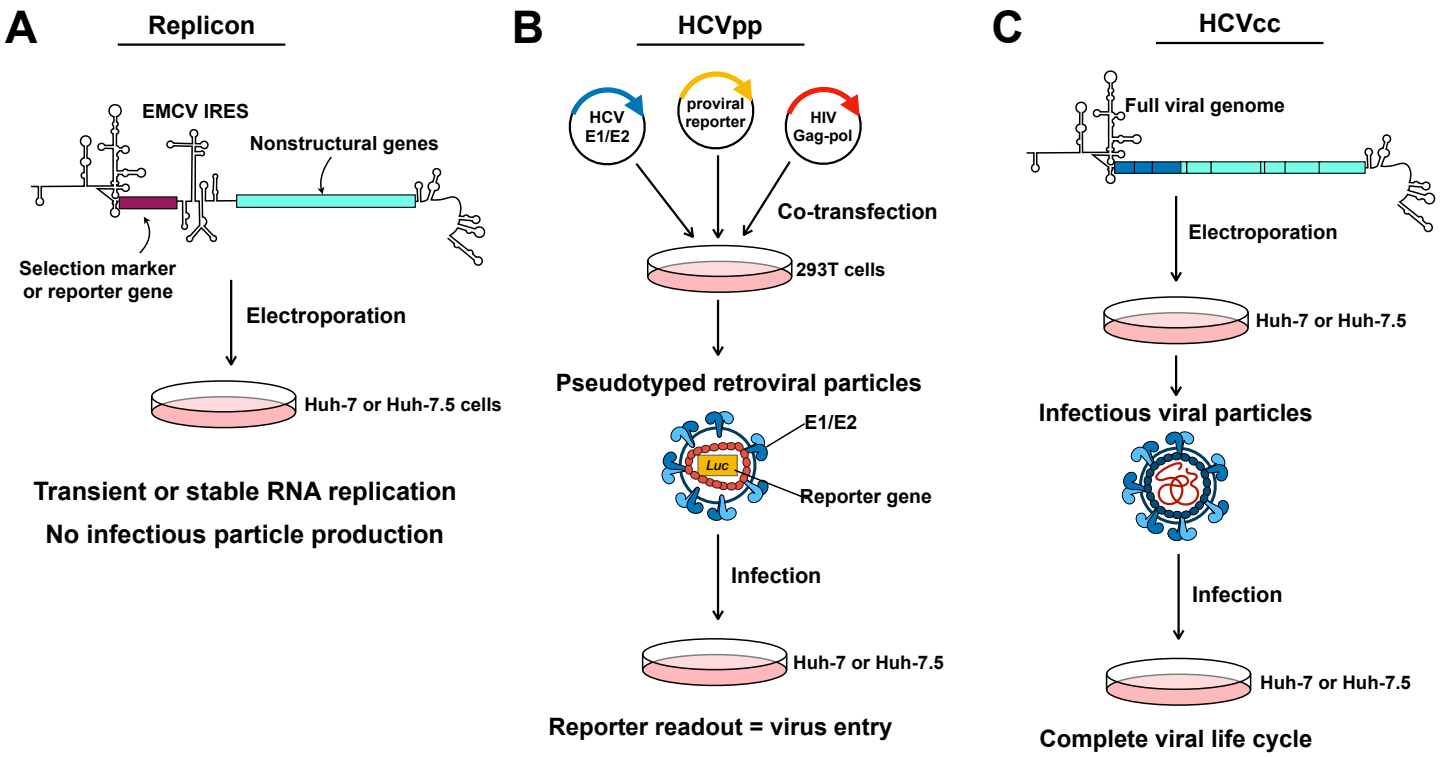

Figure 1.7. Cell culture systems for the study of HCV. (A) The subgenomic replicon system consists, at a minimum, of the viral NS3 through NS5B genes flanked by the viral 5′ and 3′ UTRs; early versions of this system consisted of a bicistronic RNA, with a selection marker expressed by the HCV IRES and the NS3-5B replicase expressed by an internal EMCV IRES. Replicons containing a selectable marker could be used to generate cell lines that stably replicate the viral replicon RNA. (**B**) The HCV pseudoparticle (HCVpp) system consists of HCV E1 and E2 glycoprotein-pseudotyped retroviral particles expressing a reporter gene (e.g. luciferase) that are generated from a producer cell line (typically 293T cells). HCVpp entry into susceptible cells is assessed by reporter gene activity. (**C**) Cell culture-derived HCV (HCVcc) is a fully infectious model of HCV infection. In this system, full-length viral RNAs are *in vitro* transcribed from a cDNA template; once these viral RNAs are introduced into cells by electroporation or transfection, they produce infectious viral particles that can complete the entire HCV life cycle.

alternative systems to study viral entry as well as the entire HCV life cycle in cell culture.

By 2003, the HCV pseudoparticle (HCVpp) system was developed to specifically assess HCV entry. HCVpp consist of lentiviral particles containing a reporter gene, such as green fluorescent protein (GFP) or luciferase, pseudotyped with the HCV E1/E2 glycoproteins. Human embryonic kidney (293T) cells are typically used to produce HCVpp, since this process requires components encoded on three separate plasmids and this cell type has a very high transfection efficiency (**Figure 1.7B**) (46, 151). However, since they are produced in a non-hepatic cell line, HCVpp are not associated with apolipoproteins and may display a different E1/E2 glycosylation pattern than authentic HCV particles. Nonetheless, HCVpp have been an extremely useful tool to study HCV entry, identify viral entry receptors and co-factors, and assess viral entry inhibitors (reviewed in (152)).

The first fully infectious HCV cell culture (HCVcc) system was established in 2005. This HCVcc system is based on the consensus sequence of the Japanese Fulminant Hepatitis-1 (JFH-1) isolate (genotype 2a strain), which was isolated from a patient that developed hepatic encephalopathy during the acute phase of HCV infection (153). When introduced into permissive cells, the JFH-1 genome can produce infectious viral particles and complete the entire HCV life cycle (**Figure 1.7C**) (143, 154). Today, HCVcc are commonly generated from chimeric viruses or cell culture-adapted variants that produce greater yields of infectious particles than the parental strain, such as JFH-1_T (which contains adaptive mutations in E2, p7 and NS2) and Jc1 (a J6/JFH-1 chimera; the coding region that spans core through the first transmembrane domain of NS2 was replaced by the J6 sequence) (155, 156). HCVcc have been crucial not only to improve our understanding of the late stages of the HCV life cycle, but also to identify lipid-dependent

HCV entry factors that could not interact with HCVpp (84, 152, 157).

1.2.4 HCV and microRNA-122

A major determinant of HCV tropism is the presence of microRNA-122 (miR-122), a highly abundant, liver-specific human microRNA (miRNA). MiRNAs are short, ~22 nt-long cellular RNAs that post-transcriptionally regulate gene expression as part of the miRNA-induced silencing complex (miRISC), which also contains an Argonaute (Ago) protein. Within miRISC, the miRNA's "seed sequence" (nucleotides 2-8), anneals to complementary messenger RNA (mRNA) sequences and thus directs the miRISC to target mRNAs (reviewed in (158)) (159-161). Interactions between miRISC and target mRNAs typically results in post-transcriptional gene silencing through translational suppression, accelerated deadenylation and mRNA decay (reviewed in (162)).

MiR-122 is a liver-specific miRNA that accounts for roughly 70% of the total microRNA content of the liver, where it normally regulates cholesterol and fatty acid metabolism, and also acts as a tumour suppressor (reviewed in (163)) (164-168). As a result of its anti-oncogenic effects, very few immortalized hepatic cell lines retain miR-122 expression. The exception is the Huh-7 cell line and its derivatives, although Huh-7 cells express miR-122 to a much lower extent than normal hepatic tissue (i.e. ~16,000 miR-122 molecules per Huh-7 cell, compared to ~135,000 miR-122 molecules per hepatocyte) (165). MiR-122 has an unusual interaction with the HCV genome, where it binds two tandem sites in the 5′ UTR. This is an unusual miRNA-target RNA interaction for three reasons: 1) while miRNAs typically interact with their targets in the 3′ UTR, miR-122 binds to the 5′ UTR of the HCV genome; 2) two miR-122-binding sites occur in close proximity, separated by only a single nucleotide; and 3) miR-122 interactions with the HCV

genome promote HCV RNA accumulation, instead of its decay (**Figure 1.8A**) (169). Recent studies have revealed that miR-122 plays at least three roles in the HCV life cycle (**Figure 1.8B**) (170). First, upon entry into the cell, the HCV 5′ UTR is thought to take on an energetically favourable conformation, termed SLII^{alt}. In this conformation, the first miR-122 binding site (site 1) is base-paired, while the second miR-122 binding site (site 2) is accessible. Thus, a Ago:miR-122 complex is initially recruited to site 2 and serves as an RNA chaperone (or "riboswitch") to re-fold the viral RNA into the functional SLII conformation. This refolding of the viral RNA reveals the site 1 miR-122 binding site, close to the 5′ terminus of the HCV genome (170-172). Second, recruitment of a second Ago:miR-122 complex to site 1 stabilizes the viral RNA by protecting the 5′ terminus (a triphosphate moiety) from recognition by cellular pyrophosphatases (DOM3Z and DUSP11), and subsequent 5′-mediated decay by exoribonucleases (Xrn1/2) (170, 173-177). The two miR-122 binding sites are in very close proximity; to accommodate the Ago:miR-122 complex at site 1, the Ago:miR-122 complex at site 2 relaxes its base-pairing interactions, but is likely further stabilized through contacts with the HCV IRES at SLII (170). Third, Ago:miR-122 interactions with the viral 5′ UTR promote HCV IRES-mediated translation, and the contacts between SLII and the Ago protein at site 2 presumably further stabilize the IRES (170, 178-180). Thus, miR-122 has been established to play important roles in the early stages of the HCV life cycle. However, whether miR-122 has additional roles in the viral life cycle, including in the switch from translation to viral RNA replication, replication complex biogenesis, or localization of the viral RNA, remains an active area of investigation.

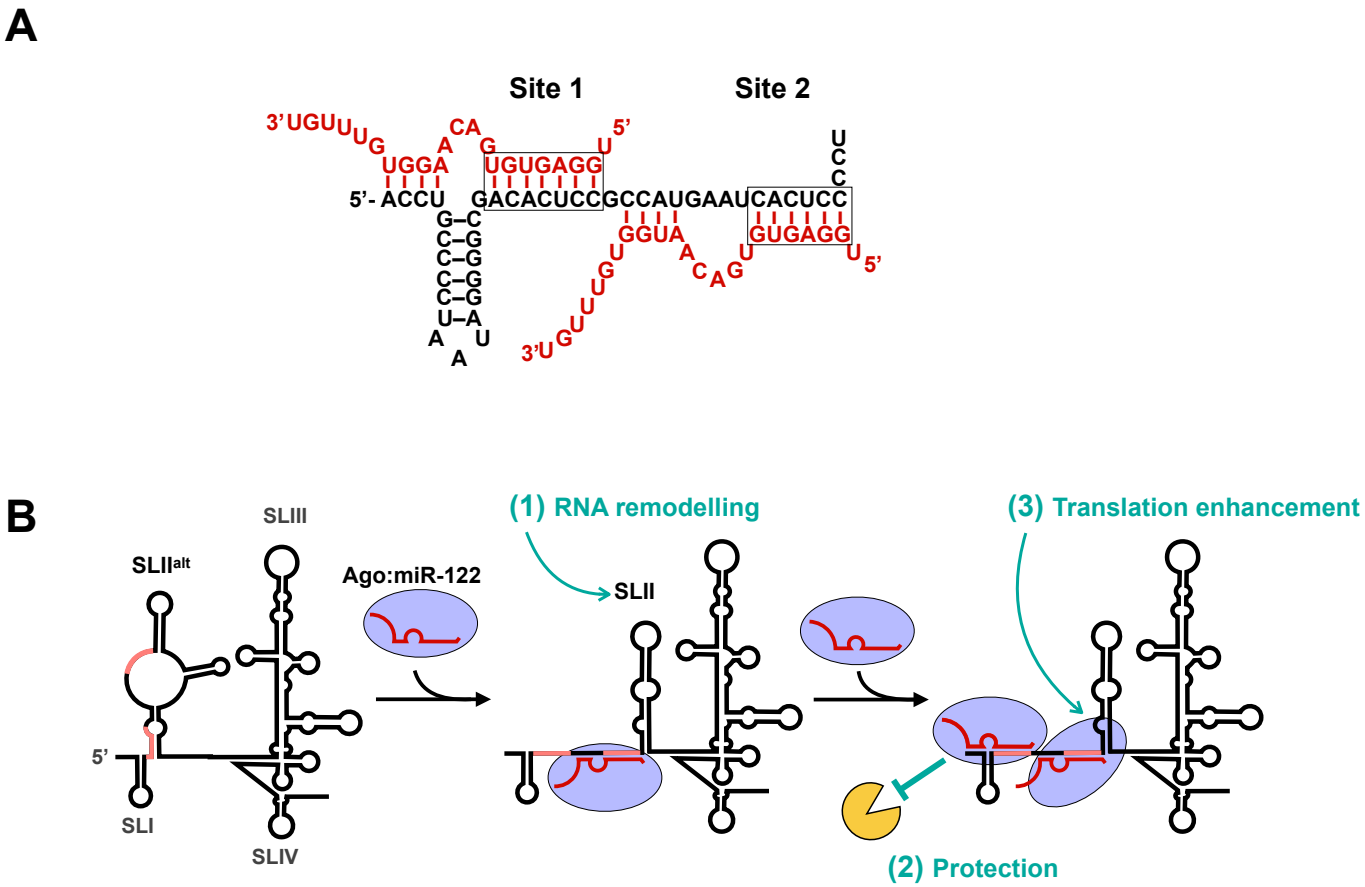

Figure 1.8. The interaction between miR-122 and HCV. (A) Model of miR-122 (red) binding to two sites at the 5′ end of the HCV genome. The miR-122 seed binding sites are indicated in boxes. Adapted from Machlin *et al.*, *PNAS*, 2011 (173) **(B)** Stepwise model of miR-122 binding to the HCV 5′ UTR, with the two miR-122 seed binding sites indicated in pink. Recent studies indicate miR-122 has at least three roles in the HCV life cycle: 1) Ago:miR-122 complex binding to site 2 acts as an RNA chaperone that destabilizes the SLII^{alt} conformation and reveals the site 1 seed binding site (RNA remodelling); 2) Ago:miR-122 binding to site 1 protects the 5′ terminus of the viral genome from degradation by cellular enzymes; and 3) these interactions enhance HCV IRES-mediated translation by further stabilizing the functional IRES structure. Adapted from Chahal *et al, Nucleic Acids Research*, 2019 (170).

1.3 Poly(rC)-binding proteins

In mammals, the poly(rC)-binding protein (PCBP) family contains five members, separated into two branches: the heterogenous nuclear ribonucloprotein (hnRNP) K branch, which contains its eponymous member (i.e. hnRNP K), and the hnRNP E branch, which includes PCBP1, PCBP2, PCBP3 and PCBP4 (181, 182). All five PCBPs are the result of ancient gene duplication events, the most recent of which occurred around 400-500 million years ago when a *PCBP2* splicing variant was retrotransposed and resulted in the *PCBP1* gene (**Figure 1.9A**) (181). All PCBPs share a common organization of three RNA-binding domains – called hnRNP K homologous (KH) domains – arranged as two N-terminal domains located in close proximity to each other, separated from the C-terminal KH domain by a linker region of no known structure (**Figure 1.9B**). The length and composition of the linker region varies between PCBPs.

The PCBP KH domains are formed of three β-sheets and three α-helices arranged in a βααββα conformation, with the RNA-binding pocket found within the core KH motif (βααβ) (**Figure 1.9C**) (183). The two alpha helices of the core motif are linked by a conserved GxxG loop, composed of two neutral-to-positively charged amino acids sandwiched between two flexible glycine residues (183-185). This GxxG loop is crucial for KH domain binding to nucleic acids, as its electrostatic interactions with the phosphate backbone of its target RNA are required to correctly orient the nucleotides within the nucleotide-binding pocket (184-186). The binding preference of a KH domain is mediated by a variable loop, just downstream of the core KH motif, that forms hydrogen bonds with the four nucleotide bases within the nucleotide-binding pocket (186). In PCBPs, the optimal KH domain binding site consists of four consecutive cytidines, but

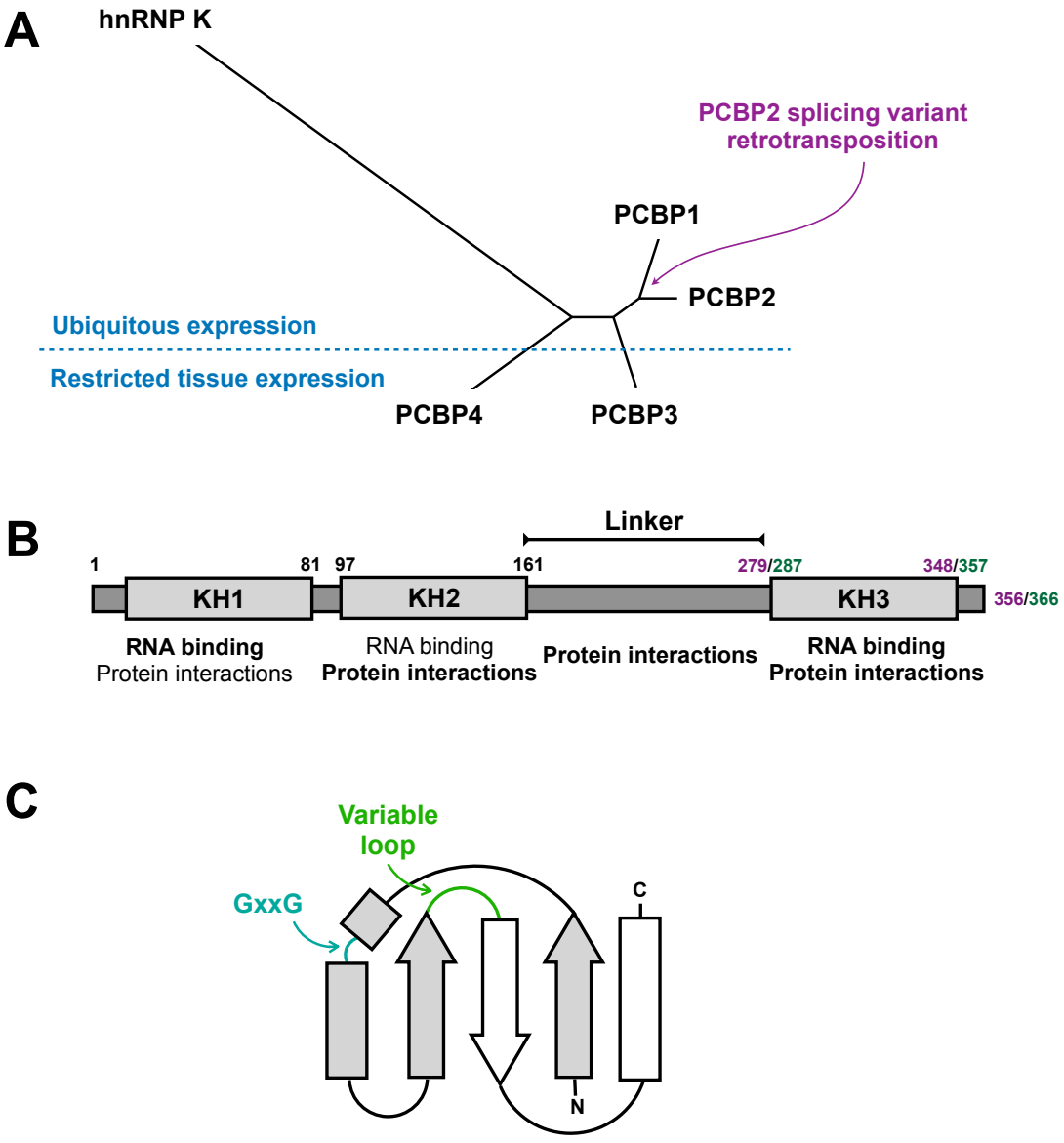

Figure 1.9. Relationships and organization of PCBPs. (A) Phylogenetic relationships of the five human PCBPs; branch length is proportional to the amino acid sequence divergence. **(B)** Domain organization of all PCBPs and indication of their functions. The length and composition of the linker region (between KH2 and KH3) varies between PCBPs. **(C)** KH domain topology. The GxxG loop and the variable loop that determines nucleotide binding affinity are labelled; the core KH motif is shaded in grey. Adapted from Pereira and Lupas, *Bioinformatics,* 2018 (183).

uridine substitutions at the first or fourth position in the binding motif can be tolerated (186, 187). While hnRNP K, PCBP1 and PCBP2 are ubiquitously and abundantly expressed, PCBP3 and PCBP4 expression is restricted to a select set of tissues that does not include the liver (188). Thus, the focus of this thesis project was on PCBP1 and PCBP2.

1.3.2 Cellular roles of PCBPs

PCBPs fine-tune cellular gene expression, both at the RNA (post-transcriptional) and protein (post-translational) level. It is difficult to generalize about the effects of PCBPs on cell function, as they can influence components of numerous cellular pathways (including those related to cell viability, cell cycle progression, and antiviral signalling), which result in variable outcomes depending on the specific cellular context in question (189-194). Generally, PCBPs help maintain homeostasis; thus, it is not surprising that their dysregulation can stall cell cycle progression, impair tissue development, or result in tumor development or progression, in various contexts (195-201). Below, I discuss some of the established roles for PCBPs in maintenance of cellular homeostasis through their RNA-binding and protein-binding activities. Additionally, I provide a brief summary of some of their interactions with RNA viruses, including HCV, which served as the rationale for the research carried out herein.

RNA binding activities

Each PCBP can bind hundreds of cellular transcripts (202-205). Most of these targets are shared between PCBPs, but some are unique; for example, both PCBP1 and PCBP2 bind their own mRNA transcript (i.e. PCBP1 binds to the PCBP1 mRNA, while PCBP2 binds to the PCBP2 mRNA), but they do not interact with to each other's mRNAs (202, 203, 206). PCBP1 or PCBP2

binding can have markedly different effects on the target transcript's stability and/or translation, depending on the precise molecular context in question (summarized below and in **Figure 1.10**).

To post-transcriptionally enhance the expression of a target transcript, PCBPs can increase the mRNA's half-life and/or stimulate its translation. PCBP binding has been shown to stabilize interactions between the poly(A)-binding protein (PABP) and the poly(A)-tail; through this mechanism, PCBPs stabilize mRNAs such as *α-globin*, *collagen α1(I)*, and *STAT2* (**Figure 1.10A**) (192-194, 207). Alternatively, PCBPs can enhance translation without altering transcript stability. Since PCBPs are not strictly necessary for canonical cap-mediated translation, the precise molecular mechanisms by which their binding to any region of an mRNA can stimulate translation are not entirely clear; still, PCBP binding to either the 5′ or 3′ UTR (such as the *μ-opioid receptor* and *tyrosine hydroxylase* mRNAs, respectively) has been shown to specifically enhance protein expression without altering the mRNA's stability (206, 208, 209) **(Figure 1.10B**). When it comes to transcripts containing cellular IRESes, which are typically found in mRNAs that need to be expressed under conditions of cell stress or mitosis, PCBP binding can enhance their IRES-mediated translation by acting as an RNA chaperone and altering the IRES' conformation to enable ribosome recruitment (such as when PCBP1, PCBP2 and hnRNP K all promote *c-myc* IRES activity; **Figure 1.10C**) (210-214).

Conversely, PCBP binding has also been shown to silence its RNA targets, which can be accomplished by destabilizing the target RNA or otherwise blocking its translation. PCBP binding to the 3′ UTR of some mRNAs has been shown to result in their destabilization (e.g. *CDK1N1A* mRNA), but the precise molecular mechanism(s) of this regulation have yet to be elucidated (**Figure 1.10D**) (195, 215). Alternatively, PCBPs can inhibit translation without

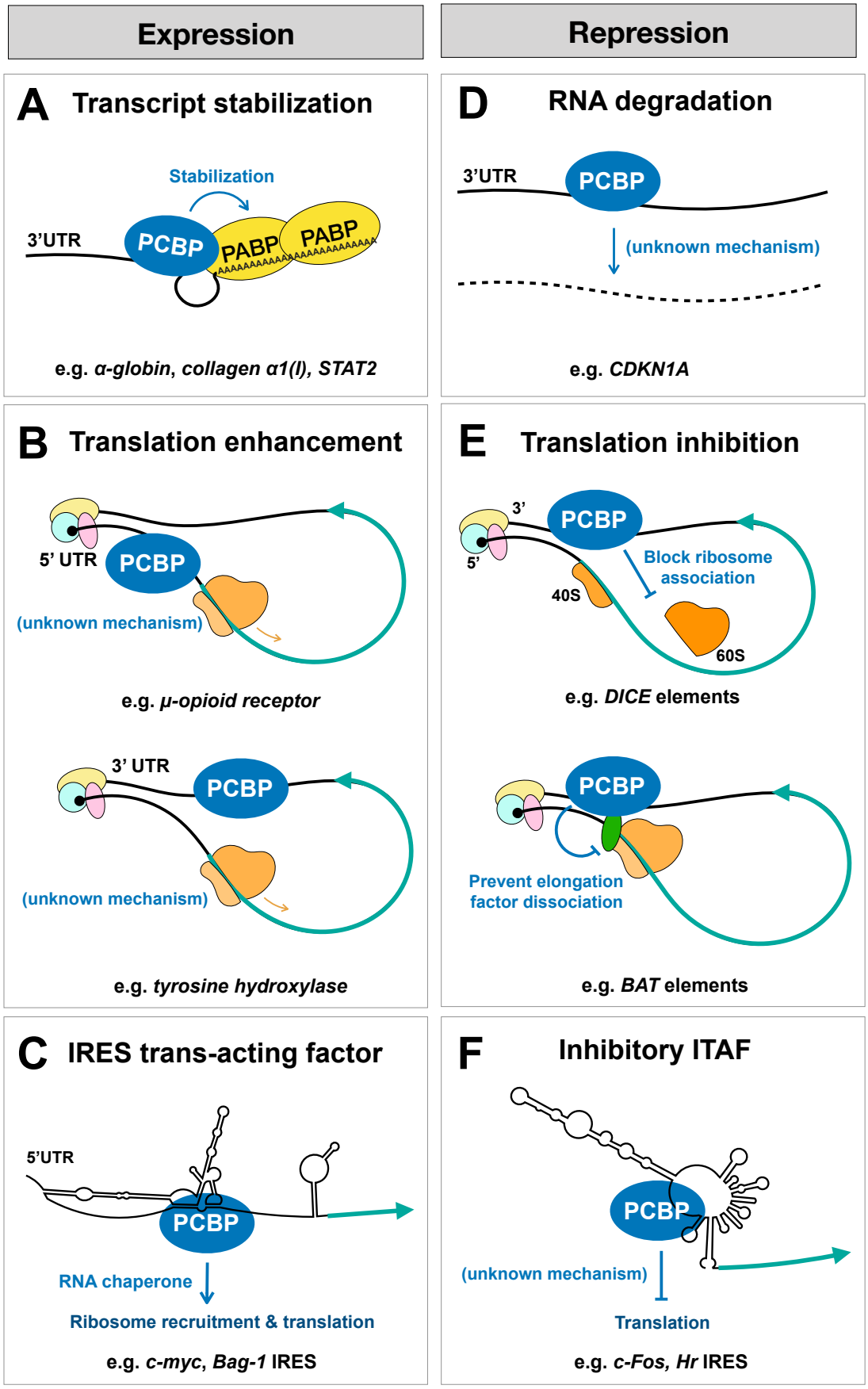
Expression
Repression
A Transcript stabilization
Stabilization
3'UTR
PCBP
PABP
PABP
e.g. α-globin, collagen α1(I), STAT2
D RNA degradation
3'UTR
PCBP
(unknown mechanism)
e.g. CDKN1A
B Translation enhancement
5' UTR
PCBP
(unknown mechanism)
e.g. μ-opioid receptor
3' UTR
PCBP
(unknown mechanism)
e.g. tyrosine hydroxylase
E Translation inhibition
3'
PCBP
5'
Block ribosome association
40S
60S
e.g. DICE elements
PCBP
Prevent elongation factor dissociation
e.g. BAT elements
C IRES trans-acting factor
5'UTR
PCBP
RNA chaperone
Ribosome recruitment & translation
e.g. c-myc, Bag-1 IRES
F Inhibitory ITAF
PCBP
(unknown mechanism)
Translation
e.g. c-Fos, Hr IRES

Figure 1.10. Mechanisms of post-transcriptional gene regulation employed by PCBPs. To enhance the expression of their target transcripts, PCBPs can employ the following mechanisms: **(A) Transcript stabilization**; PCBPs secure the poly(A)-binding protein (PABP) over a mRNA's polyadenylated tail. **(B) Cap-mediated translation enhancement**; PCBPs bind to the 5′ UTR or 3′ UTR of a target transcript, and enhance cap-mediated translation through unknown mechanisms. **(C) Activation of IRES activity**; PCBPs can act as IRES trans-acting factors, where their binding alters the secondary structure of select cellular IRESs to enable ribosome recruitment. To repress the expression of target transcripts, PCBPs can employ the following mechanisms: **(D) Transcript destabilization**; PCBP binding to a 3' UTR can lead to the degradation of the target RNA through unknown mechanisms. **(E) Cap-mediated translation inhibition**; PCBPs can prevent ribosome assembly, or block polypeptide elongation by preventing the dissociation of elongation factors from the ribosome. **(F) Inhibition of IRES activity**; PCBP binding can inhibit cellular IRES activity, though still-undescribed molecular mechanisms.

affecting RNA stability. The precise mechanism involved depends on the specific regulatory element bound by PCBPs; in some cases, PCBP binding to regulatory sites in the 3′ UTR prevents ribosome assembly over the initiation codon (such as when PCBP1 binds *DICE* elements in the *15-lipoxygenase* mRNA); in other cases, this binding blocks polypeptide elongation by anchoring translation elongation factors in place (e.g. PCBP1 binding to *BAT* elements prevents eEF1A1 release from ribosomes) (216-218). PCBP2 binding to 5′ UTRs has also been reported to inhibit both cap-mediated and cellular IRES-mediated translation (specifically, it is a negative ITAF for the *c-Fos* and *Hr* IRESes, **Figure 1.10F**), but the precise mechanism(s) involved remain unclear (198, 200, 219-221). Since PCBPs have been reported to be a dynamic constituent of a subset of stress granules and processing bodies (P bodies), membrane-less cytoplasmic organelles that mediate mRNA storage and/or turnover, it seems plausible that they could shuttle their mRNA targets to these cytoplasmic organelles for storage or decay (222, 223).

Protein interactions

Although PCBPs are primarily known for their post-transcriptional regulation of target RNAs, they have also been reported to perform functions that do not involve binding RNA, specifically by taking on roles as protein platforms or as iron chaperones. For example, either PCBP1 or PCBP2 can, via their linker region, simultaneously bind to mitochondrial antiviral signalling (MAVS) protein and to the AIP4 ubiquitin ligase; these interactions facilitate MAVS' ubiquitination and subsequent proteasomal degradation (224-226). Since MAVS is a key signal transduction protein that leads to interferon (IFN) induction after viral RNA is detected by RIG-

I-like receptors (reviewed in (227)), PCBPs can thus attenuate antiviral signalling.

As for their iron chaperone activity, PCBPs have been shown to directly bind ferrous ions (Fe^{2+}) at a micromolar affinity, and to deliver Fe^{2+} from the iron import protein DMT1 to the intracellular iron storage protein ferritin and to the iron export protein ferroportin 1 (228-231). Interestingly, these iron-binding activities are mutually exclusive with PCBPs' RNA-binding activities, as PCBPs associated with Fe^{2+} have a reduced affinity for their RNA targets (206, 232).

PCBPs were first characterized as RNA-binding proteins; as such, they have primarily been studied as post-transcriptional regulators of gene expression. Nonetheless, PCBPs have more recently been found to play roles that do not require their RNA-binding activities (i.e. as protein platforms), or that can even temporarily reduce their RNA-binding affinities (i.e. as iron chaperones). These novel interactions only emphasize how PCBPs are truly multifunctional proteins, that can help maintain cellular homeostasis through a variety of mechanisms.

1.3.3 Interactions with viruses

Although PCBPs have been reported to interact with numerous viruses, only a fraction of these interactions has been characterized in any detail. In some of these cases, PCBPs were found to directly reprise their cellular functions with viral substrates — such as when PCBP2 slows down the deadenylation and degradation of the rabies virus glycoprotein mRNA by binding its 3' UTR, or when PCBP1, PCBP2 or hnRNP K suppress the translation of the human papillomavirus 16 (HPV-16) L2 mRNA by binding its 3′ UTR (233, 234). In other cases, viral proteins enhance pre-existing PCBP activities, such as the severe acute respiratory syndrome coronavirus (SARS-CoV) ORF-9b protein's enhancement of PCBP2-mediated MAVS degradation (235).

In other contexts, viral elements have co-opted PCBPs to perform new functions. These novel functions have often been described in the context of RNA virus infections, as their use of RNA as a template for translation, replication, and packaging creates a unique molecular context and opportunity for novel PCBP interactions. The best-characterized PCBP-virus interaction takes place during the poliovirus (PV) replication cycle, where PCBPs mediate the switch between viral translation and RNA replication. Both PCBP1 and PCBP2 bind the PV 5′ UTR, but with slightly different affinities: both PCBPs bind the 5′-terminal cloverleaf, but only PCBP2 binds SLIV of the PV IRES (236-238). PCBP2 binding to SLIV is crucial for IRES-mediated translation (**Figure 1.11**) (237, 239-243). Once viral proteins accumulate, the viral 3C protease cleaves PCBPs in the linker region between their KH2 and KH3 domains, resulting in PCBP1ΔKH3 and PCBP2ΔKH3 proteins (244). While both of these truncated proteins retain their affinity for the 5′-terminal cloverleaf, PCBP2ΔKH3 can no longer bind to SLIV, effectively turning off IRES- mediated translation (244, 245). Interactions between the truncated PCBPs, the viral RNA-dependent RNA polymerase (3D), and PABP (the latter bound to the viral poly(A)-tail) circularize the viral RNA and brings the viral 3D polymerase in close proximity to the 3′ terminus of the genome, where it can initiate viral RNA synthesis (**Figure 1.11**) (241, 246, 247). Thus, poliovirus exploits a pre-existing molecular mechanism (PCBP interactions with PABP) and applies it to perform a novel function, i.e. to serve as the switch from translation to viral RNA replication in the PV life cycle. This example also further illustrates how PCBP1 and PCBP2 can play both common and unique roles within a viral life cycle.

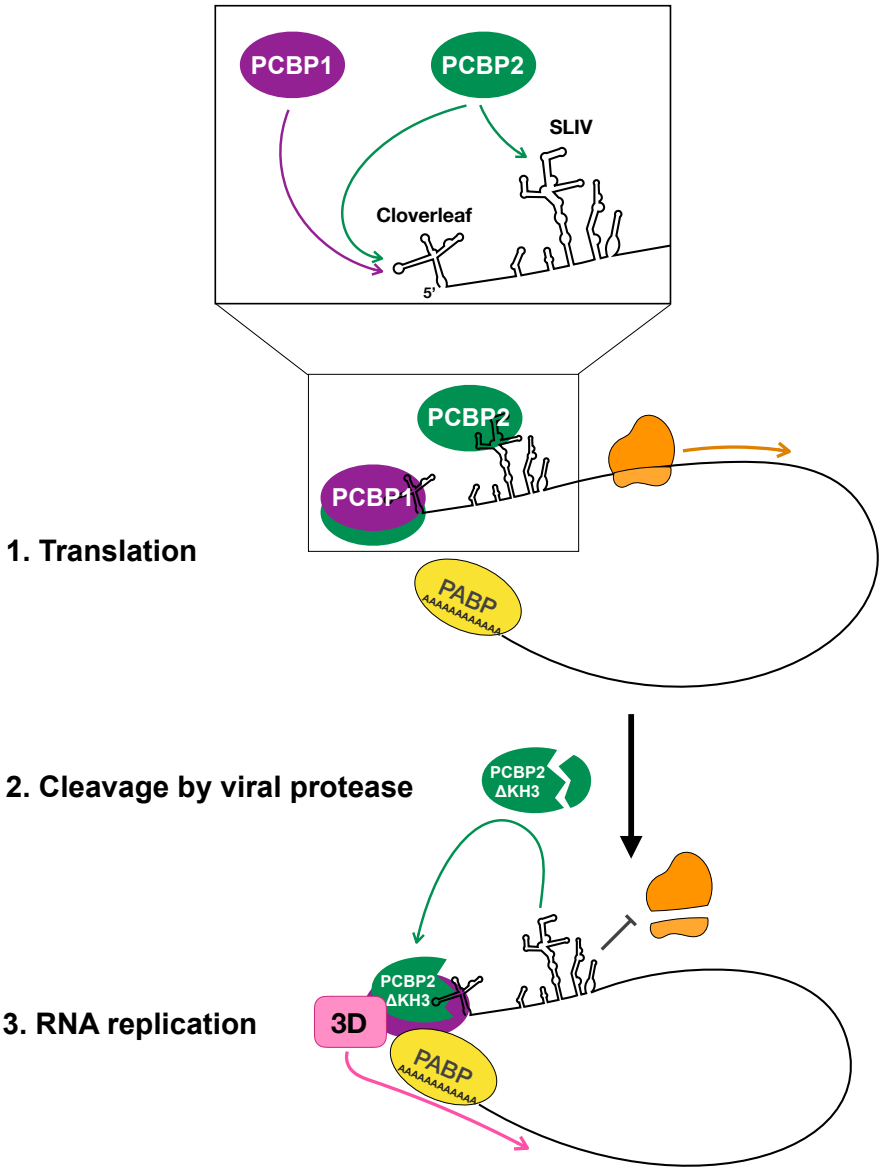

Figure 1.11. PCBP2 mediates the switch from translation to RNA replication in the poliovirus (PV) life cycle. PCBP1 and PCBP2 bind the PV 5′ UTR but have affinities for different regions of the viral genome: both PCBPs bind the 5' terminal cloverleaf, but only PCBP2 binds SLIV of the IRES (box). (1) PCBP2 binding to SLIV activates the PV IRES, which leads to viral protein synthesis. (2) As the viral proteins accumulate, the viral 3C protease cleaves PCBPs in the linker region, resulting in PCBP2ΔKH3. (3) The resulting PCBP2ΔKH3 can no longer bind to the IRES and the viral RNA is therefore cleared of ribosomes, but can still interact with the 5′ cloverleaf. At the 5′ cloverleaf, PCBPs form a ternary complex with the viral 3D RNA polymerase, facilitated by interactions with PABP on the poly(A)-tail of the viral genome. This interaction results in genome circularization, and brings the viral polymerase in close proximity to the 3′ terminus of the viral RNA, where it can then initiate viral RNA replication.

PCBPs and HCV

Although PCBP1, PCBP2 and the closely related hnRNP K have all been shown to bind to the HCV genome, their interactions with HCV have not faced equal amounts of research scrutiny (248, 249). Prior to this thesis, only three publications incidentally reported on PCBP1-HCV interactions. These studies show that PCBP1 binds the HCV 5′ UTR, that it does not participate in IRES-mediated translation within a bicistronic reporter construct, and that knockdown of PCBP1 during HCV infection resulted in an overall decrease in viral RNA accumulation (240, 248, 250).

The interactions between PCBP2 and HCV have been much more studied. PCBP2 has been shown to have six binding sites on the HCV genomic RNA, conserved across two HCV genotypes (251). Specifically, PCBP2 binds to two sites in the 5′ UTR (near SLI and overlapping the initiation codon in SLIV); two sites in the structural protein-coding region (in the *core* and *E2* genes); one site in the *NS5B*-coding region, near known *cis*-acting RNA structures; and finally, a large region in the 3′ UTR that overlaps the variable region and poly(U/UC)-tract (251). These sites include regions of the genome implicated in both viral translation (i.e. overlapping the start and stop codons), as well as viral RNA replication (i.e. SLI, cis-acting RNA elements in the *NS5B* gene, and the 3′ UTR) (**Figure 1.12**) (251). Additionally, PCBP2 has been shown to directly interact with the NS5A protein (35, 252). However, the biological significance of these interactions remains unclear, as prior studies that examined the impact of PCBP2 on viral translation, RNA replication and infectious particle production using different experimental systems have arrived at contradictory conclusions. Most studies that examined HCV IRES-mediated translation — using monocistronic or bicistronic reporter RNA constructs that were

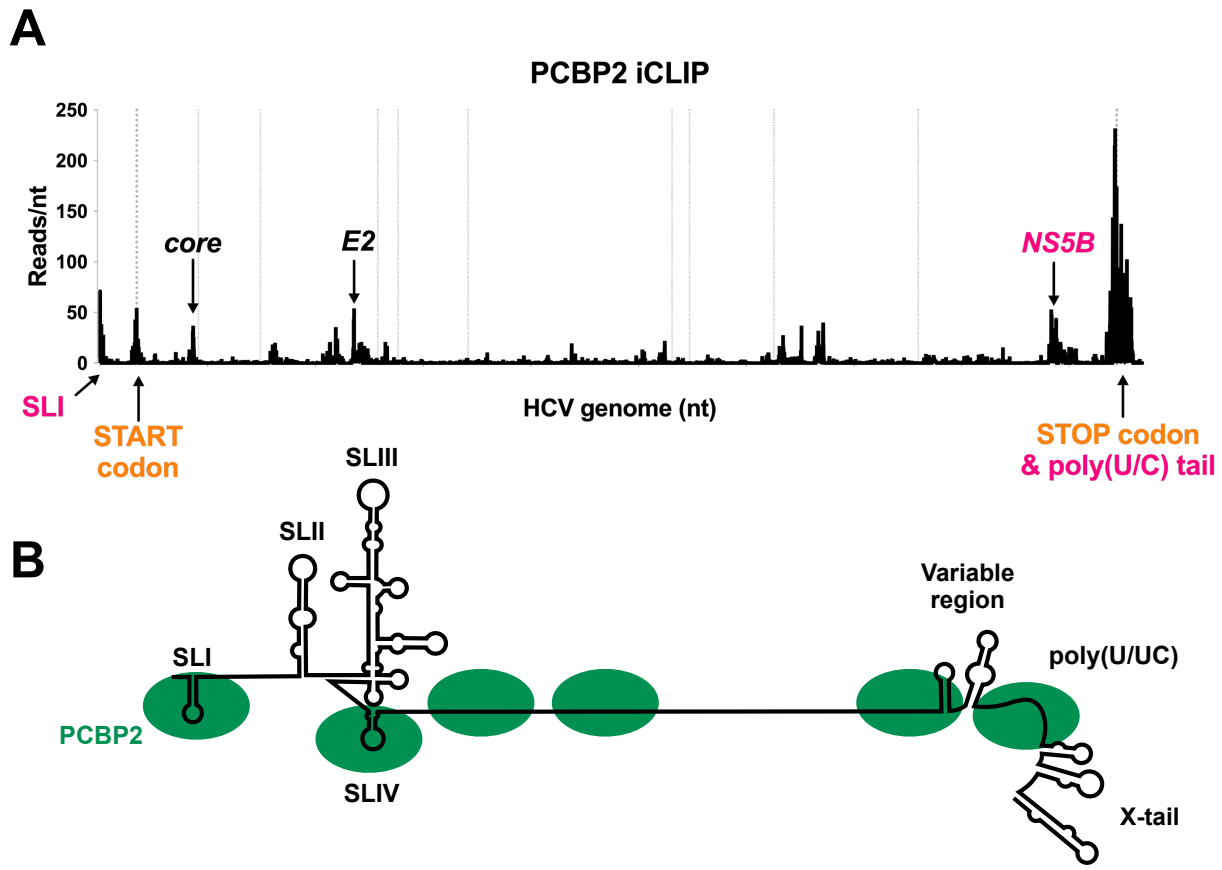

Figure 1.12. PCBP2 binding sites on the HCV genome previously identified by cross-linking immunoprecipitation and next-generation sequencing analysis. (**A**) Coverage histogram of nucleotides bound by PCBP2 across the JFH-1 (genotype 2a) genome; arrows indicate PCBP2 binding sites that were conserved with those from genotype 1. Binding sites found in regions of the genome with annotated roles in viral translation are indicated in orange, while sites in regions with annotated roles in viral RNA replication are indicated in pink. Vertical lines indicate the boundaries between the UTRs and between each protein-coding region of the genome. iCLIP data was obtained from Flynn *et al. RNA* 2015 (251) (**B**) Schematic representation of the PCBP2 binding sites across the HCV genome, including key RNA secondary structures in the 5′ and 3′ UTRs.

either used in *in vitro* translation assays, or introduced into *S. cerevisiae* or Huh-7-derived cells — found that PCBP2 was not required for viral translation (240, 253-255). However, two studies did report a role for PCBP2 in HCV translation: the first reported that PCBP2 knockdown decreased HCV IRES activity within a bicistronic reporter construct transfected into Huh-7 cells, and the second reported that PCBP2 knockdown decreased nascent protein synthesis in a cell culture model of persistent HCV infection (35, 256). However, this second study also showed that their PCBP2 knockdown decreased total viral RNA levels to the same extent as the decrease in protein synthesis; thus, it seems plausible that this decrease in viral protein synthesis could be the result of having fewer RNA templates to translate, instead of a sign that PCBP2 is important for translation *per se* (256). While this decrease in viral RNA accumulation could indicate that PCBP2 is important for RNA replication, PCBP2 knockdowns have also been previously reported to have no impact on the accumulation of a subgenomic HCV replicon that did not contain the viral structural genes (257). Finally, PCBP2 has been reported to have contradictory effects on infectious particle production; within the same study, PCBP2 knockdowns could either increase or decrease extracellular virus titers, depending on the amount of time separating the short interfering RNA (siRNA) transfection and HCV infection (250). Additionally, since the first PCBP2 binding site on the viral genome is in close proximity to the miR-122 binding sites, PCBP2 has been proposed to compete with miR-122 for the HCV 5′ UTR, although this hypothesis remains somewhat controversial (256).

Like PCBP2, hnRNP K binds the HCV 5′ UTR in close proximity to SLI (249). Furthermore, hnRNP K was shown to directly interact with endogenous miR-122 in both naïve and HCV-infected cells (258). Finally, hnRNP K has been reported to interact with both the core

and NS3 proteins, and to limit HCV virion assembly and egress without affecting viral RNA replication in a KH1-, KH2- and linker region-dependent manner (134, 259). While hnRNP K-HCV interactions are outside the scope of this thesis, these insights have informed our investigations of the other PCBPs.

1.4 Hypothesis and Specific Aims

As discussed above, PCBPs play important roles in the life cycles of several viruses. While both PCBP1 and PCBP2 have been shown to interact with the HCV genome, their precise role(s) in the HCV life cycle remain unclear. As such, we hypothesized that the interactions between PCBPs and the HCV genome are biologically significant — in other words, that PCBPs influence the HCV life cycle. Herein, we specifically aimed to identify the precise step(s) of the viral life cycle affected by both PCBP1 and PCBP2. To do so, we combined siRNA-mediated endogenous PCBP1 and PCBP2 knockdowns with HCVcc infections and assays for viral entry, translation and genome stability, replication, packaging, and egress.

In ***Chapter 2***, we present our investigation of PCBP1's role in the HCV life cycle. We found that endogenous PCBP1 knockdown decreased viral RNA accumulation during infection, but resulted in an overall increase in infectious particle production. By systematically assessing each step of the viral life cycle, we were able to rule out a role for PCBP1 in viral entry, translation, genome stability, and RNA replication in the absence of efficient virion assembly. We specifically examined virion assembly and egress by inhibiting the viral RNA-dependent RNA polymerase, and found that that even when viral RNA accumulation was inhibited, PCBP1 knockdown continued to lead to an increase in the rate of HCV particle secretion. We therefore propose a

model where endogenous PCBP1 limits virion assembly and egress, thereby indirectly enhancing viral RNA accumulation in infected cells.

In ***Chapter 3***, we turn our attention to the interactions between PCBP2 and HCV. We confirmed that knocking down endogenous PCBP2 during HCV infection was deleterious for both viral RNA accumulation and infectious particle production. By systematically assessing each step of the viral life cycle, we ruled out a role for PCBP2 in viral entry, translation, and viral RNA replication. We noticed that only RNAs that could engage in the early steps of genomic RNA packaging were sensitive to PCBP2 knockdown, and further found that PCBP2 limited the rate of virion assembly without affecting the rate of virion secretion. Additionally, we demonstrated that PCBP2 had no effect on a viral mutant (G28A) that can form the functional SLII structure independently of miR-122, suggesting that the ability to form a stable $SLII^{alt}$ structure — rather than a direct competition with miR-122 for the 5' UTR — is important for PCBP2's effects on viral RNA replication. In all, our results suggest that endogenous PCBP2 delays the early steps of HCV infectious particle assembly, thereby keeping viral genomes engaged in translation and RNA replication.

Finally, in ***Chapter 4***, we present an integrated discussion of the roles of PCBP1 and 2 in the HCV life cycle. We reconcile how both PCBP1 and 2 can act on the same step of the viral life cycle, yet result in opposite effects on secreted virus titers in cell culture. We speculate about several potential mechanisms of action that PCBPs may employ to limit infectious particle production, including direct interactions with viral RNA and/or proteins, and other potential interactions with host proteins reported to affect virion assembly and egress. Finally, we explore how PCBP2's apparent dependence on $SLII^{alt}$ raises intriguing implications about the importance

of this viral RNA structure in the HCV life cycle. In all, this thesis helps to clarify the role of PCBP1 and 2 in the HCV life cycle, and proposes that these host RNA-binding proteins influence HCV's genomic RNA usage during the viral life cycle.

CHAPTER 2: Poly(rC)-binding protein 1 limits hepatitis C virus virion assembly and egress

Sophie E. Cousineau and Selena M. Sagan.

2.1 PREFACE

As discussed in ***Chapter 1***, PCBP1 is known to bind to the HCV genomic RNA in a manner that requires the entire 5' UTR, and PCBP1 has been shown to be important for optimal viral RNA accumulation during HCV infection (240, 248, 250). Thus, we hypothesized that PCBP1 plays an important role in the HCV life cycle. Herein, we aimed to identify the precise step(s) of the HCV life cycle affected by PCBP1.

This chapter was adapted from the following manuscript: Poly(rC)-binding protein 1 limits hepatitis C virus virion assembly and egress. **Sophie E. Cousineau** and Selena M. Sagan. *bioRxiv* [preprint]: 2021 (260)

2.2 ABSTRACT

The hepatitis C virus (HCV) co-opts a number of cellular elements – including proteins, lipids, and microRNAs – to complete its viral life cycle. The cellular RNA-binding protein poly(rC)-binding protein 1 (PCBP1) had previously been reported to bind the HCV genome 5′ untranslated region (UTR), but its importance in the viral life cycle had remained unclear. Herein, we aimed to clarify the role of PCBP1 in the HCV life cycle. Using the HCV cell culture (HCVcc) system, we found that endogenous PCBP1 knockdown decreased viral RNA accumulation yet increased extracellular virus titers. To dissect PCBP1's specific role in the viral

life cycle, we carried out assays for viral entry, translation, genome stability, RNA replication, virion assembly and egress. We found that PCBP1 did not affect viral entry, translation, RNA stability, or RNA replication in the absence of efficient virion assembly. To specifically examine virion assembly and egress, we inhibited viral RNA replication with an RNA-dependent RNA polymerase inhibitor and tracked both intracellular and extracellular viral titers over time. We found that when viral RNA accumulation was inhibited, knockdown of PCBP1 still resulted in an overall increase in HCV particle secretion. We therefore propose a model where endogenous PCBP1 limits virion assembly and egress, thereby indirectly enhancing viral RNA accumulation in infected cells. This model furthers our understanding of how cellular RNA-binding proteins modulate HCV genomic RNA utilization during the viral life cycle.

IMPORTANCE

Hepatitis C virus (HCV) is a positive-sense RNA virus, and as such, its genome must be a template for multiple mutually exclusive steps of the viral life cycle, namely translation, RNA replication, and virion assembly. However, the mechanism(s) that regulate how the viral genome is used throughout the viral life cycle still remain unclear. A cellular RNA-binding protein – PCBP1 – had previously been reported to bind the HCV genome, but its precise role in the viral life cycle was unknown. In this study, we found that depleting PCBP1 decreased viral RNA accumulation but increased virus secretion. We ruled out a role for PCBP1 in virus entry, translation, genome stability or RNA replication, and demonstrate that PCBP1 knockdown enhances virus secretion when RNA replication is inhibited. We conclude that PCBP1 normally prevents virus assembly and egress, which allows more of the viral genomic RNA to be available for translation and viral RNA replication.

2.3 INTRODUCTION

The hepatitis C virus (HCV) is an enveloped virus of the *Flaviviridae* family (genus: hepacivirus) that typically causes a persistent liver infection (44). Its ~9.6 kb single-stranded, positive-sense RNA genome contains a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). A highly structured, type 3 internal ribosomal entry site (IRES) in the 5′ UTR drives the translation of the viral polyprotein, which is subsequently processed into 10 mature viral proteins: 3 structural proteins (core, E1 and E2 glycoproteins), and 7 nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (261, 262). While the structural proteins form the nucleocapsid and viral envelope, the NS3-5B form the viral replicase, required for viral RNA replication (263). The p7, NS2, NS3 and NS5A proteins have also been implicated in viral genome packaging and the assembly of new virion particles (59, 126, 129, 264). As a positive-sense RNA virus, the HCV genome itself must serve as a template for viral translation, genome replication, and packaging; however, the mechanisms that determine which process each viral RNA is engaged in at any given time have not been defined. Due to their limited coding capacity, viruses are highly dependent on the molecular machinery of the host cell; thus, it is likely that cellular components participate in regulation of the viral RNA during the HCV life cycle. While a number of cellular proteins and RNAs have been shown to interact with the HCV genome, their precise role(s) in the viral life cycle have yet to be defined.

The poly(rC)-binding protein 1 (PCBP1) is one of the three most abundant cellular RNA-binding proteins with a strong affinity for poly(rC), along with its paralogs hnRNP K and PCBP2 (181, 265). These multifunctional proteins can regulate translation and enhance the stability of their cellular mRNA targets, which they interact with through their hnRNP K homologous (KH)

domains (184). Notably, all three paralogs have been reported to bind to the HCV 5′ UTR (248, 249, 251). However, the degree to which each protein has been studied in the context of HCV infection varies significantly – while hnRNP K and PCBP2 have been fairly extensively studied and reported to play markedly different roles in the viral life cycle, the role of PCBP1 in the HCV life cycle has not been investigated in detail (35, 134). Beyond its interactions with the 5′ UTR, previous reports suggested that PCBP1 was not necessary for HCV IRES-mediated translation, but that knockdown of PCBP1 decreases HCV RNA accumulation during infection (240, 250).

Herein, we sought to clarify the role of PCBP1 in the HCV life cycle. Using a cell culture-adapted strain of HCV, we found that PCBP1 knockdown decreased viral RNA accumulation, yet led to an increase in virus secretion. By examining individual steps of the viral life cycle, we ruled out a role for PCBP1 in viral entry, translation, genome stability and viral RNA replication. Further analysis of the assembly step revealed that, similarly to its paralog hnRNP K, endogenous PCBP1 limits HCV virion assembly and release.

2.4 MATERIALS AND METHODS

Cell culture

Huh-7.5 human hepatoma cells were obtained from Charlie Rice (Rockefeller University) and maintained in complete media: Dulbecco's Modified Eagle Media (DMEM) supplemented with inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1X MEM non-essential amino acids. Human embryonic kidney (293T) cells were kindly provided by Martin J. Richer (McGill University, Montreal, QC, Canada) and were maintained in DMEM supplemented with 10% FBS. All cell lines were maintained at 37°C/5% CO_2 and were routinely screened for mycoplasma

contamination.

Plasmids and viral RNAs

The pJFH-1_T plasmid, encoding a cell culture-adapted Japanese Fulminant Hepatitis (JFH-1; HCV genotype 2a) with three adaptive mutations that increase viral titers in cell culture, was a gift from Rodney Russell (Memorial University of Newfoundland) (155). Plasmids pJ6/JFH1 FL RLuc WT ("RLuc-wt") and pJ6/JFH-1 FL RLuc GNN ("RLuc-GNN") bear full-length viral sequence derived from the J6 (structural genes) and JFH-1 (NS genes) isolates of HCV, with a *Renilla* luciferase (RLuc) reporter (59). The pJ6/JFH-1 mono RLuc-NS2 plasmid ("Δcore-p7") – a truncated version of the *Renilla* reporter virus with a deletion of the structural genes through p7 – was a gift from Joyce Wilson (University of Saskatchewan) (266). The pJ6/JFH-1 FL RLuc-NS5A-GFP ("NS5A-GFP") was created via overlapping PCR and subcloned using the *AvrII* and *XbaI* restriction sites, as previously described (267).

To make full-length uncapped viral RNAs, all plasmid templates were linearized and *in vitro* transcribed as previously described (174). The firefly luciferase (FLuc) mRNA was transcribed from the Luciferase T7 Control DNA plasmid (Promega) linearized using *XmnI* and *in vitro* transcribed using the mMessage mMachine T7 Kit (Life Technologies) according to the manufacturer's instructions.

Generation of infectious HCV stocks

To generate viral stocks, 30 μg of *in vitro* transcribed JFH-1_T RNA was transfected into Huh-7.5 cells using the DMRIE-C reagent (Life Technologies) according to the manufacturer's instructions. Four days post-transfection, infectious cell supernatants were passed through a 0.45

μm filter and infectious viral titers were determined by focus-forming unit assay (155). Infectious virus was amplified for two passages through Huh-7.5 cells at a MOI of 0.1. Viral stocks were aliquoted and stored at -80°C until use.

Focus-forming unit (FFU) assays

One day prior to infection, 8-well chamber slides (Lab-Tek) were seeded with 4 x 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, while intracellular virus titers were determined after cell pellets were subjected to lysis via four freeze-thaw cycles, removal of cellular debris via centrifugation, and recovery of virus-containing supernatants.

MicroRNAs and siRNA sequences

siGL3 (siCTRL): 5'-CUU ACG CUG AGU ACU UCG AUU-3', siGL3* : 5'-UCG AAG UAC UCA GCG UAA GUU-3', $miR122_{p2\text{-}8}$ (siCTRL for luciferase experiments): 5'-UAA UCA CAG ACA AUG GUG UUU GU-3', $miR122_{p2\text{-}8}$*: 5'-AAA CGC CAU UAU CUG UGA GGA UA-3' (173), siPCBP1: 5'-CUG UGU AAU UUC UGG UCA GUU-3', siPCBP1*: 5'-CUG ACC AGA AAU UAC ACA GUU-3' (250) were all synthesized by Integrated DNA Technologies.

All microRNA and siRNA 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), and annealed at 37°C for 1 h and stored at -20°C. For all knockdown experiments, 50 nM siRNA transfections were conducted 2 days prior to infection or electroporation of viral RNAs. Transfections were conducted using the Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions with the modification that 20 μL of reagent were used to transfect a 10-cm dish of cells.

HCV and VSV pseudoparticles (HCVpp and VSVpp)

HCVpp consisting of a FLuc reporter lentiviral vector pseudotyped with the HCV E1 and E2 glycoprotein (from the H77, a genotype 1a strain) were a kind gift from John Law (University of Alberta) (46). To generate lentiviral vectors pseudotyped with the VSV-G glycoprotein (VSVpp), a 90% confluent 10-cm dish of 293T cells were transfected with 10 μg pPRIME-FLuc, 5 μg psPAX.2, and 2.5 μg pVSV-G plasmid with 10 μL Lipofectamine 2000 (Invitrogen) diluted in 4 mL Opti-MEM. Media was changed 4, 20, and 28 h post-transfection. At 48 h post-transfection, the cell culture media was passed through a 0.45 μm filter and stored at -80°C.

To assay for cell entry, HCVpp and VSVpp were diluted 1/3 in dilution media (1X DMEM, 3% FBS, 100 IU penicillin and 100 μg/mL streptomycin) with 20 mM HEPES and 4 μg/μL polybrene, and then introduced to Huh-7.5 cells by spinoculation at 1,200 rpm for 1 h at room temperature. The cells were left to recover at 37°C for at least 5 h before the pseudoparticle-containing media was changed for fresh complete Huh-7.5 media. In parallel, cells seeded in a 6-well plate were transfected with 1 μg of pPRIME-FLuc plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three days post-spinoculation and

transfection, cells were lysed in passive lysis buffer (Promega) and FLuc activity was assayed using the Dual Reporter Luciferase kit (Promega).

Electroporations

For each electroporation, 400 µL of resuspended cells (1.5 x 10^7 cell/mL) were mixed with 2 µg of FLuc mRNA and 5 µg of replicating (WT, Δcore-p7 or NS5A-GFP J6/JFH-1 RNA) or 10 µg GNN J6/JFH-1 RNA, and electroporated in 4-mm cuvettes at 270 V, 950 µF, and infinite resistance, optimized for the Bio-Rad GenePulser XCell (Bio-Rad). Electroporated cells were resuspended in complete Huh-7.5 media and transferred to 6-well plates for luciferase assays and protein analysis.

Inhibition of RNA replication by 2´CMA

Two days post-siRNA transfection, Huh-7.5 cells were infected with JFH-1_T at a MOI of 0.05. Four to five hours post-infection, each plate of infected cells was split into 6-well plates. Three days post-infection, the media on these cells was changed for complete Huh-7.5 media with 5 µM 2´CMA (2´C-methyladenosine, Carbosynth), an HCV NS5B polymerase inhibitor, or DMSO control (268). Total RNA and intracellular virus samples were collected at 0, 6 and 12 h post-treatment, while cell culture supernatants were collected 6 and 12 h post-treatment. Protein samples were collected from untreated plates to assess PCBP1 knockdown efficiency by Western blot.

Western blot analysis

To collect total intracellular protein samples, cells were lysed in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, Complete

Protease Inhibitor Cocktail (Roche)) and frozen at -80°C. Cellular debris was pelleted by centrifugation at 16,000 x g for 30 min at 4°C, and the supernatant was quantified by BCA Protein Assay (ThermoScientific). Ten micrograms of sample were loaded onto 10-12% SDS-PAGE gels. Samples were transferred onto Immobilon-P PVDF membranes (Millipore), blocked in 5% milk, and incubated overnight with primary antibodies diluted in 5% BSA: rabbit anti-PCBP1 (clone EPR11055, Abcam ab168378, 1:10,000); rabbit anti-actin (A2066, Sigma, 1:20,000); mouse anti-HCV core (clone B2, Anogen MO-I40015B, diluted 1:7,500); mouse anti-JFH-1 NS5A (clone 7B5, BioFront Technologies, 1:10,000). Blots were incubated for 1 hour with HRP-conjugated secondary antibodies diluted in 5% skim milk: anti-mouse (HAF007, R&D Systems, 1:25,000); anti-rabbit (111-035-144, Jackson ImmunoResearch Laboratories, 1:50,000) and visualized using enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, Fisher Scientific).

RNA isolation and Northern blot analysis

Total RNA was harvested using TRIzol Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Ten micrograms of total RNA were separated on a 1% agarose gel containing 1X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and 2.2 M formaldehyde and transferred to a Zeta-probe membrane (Bio-Rad) by capillary transfer in 20X SSC buffer (3 M NaCl, 0.3 M sodium citrate). Membranes were hybridized in ExpressHyb Hybridization Buffer (ClonTech) to random-primed ^{32}P-labeled DNA probes (RadPrime DNA labelling system, Life Technologies) complementary to HCV (nt 84-374) and γ-actin (nt 685-1171). Autoradiograph band densities were quantified using Fiji (269).

RT-qPCR analysis

The iTaq Universal Probes One-Step kit (Bio-Rad) was used to perform duplex assays probing for the HCV genome (NS5B-FW primer: 5'-AGA CAC TCC CCT ATC AAT TCA TGG C-3'; NS5B-RV primer: 5'-GCG TCA AGC CCG TGT AAC C-3'; NS5B-FAM probe: 5'-ATG GGT TCG CAT GGT CCT AAT GAC ACA C-3') and the GAPDH loading control (PrimePCR Probe assay with HEX probe, Bio-Rad). 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. RT-PCR reactions were conducted in a CFX96 Touch Deep Well Real-Time PCR system (Bio-Rad). Genome copies were calculated using a standard curve and fold-differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (270).

Luciferase assays

For translation and replication assays, cells were washed in PBS and harvested in 100 µL of 1X passive lysis buffer (Promega). The Dual-Luciferase Assay Reporter Kit (Promega) was used to measure both *Renilla* and firefly luciferase activity according to the manufacturer's instructions with the modification that 25 µL of reagent were used with 10 µL of sample. All samples were measured in triplicate.

Data analysis

Statistical analyses were performed using GraphPad Prism v9 (GraphPad, USA). Statistical significance was determined by paired t-test to compare results obtained from multiple experiments, and by two-way ANOVA with Geisser-Greenhouse and Bonferroni corrections when more than two comparisons were applied at once. To calculate half-lives, a one-step decay

curve using the least-squares regression was used, and error was reported as the asymmetrical (profile likelihood) 95% confidence interval of the half-life. To calculate virus accumulation and virus secretion rates, a simple linear regression was performed using the least squares regression method using data reported as FFU/mL (y-axis) measured over several hours (x-axis). The slope and standard error calculated for each regression represents the rate of virus accumulation or secretion (in FFU/h).

2.5 RESULTS

PCBP1 plays a role in the HCV life cycle

The PCBP1 protein was previously reported to directly interact with the 5´ UTR of the HCV genome, and in an siRNA screen PCBP1 knockdown resulted in a reduction in HCV RNA accumulation (248, 250). Thus, we sought to further characterize the role of PCBP1 in the HCV life cycle. We began by assessing how PCBP1 knockdown affected the accumulation of cell culture-derived HCV (HCVcc), using the cell-culture adapted JFH-1_T strain. Compared to the parental JFH-1 strain, JFH-1_T has three adaptive mutations in the E2, p7 and NS2 coding-region, which enable it to produce higher viral titers in cell culture (155). We found that knockdown of endogenous PCBP1 resulted in an approximately 2.2-fold decrease in viral protein and RNA accumulation in Huh-7.5 cells (**Figure 2.1A-C and S2.1**). Interestingly, when we quantified intracellular and extracellular virions, we found that while intracellular titers were not significantly different between the PCBP1 knockdown and control conditions, extracellular titers were elevated, with an average increase of approximately 3.90-fold in the PCBP1 knockdown condition (**Figure 2.1D-E**). Thus, in line with previous findings, we found that PCBP1

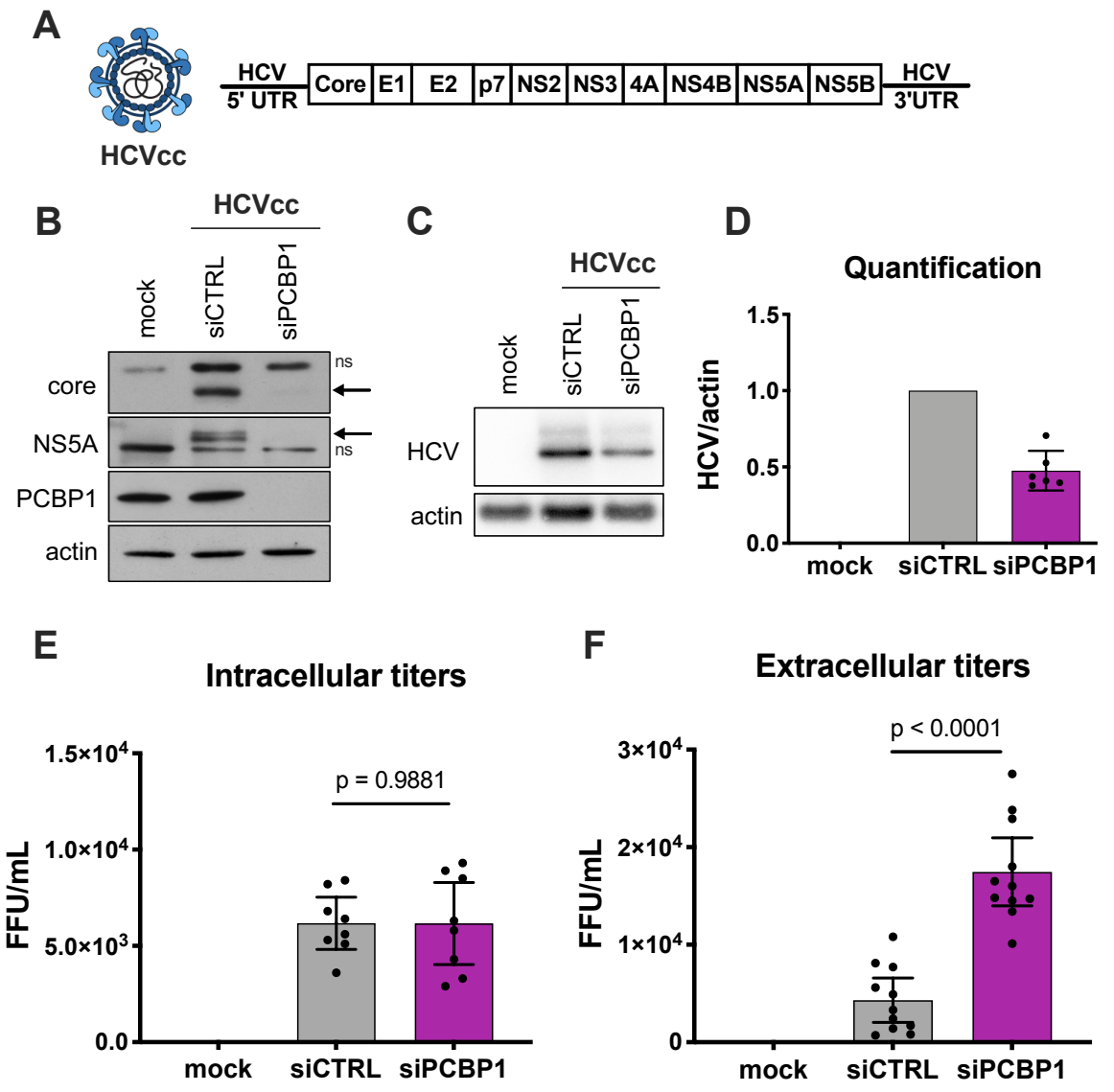

Figure 2.1. PCBP1 knockdown decreases viral protein expression and intracellular viral RNA accumulation, but increases secreted virus titers. (A) Schematic representation of the HCVcc (JFH-1_T) infectious particles and genomic RNA used in infections. Huh-7.5 cells were transfected with siPCBP1 or siCTRL at day -2, and at day 0 were infected with JFH-1_T (MOI = 0.05). Viral protein, Total RNA and intracellular and extracellular infectious virus were harvested at day 3 post-infection. **(B)** Viral protein expression analysis by Western blot (ns indicates non-specific band; and arrow indicates core and NS5A bands). **(C)** Viral RNA accumulation analysis by Northern blot, and **(D)** quantification by densitometry. **(E)** Intracellular and **(F)** extracellular (secreted) virus titers, quantified by FFU assay. All data are representative of three independent replicates, and error bars represent the standard deviation of the mean. P-values were calculated by paired t-test.

knockdown decreased viral protein expression and intracellular viral RNA accumulation. Yet, despite this overall decrease, we observed an increase in extracellular (secreted) virus titers. These results imply that endogenous PCBP1 indeed plays a role in the HCV life cycle, although the precise step(s) influenced by PCBP1 remain unclear.

PCBP1 knockdown has no impact on HCV entry

Firstly, we explored whether PCBP1 knockdown had any effect on virus entry. To this end, we made use of the HCV pseudoparticle (HCVpp) system, which consists of lentiviral vectors with a firefly luciferase reporter gene pseudotyped with the HCV E1 and E2 glycoproteins (46). HCVpp enter cells by clathrin-mediated endocytosis after engaging with HCV-specific entry receptors; thus, to account for any changes in clathrin-mediated endocytosis, we used a vesicular stomatitis virus (VSV) pseudoparticle system (VSVpp) as a control. In addition, to verify that PCBP1 knockdown did not affect luciferase reporter gene expression, we assessed firefly luciferase expression from cells directly transfected with a FLuc reporter plasmid. In all cases, we found that depleting endogenous PCBP1 had no impact on luciferase activity (**Figure 2.2**). This suggests that PCBP1 knockdown does not affect FLuc reporter expression, clathrin-mediated endocytosis, or the HCV entry process.

PCBP1 knockdown has no impact of HCV translation or genome stability

PCBP1 was previously reported to bind the HCV 5′ UTR, which contains the viral IRES that drives translation of the viral polyprotein in the absence of most canonical translation initiation factors (37, 248, 261, 262). Furthermore, PCBP1 has been reported to contribute to the IRES-mediated translation of some cellular mRNAs such as Bag-1 and *c-myc* (212-214). Thus, it was

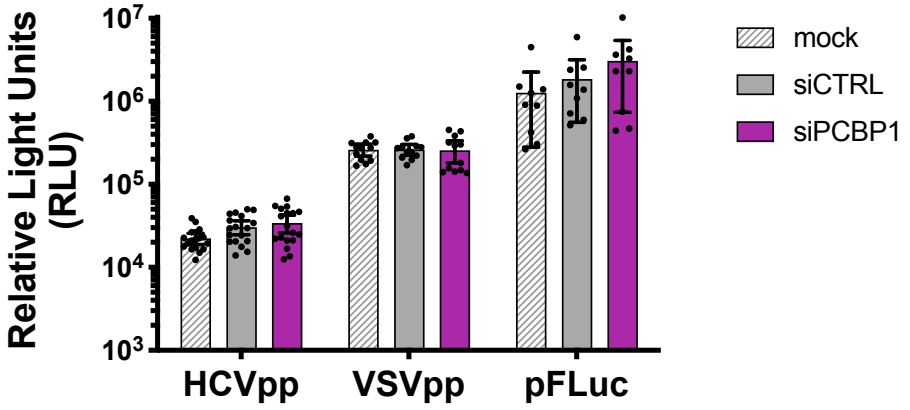

Figure 2.2. PCBP1 knockdown has no effect on HCV entry. Two days post-siRNA transfection, cells were spinoculated with luciferase reporter pseudoparticles expressing the HCV E1/E2 glycoproteins (HCVpp) or the VSV-G glycoprotein (VSVpp). In parallel, cells were transfected with a firefly luciferase expression plasmid. Samples were harvested 3 days post-infection/transfection, and analyzed by luciferase assay. The HCVpp and pFLuc data is representative of three independent replicates, while the VSVpp data is representative of two independent replicates. Error bars represent the standard deviation of the mean.

plausible that PCBP1's interactions with the viral 5′ UTR could affect viral protein expression by altering IRES-mediated translation. To assess PCBP1's impact on HCV translation, we used full-length J6/JFH-1 RLuc reporter RNAs containing an inactivating mutation in the NS5B polymerase gene (GNN). The RLuc activity thus served as a direct measure of HCV IRES-mediated translation, and over time, this signal also served as a proxy measure for viral RNA stability. We found that the siPCBP1 and siCTRL conditions had similar RLuc activity at all timepoints (**Figure 2.3**). Moreover, the luciferase signal half-lives were nearly identical, with 2.68 h (95% CI 1.64 – 4.64) for the siPCBP1 condition and 2.71 h (95% CI 1.43 – 5.50) for the siCTRL condition. Thus, PCBP1 knockdown does not appear to affect either viral IRES-mediated translation or genome stability.

PCBP1 knockdown does not affect viral RNA replication

PCBP1 has been reported to promote viral RNA replication by binding to the 5′ UTR of several RNA viruses (242, 271). Thus, to specifically assess whether PCBP1 knockdown has an effect on HCV RNA replication, we assessed RLuc expression of replication-competent but assembly-deficient reporter RNAs (**Figure 2.4**). This includes the full-length WT J6/JFH-1 RLuc RNA, a subgenomic J6/JFH-1 replicon RNA containing a deletion of the structural protein genes (Δcore-p7), and a full-length J6/JFH-1 genome with a GFP insertion in the NS5A gene (NS5A-GFP), the latter previously shown to impair virion assembly without impairing viral RNA replication (126, 267). In all cases, we did not observe any significant differences in viral RNA accumulation between the siPCBP1 and siCTRL conditions (**Figure 2.4A-C**). This is seemingly in contrast to our findings using the JFH-1_T system, where PCBP1 depletion decreased viral RNA accumulation (**Figure 2.1B-C and S2.1**). However, it is notable that all of the viral RNAs used in **Figure 2.4** are

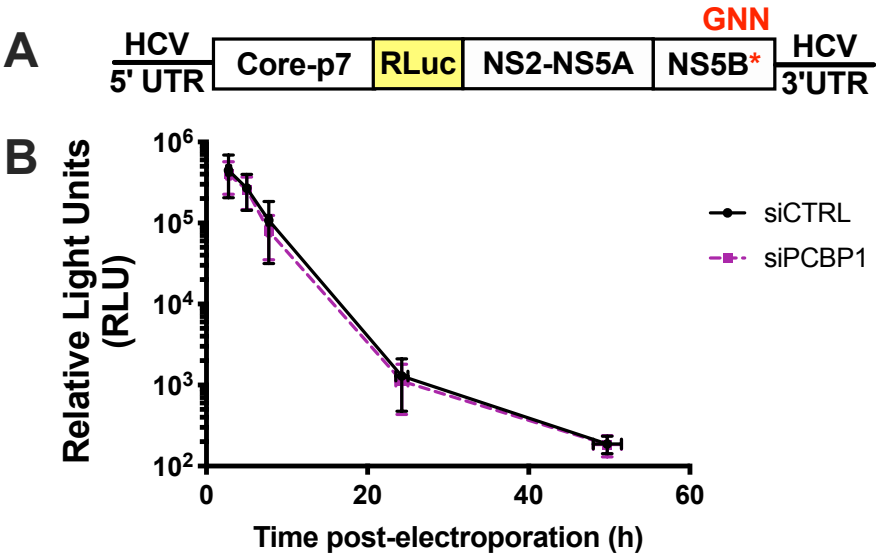

Figure 2.3. PCBP1 knockdown has no impact on HCV translation or genome stability. (A) Schematic representation of the J6/JFH-RLuc-GNN reporter RNA. **(B)** Huh-7.5 cells were electroporated with J6/JFH-1-RLuc-GNN reporter RNAs and FLuc control mRNA, and luciferase activity was monitored at several timepoints post-electroporation. All data are representative of three independent replicates. Error bars on the y-axis represent the standard deviation of the mean, while error bars on the x-axis indicate the range of sample harvest times across replicates.

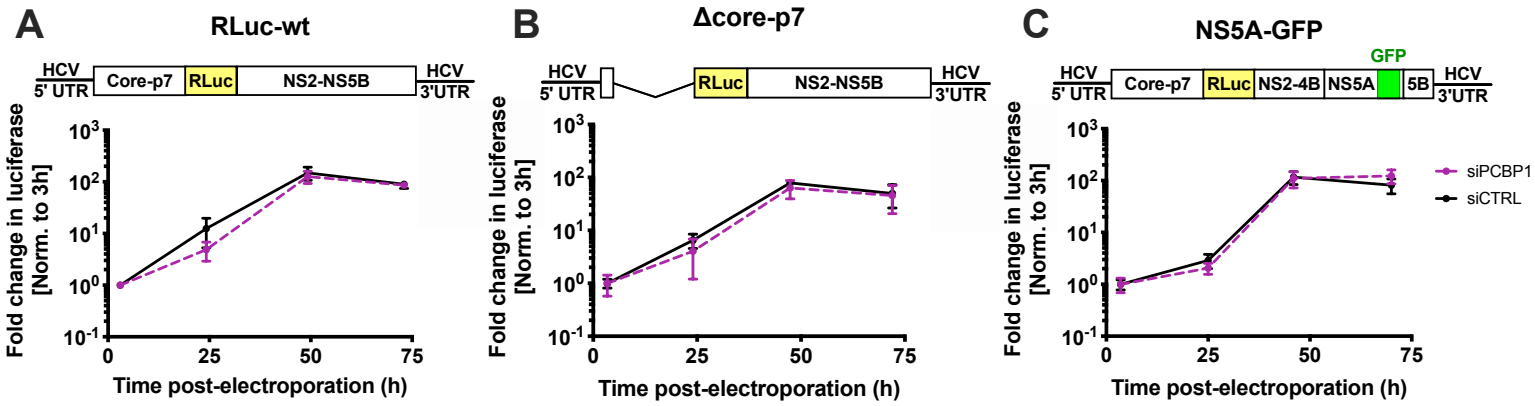

Figure 2.4. PCBP1 knockdown does not affect viral RNA replication. Two days post-siRNA transfection, Huh-7.5 cells were electroporated with **(A)** full-length RLuc WT J6/JFH-1 RNA, **(B)** Δcore-p7 J6/JFH-1 RLuc RNA, or **(C)** full-length NS5A-GFP J6/JFH-1 RLuc RNA. RLuc values were normalized to the early timepoint (3h), to control for disparities in electroporation efficiency between experiments. All data are representative of three independent replicates and error bars represent the standard deviation of the mean.

defective in viral packaging, including the full-length WT J6/JFH-1 RLuc RNA (**Figure 2.4A**), likely due to the large RLuc reporter gene insertion (**Figure S2.2**). Thus, taken together, our data suggests that PCBP1 has no effect on the viral RNA replication step of the HCV life cycle.

PCBP1 knockdown limits virus egress

Finally, since knockdown of PCBP1 did not seem to have a significant impact on HCV entry, translation, genome stability or viral RNA replication, we reasoned that PCBP1 was likely to be exerting an effect on virion assembly and/or secretion. We had previously established that PCBP1 knockdown did not have a significant effect on intracellular viral titers yet resulted in an overall increase in extracellular (secreted) virions (**Figure 2.1D-E**). Thus, to further investigate whether PCBP1 plays a role in viral packaging and/or egress, we used a nucleoside analog, 2´C-methyladenosine (2´CMA), to block viral RNA synthesis by the HCV NS5B RNA-dependent RNA polymerase and monitored viral titers over time (268). Similar to related studies using Zika virus, we reasoned that blocking viral RNA synthesis could allow us to assess viral packaging and egress in the absence of genomic RNA production (272).

To this end, we infected cells with JFH-1_T and, three days post-infection, we replaced the culture media with media containing 5 μM 2´CMA (or DMSO, as a control) and collected total RNA, intracellular virions and extracellular virions over the next 12 h (**Figure 2.5A**). In agreement with our previous findings, we observed an overall reduction in JFH-1_T viral RNA accumulation by day 3 post-infection in the siPCBP1 condition (0 h timepoint, **Figure 2.5B**). In addition, the 2´CMA treatment efficiently blocked viral RNA accumulation, which continued to increase under the control (DMSO) condition (6-12 h timepoints, **Figure 2.5B**). Additionally, we observed similar initial intracellular titers as well as similar 2´CMA-induced decreases in

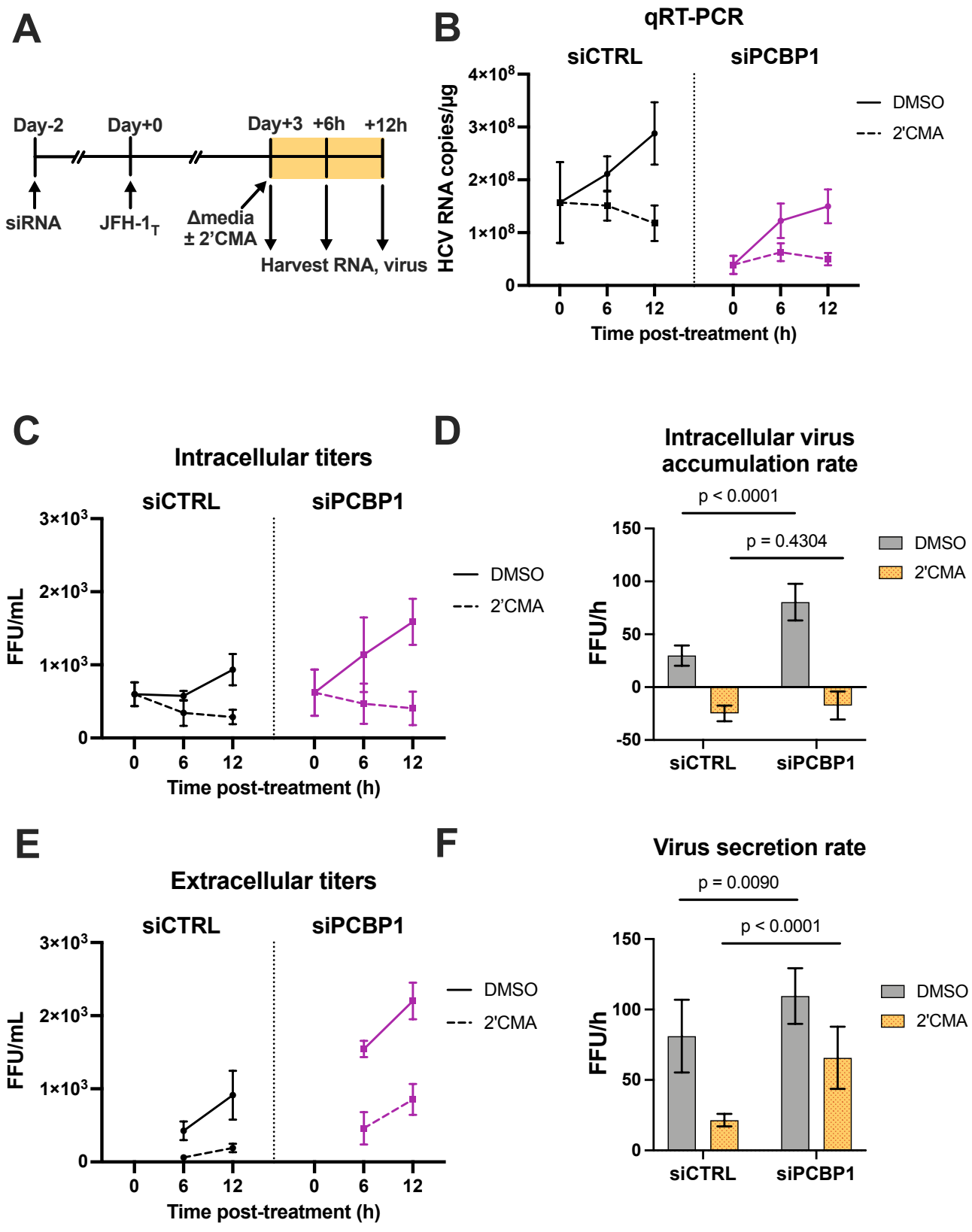
A
Day-2
Day+0
Day+3
+6h
+12h
siRNA
JFH-1T
Δmedia
± 2'CMA
Harvest RNA, virus
B
qRT-PCR
siCTRL
siPCBP1
HCV RNA copies/µg
4×10^8
3×10^8
2×10^8
1×10^8
0
DMSO
2'CMA
0
6
12
Time post-treatment (h)
C
Intracellular titers
FFU/mL
3×10^3
2×10^3
1×10^3
Time post-treatment (h)
D
Intracellular virus accumulation rate
p < 0.0001
p = 0.4304
FFU/h
150
100
50
-50
E
Extracellular titers
F
Virus secretion rate
p = 0.0090
p < 0.0001

Figure 2.5. Viral particle production after 2´CMA treatment confirms that PCBP1 knockdown enhances virion secretion. **(A)** Schematic representation of the experimental approach for 2´CMA experiments: two days post-siRNA transfection, Huh-7.5 cells were infected with JFH-1_T (MOI = 0.05). Three days post-infection, cells were treated with 2´CMA or DMSO (control). Total RNA, intracellular and extracellular virus was collected at t = 0, 6 and 12 h post-treatment. **(B)** Quantitative RT-PCR analysis after 2´CMA treatment. **(C)** Intracellular viral titers and **(D)** intracellular virus accumulation rate, calculated by linear regression from the slopes in panel (C). **(E)** Extracellular viral titers and **(F)** virus secretion rates calculated by linear regression from the slopes in panel (E). All data are representative of three independent replicates and error bars in (B), (C) and (E) represent the standard deviation of the mean. Error bars in (D) and (F) represent the slopes of the linear regressions ± standard error. P-values were calculated by two-way ANOVA.

intracellular titers under both the siPCBP1 and siCTRL conditions (**Figure 2.5C and D**). In contrast, we observed a continued rise in extracellular titers as packaged viruses continued to egress out of the cell, with the PCBP1 knockdown resulting in a >3-fold greater virus secretion rate than the siCTRL condition during 2´CMA treatment (**Figure 2.5E and F**). Notably, since inhibiting viral RNA synthesis equalized the intracellular virus accumulation rates yet failed to equalize the virus secretion rates, these results suggest that PCBP1 modulates the virion assembly and egress step of the HCV life cycle.

2.6 DISCUSSION

Herein, we investigated the role of PCBP1 in the HCV life cycle. We found that PCBP1 knockdown did not directly affect virus entry, translation, genome stability or viral RNA replication; but resulted in an increase in infectious particle secretion in cell culture. Furthermore, since PCBP1 knockdown does not alter intracellular infectious particle accumulation yet reduces total intracellular viral RNA, our results suggest that PCBP1 knockdown promotes virion assembly and egress.

Previous studies had found that PCBP1 interacts with the HCV 5´ UTR *in vitro* (248). Notably, the full 5´ UTR appeared to be critical for PCBP1 binding, as truncated fragments of the 5´ UTR were unable to bind to PCBP1 (240, 248). Somewhat more recently, PCBP1 was one of many host factors evaluated in a siRNA screen targeting cellular proteins predicted to interact with HCV (250). This screen revealed that PCBP1 knockdown decreased viral RNA accumulation approximately 2.3-fold, a result consistent with our observation that PCBP1 knockdown decreased JFH-1_T viral RNA accumulation by approximately 2.2-fold (**Figure 2.1D**)

(250). However, our systematic evaluation of the effect of PCBP1 knockdown on viral translation and viral RNA replication revealed that PCBP1 does not modulate either of these steps in the viral life cycle. Moreover, we did not observe any significant effects on viral entry or genome stability.

Initially, we were quite puzzled to find that PCBP1 did not affect viral RNA accumulation of a full-length WT J6/JFH-1 RLuc RNA; however, this RNA generates far fewer infectious viral particles than the JFH-1_T strain we used for our infection experiments, likely due to the increased genome size due to the RLuc insertion resulting in reduced packaging efficiency (**Figure S2.2**) (134). The fact that PCBP1 knockdown failed to have a significant impact on the luciferase reporter RNA is therefore consistent with a model whereby PCBP1 primarily acts at the level of virion assembly and egress. Our findings suggest a model whereby PCBP1 limits both the virion assembly and egress steps of the viral life cycle (**Figure 2.6**). As such, knockdown of PCBP1 results in an overall decrease in the translating/replicating pool of viral RNAs and a concomitant increase in extracellular (secreted) virions. Since intracellular viral titers remained similar in PCBP1 knockdown and control conditions, a greater proportion of the intracellular viral RNA must represent viral RNA packaged into virions, implying that endogenous PCBP1 normally limits virion assembly. This model is further supported by our finding that 2´CMA equalized the rate of intracellular virus accumulation yet resulted in a virus secretion rate >3-fold greater during PCBP1 knockdown, suggesting that endogenous PCBP1 limits virion egress. Moreover, this model is consistent with the observed decreases in viral protein expression and negative-strand replicative intermediate accumulation (**Figure 2.1B and S2.1**), since increasing viral genome packaging into new virions would sequester the RNA from the translation and RNA

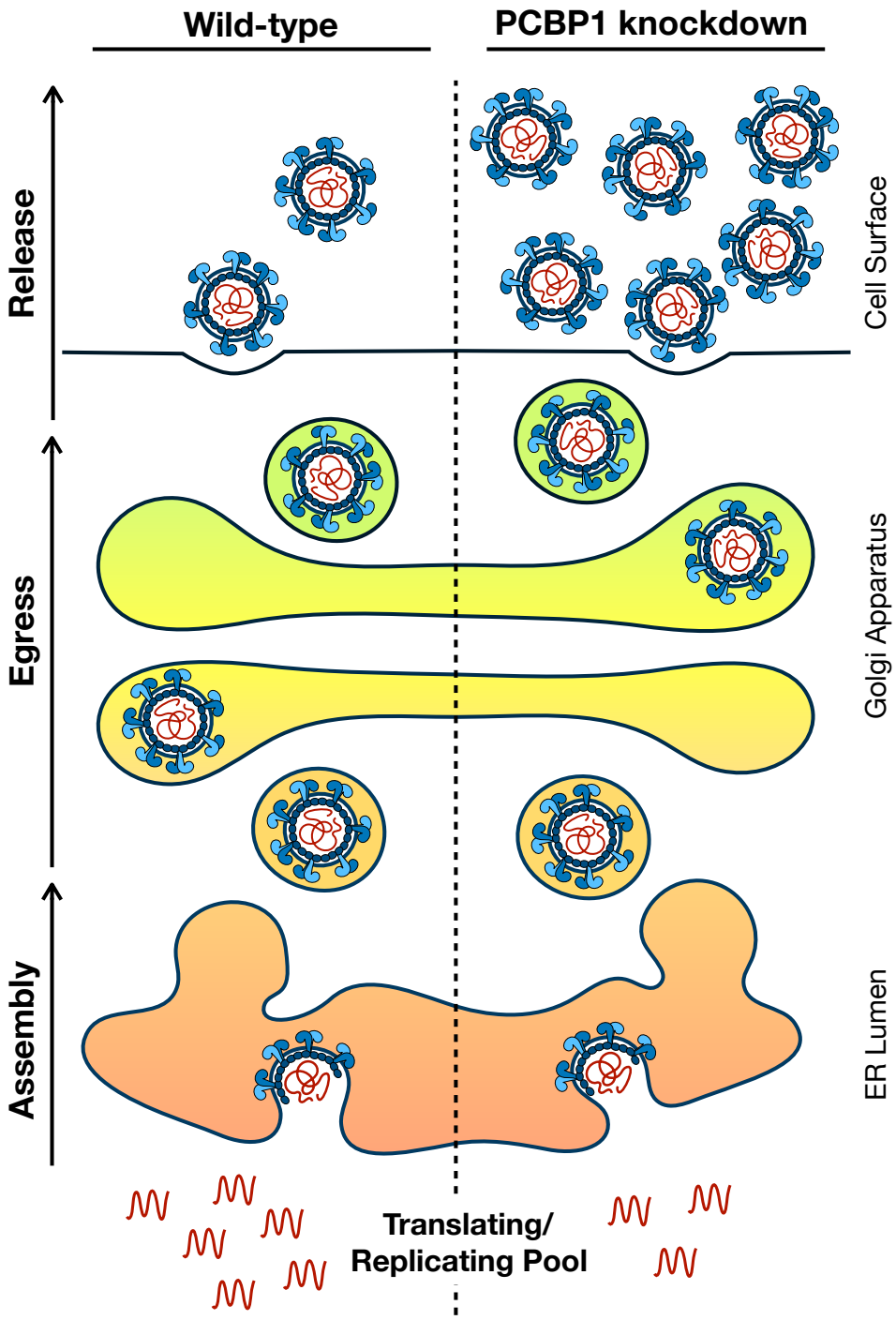

Figure 2.6. Model for PCBP1's effects on HCV assembly and egress. Under wild-type conditions, PCBP1 inhibits HCV assembly and egress, leaving the majority of intracellular viral RNAs to engage in protein translation and viral genome replication. However, during PCBP1 knockdown, a disinhibition of assembly and egress leads to an overall decrease in the translating/replicating pool of viral RNAs, and a concomitant increase in viral infectious particle production.

replication machinery. Thus, endogenous PCBP1 normally limits viral assembly and egress, and PCBP1 knockdown therefore indirectly impairs viral protein synthesis and viral RNA replication by liberating the RNA for virion assembly and egress.

Notably, PCBP1 has been previously implicated in turnover of MAVS, a signal transduction protein directly downstream of RIG-I, which is known to limit HCV infection (225). However, all of our conclusions were drawn from experiments conducted in the Huh-7.5 cell line, which has well-documented defects in the RIG-I antiviral signalling pathway, and our own brief explorations of interferon induction and MAVS turnover did not reveal any significant differences between PCBP1 knockdown and control conditions (data not shown) (146). Moreover, the HCV NS3-4A protease also inactivates MAVS during infection to reduce antiviral signaling and recognition of the viral RNA (68, 273). However, as hepatocytes typically express RIG-I and MAVS, we cannot rule out the possibility that PCBP1 may modulate this pathway during infection *in vivo*.

Finally, PCBP1's closely related paralogs, hnRNP K and PCBP2, have been more extensively studied during HCV infection. While the hnRNP K protein was shown to restrict infectious virion production, the PCBP2 protein has been suggested to modulate viral translation and RNA replication (35, 134). Since the PCBP1 amino acid identity is far more similar to PCBP2 (~80% identity) than hnRNP K (~24% identity), we were initially surprised that our findings for PCBP1 in HCV infection closely matched those reported for hnRNP K. Yet, while PCBP1 and PCBP2 have been shown to perform similar functions, they have also been reported to play distinct roles during poliovirus, VSV, and human immunodeficiency virus infection (242, 274, 275). In addition to the similarities we observed with PCBP1 and those previously reported

with hnRNP K during HCV infection, our results and conclusions also echo those reported for the IGF2BP2/YBX-1 complex, METTL3/METTL14 *N*6-methyladenosine (m^6A) writers, and the YTHDF m^6A-binding proteins (131, 134, 276). These have all been reported to inhibit HCV infectious particle production, with no effect on viral translation or RNA replication – with the exception of the IGF2BP2/YBX-1 complex, which plays an additional role in facilitating viral RNA replication (276). It is currently unclear if PCBP1, hnRNP K, the YBX-1 complex or m^6A modifications of the HCV genome inhibit virion assembly as components of a common pathway, or through distinct and/or additive mechanisms. Interestingly, high-throughput affinity capture and proximity ligation studies have found that PCBP1 interacts with hnRNP K, IGF2BP2, YBX-1, METTL3 and METTL14; although this has been demonstrated only in non-hepatic cell lines to date (277-279). Should these interactions be conserved during HCV infection, it seems plausible that PCBP1 could participate in these virion assembly inhibition pathways – or, conversely, that it may have an inhibitory role to promote one pathway over another. Future investigations will reveal whether these proteins function in an overlapping or a distinct manner, and are likely to improve our understanding of HCV virion assembly and how this process is regulated by cellular RNA binding proteins.

Taken together, our results support a model where endogenous PCBP1 limits HCV infectious particle production. By preventing virion assembly and egress, PCBP1 indirectly enhances viral RNA accumulation. The model presented herein helps to inform understanding of how cellular RNA-binding proteins modulate HCV genomic RNA utilization during the viral life cycle, specifically as it pertains to virion assembly. While the precise molecular mechanism employed by PCBP1 to inhibit HCV assembly and egress remains to be characterized, similar

phenotypes reported for hnRNP K, IGF2BP2/YBX-1 and m^{6}A modifications of the HCV genome offer promising leads for future investigations.

2.7 ACKNOWLEDGEMENTS

We would like to acknowledge Charlie Rice (Rockefeller University) for kindly providing the Huh-7.5 cells, pJ6/JFH FL RLuc WT and GNN plasmids; Rodney Russell (Memorial University) for providing JFH-1$_T$; Mamata Panigrahi and Joyce Wilson (University of Saskatchewan) for the pJ6/JFH mono RLuc NS2 plasmid; Martin J Richer (McGill University) for the 293T cells; John Law (University of Alberta) for the kind gift of HCVpp. We are also grateful to Nathan Taylor, Julie Magnus and Carolina Camargo (McGill University) for technical support.

2.8 FUNDING INFORMATION

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2.9 AUTHOR CONTRIBUTIONS

S.E.C. and S.M.S. designed the study; S.E.C. performed the experiments and analyzed the data, and S.E.C and S.M.S. wrote and edited the manuscript.

2.10 SUPPLEMENTARY METHODS

Strand-specific RT-qPCR

The iTaq Universal Probes One-Step kit (Bio-Rad) was used to perform duplex assays probing for the HCV genome (NS5B-FW primer: 5'-AGA CAC TCC CCT ATC AAT TCA TGG C-3'; NS5B-RV primer: 5'-GCG TCA AGC CCG TGT AAC C-3'; NS5B-FAM probe: 5'-ATG GGT TCG CAT GGT CCT AAT GAC ACA C-3') and the GAPDH loading control (PrimePCR Probe assay with HEX probe, Bio-Rad). For the reverse transcription step, each 18.75 µL reaction contained 500 ng of total RNA, 0.5 µL of the GAPDH primers and probe, and 10 pmol (0.25 µL of a 40 µM stock) of only one of the HCV primers (NS5B-FW for the negative-strand reaction, and NS5B-RV for the positive-strand reaction). Reverse transcription took place at 50°C for 10 minutes, after which 0.25 µL of the complementary primer and 1 µL of HCV primers pre-mixed with the probe were added to each reaction. The same PCR cycling protocol was followed as it would be for a typical HCV RT-qPCR, in a CFX90 Touch Deep Well Real-Time PCR system (Bio-Rad). Genome copies were calculated using a standard curve and fold-differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (270).

2.11 SUPPLEMENTARY FIGURES

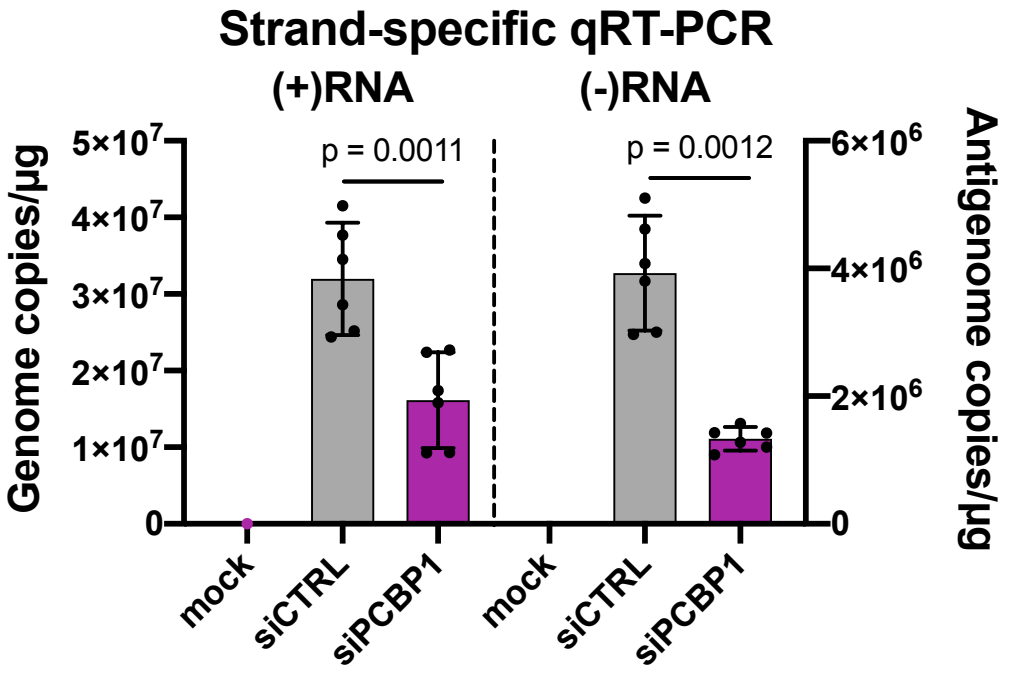


Figure S2.1. PCBP1 knockdown decreases both positive-sense and negative-sense viral RNA accumulation. Two days post-siRNA transfection, Huh-7.5 cells were infected with JFH-1_T (MOI = 0.05). Total RNA was harvested 3 days post-infection and analysed by strand-specific RT-qPCR. All data is representative of three independent replicates and error bars represent the standard deviation. P-values were calculated by paired t-test.

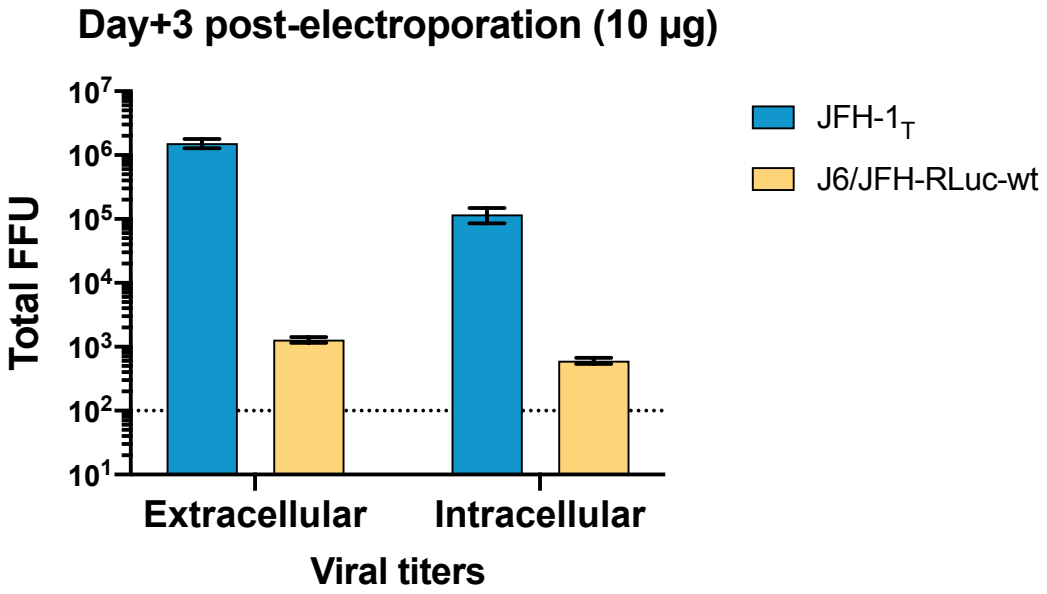


Figure S2.2. The J6/JFH FL Rluc WT can produce infectious viral particles, but much less efficiently than JFH-1_T. Three days post-electroporation of viral RNA, intracellular and extracellular viral titers were harvested and assayed by focus-forming unit assay. Data is representative of one independent replicate measured in duplicate. The limit of detection is indicated (dotted line).

CHAPTER 3: The cellular poly(rC)-binding protein 2 prevents the early steps of HCV virion assembly

Sophie E. Cousineau and Selena M. Sagan.

3.1 PREFACE

As discussed in ***Chapter 1***, PCBP2 has six conserved binding sites on the HCV genome, located in regions of the viral RNA with documented roles in the viral life cycle; however, the impact of PCBP2 interactions with the viral genome was not clear, as prior studies that examined PCBP2's role in viral translation and RNA replication had arrived at contradictory conclusions (35, 250, 251, 253, 257). We thus aimed to clarify PCBP2's specific role in the viral life cycle, using many of the same experimental approaches described in ***Chapter 2***. Additionally, since miR-122 had previously been reported to depend on PCBP2 to stimulate viral RNA synthesis, we sought to explore if PCBP2 could still affect a viral mutant (G28A) that could form the functional SLII structure independently of miR-122 (170, 256).

This chapter was adapted from the following manuscript: The cellular poly(rC)-binding protein 2 prevents the early steps of HCV virion assembly. **Sophie E. Cousineau** and Selena M. Sagan. *Manuscript in preparation*

3.2 ABSTRACT

Hepatitis C virus (HCV) co-opts many cellular factors – including proteins and microRNAs – to complete its viral life cycle. A cellular RNA-binding protein, poly(rC)-binding protein 2 (PCBP2), binds multiple sites across the HCV genome, but its precise role in the viral life cycle

has remained unclear. Using the HCV cell culture (HCVcc) system, we confirmed that endogenous PCBP2 was necessary for optimal viral protein expression, RNA accumulation and infectious particle production. Using assays to systematically examine each step of the viral life cycle, we found that PCBP2 did not directly affect viral entry, translation, RNA stability, viral RNA replication or viral egress. However, PCBP2 depletion reduced the accumulation of viral RNAs that expressed both the wild-type core and NS5A proteins, suggesting that PCBP2 may modulate early steps of genomic RNA packaging. Indeed, when we specifically assessed virion assembly and egress, we found that PCBP2 knockdown enhanced the rate of intracellular virion accumulation without affecting the rate of virion secretion. Additionally, we observed that only viral RNAs that could form the alternative $SLII^{alt}$ conformation were sensitive to PCBP2 knockdown. Collectively, our data suggest that PCBP2 regulates HCV genome packaging, thereby altering the balance between viral RNAs in the translating/replicating pool and those engaged in virion assembly.

IMPORTANCE

Hepatitis C virus (HCV) is a positive-sense RNA virus; as such, its genome must serve as a template for multiple mutually exclusive steps of the viral life cycle (i.e. translation, RNA replication, and virion assembly). The cellular poly(rC)-binding protein 2 (PCBP2) was previously shown to bind the HCV genome, but its precise role in the HCV life cycle was unclear. We systematically ruled out a role for PCBP2 in all steps of the viral life cycle other than virion assembly, and showed that the core and NS5A proteins, involved in the early steps of genomic RNA packaging, are necessary for PCBP2 to affect viral RNA accumulation. We propose that PCBP2 normally delays virion assembly, thereby keeping viral genomes available for translation

and RNA replication. These insights clarify PCBP2's controversial role in the HCV life cycle, but also improve our understanding of how a RNA virus can regulate its genome usage.

3.3 INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-sense RNA virus of the *Flaviviridae* family (genus: hepacivirus) that typically causes a persistent liver infection (44). Its ~9.6 kb genome is single-stranded RNA containing a single open reading frame flanked by highly structured 5′ and 3′ untranslated regions (UTRs). The 5' UTR contains stem-loop (SL) structures necessary for RNA replication (SLI-SLII) and translation (SLII-IV), while the 3' UTR is composed of a short, hypervariable region followed by a poly(U/UC) tract and a highly conserved triple loop structure termed the "X-tail", all necessary for RNA replication. In the 5′ UTR, SLII-IV form an internal ribosomal entry site (IRES) that drives the translation of the viral polyprotein, which is subsequently processed into 10 mature viral proteins: 3 structural proteins (core, E1 and E2 glycoproteins), and 7 nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (261, 262). The structural proteins form the nucleocapsid and viral envelope, while the NS3-5B proteins form the viral replicase, required for viral RNA replication (263). Additionally, the p7, NS2, NS3, NS4A and NS5A proteins have all been implicated in viral genome packaging and the assembly of new progeny viral particles (59, 71, 126, 129, 264).

Like all viruses, HCV is highly dependent on its host cell and co-opts cellular proteins, RNAs and lipids to complete its life cycle. One such cellular element is the liver-specific microRNA, miR-122, which binds to two sites in the 5′ UTR of the HCV genome (reviewed in (280) and (281))(169, 282). These interactions are crucial for HCV RNA accumulation, as miR-

122 has been shown to have at least three roles in the viral life cycle: 1) it acts as a RNA chaperone or riboswitch to suppress an energetically stable RNA secondary structure (termed $SLII^{alt}$) allowing the functional IRES (SLII-IV) to form; 2) it protects the 5′ terminus of the genome from pyrophosphatase activity and subsequent exoribonuclease-mediated decay; and 3) it promotes translation through interactions between the Argonaute (Ago)-miR-122 complex at site 2 and the HCV IRES (170, 171, 174, 175, 178, 179). In addition, miR-122 has been proposed to modulate viral RNA replication, potentially by promoting the switch from translation to viral RNA replication, although this mechanism has not been clearly elucidated to date (256, 283, 284).

The poly(rC)-binding protein 2 (PCBP2) is one of the three most abundant cellular RNA-binding proteins with a strong affinity for poly(rC), along with its paralogs hnRNP K and PCBP1 (181, 265). PCBP2 is a multifunctional protein that can bind to hundreds of cellular mRNAs, including its own mRNA transcript (202). Depending on the context of its binding site and binding partners, PCBP2 modulates the stability or translation of its mRNA targets (192, 285). Beyond its known cellular target mRNAs, PCBP2 has also been reported to interact with numerous viral RNAs, and has been demonstrated to participate in viral translation, RNA stability, replication and/or viral assembly (233, 234, 275, 286-291). Its role in the poliovirus (PV) life cycle is particularly well-characterized, where it mediates the switch from translation to replication. Briefly, PCBP2 binds to the 5′ cloverleaf and PV IRES, and promotes viral translation. As viral proteins accumulate, the viral protease cleaves PCBP2 resulting in it losing its affinity for the IRES, such that it can no longer promote translation and the viral RNA is cleared of ribosomes. Still, cleaved PCBP2 maintains its affinity for the 5′ cloverleaf, and

interactions between the cleaved PCBP2, the viral RNA-dependent RNA polymerase, and the poly(A)-binding protein (PABP) at the 3′ end of the viral genome promote genome circularization and viral RNA replication (237-239, 243-245).

While PCBP2 has been known to bind the HCV 5′ UTR for more than two decades, its precise binding sites were only recently mapped by cross-linking immunoprecipitation (iCLIP), which identified six conserved PCBP2 binding sites across two HCV genotypes (248, 251). Two of these binding sites are located in the 5′ UTR (near SLI, and over the initiation codon in SLIV of the IRES), three sites are located in the polyprotein-coding region (in the *core* and *E2* structural genes, and in the *NS5B* gene), and the strongest binding site is located in the 3' UTR (i.e. a large binding area in the 3′ UTR that overlaps the poly(U/UC) tract and the stop codon within the variable region) (251). However, the importance of PCBP2 interactions with the HCV genome have remained somewhat elusive.

In a previous siRNA screen, PCBP2 knockdown was found to initially enhance HCV titers and viral RNA levels, followed by a sharp decline in titers and viral RNA accumulation (250). However, the precise mechanism by which PCBP2 modulates HCV RNA accumulation and viral particle production is still unclear, as studies examining viral translation and RNA replication using different experimental systems have arrived at contradictory conclusions (35, 240, 253-256). In addition, while miR-122's promotion of viral RNA accumulation was reported to rely on endogenous PCBP2, the relationship between PCBP2 and miR-122, and whether PCBP2's role in the HCV life cycle depends on miR-122, remains unclear (256).

Herein, we aimed to clarify the role of PCBP2 in the HCV life cycle. Using a cell culture-adapted strain of HCV, we found that endogenous PCBP2 was important for optimal HCV

replication and infectious particle production in cell culture. By examining individual steps of the viral life cycle, we ruled out a direct role for PCBP2 in viral entry, translation, genome stability and viral RNA replication in the absence of virion assembly. We discovered that only viral RNAs that undergo the early steps of viral packaging, and those that are able to form the alternative $SLII^{alt}$ conformation, were sensitive to PCBP2 knockdown. Thus, we propose a model where PCBP2 modulates HCV genome packaging, thereby altering the balance between viral RNAs in the translating/replicating pool and those engaged in virion assembly.

3.4 MATERIALS AND METHODS

Cell culture

Huh-7.5 human hepatoma cells were provided by Charlie Rice (Rockefeller University) and miR-122 knockout (miR122-KO) Huh-7.5 cells were provided by Matthew Evans (Icahn School of Medicine at Mount Sinai). Both Huh-7.5 and miR122-KO cells were maintained in complete media: Dulbecco's Modified Eagle Media (DMEM) supplemented with inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1X MEM non-essential amino acids. Human embryonic kidney cells (293T) were provided by Dr. Martin J Richer (McGill University) and maintained in DMEM supplemented with 10% FBS. All cell lines were incubated at 37°C/5% CO_2 and were routinely screened for mycoplasma contamination.

Plasmids and viral RNAs

The pJFH-1_T plasmid, which encodes a cell culture-adapted Japanese Fulminant Hepatitis (JFH-1; HCV genotype 2a) with three adaptive mutations that increase viral titers in cell culture, was a

gift from Rodney Russell (Memorial University of Newfoundland) (155). The pJ6/JFH1 FL RLuc WT ("RLuc-wt") and pJ6/JFH-1 FL RLuc GNN ("RLuc-GNN") plasmids contain full-length viral sequences derived from the J6 (structural genes) and JFH-1 (nonstructural genes) isolates of HCV, with a *Renilla* luciferase (RLuc) reporter (59). The pJ6/JFH-1 mono RLuc-NS2 ("Δcore-p7") and pJ6/JFH-1 E1-p7 del ("ΔE1-p7") plasmids – truncated versions of the *Renilla* reporter virus with deletions of structural genes through p7 – were a gift from Joyce Wilson (University of Saskatchewan, Saskatoon, SK, Canada) (266). The pJ6/JFH Δcore ("Δcore") plasmid consists of a truncated version of the *Renilla* reporter virus with a deletion of the core-coding gene that still retained the first 15 codons (necessary for a functional HCV IRES) and the final 14 codons (which orient the E1 protein in the ER) of the core-coding sequence. To clone Δcore, the *EcoRI* to *KpnI* fragment was subcloned into a temporary plasmid, PCR amplified with the Q5 high fidelity DNA polymerase (NEB) using the dCore-BamHI-FW (5'-TTT CTG GAT CCT TGC TGG CCC TGC TGT CCT GCA TC-3') and dCore-BamHI-RV (5'-GGG CGG GAT CCG GTG TTT CTT TTG GTT TTT CTT TGA G-3') primers, and auto-ligated after *BamHI* digestion. The *EcoRI* to *KpnI* fragment was then subcloned back into the parental pJ6/JFH-RLuc plasmid. The pJ6/JFH-1 FL RLuc-NS5A-GFP ("NS5A-GFP") and pJ6/JFH-RLuc-G28A plasmid were created by overlapping PCR as previously described (260).

To make full-length uncapped viral RNAs, all plasmid templates were linearized with *XbaI* and *in vitro* transcribed as previously described (174, 260). The firefly luciferase (FLuc) mRNA was transcribed from the Luciferase T7 Control DNA plasmid (Promega) linearized using *XmnI* and *in vitro* transcribed using the mMessage mMachine T7 Kit (Life Technologies) according to the manufacturer's instructions.

Generation of infectious HCV stocks

To generate viral stocks, Huh-7.5 cells were transfected with 30 μg of *in vitro* transcribed JFH-1_T RNA, using the DMRIE-C reagent (Life Technologies) according to the manufacturer's instructions. Four days post-transfection, infectious cell supernatants were filtered with a 0.45 μm filter and infectious viral titers were subsequently determined by focus-forming unit assay (155). Infectious virus was amplified for up to two passages through Huh-7.5 cells at a MOI of 0.1. Viral stocks were aliquoted and stored at -80°C until use.

Focus-forming unit (FFU) assays

Infectious viral titers were determined as previously described (260). Briefly: 8-well chamber slides (Lab-Tek) were seeded with 4 x 10^5 Huh-7.5 cells/well one day prior to infection. Each well was infected with 100 μL of viral samples serially diluted 10-fold; 4 hours post-infection, the supernatant was replaced with fresh media. Three days post-infection, slides were fixed in 100% acetone, stained with anti-HCV core antibody (1:100, clone B2, Anogen), and subsequently stained with the AlexaFluor-488-conjugated anti-mouse antibody (1:200, ThermoFisher Scientific) for immunofluorescence analysis. Each focus was defined as a cluster of at least three core-positive cells. Viral titers are expressed as the number of focus-forming units (FFU) per mL.

As previously described, extracellular virus titers were determined directly from cell supernatants, while intracellular virus titers were harvested by subjecting infected cell pellets to lysis via four freeze-thaw cycles, removing cellular debris by centrifugation and recovering the virus-containing supernatants (260).

MicroRNAs and siRNA sequences

siGL3 (siCTRL): 5'-CUU ACG CUG AGU ACU UCG AUU-3', siGL3* : 5'-UCG AAG UAC UCA GCG UAA GUU-3', miR122$_{p2\text{-}8}$ (siCTRL for luciferase experiments): 5'-UAA UCA CAG ACA AUG GUG UUU GU-3', miR122$_{p2\text{-}8}$*: 5'-AAA CGC CAU UAU CUG UGA GGA UA-3' (173), siPCBP2-1: 5'-UCC CUU UCU GCU GUU CAC CUU-3', siPCBP2-1*: 5'-GGU GAA CAG CAG AAA GGG AUU-3', siPCBP2-2: 5'-GGA CAG UAU GCC AUU CCA CUU-3', and siPCBP2-2*: 5'-GUG GAA UGG CAU ACU GUC CUU-3'(250) were all synthesized by Integrated DNA Technologies.

To anneal microRNA and siRNA duplexes, guide and passenger strands were diluted together to 20 μM in RNA annealing buffer (150 mM HEPES pH 7.4, 500 mM potassium acetate, 10 mM magnesium acetate), annealed at 37°C for 1 h, and stored at -20°C. Annealed siPCBP2-1 and siPCBP2-2 were mixed together at a 1:1 ratio prior to transfection. For all knockdown experiments, 50 nM siRNA transfections were conducted 2 days prior to infection or electroporation of viral RNAs. MicroRNAs and siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, with the modification that 20 μL of reagent were used to transfect a 10-cm dish of cells.

HCV and VSV pseudoparticles (HCVpp and VSVpp)

HCVpp consisting of a Firefly luciferase reporter lentiviral vector pseudotyped with the HCV E1 and E2 glycoprotein (from the H77, a genotype 1a strain) were a kind gift from Dr. John Law (University of Alberta) (46). Lentiviral vectors pseudotyped with the VSV-G glycoprotein (VSVpp) were generated as previously described (260). Briefly, 90% confluent 10-cm dishes of 293T cells were transfected with 10 μg pPRIME-FLuc, 5 μg psPAX.2, and 2.5 μg pVSV-G plasmid

with 10 μL Lipofectamine 2000 (Invitrogen) diluted in 4 mL Opti-MEM. Media was changed to complete media 4 h, 20 h, and 28 h post-transfection. At 48 h post-transfection, the cell culture media was filtered with a 0.45 μm filter and stored at -80°C.

To assay for cell entry, HCVpp and VSVpp were diluted 1/3 in dilution media (1X DMEM, 3% FBS, 100 IU penicillin and 100 μg/mL streptomycin) with 20 mM HEPES and 4 μg/μL polybrene, then spinoculated into Huh-7.5 cells at 1,200 rpm for 1 h at room temperature. After the cells recovered for at least 5 h at 37°C, the pseudoparticle-containing media was changed for fresh complete media. In parallel, cells seeded in a 6-well plate were transfected with 1 μg of pPRIME-FLuc plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three days post-spinoculation and transfection, cells were lysed in passive lysis buffer (Promega), and firefly luciferase activity was assayed using the Dual Reporter Luciferase kit (Promega).

Electroporations

Electroporations were conducted as previously described (260). Briefly, 5 μg of replication-competent or 10 μg of nonreplicative GNN RLuc RNA were mixed with 2 μg of FLuc mRNA and applied to 400 μL of resuspended cells (1.5 x 10^7 cell/mL) and electroporated in 4-mm cuvettes at 270 V, 950 μF, infinite resistance optimized for the Bio-Rad GenePulser XCell (Bio-Rad). Electroporated cells were resuspended in complete Huh-7.5 media and plated in 6-well plates (for luciferase assays and protein analysis), or in a 10-cm dish (to assess infectious virus production).

Western blot analysis

To collect total intracellular protein samples, cells were lysed in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, Complete Protease Inhibitor Cocktail (Roche)) and frozen at -80°C. Cellular debris was pelleted by centrifugation at 16,000 x g for 30 min at 4°C, and the supernatant was quantified by BCA Protein Assay (ThermoScientific). Ten micrograms of sample were separated in 10-12% SDS-PAGE gels. Samples were transferred onto Immobilon-P PVDF membranes (Millipore), blocked in 5% milk, and incubated overnight with primary antibodies diluted in 5% BSA: mouse anti-PCBP2 (clone 5F12, Abnova H00005094-M07, 1:30,000); rabbit anti-actin (A2066, Sigma, 1:20,000); mouse anti-HCV core (clone B2, Anogen MO-I40015B, diluted 1:7,500); mouse anti-JFH-1 NS5A (clone 7B5, BioFront Technologies, 1:10,000). Blots were incubated for 1 hour with HRP-conjugated secondary antibodies diluted in 5% milk: anti-mouse (HAF007, R&D Systems, 1:25,000); anti-rabbit (111-035-144, Jackson ImmunoResearch Laboratories, 1:50,000) and visualized using enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, Fisher Scientific).

RNA isolation and Northern blot analysis

Total RNA was collected using TRIzol Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Ten micrograms of total RNA were separated on a 1% agarose gel containing 1X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and 2.2 M formaldehyde and transferred to a Zeta-probe membrane (Bio-Rad) by capillary transfer in 20X SSC buffer (3 M NaCl, 0.3 M sodium citrate). Membranes were hybridized in ExpressHyb Hybridization Buffer (ClonTech) to random-primed ^{32}P-labeled DNA probes (RadPrime DNA labelling system, Life

Technologies) complementary to HCV (nt 84-374) and γ-actin (nt 685-1171). Autoradiograph band densities were quantified using Fiji (269).

RT-qPCR analysis

Duplex assays probing for the HCV genome (NS5B-FW primer: 5'-AGA CAC TCC CCT ATC AAT TCA TGG C-3'; NS5B-RV primer: 5'-GCG TCA AGC CCG TGT AAC C-3'; NS5B-FAM probe: 5'-ATG GGT TCG CAT GGT CCT AAT GAC ACA C-3') and the GAPDH loading control (PrimePCR Probe assay with HEX probe, Bio-Rad) were performed using the iTaq Universal Probes One-Step kit (Bio-Rad) and a CFX96 Touch Deep Well Real-Time PCR system (Bio-Rad). Each reaction (20 µL) contained 500 ng of total RNA, 1.5 µL of HCV primers and probe, and 0.5 µL of GAPDH primers and probe. Genome copies were calculated using a standard curve, and fold-differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (270).

Luciferase assays

For translation and replication assays, luciferase activity was assayed as previously described (260). Briefly, cells plated in 6-well plates were harvested in 100 µL of 1X passive lysis buffer (Promega). Both *Renilla* and firefly luciferase activity was measured using the Dual-Luciferase Assay Reporter Kit (Promega) according to the manufacturer's instructions, with the modification that 25 µL of reagent were used with 10 µL of sample. All samples were measured in triplicate.

Data analysis

Statistical analyses were performed using GraphPad Prism v9 (GraphPad, USA). Statistical

significance was determined by paired t-test to compare results obtained from multiple experiments, and by two-way ANOVA with Geisser-Greenhouse and Bonferroni corrections when more than two comparisons were applied at once. Half-lives were calculated using a one-step decay curve using the least-squares regression, and error was reported as the asymmetrical (profile likelihood) 95% confidence interval of the half-life. Virus accumulation and virus secretion rates were calculated by performing a simple linear regression using the least squares regression method on data reporting virus titers (in FFU/mL) over time (in hours). The resulting slope and error calculated for each regression represents the rate of virus accumulation or secretion (in FFU/h).

3.5 RESULTS

PCBP2 is necessary for optimal HCV replication in cell culture

A previous siRNA screen previously identified PCBP2 as an important cellular factor for optimal HCV replication in cell culture (250). To further investigate the role of PCBP2, we performed PCBP2 knockdown in Huh7.5 cells and assessed viral protein expression, viral RNA accumulation, as well as intracellular and extracellular viral titers following infection with the JFH-1_T strain of cell culture-derived HCV (HCVcc) (**Figure 3.1**). While PCBP2 knockdown was efficient, siRNA transfection generally led to a transient increase in PCBP2 protein levels at day 1 post-transfection, followed by a sustained knockdown from days 2-5 post-transfection (**Figure S3.1**). Thus, all HCV infections were carried out at day 2 post-siRNA transfection. Endogenous PCBP2 knockdown resulted in an approximately 2.3-fold decrease in viral protein and RNA accumulation in Huh-7.5 cells (**Figure 3.1A-D and S3.2**). Consistent with the reduction in viral

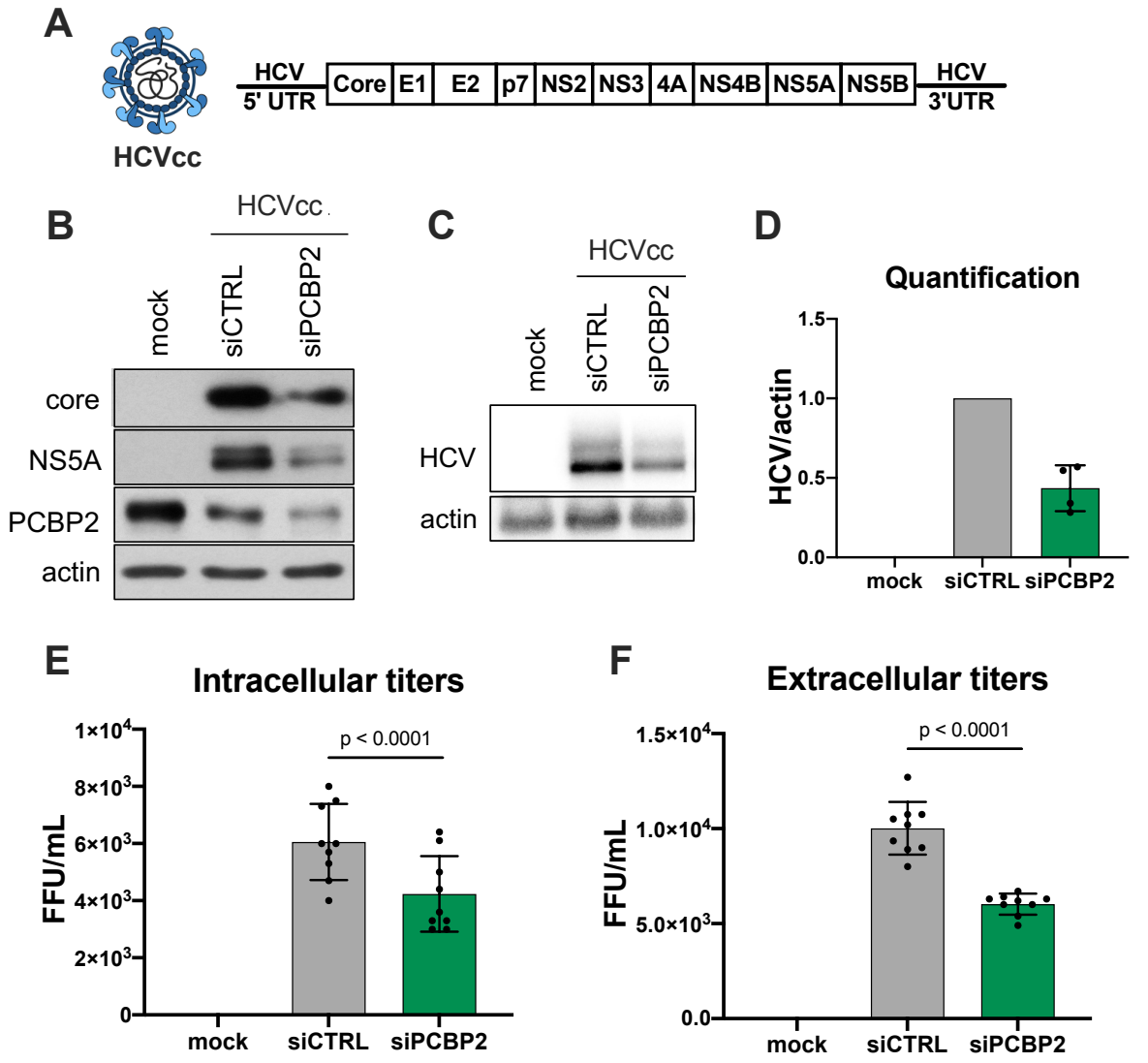


Figure 3.1. PCBP2 is required for optimal RNA accumulation and infectious virus production in cell culture. **(A)** Schematic representation of the HCVcc (JFH-1_T) infectious particles and genomic RNA used in infections. Huh-7.5 cells were transfected with siPCBP2 or siCTRL 2 days prior to infection with JFH-1_T (MOI = 0.05). Viral protein, total RNA and intracellular and extracellular infectious virus were harvested 3 days post-infection. **(B)** Viral protein expression analysis by Western blot. **(C)** Viral RNA accumulation analysis by Northern blot, and **(D)** quantification by densitometry. **(E)** Intracellular and **(F)** extracellular (secreted) virus titers, quantified by FFU assay. All data are representative of three independent replicates, and error bars represent the standard deviation of the mean. P-values were calculated by paired t-test.

protein and RNA accumulation, we also observed 1.4- and 1.7-fold decreases in intracellular and extracellular (secreted) virus titers, respectively (**Figure 3.1E-F**). Thus, in line with previous findings, we found that endogenous PCBP2 is necessary for optimal HCV replication in cell culture, although the precise step(s) of the viral life cycle modulated by PCBP2 remained unclear.

PCBP2 knockdown has no effect on viral entry

Firstly, we explored whether PCBP2 knockdown had any effect on virus entry. To do so, we used the HCV pseudoparticle (HCVpp) system, which consists of lentiviral vectors with a firefly luciferase reporter gene pseudotyped with the HCV E1 and E2 glycoproteins (46). HCVpp enters cells by clathrin-mediated endocytosis after engaging with HCV-specific entry receptors; thus, we used vesicular stomatitis virus (VSV) pseudoparticles (VSVpp) as a control for clathrin-mediated endocytosis. Additionally, to verify that PCBP2 knockdown did not affect luciferase reporter gene expression, we assessed firefly luciferase expression from cells directly transfected with a FLuc reporter plasmid. In all cases, we found that PCBP2 knockdown had no impact on luciferase activity (**Figure 3.2**). This suggests that endogenous PCBP2 does not affect FLuc reporter expression, clathrin-mediated endocytosis, or the HCV entry process.

PCBP2 knockdown has no impact on HCV translation or genome stability

PCBP2 is a known IRES trans-acting factor for several cellular and viral IRESes (208, 212, 221, 291). However, whether PCBP2 affects HCV translation is still unclear, as studies using a variety of experimental systems have arrived at contradictory conclusions (35, 240, 253-256). Thus, to specifically assess PCBP2's effect on viral translation, we used full-length J6/JFH-1 RLuc reporter RNAs with an inactivating mutation in the NS5B polymerase active site (GNN). As such, RLuc

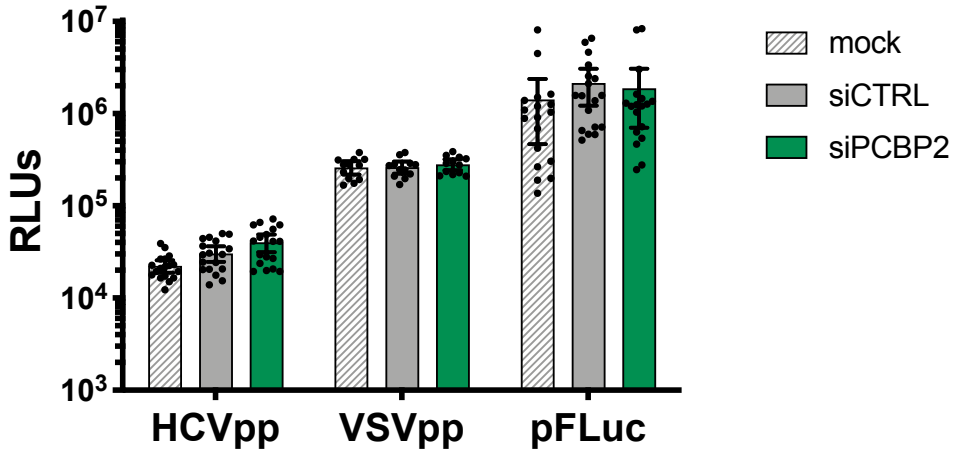


Figure 3.2. PCBP2 knockdown has no effect on HCV entry. Two days post-siRNA transfection, cells were spinoculated with luciferase reporter pseudoparticles bearing the HCV E1/E2 glycoproteins (HCVpp) or the VSV-G glycoprotein (VSVpp). In parallel, cells were transfected with a firefly luciferase expression plasmid. Samples were harvested 3 days post-infection/transfection, and analyzed by luciferase assay. The HCVpp and pFLuc data is representative of three independent replicates, while the VSVpp data is representative of two independent replicates. Error bars represent the standard deviation of the mean.

activity serves as a direct measure of HCV IRES-mediated translation, and over time this signal also serves as a proxy measure for viral RNA stability. We found that the siPCBP2 and siCTRL conditions had similar RLuc activity at all timepoints (**Figure 3.3**). Moreover, the luciferase signal half-lives were nearly identical, with 1.59 h (95% CI 1.13-2.21) for the siPCBP2 condition and 1.44 h (95% CI 1.11-1.85) for the siCTRL condition. In addition, we also assessed HCV IRES-mediated translation using luciferase reporter RNAs, and confirmed that PCBP2 knockdown had no effect on HCV IRES-mediated translation by itself (**Figure S3.3**). Thus, PCBP2 knockdown does not appear to modulate either HCV IRES-mediated translation or genome stability.

PCBP2 knockdown only has an effect on packaging-competent viral reporter RNAs

PCBP2 was previously reported to bind to and promote the replication of viral RNAs through interactions with their 5′ UTRs (242, 287). Since we found that endogenous PCBP2 was important for HCV RNA accumulation during infection yet did not affect HCV IRES-mediated translation or genome stability (**Figures 3.1C and 3.3**), we next examined if PCBP2 knockdown had an effect on viral RNA replication. To this end, we assessed RLuc expression of replication-competent reporter RNAs, starting with the full-length WT J6/JFH-1 RLuc RNA (**Figure 3.4**). Consistent with our results with HCVcc infection, we found that PCBP2 knockdown decreased luciferase activity of the WT J6/JFH-1 RLuc viral RNA during the first two days post-electroporation by approximately 2.6-fold (**Figure 3.4A**). However, while the large RLuc gene insertion severely impairs the WT J6/JFH-1 RLuc RNA packaging efficiency, some viral RNA can still exit cells as infectious viral particles (134, 260, 292). Thus, to specifically examine viral RNA replication in the complete absence of infectious particle production, we assessed luciferase

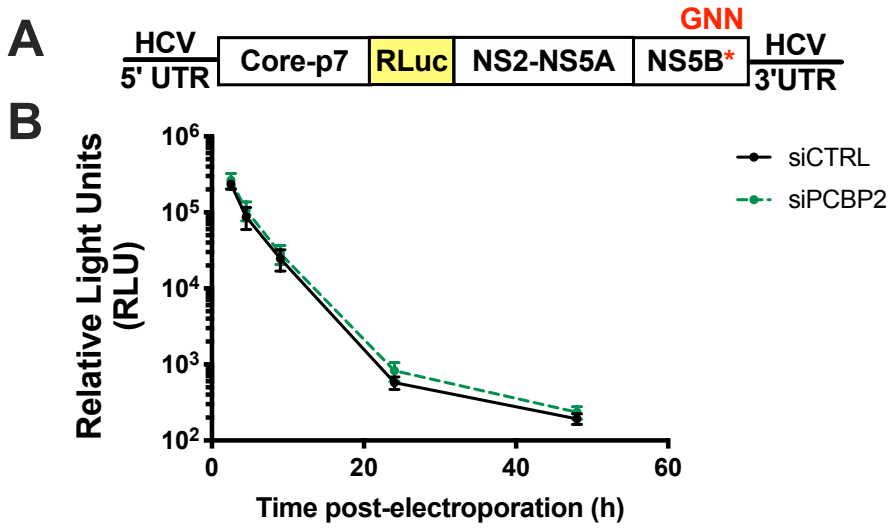


Figure 3.3. PCBP2 knockdown has no impact on HCV translation or genome stability. (A) Schematic representation of the J6/JFH-RLuc-GNN reporter RNA. **(B)** Huh-7.5 cells were electroporated with J6/JFH-RLuc-GNN reporter RNAs and a FLuc control mRNA, and luciferase activity was monitored at several timepoints post-electroporation. All data are representative of three independent replicates. Error bars represent the standard deviation of the mean.

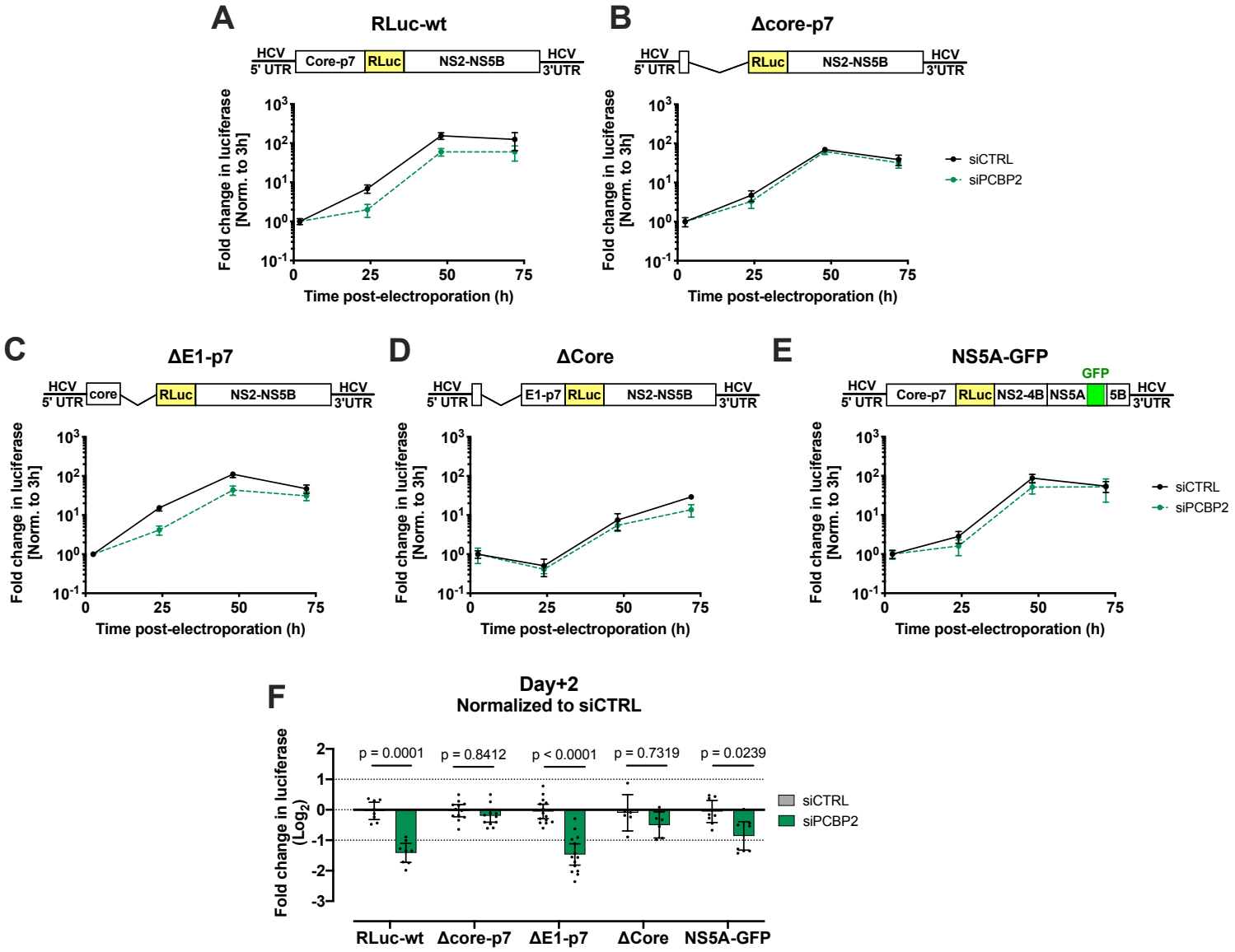


Figure 3.4. PCBP2 knockdown affects reporter viruses when the core-NS5A interaction is conserved. Two days post-siRNA transfection, Huh-7.5 cells were electroporated with **(A)** full-length RLuc WT J6/JFH-1 RNA, **(B)** Δcore-p7 J6/JFH-1 RLuc RNA, **(C)** ΔE1-p7 J6/JFH-1 RLuc RNA, **(D)** ΔCore J6/JFH-1 RLuc RNA, or **(E)** full-length NS5A-GFP J6/JFH-1 RLuc RNA. RLuc values were normalized to the early timepoint (3 h), to control for disparities in electroporation efficiency between experiments. **(F)** Comparison of luciferase values obtained 2 days post-electroporation, normalized to the siCTRL condition. Data are representative of three independent replicates for RLuc-wt, Δcore-p7, ΔE1-p7 and NS5A-GFP, and two independent experiments for ΔCore; error bars represent the standard deviation of the mean. P-values were calculated by two-way ANOVA.

activity of a subgenomic J6/JFH-1 replicon RNA lacking all of the structural protein genes (Δcore-p7, **Figure 3.4B**). We found that PCBP2 knockdown no longer had an impact on Δcore-p7 luciferase reporter gene expression or RNA accumulation, suggesting that PCBP2 does not modulate viral RNA replication (**Figure 3.4B and S3.4A)**. However, as a previous iCLIP study had identified two conserved PCBP2 binding sites in this region of the genome (i.e. *core* and *E2*), it was unclear whether these results suggested that the effect of PCBP2 knockdown on HCV RNA accumulation depended upon the core-p7 region of the viral RNA, or if this was related to a specific effect on viral assembly and/or egress. Thus, to further investigate why PCBP2 knockdown is detrimental to full-length viral RNA replication yet has no impact on the Δcore-p7 RNA, we attempted to map the structural gene requirements for PCBP2 sensitivity. Specifically, we made use of subgenomic viral RNAs with deletions of the E1-p7 (ΔE1-p7) or the core-coding (ΔCore) region (**Figure 3.4C and D**). We found that ΔE1-p7 was sensitive to PCBP2 knockdown, with a 2.5-fold decrease in luciferase activity during the first two days post-electroporation, which corresponded to a decreased RNA accumulation (**Figure 3.4C and S3.4C**). In contrast, while ΔCore exhibited delayed replication kinetics, PCBP2 knockdown did not result in any further impairment of viral RNA replication (**Figure 3.4D**). Taken together, these results suggest that PCBP2 knockdown only delays the replication of viral RNAs that contain the *core* gene.

To further explore if PCBP2 modulated viral assembly, we made use of a packaging-defective viral RNA that retains the *core* gene, specifically a full-length J6/JFH-1 RLuc NS5A-GFP reporter RNA (**Figure 3.4E**). While HCV virion assembly is still an incompletely understood process, the NS5A protein is known to play crucial roles in both viral replication and assembly (126, 127). Previous studies have identified key residues in NS5A domain III that must be

phosphorylated for NS5A to interact with the core protein and enable nucleocapsid assembly, and disruption of domain III by a GFP insertion has been shown to reduce viral assembly without impacting viral RNA replication (126, 127, 267). Moreover, PCBP2 has been reported to interact with the NS5A protein, at least *in vitro* (35). We therefore hypothesized that PCBP2 may regulate viral assembly by modulating core-NS5A interactions, which could explain why only RNAs that contained the *core* gene were sensitive to PCBP2 knockdown. In agreement with this hypothesis, PCBP2 knockdown did not significantly alter NS5A-GFP luciferase activity (**Figure 3.4E and F**). Thus, taken together, our results suggest that PCBP2 knockdown does not impair viral RNA replication, and only modulates the accumulation of packaging-competent viral RNAs. Specifically, our results suggest that PCBP2 likely plays a role in the early steps of viral assembly, during transfer of the viral RNA from NS5A to the core protein for packaging.

PCBP2 knockdown does not affect virus egress

As we established that PCBP2 knockdown primarily affected the viral assembly and packaging step of the viral life cycle, we wanted to understand if PCBP2 knockdown also had an impact on virion egress. Thus, to examine virion assembly and egress in isolation from earlier steps of the viral life cycle, we inhibited viral RNA replication with the nucleoside analog 2′ C-methyladenosine (2′CMA) and monitored viral titers over time (268). Three days post-infection, we replaced the culture media with media containing 5 μM 2′CMA (or DMSO, as a control) and collected total RNA, intracellular virions and extracellular virions over the next 12 h (**Figure 3.5A**). In agreement with our previous findings, we observed an overall reduction in viral RNA accumulation during PCBP2 knockdown (0 h timepoint, siCTRL vs. siPCBP2, **Figure 3.5B**). Importantly, the 2′CMA treatment efficiently blocked viral RNA accumulation, which continued

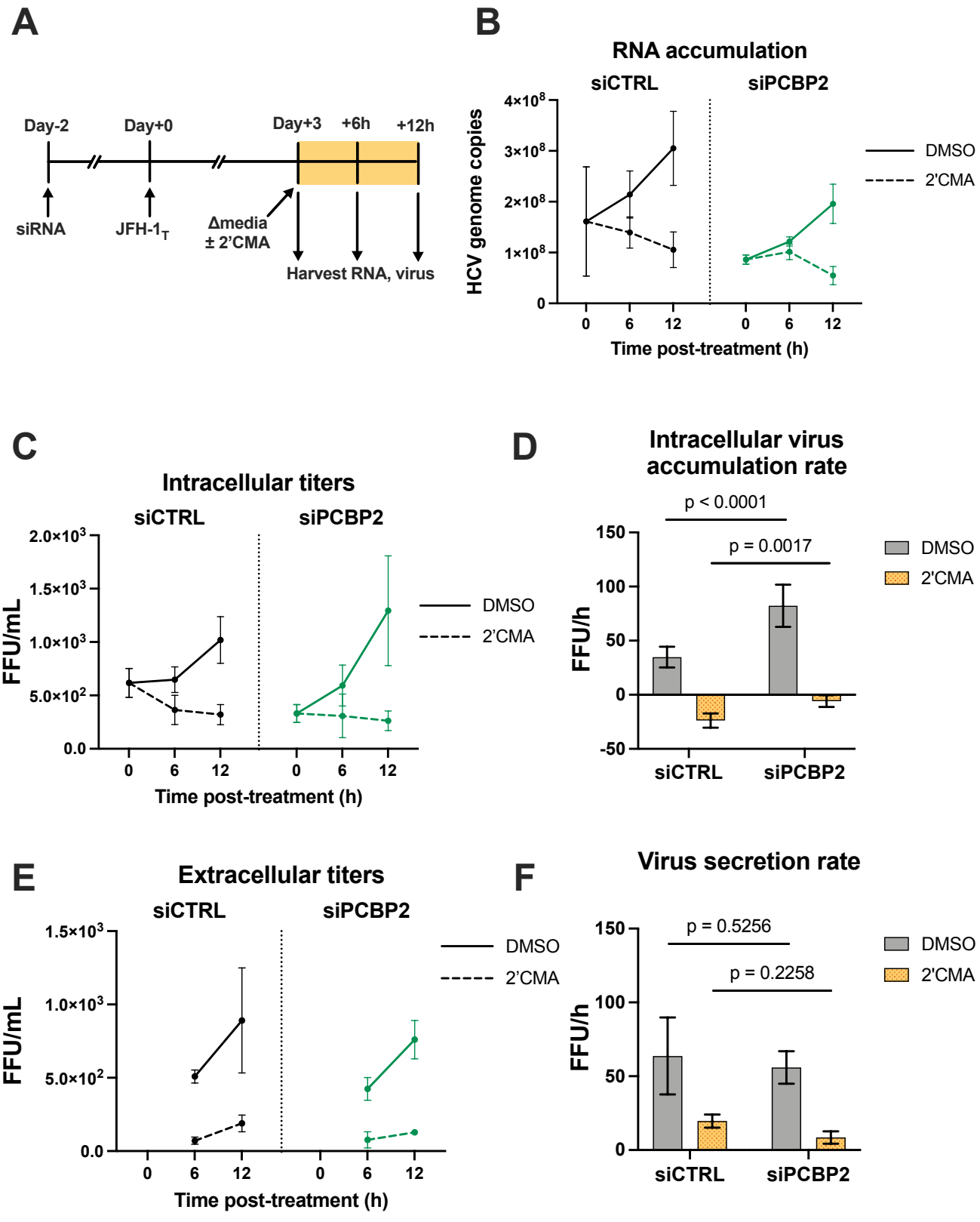

A
Day-2
Day+0
Day+3
+6h
+12h
siRNA
JFH-1T
Δmedia
± 2'CMA
Harvest RNA, virus
B
RNA accumulation
siCTRL
siPCBP2
HCV genome copies
4×10^8
3×10^8
2×10^8
1×10^8
0
0
6
12
Time post-treatment (h)
DMSO
2'CMA
C
Intracellular titers
siCTRL
siPCBP2
FFU/mL
2.0×10^3
1.5×10^3
1.0×10^3
5.0×10^2
0.0
Time post-treatment (h)
DMSO
2'CMA
D
Intracellular virus accumulation rate
p < 0.0001
p = 0.0017
FFU/h
150
100
50
0
-50
siCTRL
siPCBP2
DMSO
2'CMA
E
Extracellular titers
siCTRL
siPCBP2
FFU/mL
1.5×10^3
1.0×10^3
5.0×10^2
0.0
Time post-treatment (h)
DMSO
2'CMA
F
Virus secretion rate
p = 0.5256
p = 0.2258
FFU/h
150
100
50
0
siCTRL
siPCBP2
DMSO
2'CMA

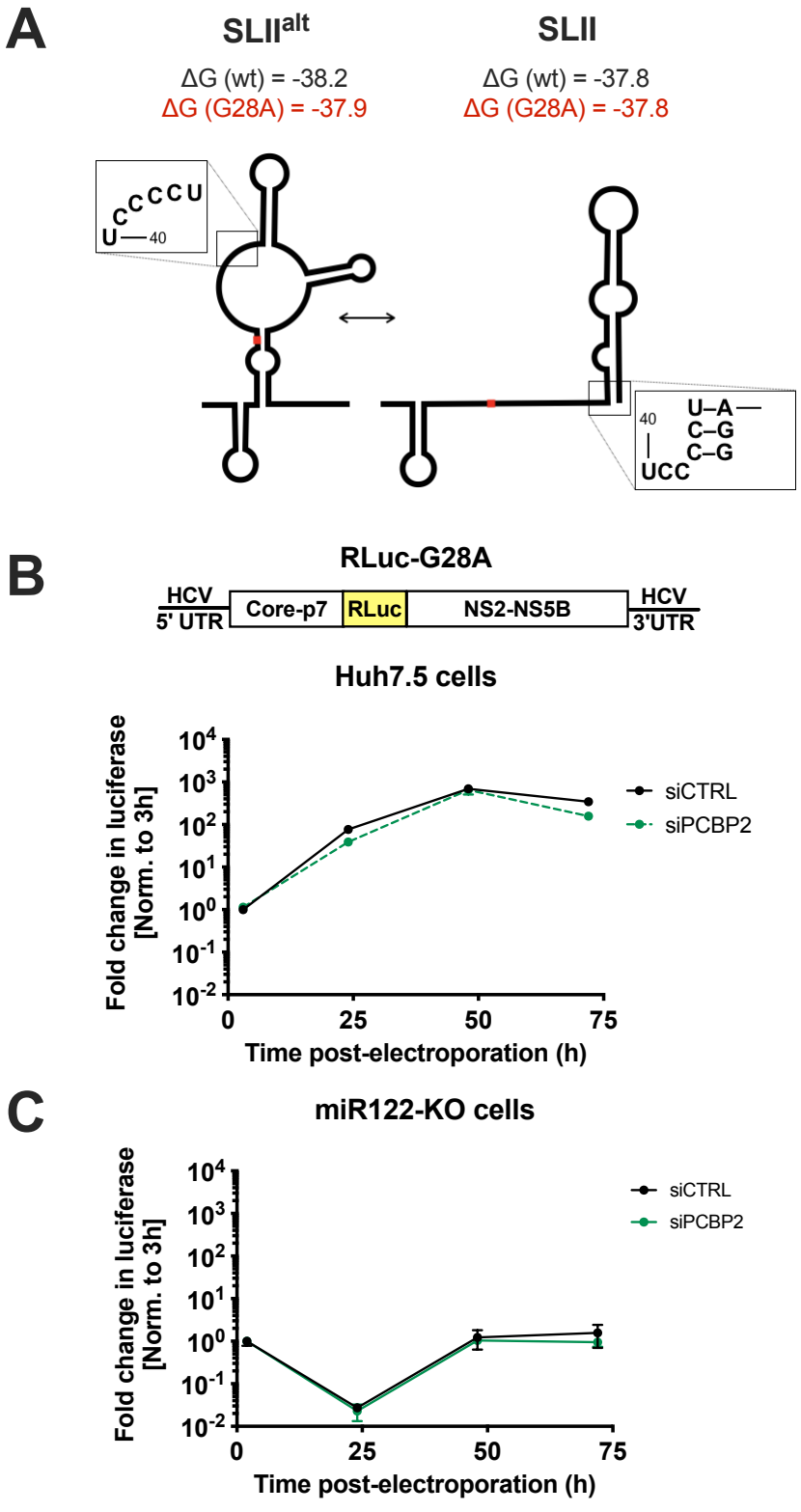
Figure 3.5. PCBP2 knockdown affects virion assembly, but not egress. **(A)** Schematic representation of the experimental approach for 2´CMA experiments: two days post-siRNA transfection, Huh-7.5 cells were infected with JFH-1_T (MOI = 0.05). Three days post-infection, cells were treated with 2´CMA or DMSO (control). Total RNA, intracellular and extracellular virus was collected at t = 0, 6 and 12 h post-treatment. **(B)** Quantitative RT-PCR analysis after 2´CMA treatment. **(C)** Intracellular viral titers and **(D**) intracellular virus accumulation rate calculated by linear regression. **(E)** Extracellular viral titers and **(F)** virus secretion rates calculated by linear regression. All data are representative of three independent replicates and error bars in (B), (C) and (E) represent the standard deviation of the mean. Error bars in (D) and (F) represent the slopes of the linear regressions ± standard error. P-values were calculated by two-way ANOVA.

to increase under the control (DMSO) condition (6-12 h timepoints, **Figure 3.5B**). When we evaluated intracellular viral titers, we observed lower initial titers in the siPCBP2 condition, yet both siRNA conditions arrived at nearly identical viral titers under both treatment conditions by the 12 h timepoint (**Figure 3.5C**). Thus, compared to siCTRL, PCBP2 knockdown resulted in a steeper increase in intracellular virion accumulation during DMSO treatment and a shallower decrease following 2′CMA treatment, which are reflected in the intracellular virus accumulation rates (**Figure 3.5D**). Yet, with regards to extracellular (secreted) viral titers, we did not observe any significant differences between the PCBP2 knockdown or control conditions (**Figure 3.5E-F**). Thus, our data suggests that in the absence of ongoing viral RNA replication, viruses are secreted from wild-type and PCBP2 knockdown cells at the same rate. However, while virus egress depletes the intracellular virion pool under wild-type conditions, PCBP2 knockdown appears to enhance viral assembly, leading to an overall increase in the pool of intracellular viral particles. Taken together, our findings suggest that PCBP2 knockdown does not alter virus egress, but rather acts specifically at the virion assembly step of infectious viral particle production.

The miR-122-independent G28A variant is also PCBP2-independent

PCBP2 has previously been reported to bind the HCV 5′ UTR in close proximity to the two miR-122 binding sites (35, 251). Previously, Masaki *et al.* reported that PCBP2 and miR-122 directly compete for binding to the HCV 5′ UTR, and that this competition regulates the proportion of viral genomes engaged in translation versus viral RNA replication (256). Specifically, they suggested a model where PCBP2 promotes translation of the viral RNA, while miR-122 promotes viral RNA synthesis. While our data do not support a role for PCBP2 in viral translation, our

data thus far cannot rule out the possibility that PCBP2 competes with miR-122 for binding to the HCV 5′ UTR. Based on the previous iCLIP data and due to the affinity of PCBP2 for single-stranded poly(rC) sequences, we hypothesized that the primary PCBP2 binding site on the 5' UTR is likely to be a 4-nt cytidine stretch from nt 41-44 in the HCV 5′ UTR (**Figure 3.6A**). This site is single-stranded in the SLII^{alt} conformation, but is engaged in base-pairing interactions in the functional SLII structure (170, 293). Interestingly, the miR-122-independent G28A mutant was recently shown to favor the functional SLII structure, even in the absence of miR-122 (170, 171, 293). We therefore hypothesized that G28A may be less susceptible to PCBP2 knockdown because the primary PCBP2 binding site in the 5′ UTR is less accessible in this mutant. In agreement with this hypothesis, we found that G28A accumulation was not affected by PCBP2 knockdown in either Huh-7.5 or miR122-KO cells (**Figure 3.6B-C**). Since the G28A mutation is the only difference between PCBP2-sensitive (RLuc-WT, **Figure 3.4A)**, and PCBP2-insensitive viral RNA accumulation (RLuc-G28A, **Figure 3.6B**), our results suggests that it is not miR-122 binding itself, but another property of the RNA — such as its ability to form SLII^{alt} or its packaging efficiency — that is required for PCBP2 to exert an effect on the HCV life cycle.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SLIIalt
SLII
ΔG (wt) = -38.2
ΔG (G28A) = -37.9
ΔG (wt) = -37.8
ΔG (G28A) = -37.8
UCCCCCU
U—40
40
U–A
C–G
C–G
UCC
B
RLuc-G28A
HCV 5' UTR
Core-p7
RLuc
NS2-NS5B
HCV 3'UTR
Huh7.5 cells
Fold change in luciferase [Norm. to 3h]
Time post-electroporation (h)
siCTRL
siPCBP2
C
miR122-KO cells
Fold change in luciferase [Norm. to 3h]
Time post-electroporation (h)
siCTRL
siPCBP2

Figure 3.6. PCBP2 knockdown does not affect the G28A resistance-associated variant. **(A)** Schematic representation of the HCV 5′ UTR and the SLII^{alt} and SLII conformations, including the free energies (ΔG) for WT and G28A. The location of the G28A mutation is indicated (red). The stretch of single-stranded cytidine residues (nt 41-44) in the SLII^{alt} and SLII conformations are also indicated (insets). **(B)** Two days post-siRNA transfection, the full-length G28A J6/JFH-1-RLuc RNA was electroporated into Huh-7.5 cells, and luciferase activity was monitored at several timepoints post-electroporation. **(C)** Two days post-siRNA transfection, the same full-length G28A J6/JFH-1-RLuc RNA was electroporated into miR-122-knockout (miR122-KO) Huh7.5 cells, and luciferase activity was monitored post-electroporation. Data are representative of three independent replicates (Huh-7.5 cells) or two independent replicates (miR122-KO cells), and error bars represent the standard deviation of the mean.

3.6 DISCUSSION

Herein, we investigated the role of PCBP2 in the HCV life cycle. We found that PCBP2 was not necessary for viral entry, translation, genome stability or RNA replication in the absence of virion assembly; rather, we found that endogenous PCBP2 was only necessary for the optimal accumulation of viral RNAs that could complete early steps of the genome packaging process. Accordingly, we observed that PCBP2 knockdown increased the virion assembly rate without affecting virion egress. Finally, we found that the G28A mutation renders viral RNAs PCBP2-independent, in the presence or absence of miR-122. Thus, we propose that PCBP2's effect on the viral life cycle is not strictly due to its competition with miR-122 to bind to the HCV 5′ UTR, but is instead determined by another property of the viral RNA, i.e. its ability to form the $SLII^{alt}$ secondary structure and/or its packaging efficiency.

Although HCV packaging and assembly are still incompletely understood, data to date suggest that it is a two-step process: 1) NS5A transfers the viral genome to the core protein at the surface of lipid droplets; and 2) a protein complex involving NS2, NS3, NS4A and the structural proteins simultaneously assemble and envelope the nucleocapsid as it buds into the ER lumen (59, 71, 83, 126, 127, 129, 264, 294). As such, while viral RNAs are not packaged into true nucleocapsids in the absence of the E1/E2 glycoproteins, it is likely that the core protein can still associate with the viral RNA in their absence. Thus, based on our findings, we propose that endogenous PCBP2 normally interferes with the transfer of the viral genomic RNA to core by the NS5A protein, thereby preventing premature virion assembly, and indirectly enhancing viral translation and RNA replication (**Figure 3.7**).

Importantly, this model is based on our observation that PCBP2 knockdown only delays

viral RNA replication when the NS5A-core interaction is intact. While WT or ΔE1-p7 luciferase reporter RNAs display delayed viral RNA replication during PCBP2 silencing, RNAs that do not express the viral core protein (Δcore-p7 and ΔCore) or that express a GFP-tagged NS5A protein (NS5A-GFP), reported to impair the core-NS5A interaction, are far less sensitive to PCBP2 knockdown (**Figure 3.4**) (126). Furthermore, we propose that PCBP2 specifically interferes with the core-NS5A interaction step rather than impairing the overall process of virion assembly, because ΔE1-p7 RNAs are just as sensitive to PCBP2 knockdown as the wild-type viral RNA but cannot form enveloped viral particles due to the lack of E1/E2 glycoproteins (**Figure 3.4A, C**). Interestingly, other groups have reported that some core protein can be secreted from infected cells in the absence of the E1 and E2 glycoproteins, albeit less efficiently than when virion assembly is intact (71, 264). Thus, it seems plausible that when ΔE1-p7 RNA is efficiently loaded onto the core protein by NS5A during PCBP2 knockdown, a portion of these core-associated RNAs may be removed from the cell through non-enveloped core oligomer secretion. This is consistent with our observation that PCBP2 knockdown decreases positive-sense ΔE1-p7 genomic RNA accumulation, but not the accumulation of the negative-strand replication intermediate (**Figure S3.4D**).

The exact molecular mechanism by which PCBP2 interferes with the core-NS5A interaction is unknown. Two possibilities are that PCBP2 may directly interact with NS5A, and/or that PCBP2's binding to the viral RNA prevents NS5A or core from binding the viral genome. PCBP2 was previously shown to bind NS5A *in vitro* and to co-precipitate with exogenously overexpressed NS5A (35, 252). Thus, we speculate that PCBP2 might interact with NS5A in a manner that directly blocks the NS5A-core interaction, or that interferes with the

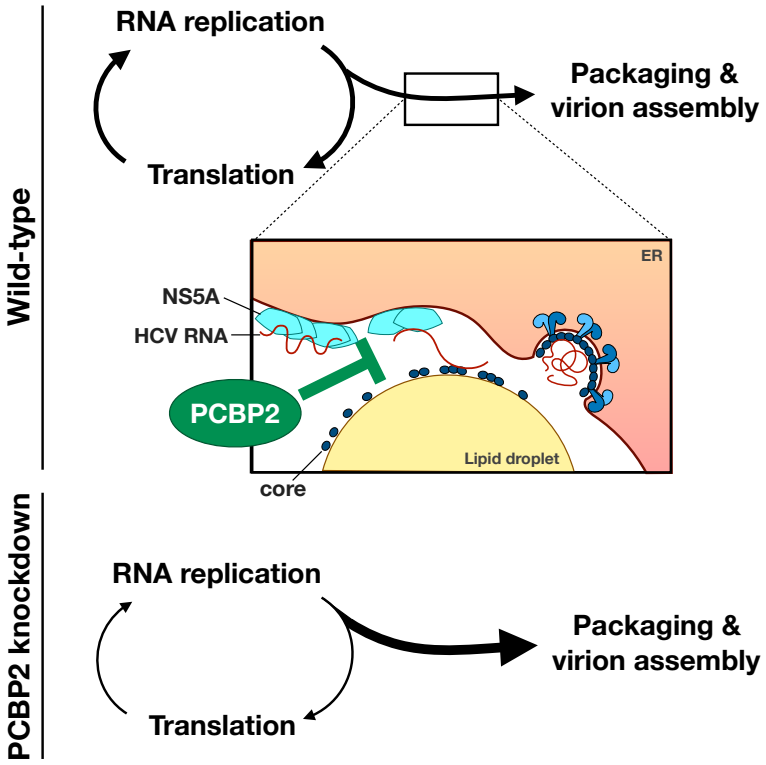

Figure 3.7. Model of PCBP2's effects on the HCV life cycle. Normally, PCBP2 prevents the early steps of virion packaging mediated by core and NS5A (box); this interference enables viral RNAs to engage in translation and RNA replication, and ultimately increases long-term virion production. In contrast, when endogenous PCBP2 is silenced, NS5A is free to transfer the viral RNA to the core protein for packaging; this depletes the pool of viral RNAs engaged in translation and replication, and ultimately impairs viral RNA accumulation and infectious particle production.

phosphorylation of NS5A that mediates its interactions with core or the HCV RNA (128). However, distinguishing between these possibilities will require further mapping of the PCBP2-NS5A and core-NS5A interfaces. An alternative possibility is that PCBP2 interferes with NS5A's or core's interactions with the viral RNA. The PCBP2 iCLIP study showed that its dominant binding site was at the poly(U/UC) tract, a region of the genome known to be bound by NS5A (251, 295). It is currently unclear if PCBP2 could block or displace NS5A by binding this poly(U/UC) tract, or if these two proteins could simultaneously bind the same region of the viral genome. As for the core protein, it has been proposed to bind numerous low-affinity packaging signals located all over the viral genome (47, 296-298). While PCBP2 binds most strongly to the poly(U/UC) tract, it still binds sites that span multiple regions of the genome; whether PCBP2 could directly block core binding by occupying sites that overlap with core's packaging signals — or if PCBP2 binding could alter the local RNA secondary structure and disrupt the structural requirements of these packaging signals — still remains to be examined.

While very few studies investigating PCBP2-HCV interactions used a fully infectious system, previous reports using fully infectious virus are consistent with our model that PCBP2 modulates the viral packaging step. For example, Randall *et al.* assessed PCBP2's effect on a cell culture-competent strain of HCV in the context of a large siRNA screen (250). Notably, this screen reported markedly different effects on HCV replication depending on the timing between siRNA transfection and infection with HCVcc, i.e. infecting cells three days post-PCBP2 siRNA transfection decreased both viral RNA accumulation (1.8-fold) and infectious particle production (3.1-fold), while infecting cells at earlier timepoints (i.e. two days post-transfection), resulted in an increase in viral RNA accumulation (1.8-fold) and virion production (3.4-fold) (250). This is

consistent with our finding that siPCBP2 transfection led to a transient increase in PCBP2 protein expression prior to its sustained knockdown (**Figure S3.1B**). Thus, infecting cells at a timepoint when PCBP2 levels are elevated would initially enhance viral replication by preventing genomic RNA packaging; at later timepoints, when PCBP2 is effectively silenced and virion assembly increases (as observed in **Figure 3.5D)**, this larger pool of viral RNAs would make it possible to produce a greater quantity of infectious particles.

Importantly, our model does not require PCBP2 to play a direct role in translation or viral RNA replication to enhance viral protein expression or intracellular RNA accumulation. Indeed, we found that PCBP2 had no direct effects on viral translation when assessed in isolation from the rest of the viral life cycle, which is consistent with prior studies that examined PCBP2's effect on HCV IRES-mediated translation (240, 253-255). Furthermore, we found that PCBP2 was not necessary for viral RNA replication, as the Δcore-p7 and Δcore subgenomic RNAs were not sensitive to PCBP2, consistent with a prior report that PCBP2 knockdown does not affect the replication of a subgenomic HCV replicon that did not contain the *core* through *NS2* genes (257). Although our results are largely in agreement with prior studies, our findings are seemingly in contrast with those reported by Masaki *et al.*, who found that silencing PCBP2 reduced nascent viral protein synthesis in a stably infected Huh-7.5 cell line (256). However, a direct effect on protein synthesis is not necessarily supported by their data, which shows that PCBP2 knockdown impairs viral translation but also decreases viral RNA accumulation to a similar extent, thereby reducing the overall quantity of templates available for translation (256). Interestingly, PCBP2 knockdown does not reduce the rate of nascent viral RNA synthesis, suggesting that a similar number of viral replication complexes are present under PCBP2 knockdown and control

conditions. This observation is compatible with our model: while PCBP2 knockdown decreases the total intracellular RNA pool by promoting virion packaging, it is unlikely to disrupt the already established replication complexes of a persistently HCV-infected cell population.

In addition, Masaki *et al.* previously reported that miR-122 supplementation stimulates nascent viral RNA synthesis in a PCBP2-dependent manner, and that miR-122 and PCBP2 compete for the 5′ terminus of the viral RNA (256). However, our study suggests that replication of the G28A mutant – which can still interact with miR-122 but favours the functional SLII structure over the SLII^{alt} conformation – was unaffected by PCBP2 knockdown in both the presence and absence of miR-122 (**Figure 3.6B-C**). Thus, it seems that the ability to form SLII^{alt}, rather than direct competition with miR-122, is important for PCBP2's effects on the HCV life cycle. Notably, this is consistent with the accessibility of the predicted PCBP2 binding site (nts 41-44) in the 5′ terminus, as these nucleotides are predicted to be single-stranded in the SLII^{alt} conformation, but partially base-paired and thus inaccessible for PCBP2 binding in the functional SLII conformation (**Figure 3.6A**). Moreover, the findings by Masaki *et al.* are likely further confounded by their choice of HCV "bait" RNA in competition assays, which consisted of nts 1-46 of the genotype 1 HCV genome that forces the putative PCBP2 binding site residues into a single-stranded conformation and is too short to form the SLII^{alt} or functional SLII structures, thereby creating an "artificial" PCBP2 binding site (256). Thus, while it is still possible that miR-122 and PCBP2 compete for binding to this region of the genome, more research is needed to determine whether miR-122 acts as a competitive inhibitor (i.e. by competing with PCBP2 for the 5′ UTR) or an allosteric inhibitor (i.e. by preventing PCBP2 interactions by re-folding the viral RNA into SLII). Nevertheless, our findings suggest that the conformation of the viral RNA,

rather than miR-122 binding *per se*, appears to be the main determinant of PCBP2 susceptibility.

Finally, it is possible that the SLII^{alt} conformation itself is important for recruitment of the viral RNA into the assembly pathway, through interactions with PCBP2 and/or other host or viral proteins. We cannot yet rule out the possibility that G28A is not sensitive to PCBP2 knockdown because it is simply not packaged into virions. However, this seems unlikely given that G28A is still able to form both SLII^{alt} and SLII conformations with similar free energies, and that the G28A polymorphism is already present in many HCV isolates (299). The importance of SLII^{alt} in the assembly of HCV particles is likely to be further clarified by examining fully riboswitched mutants (i.e. those only able to form SLII), such as the recently reported U4C mutant (300). Thus, further study is needed to clarify whether the SLII^{alt} conformation is important for HCV packaging.

Taken together, our results support a model where endogenous PCBP2 blocks the early steps of virion assembly mediated by the core and NS5A proteins. By preventing viral genome sequestration by the core protein, PCBP2 indirectly promotes viral RNA retention in the translating/replicating pool, which explains the discrepancies with respect to PCBP2's reported effects on viral translation and RNA replication. Furthermore, the G28A mutant – known to preferentially form the functional SLII conformation even in the absence of miR-122 – is insensitive to PCBP2 knockdown, suggesting that the conformation of the RNA is important for mediating PCBP2 interactions. However, future studies will be needed to reveal whether the SLII^{alt} conformation is absolutely required for HCV RNA packaging. The model presented herein helps clarify the role of PCBP2 in the viral life cycle and further informs our understanding of how cellular RNA binding proteins regulate viral genomic RNA utilization during the HCV life

cycle.

3.7 ACKNOWLEDGEMENTS

We would like to acknowledge Charlie Rice (Rockefeller University) for kindly providing the Huh-7.5 cells, pJ6/JFH FL RLuc WT and GNN plasmids; Matthew Evans (Icahn School of Medicine at Mount Sinai) for the miR-122 knockout (miR122-KO) Huh-7.5 cells; Rodney Russell (Memorial University) for providing JFH-1_T; Mamata Panigrahi and Joyce Wilson (University of Saskatchewan) for the pJ6/JFH mono RLuc NS2 and pJ6/JFH-1 E1-p7 del plasmids; Martin J Richer (McGill University) for the 293T cells; John Law (University of Alberta) for the kind gift of HCVpp. We are also grateful to Nathan Taylor, Julie Magnus, Carolina Camargo and Marylin Rheault (McGill University) for technical support.

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3.9 AUTHOR CONTRIBUTIONS

S.E.C. and S.M.S. designed the study; S.E.C. performed the experiments and analyzed the data, and S.E.C and S.M.S. wrote and edited the manuscript.

3.10 SUPPLEMENTARY METHODS

Strand-specific RT-qPCR

The iTaq Universal Probes One-Step kit (Bio-Rad) was used to perform duplex assays probing for the HCV genome (NS5B-FW primer: 5'-AGA CAC TCC CCT ATC AAT TCA TGG C-3'; NS5B-RV primer: 5'-GCG TCA AGC CCG TGT AAC C-3'; NS5B-FAM probe: 5'-ATG GGT TCG CAT GGT CCT AAT GAC ACA C-3') and the GAPDH loading control (PrimePCR Probe assay with HEX probe, Bio-Rad). For the reverse transcription step, each 18.75 µL reaction contained 500 ng of total RNA, 0.5 µL of the GAPDH primers and probe, and 10 pmol (0.25 µL of a 40 µM stock) of only one of the HCV primers (NS5B-FW for the negative-strand reaction, and NS5B-RV for the positive-strand reaction). Reverse transcription took place at 50°C for 10 minutes, after which 0.25 µL of the complementary primer and 1 µL of HCV primers pre-mixed with the probe were added to each reaction. The same PCR cycling protocol was followed as it would be for a typical HCV RT-qPCR, in a CFX90 Touch Deep Well Real-Time PCR system (Bio-Rad). Genome copies were calculated using a standard curve and fold-differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (270).

IRES-specific translation assays

Cells

HeLa cervical epithelial adenocarcinoma cells obtained from the ATCC (CCL-2) were maintained in DMEM supplemented with 10% FBS, at 37°C/5% CO_2, and were routinely screened for mycoplasma contamination.

Plasmids

Plasmids encoding a firefly luciferse (FLuc) reporter gene under the translational control of a poliovirus IRES (PV IRES, nt 71-732), HCV IRES (nt 40-372) or encephalomyocarditis virus IRES (EMCV IRES, nt 281-848) were a kind gift from Drs. Yuri Svitkin and Nahum Sonenberg (301). These plasmid templates were linearized with *BamHI* and *in vitro* transcribed using T7 RNA polymerase as previously described (174). The Renilla luciferase (RLuc) mRNA was transcribed from the pRL-TK plasmid (Promega) linearized using *BglII* and *in vitro* transcribed using the mMessage mMachine T7 Kit (Life Technologies) according to the manufacturer's instructions.

Transfection and luciferase assay

HeLa cells were plated at a density of 5 x 10^5 cells/10-cm dish and transfected with 50 nM of siPCBP2 or siCTRL (miR-$122_{p2\text{-}8}$) siRNA duplexes, as described in the main text's methods. The next day, transfected cells were seeded into 6-well plates at a density of 2.5 x 10^5 cells per well. On the second day post-siRNA transfection, each well was co-transfected with 1.5 μg of IRES-FLuc IVT and 2.5 μg of RLuc mRNA, resuspended into 1 mL OptiMEM with 2.5 μL of Lipofectamine 3000 (Invitrogen). Four hours post-transfection, cell culture media was changed for DMEM with 10% FBS.

The next day, each well was harvested in 100 μL of 1X passive lysis buffer (Promega). Renilla and firefly luciferase activity was measured using the Dual-Luciferase Assay Reporter Kit (Promega), following the manufacturer's instructions with the modification that 25 μL of reagent were used with 10 μL of sample. All samples were measured in triplicate.

3.11 SUPPLEMENTARY FIGURES

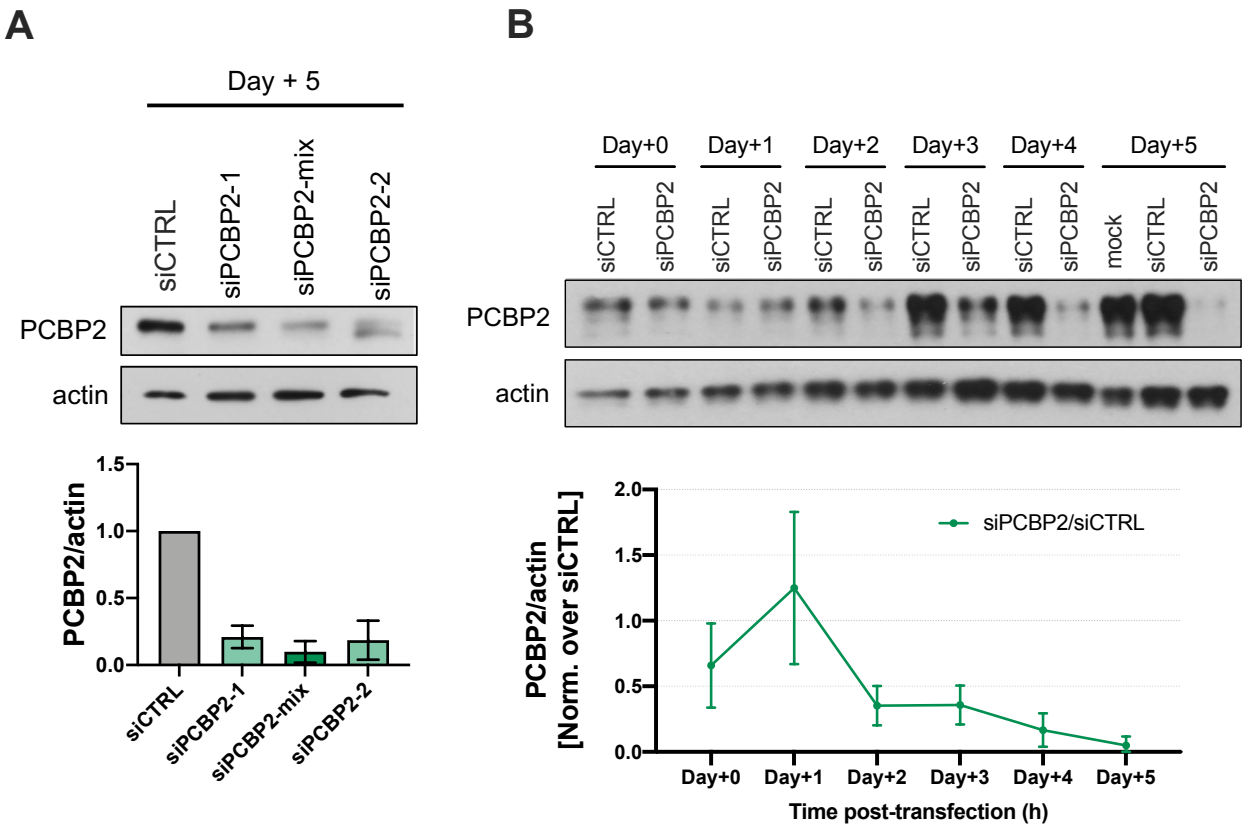

Figure S3.1. Optimization of the anti-PCBP2 siRNA transfection. (A) Comparison between single-siRNA transfections and the 1:1 combination of both siRNAs (siPCBP2-mix). The error bars (below) represent the standard deviation from two independent experiments. **(B)** Analysis of PCBP2 knockdown over time, compared to the siCTRL condition. PCBP2/actin band densities were compared to the same day's siCTRL condition (line graph, below); error bars represent the standard deviation from two independent replicates.

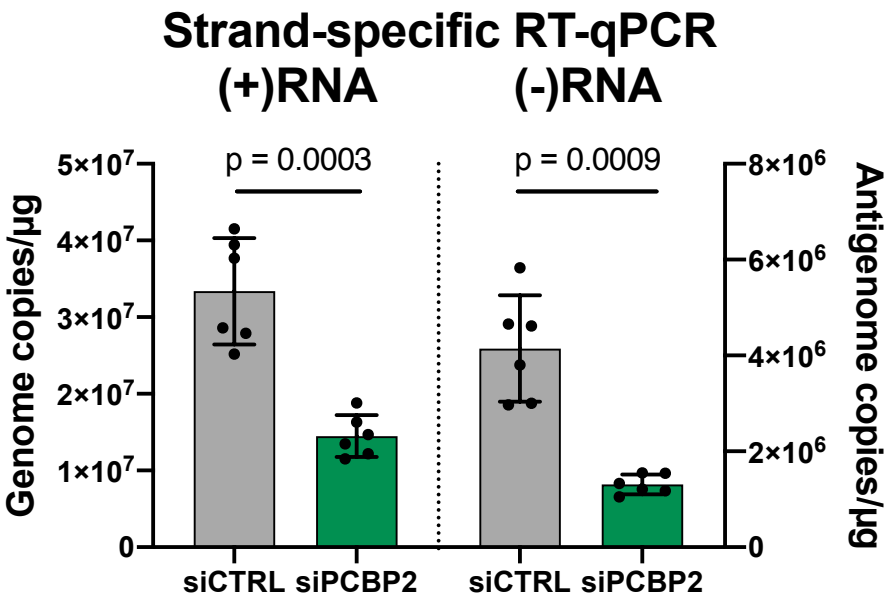

Figure S3.2. PCBP2 knockdown decreases both positive-sense and negative-sense viral RNA accumulation. Two days post-siRNA transfection, Huh-7.5 cells were infected with JFH-1_T (MOI = 0.05). Total RNA was harvested 3 days post-infection and analyzed by strand-specific RT-qPCR. Error bars represent the standard deviation from three independent replicates. P-values were calculated by paired t-test.

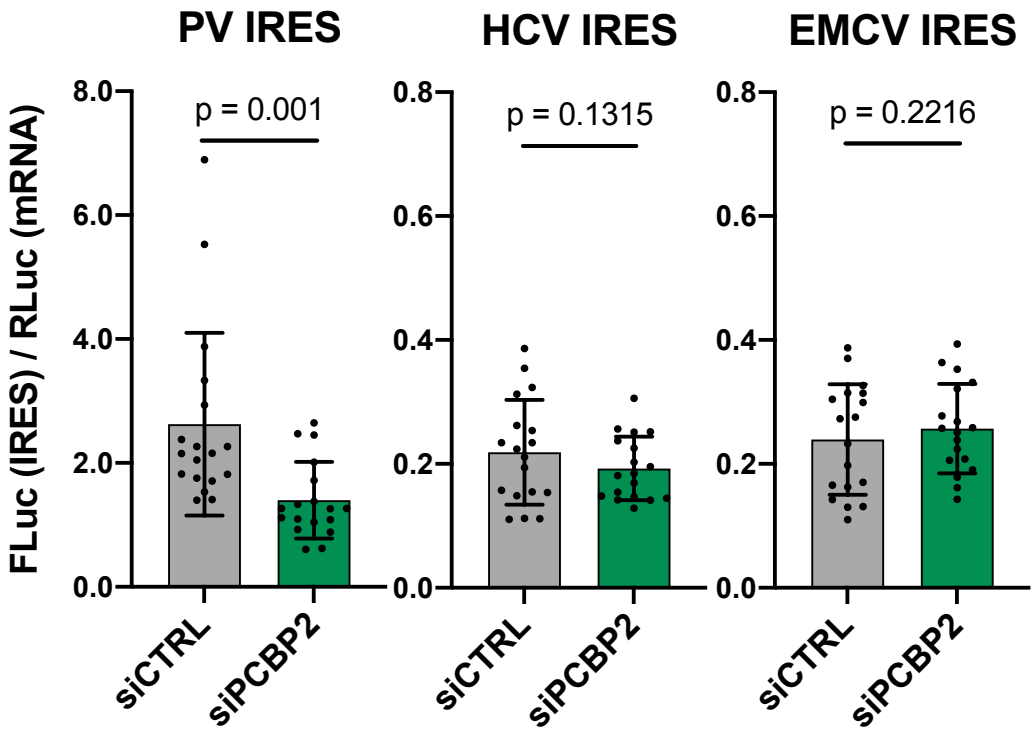

Figure S3.3. PCBP2 knockdown decreases PV IRES-mediated translation, but has no effect on HCV or EMCV IRES-mediated translation. Two days post-siRNA transfection, HeLa cells were co-transfected with 2.5 µg of Renilla luciferase mRNA and 1.5 µg of RNAs comprised of a firefly luciferase (FLuc) gene under the control of either the PCBP2-sensitive poliovirus (PV) IRES, the PCBP2-insensitive encephalomyocarditis virus (EMCV) IRES, or the hepatitis C virus (HCV) IRES. Total protein samples were harvested in passive lysis buffer one day later, and luciferase activity was determined using the Dual Luciferase Reporter Assay kit. The IRES-mediated translation signal (FLuc) was normalized to the transfection efficiency control (RLuc) signal. Bars represent the mean ± standard deviation of four independent replicates. P-values were determined by paired t-test.

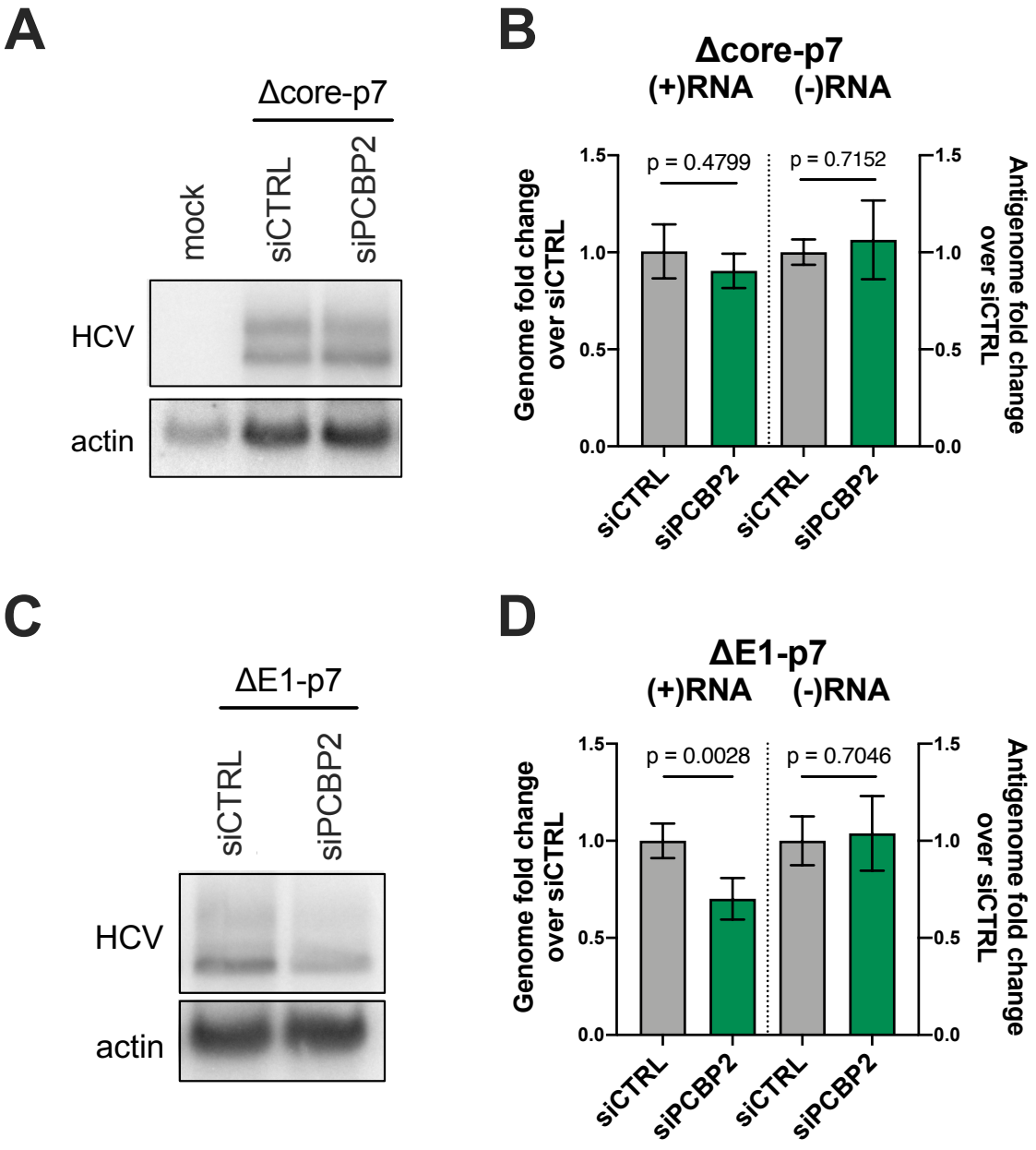

Figure S3.4. PCBP2 knockdown does not affect Δcore-p7 RNA accumulation, and only decreases positive-sense ΔE1-p7 RNA accumulation. **(A)** Representative Northern blot of of Δcore-p7 RNA accumulation, 2 days post-electroporation into Huh-7.5 cells. **(B)** Strand-specific RNA accumulation of Δcore-p7, assessed by strand-specific RT-qPCR. **(C)** Representative Northern blot of of ΔE1-p7 RNA accumulation, 2 days post-electroporation. **(D)** Strand-specific ΔE1-p7 RNA accumulation, 2 days post-electroporation. Error bars represent the standard deviation of two independent Δcore-p7 replicates or three independent ΔE1-p7 replicates. P-values were determined by paired t-test.

CHAPTER 4: Discussion

4.1 PCBP1 and PCBP2 regulate infectious particle assembly and secretion

PCBP1 and PCBP2 are among the most abundant human RNA-binding proteins, with high expression levels in most human tissues, including liver cells (188, 302). In the previous chapters, we reported that PCBP1 and PCBP2 were each necessary for optimal HCV RNA accumulation in cell culture — but resulted in opposite effects on extracellular (secreted) viral titers, with PCBP1 knockdown resulting in an increase in viral titer, while PCBP2 knockdown resulted in a reduction in viral titer. Neither PCBP1 or PCBP2 had a direct impact on viral entry, translation, RNA stability or viral RNA replication. Instead, we found that PCBP1 limited virion assembly and egress, while PCBP2 delayed virion assembly without a significant effect on virion secretion (summarized in **Figure 4.1**).

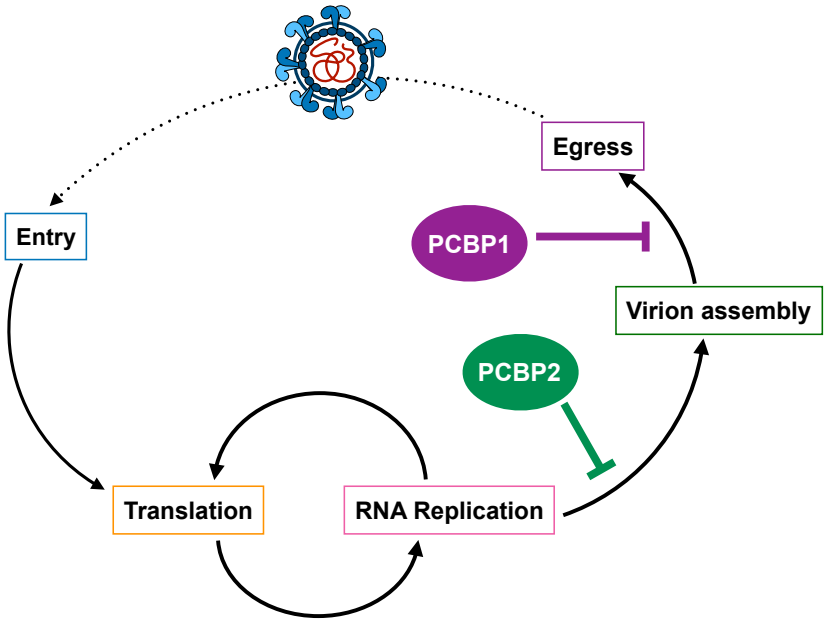

Figure 4.1. The roles played by PCBP1 and PCBP2 in the HCV life cycle. Endogenous PCBP1 limits HCV virion assembly and secretion, while PCBP2 blocks the early steps of viral RNA packaging into virions. As a result, both of these proteins normally enhance translation and RNA replication, without a direct impact on these steps of the viral life cycle.

4.1.1 PCBPs promote a "staying-at-home" strategy of viral replication

The idea that limiting virion assembly can increase viral titers may seem counterintuitive, since assembly is a necessary step to produce infectious viral particles. Yet mathematical modelling studies of HCV replication have shown that to maximize its viral progeny, HCV should prioritize RNA replication over virion assembly — an approach some have called a "staying-at-home" strategy, since nascent viral RNAs preferentially stay within their producer cell (303, 304). This strategy is in contrast to a "leaving-home" strategy, which allocates a greater proportion of viral genomes to infectious particle production (**Figure 4.2A**) (304). Compared to staying-at-home, leaving-home results in an early burst of secreted viral particles and a concomitant loss of intracellular genomic RNA as it is packaged into new infectious virions. These new virions are able to establish new infections in naïve cells, but the multi-step process of establishing a new infection (i.e. assembling and secreting virions, infecting a new cell, then undergoing translation and RNA replication) necessarily takes more time than the intracellular steps of the viral life cycle (i.e. translation and RNA replication). Thus, by the time a virus that has left home has generated its first RNA copies from a new infection, a virus that stayed at home will have completed multiple cycles of translation and RNA replication and generated an exponentially larger pool of viral RNAs available for packaging into new virions. Assuming that a virus following a staying-at-home strategy still assembles virions, that viral replication does not kill host cells (to note: HCV is non-lytic), and that naïve cells are always available for infection, the staying-at-home strategy is thus predicted to eventually lead to a greater amount of progeny infectious particles than the leaving-home strategy (**Figure 4.2B**). The time required to reach this crossover point, where staying-at-home produces more virions than leaving-home, depends on

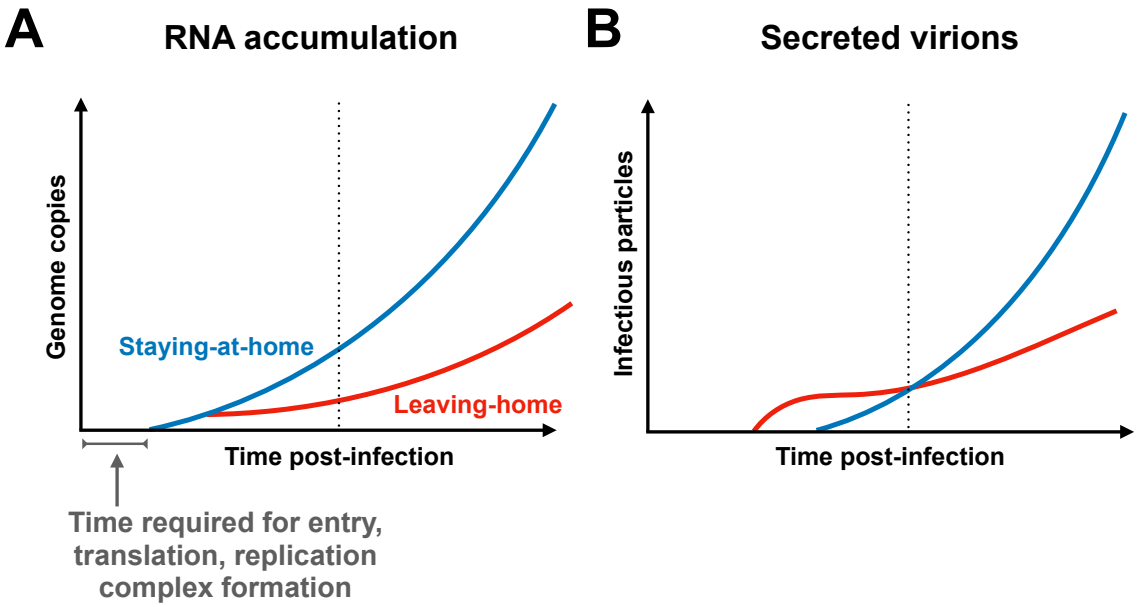

Figure 4.2. How the "staying-at-home" and "leaving-home" viral replication strategies affect viral RNA accumulation and virion secretion. (A) Summary of how prioritizing RNA replication ("staying-at-home") or preferentially forming infectious viral particles ("leaving-home") affects RNA accumulation. **(B)** Impact of the two strategies on infectious particle secretion. The vertical dotted line represents the crossover point, where the staying-at-home strategy produces a greater viral progeny than the leaving-home strategy. This model assumes that the staying-at-home strategy produces some viral particles, that viral RNA accumulation does not trigger cell death, and that there is an infinite number of naïve cells available for secreted virions to infect.

the relative proportion of genomes engaged in virion assembly; two viruses that take on similar staying-at-home strategies (with one virus leaning slightly more towards leaving home), will take longer to reach this crossover point than two viruses that take on more extreme staying-at-home and leaving-home strategies (**Figure 4.3**).

The data presented in this thesis suggest that endogenous PCBPs normally help HCV employ a staying-at-home strategy. In ***Chapter 3***, we reported that PCBP2 knockdown increases the virion assembly rate and, by day 3 post-infection, results in a reduction in total viral RNA accumulation and infectious particle production. These observations are consistent with the

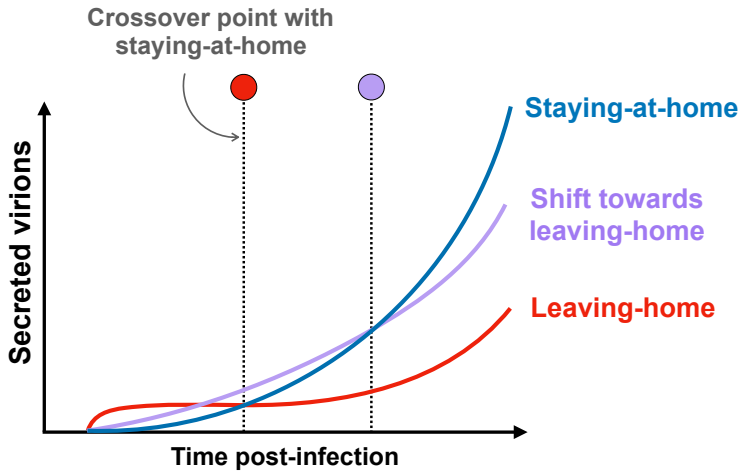

Figure 4.3. How an intermediate strategy delays the crossover point of virion production. A staying-at-home virus (blue) will take longer to out-compete the virion progeny of a virus taking on an intermediate strategy (purple) than a virus that takes on a more extreme leaving-home strategy (red). The point at which the staying-at-home strategy out-competes each strategy is indicated with a dotted line, with the associated colour indicated above. This model assumes that the staying-at-home strategy produces some viral particles, that viral RNA accumulation does not cause cell death, and that there is an infinite number of naïve cells available for secreted virions to infect.

right-hand side of the **Figure 4.2B** graph, past the point at which the staying-at-home virion progeny has surpassed the leaving-home progeny. Since PCBP2 knockdown favours a leaving-home strategy, endogenous PCBP2 logically helps to support the staying-at-home strategy. In contrast, in ***Chapter 2***, we reported that depleting endogenous PCBP1 increased both virion assembly and egress. Furthermore, by day 3 post-infection, PCBP1 knockdown results in a reduction in total viral RNA accumulation, yet enhances secreted viral titers. Within the framework of the staying-at-home versus leaving-home strategies, there are two possible explanations for this relative increase in extracellular infectious particles, either: 1) PCBP1 knockdown provided a more subtle shift towards the leaving-home strategy than PCBP2 knockdown; or 2) PCBP1 knockdown enhanced infectious particle production so effectively that

no naïve cells remained to be infected in the culture.

In the first scenario, PCBP1 knockdown only gently nudges HCV towards a leaving-home strategy; in other words, the PCBP1 knockdown would result in an intermediate strategy, somewhere between the normal staying-at-home strategy and the leaving-home strategy adopted during PCBP2 knockdown (**Figure 4.3**). This could plausibly delay the crossover point past day 3 post-infection (i.e. day 3 would be to the left of the dotted line in **Figure 4.2**). However, if this were the case, we would expect that PCBP1 knockdown would result in an intermediate level of viral RNA accumulation, somewhere between PCBP2 knockdown and control conditions. Yet, we observed more HCV RNA accumulation during PCBP2 knockdown (~8.62 x 10^7 genomes/μg) than during PCBP1 knockdown (~3.91 x 10^7 genomes/μg). Thus, it seems unlikely that the PCBP1 knockdown exerts less of a shift towards the leaving-home strategy than PCBP2 knockdown.

The second possibility is that the PCBP1 knockdown was so effective at producing infectious particles that it broke some of the assumptions of the model shown in **Figure 4.2**. The leaving-home strategy increases virus spread, so within a finite system (such as a tissue culture dish) a virus engaged in a leaving-home strategy will infect all available naive cells faster than a staying-at-home virus (**Figure 4.4A**, dotted line). This poses a challenge to further viral growth, since HCV infection renders cells refractory to subsequent infection, an ability known as superinfection exclusion. This superinfection exclusion was initially proposed to occur at a post-entry step of the viral life cycle such as translation or RNA replication, since HCVpp can generally enter cells bearing HCV replicons (140, 141). However, more recent studies have reported that HCVcc infection leads to the downregulation of some of its entry receptors

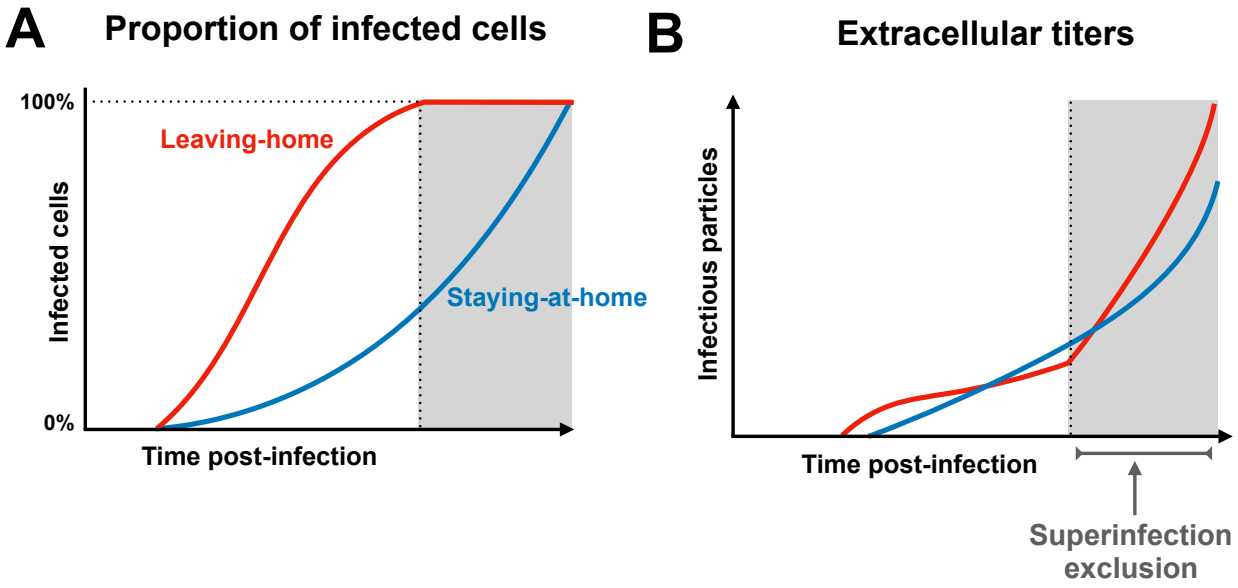

Figure 4.4. How a fixed cell population size affects the staying-at-home and leaving-home strategies of viral replication. **(A)** Virus spread within a finite cell population; a virus prioritizing assembly ("leaving-home") will infect to all potential hosts more quickly than one prioritizing RNA replication ("staying-at-home"). The vertical dotted line indicates the point at which all potential hosts have been infected by the leaving-home virus. **(B)** Impact of the two strategies on secreted infectious particle accumulation in the cell culture medium. Virions can leave the extracellular virus pool by infecting new cells; however, if superinfection exclusion prevents virion entry into already-infected cells, virions produced after all permissive cells have been infected (grey shading) will simply accumulate in the culture media. This model assumes that the staying-at-home strategy produces some infectious viral particles and that viral RNA accumulation does not trigger cell death.

(specifically CLDN1, OCLN, and NPC1L1), which may further prevent HCV superinfection by preventing infectious particle entry (95, 138, 139). Thus, if all cells are infected within a tissue culture dish, newly secreted virions would simply accumulate in the culture media because they have nowhere to go — and the leaving-home strategy would result in an overall increase in extracellular viral titers relative to a staying-at-home strategy (**Figure 4.4B**, right of the dotted line). We currently do not know if PCBP1 knockdown alters the percentage of cells infected by day 3 post-infection, if it somehow impairs viral entry into already-infected cells, or even if

superinfection exclusion results in a continued accumulation of infectious particles in the culture media. However, our data is compatible with this scenario, where PCBP1 knockdown leads to a rapid infection of all available naive cells.

4.1.2 How do PCBPs limit HCV assembly and egress?

Although I have identified the steps of the HCV life cycle that are regulated by PCBPs, the precise molecular mechanisms used by PCBPs to limit virion assembly and egress are still unclear. It seems likely that PCBPs could influence virion assembly through direct interactions with the viral genome and/or proteins, although their interactions with other cellular proteins may contribute to their roles in the HCV life cycle.

Direct interactions with HCV RNA

Firstly, it is possible that PCBPs could limit virion assembly by directly binding the viral RNA itself, as both PCBPs have been reported to bind directly to the HCV genomic RNA (248). Specifically, PCBP1 was shown to bind to the viral 5′ UTR, and this binding required the full HCV 5′ UTR; however, it is not known whether PCBP1 binds to other regions of the viral genome, as this has not yet been examined in detail (240, 248). Meanwhile, several RNA-binding studies have reported that PCBP2 can interact directly with the HCV genome, and iCLIP analysis mapped out six PCBP2 binding sites conserved across two HCV genotypes (35, 248, 251, 257). This included two sites within the 5′ UTR (near SLI and SLIV), three within the polyprotein-coding sequence (within the *core*, *E2* and *NS5B* genes), and the most extensive binding site covered both the variable region and poly(U/UC) tract in the 3′ UTR (251). Given that both

proteins have a preference for C-rich RNA sequences, it is not yet clear if these sites are unique to PCBP2, or if they may also be similarly bound by PCBP1.

The molecular mechanisms by which PCBP interactions with the viral RNA limit its packaging are still unclear, but one could envisage several possible scenarios. Firstly, PCBPs might block core's association with the viral genomic RNA. Current evidence suggests that the HCV genome does not contain a singular packaging signal, but that the core protein has to simultaneously bind the 3′ UTR and multiple low-affinity sites scattered across the genome (296, 297, 305-307). Studies that examined core's RNA-binding affinity have identified these binding sites as stem-loop structures with G-rich loops; although mutations that unzip individual stem-loop structures do not abolish virion assembly, simultaneously disrupting multiple stem-loop structures can delay or even impair infectious particle production (33, 296, 308). Since PCBPs do not bind to G-rich RNA, they are unlikely to occupy the same sites on the viral genome as the core protein. Nonetheless, it is possible that PCBP binding could mask core-binding sites through steric interference or by altering local RNA structures, in a manner reminiscent of the RNA chaperone activity whereby PCBPs activate certain cellular IRES elements (212-214).

Alternatively, PCBPs might compete with viral proteins that bind to the viral genome to participate in virion assembly, such as the NS3 or NS5A proteins. While the exact mechanism by which NS3 facilitates virion assembly is still unclear, its helicase domain has been shown to be crucial for this activity, and it has been proposed that NS3 may unwind secondary structures within the viral RNA to facilitate its packaging within a tight capsid (129, 294, 309, 310). As for NS5A, its interactions with both the viral genomic RNA and the core protein are necessary to initiate packaging, as NS5A controls the transfer of the viral RNA to core (126, 128). The

poly(U/UC) tract is the major binding site for both NS3 and NS5A and has also been shown to be a major PCBP2 binding site (as mapped by iCLIP analysis) (119, 251). Thus, it seems plausible that, by occupying the poly(U/UC) tract, PCBPs might block NS3 and/or NS5A binding to the viral genome and thus prevent virion assembly.

While it is clear that PCBP1 and PCBP2 can physically interact with the HCV genomic RNA, the precise molecular details of these interactions, including which of the PCBPs' three RNA-binding domains engage with the viral RNA, are still unclear. In other contexts, the different KH domains have been shown to differ in their affinity for RNA substrates. For example, in the context of poliovirus infection, the KH1 domain of either PCBP is sufficient to interact with the 5′ terminal cloverleaf of the polivirus genomic RNA, but the PCBP2 linker region and KH3 domain are required to bind SLIV of the poliovirus IRES (241-244, 311). The RNA-binding affinity of these two KH domains is important for poliovirus replication, as poliovirus protease-mediated cleavage of PCBP2 in the linker region (which eliminates PCBP2's affinity for the IRES) is required to mediate the switch from translation to RNA replication in the poliovirus life cycle (242, 245). Although the PCBPs are not substrates for the HCV protease, identification of the specific KH domains that interact with HCV RNA could provide insight into how these two closely related proteins exert different effects on the viral life cycle. To this end, we have constructed PCBP1 and PCBP2 overexpression plasmids with mutations in each of the three KH domains that abolish RNA-binding activity. Specifically, to minimize domain structure alterations, we mutated the two polar residues in the GxxG loop of each KH domain to alanine (i.e. GAAG), as these residues normally interact with the phosphate backbone of RNA targets to correctly orient bases within the RNA-binding pocket (185, 186). In the future, we plan to

perform co-immunoprecipitation experiments using these mutant PCBPs to help identify the domains that directly interact with the HCV RNA. Additionally, using a triple KH domain mutant in knockdown-and-rescue infection experiments will clarify whether the RNA-binding activity of the PCBPs is necessary to limit virion assembly and/or egress, or if these functions are primarily mediated by protein-protein interactions.

Interactions with viral proteins

In addition to directly binding the HCV RNA, it also possible that PCBPs could limit HCV virion assembly by interacting with one or more of the viral proteins. Whether PCBP1 interacts with any HCV proteins has not yet been assessed. As for PCBP2, while its interactions with the structural proteins or with p7 or NS2 have not yet been assessed, it has been reported to co-precipitate with NS5A *in vitro*, in the absence of viral RNA; notably, this interaction was only observed with NS5A and not with any other replicase protein (35). Although this PCBP2-NS5A interaction has not yet been confirmed in the context of an HCV infection, circumstantial evidence suggests it may be taking place on the poly(U/UC) tract, as this region of the genome is a common binding site for both PCBP2 and NS5A (119, 251). The possibility of a PCBP2-NS5A interaction is particularly intriguing, as NS5A is also implicated in regulation of viral genome usage during infection, i.e. NS5A binding to the poly(U/UC) tract is crucial for HCV RNA replication and has also been proposed to downregulate viral translation, and NS5A is responsible for transferring the genomic RNA to core in the early steps of viral packaging (74, 78, 119, 126, 264). Both the interaction between NS5A and the core protein, as well as its transfer of the viral RNA, are regulated by phosphorylation events in domain III of NS5A (126-128).

Consistent with these roles, NS5A mutations that enhance RNA replication in cell culture typically pose a trade-off for genomic packaging efficiency; likewise, treating cells with kinase inhibitors that prevent NS5A hyperphosphorylation results in a reduction of viral RNA packaging and a concomitant enhancement of viral RNA replication (132, 133, 312). Keeping these roles in mind, it seems plausible that PCBP2 could be limiting virion assembly by interacting with NS5A in a manner that prevents its packaging activities. This may include blocking the interaction between NS5A and the core protein, or preventing the phosphorylation events that lead to NS5A-core interactions and viral RNA transfer. Thus, additional studies are needed to further assess whether PCBP2 and NS5A interact during HCV infection and to map out the PCBP2 and NS5A residues involved in this interaction. Moreover, an examination of the phosphorylation status of NS5A during PCBP2 knockdown, and co-immunoprecipitation studies to assess whether PCBPs can interact with core (or any of the other viral proteins), would further shed light onto the mechanism of action of the PCBPs in the HCV life cycle.

Modulation of other host proteins

In addition to direct interactions with the viral RNA or proteins, it is also possible that PCBPs exert their effects on the HCV life cycle indirectly, through interactions with other host proteins implicated in antiviral surveillance, viral assembly, and/or egress. Firstly, both PCBP1 and PCBP2 have been implicated in shutting off antiviral signalling pathways that lead to interferon (IFN) production, and in amplifying the response to IFN stimulation. Specifically, they have been implicated in the turnover of MAVS, a signal transduction protein, downstream of the RIG-I-like helicases, which are sensors of dsRNA, a key intermediate in the life cycles of a variety of RNA

viruses (224, 225, 313). Moreover, PCBPs have also been shown to stabilize the *STAT1* and *STAT2* mRNAs, which results in an enhanced antiviral activity after IFN treatment (207, 285). However, these activities are unlikely to be relevant in the context of the experiments presented in this thesis; all of our experiments were conducted in Huh-7.5 cells that harbour several well-documented defects in antiviral detection and signalling pathways, including an inactivating mutation in RIG-I (146, 314, 315). In addition, the HCV NS3-4A protease inactivates MAVS during infection, and our own brief explorations of MAVS protein expression did not reveal any differences in MAVS expression levels between PCBP knockdown and control conditions, or any interferon expression during HCV infection (data not shown) (68, 273). However, since hepatocytes typically express functional RIG-I and MAVS, and can produce IFN, it is possible that PCBPs modulate this pathway during HCV infection *in vivo*, in addition to their roles in virion assembly and egress.

A second, separate possibility is that PCBPs modulate the HCV life cycle in collaboration with other cellular proteins that affect virion assembly. These include hnRNP K, the YBX-1 and IGF2BP2 protein complex, and the *N*6-methyladenosine (m^6A) writing and reading machinery (specifically the METTL3/METTL14 m^6A writers and YTHDF m^6A readers) (131, 134, 276). During HCV infection, hnRNP K accumulates on the surface of lipid droplets and interacts with both the core and NS3 proteins, but notably does not physically interact with NS5A (134, 259). Its knockdown increases secreted HCV titers without affecting RNA replication, which is remarkably similar to the phenotype that results from PCBP1 knockdown (134, 260). Notably, while hnRNP K has been reported to interact with both PCBP1 and PCBP2 in several cellular contexts, hnRNP K and PCBP1 have been reported to occasionally collaborate without PCBP2;

for example, hnRNP K and PCBP1 together enhance μ-opioid receptor translation and inhibit 15-lipooxygenase translation (217, 277, 278, 316, 317). Thus, it seems plausible that hnRNP K and PCBP1 could work together to limit HCV virion assembly.

A second potential PCBP partner is a protein complex comprised of Y-box binding protein 1 (YBX-1), a multifunctional RNA-binding protein, and insulin growth factor 2 mRNA binding protein 2 (IGF2BP2), another KH domain-containing protein. During HCV infection, the YBX-1/IGF2BP2 complex accumulates on lipid droplets, interacts with the NS3 protein, and inhibits infectious particle assembly and egress while enhancing viral RNA replication (276, 318). Both YBX-1 and IGF2BP2 have individually been reported to interact with PCBP1 and with PCBP2 in various cellular contexts; and, during HCV infection, PCBP2 and YBX-1 co-precipitate (277, 278, 319). This direct interaction raises the possibility that PCBP2 may normally collaborate with the YBX-1/IGF2BP2 complex to limit virion assembly.

Finally, m^6A modifications of the HCV RNA have been proposed to control viral RNA packaging into new virions (131). Recent studies have suggested that HCV RNA that is packaged into virions contains fewer m^6A modifications than viral RNAs that are retained in cells (131). Furthermore, silencing either the METTL3 and METTL14 m^6A writers or the YTHDF reader proteins that interact with m^6A-modified RNAs results in an increase in infectious particle production, without affecting HCV RNA replication (131). Interestingly, PCBP1, PCBP2 and hnRNP K have all been reported to interact with METTL3 and METTL14, and YBX-1 has been shown to interact with METTL14, although it is unclear if these interactions occur during HCV infection (279). Since the PCBPs and hnRNP K all bind to C-rich RNA, m^6A modifications are unlikely to alter their RNA-binding activities; however, IGF2BP2 has recently been shown to

preferentially bind m^{6}A-modified RNA over un-methylated RNA (320). Thus, it is possible that m^{6}A modifications could help recruit the YBX-1/IGF2BP2 complex to the viral RNA; however, it is not yet clear if this complex recruitment is already part of the mechanism by which YTHDF proteins inhibit virion assembly, or if it could enhance YTHDF activity. Collectively, these interactions raise the possibility that PCBPs and the YBX-1/IGF2BP2 complex may be limiting HCV virion assembly through a common pathway that centers around m^{6}A modifications of the viral RNA. However, as intriguing as this may be, we cannot yet rule out the possibility that these proteins are acting through complementary, but independent mechanisms.

Do PCBPs use common or complementary mechanisms?

While both PCBP1 and PCBP2 limit HCV assembly, it is not yet clear if they function in an additive or redundant manner during HCV infection. In other contexts, PCBP1 and PCBP2 have been shown to dimerize with each other — but it is unclear if they do so as they fulfill their roles in the HCV life cycle (194, 239, 317, 321). Our data suggests that the PCBPs are using independent, complementary mechanisms of action to influence the same step of the viral life cycle, since PCBP1 knockdown had unique effects on virus egress that were not shared with PCBP2. This hypothesis could be assessed by simultaneously depleting of both PCBP1 and PCBP2; however, this is challenging as cells do not tolerate double PCBP knockdowns well, due to their role in cell cycle regulation. Normally, PCBPs repress the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A), a kinase inhibitor whose expression leads to an arrest in cell cycle progression at the G1 phase (322-324). Since PCBPs target the *CDKN1A* mRNA for degradation in a redundant manner, knocking down a single PCBP does not significantly alter

CDKN1A mRNA stability, but silencing both PCBPs simultaneously de-represses *CDKN1A*, leading to cell cycle arrest (195). Thus, instead of using siRNA knockdown to silence PCBPs, the establishment of a conditional protein degradation system (e.g. auxin-inducible or blue-light inducible degron tags) which more quickly leads to the tagged protein's proteasomal degradation after its respective stimulus is applied to cells, may be a better system to address double PCBP depletions during HCV infection (325-327).

4.2 PCBP2 and miR-122: antagonists or allies?

MiR-122 is a liver-specific microRNA that binds two tandem sites in the 5′ UTR of the HCV genome (169, 282). Recent studies suggest that miR-122 has at least three roles in the HCV life cycle: 1) it acts as an RNA chaperone to suppress a more energetically favorable secondary structure ($SLII^{alt}$) and promote the formation of the functional IRES conformation (SLII-IV); 2) it promotes viral RNA stability by protecting the 5′ terminus from pyrophosphatase recognition and exoribonuclease-mediated decay; and 3) it promotes translation through contacts between the Ago:miR-122 complex at site 2 and the HCV IRES at SLII-III (170, 171, 173-175, 178, 179, 328). Additionally, miR-122 has been proposed to contribute to the formation of viral RNA replication complexes, but has been shown to be dispensable for viral RNA synthesis once replication complexes have been formed (256, 280, 283, 284).

Previously, Masaki *et al.* proposed that PCBP2 and miR-122 competed for binding to the HCV 5′ UTR, and that this competition altered the balance of viral RNAs engaged in translation or RNA replication (256). However, these conclusions are not entirely supported by their data. Firstly, to show that PCBP2 and miR-122 competed against each other, they performed *in vitro*

binding assays with a RNA probe composed of the first 46 nt of the HCV genome, and demonstrated that PCBP2 binding to this RNA could be abolished by pre-annealing the probe with miR-122 (329). However, this approach has some major flaws, including the fact that the 46 nt probe artificially reveals a single-stranded stretch of four cytidines (nt 41-44), which are partially base-paired in the SLII conformation (illustrated in **Figure 4.5A**) (256). There remains a possibility that PCBP2 could bind this putative binding site when the viral RNA takes a $SLII^{alt}$ conformation, since it reveals this C41-44 stretch, but this binding site has not yet been tested (251, 293). Secondly, Masaki *et al.* concluded that miR-122 and PCBP2 altered the balance of genomes involved in RNA replication versus translation, since miR-122 supplementation could stimulate viral RNA synthesis in a cell culture model of persistent HCV infection, but this supplementation no longer increased nascent RNA levels if translation was inhibited or if endogenous PCBP2 was knocked down (256). The authors therefore concluded that miR-122 stimulated RNA synthesis by shifting viral genome usage towards RNA replication, and they proposed that since PCBP2 and miR-122 were competitors, PCBP2 must normally shift genome usage towards translation. However, their data only demonstrated that miR-122 supplementation required endogenous PCBP2 to stimulate RNA replication, and they did not definitively rule out whether PCBP2 and miR-122 could collaborate to stimulate RNA synthesis.

We initially aimed to explore if PCBP2's effects on the viral life cycle depended on miR-122 by assessing if PCBP2 knockdown affected HCV RNA accumulation in the absence of miR-122. Since miR-122 is essential for wild-type viral RNA accumulation, these experiments made use of the G28A mutant, which can replicate to low levels independently of miR-122. The G28A mutation has been identified in patients with extrahepatic symptoms of HCV infection,

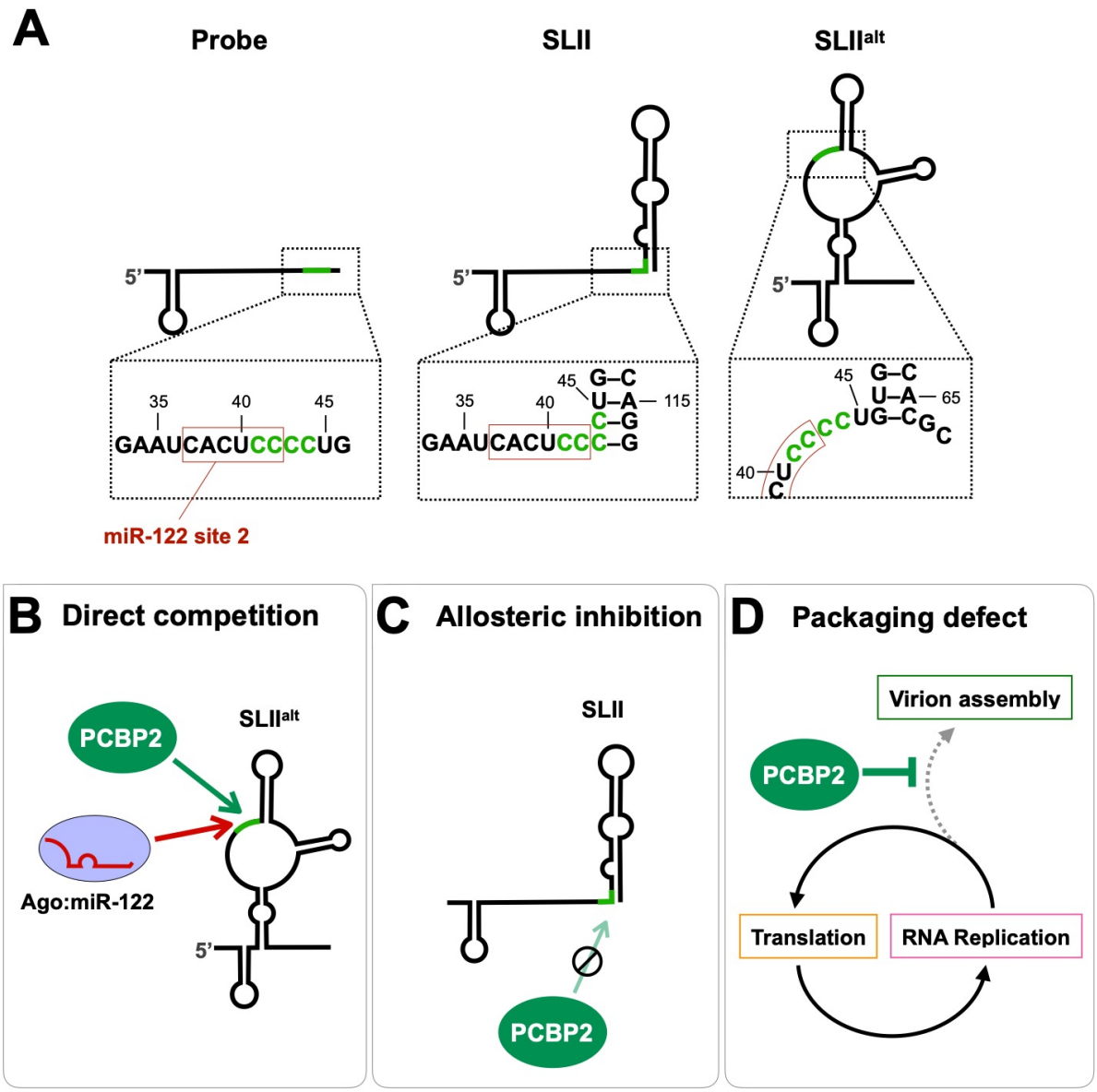

Figure 4.5 A potential PCBP2 binding site in the 5' UTR, and three potential mechanisms of competition between miR-122 and PCBP2. (A) The 46 nt probe of the HCV 5′ UTR used in prior *in vitro* binding assays revealed a C41-44 stretch of nucleotides (green) that partially overlaps with the second miR-122 binding site (red box). This putative PCBP2 binding site is partially base-paired when the viral RNA takes on the functional IRES (SLII-IV) conformation, but is exposed (i.e. single-stranded) in the SLII^{alt} conformation. **(B) Direct competition**: PCBP2 and miR-122 may be directly competing to bind the HCV 5′ UTR, when the SLII^{alt} conformation reveals a putative PCBP2 binding site (green). **(C) Allosteric inhibition**: the functional SLII RNA structure could prevent PCBP2 binding by masking the putative PCBP2 binding site (green). **(D) Packaging defect**: miR-122-independent viral RNAs may be defective in virion assembly, the step of the viral life cycle inhibited by PCBP2.

and has also been identified in cell culture studies that used miR-122 inhibitors or miR122-KO cells to select viral variants that can replicate in miR-122-limited conditions (283, 293, 299, 328). Notably, this residue is already an A in genotypes 1, 3, 5 and 6, but is a G in genotype 2 isolates (173). Recently, our laboratory has shown that this mutation in JFH-1 destabilizes $SLII^{alt}$, which makes the functional SLII structure similarly energetically favorable to $SLII^{alt}$, even in the absence of miR-122 (170). Thus, a G28A JFH-1 mutant no longer requires miR-122 to fold its RNA into the functional IRES structure, although miR-122 can still bind to this viral RNA to enhance its stability and further promote translation (170). Our laboratory also demonstrated that G28A mutants are able to replicate to low levels in miR122-KO cells (300). Thus, we believed that the G28A mutant would be a useful tool to assess the impact of PCBP2 knockdown on HCV replication in the absence or presence of miR-122. We found that the G28A virus was insensitive to PCBP2 in miR122-KO cells, which is consistent with a direct competition model, whereby PCBP2 would need to compete against miR-122 to influence HCV (**Figure 4.5B**). However, in wild-type Huh-7.5 cells (i.e. in the presence of miR-122), the G28A virus was still PCBP2-insensitive. This suggests that a property of the G28A virus itself is likely to be responsible for its PCBP2-independence, rather than a direct competition between miR-122 and PCBP2. In line with this, it is possible that the change in RNA structure caused by the G28A mutation, which favours the functional SLII conformation, may have incidentally abolished the PCBP2 binding site, as nucleotides 41-44 are base-paired in the SLII conformation but are accessible in $SLII^{alt}$ conformation (**Figure 4.5C**) (170). Furthermore, this change in RNA structure reveals the miR-122 site 1; miR-122 binding to site 1 would prevent the viral RNA from refolding into $SLII^{alt}$, and could thus continue to prevent PCBP2 binding without directly occupying the PCBP2 binding

site (170). This mechanism is reminiscent of how allosteric inhibitors can block an enzyme's activity without directly binding its active site, but by inducing a structure change. To clarify if the SLII^{alt} conformation reveals the primary PCBP2 binding site in the 5' UTR, our laboratory will perform *in vitro* PCBP2 binding assays that compare its binding affinity to viral RNAs that preferentially form SLII^{alt} (WT), a mixture of SLII^{alt} and SLII (G28A), or only the functional SLII structure (U4C) (300).

An assumption of the competition hypotheses described above is that PCBP2 binding to the 5′ UTR is important for its role in the viral life cycle. However, it is possible that the G28A mutant's PCBP2-insensitivity is unrelated to PCBP2 binding to the 5′ UTR, but is instead related to its packaging efficiency (or lack thereof) (**Figure 4.5D**). Other research groups have proposed that SLII^{alt} plays a role at a post-translation step of the viral life cycle, since viral genomes that form SLII^{alt} have a fitness advantage over genomes that form SLII; i.e. in cell culture competition assays, wild-type HCV out-competes G28A mutants in miR-122-replete conditions (283, 328). Since our data suggests that PCBP2 acts at the early steps of virion assembly, it is possible that the SLII^{alt} conformation could be important for viral RNA packaging into virions. If this were the case, we would predict that G28A mutants would be PCBP2-insensitive because they would not appropriately engage in viral assembly. To assess this possibility, future studies will introduce the G28A mutation into a packaging competent virus (i.e. JFH-1_T), and assess the impact of the G28A mutation on viral packaging and infectious particle production.

If SLII^{alt} is necessary for virion assembly, then miR-122 interactions with the HCV genome, which re-fold the SLII^{alt} conformation into the functional SLII conformation, would be predicted to have a negative impact on virion assembly (170). In other words, miR-122 binding

would be predicted to promote a staying-at-home strategy. This parallels the roles played by PCBP2 and PCBP1 in the viral life cycle, except that while PCBPs indirectly enhance translation and replication by directly blocking assembly, miR-122 binding would directly enhance translation at the expense of viral assembly. Interestingly, PCBPs and Ago proteins have been reported to interact in several cellular contexts, and since miR-122 normally binds to the viral RNA as part of an Ago:miR-122 complex, it would not be surprising if PCBPs and the Ago:miR-122 complexes collaborate to limit viral assembly (170, 278, 330-332). Thus, further assessment of the proteins associated with the Ago:miR-122 complexes on the HCV genome may help clarify whether PCBPs participate in this miR-122-mediated enhancing complex during HCV infection.

4.4. Concluding remarks

In summary, we found that PCBP1 and PCBP2 both modulate steps of the HCV life cycle that pertain to infectious particle production. Specifically, PCBP2 delays the early steps of virion assembly, and PCBP1 further limits virion secretion. By preventing viral genome sequestration into nucleocapsids, PCBPs indirectly facilitate viral translation and RNA replication without directly promoting these steps of the viral life cycle. Overall, these activities help HCV to engage in a "staying-at-home" strategy that maximizes its viral progeny. Additionally, we found that a resistance-associated variant to miR-122 inhibitors, the G28A mutant, renders HCV insensitive to PCBP2 knockdown. It is currently unclear if this insensitivity is the result of G28A's ability to preferentially form SLII, thereby abolishing the putative primary PCBP2 binding site in the 5′ UTR, or if this mutant has a reduced packaging efficiency, and thus does not engage in the step of the viral life cycle affected by PCBP2. Clarifying this point will be the focus of future studies.

Collectively, our research reveals how host RNA-binding proteins influence HCV's genome usage during the viral life cycle. As the host proteins in question are ubiquitously expressed and well-conserved between mammalian species, it is possible that the mechanisms identified herein may be applicable to related human and veterinary viral pathogens.

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