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The Yin and Yang of Hepatitis C: Synthesis and Decay of HCV RNA

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PREFACE

Hepatitis C virus (HCV) is an unusual RNA virus that possesses a striking capacity to persist for life in the majority of infected individuals. In order to persist, HCV must balance viral RNA synthesis and decay in infected cells. In this Review, we focus on interactions between the positive-sense RNA genome of HCV and host RNA-binding proteins and microRNAs (miRNAs) that influence the competing processes of viral RNA synthesis and decay to achieve stable, long-term persistence of the viral genome. We discuss how these processes impact HCV pathogenesis and therapeutic strategies against the virus.

INTRODUCTION

The pathogenesis of hepatitis C is linked intimately to the capacity of hepatitis C virus (HCV) to persist for decades in most infected persons. Mechanism(s) underlying this long-term HCV persistence remain incompletely characterized, but include multiple viral assaults on the host innate immune system coupled with defective adaptive immunity^{1, 2}. Although interferon-based therapies, at best only modestly effective, are being rapidly supplanted by highly potent interferon-sparing regimens containing multiple direct-acting antiviral agents (DAAs)³ (Box 1), HCV will remain a public health concern for many years. Many individuals are unaware of their infection, and access to curative therapies is limited by a variety of economic and social issues⁴. In addition, those fortunate enough to be cured of chronic hepatitis C with DAAs may remain at risk for liver cancer for an indefinite period.

Box 1

Host-targeting and direct-acting antivirals (DAAs)

Unlike the human immunodeficiency virus-1 (HIV-1), hepatitis C virus (HCV) does not integrate its genome within the host genome, and thus infections can be completely cured and the virus eradicated by effective antiviral therapies. Prior to 2011, the standard-of-

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care for treatment of hepatitis C was a combination of weekly injections of pegylated recombinant interferon- α and daily oral ribavirin. This therapy was continued for periods of 6–12 months and was often associated with substantial side effects. Cure (“sustained antiviral response”, or SVR) was achieved in only 50% or fewer patients infected with the most difficult to treat HCV genotype, genotype 1.

However, the outlook for infected patients has been radically altered in the past 5 years with the introduction of direct-acting antivirals (DAAs) (see the figure, brown boxes). These small molecules target specific proteins expressed by HCV, can be administered orally, and (with newer formulations) have minimal side effects. There are 4 major classes of DAAs³. Two of these classes target active sites in viral enzymes essential for replication: the NS3/4A protease and the NS5B polymerase. A third major class targets the NS5A protein and dually inhibits its activities in biogenesis of the membranous web and in viral assembly^{38, 39}. The fourth class is represented by allosteric inhibitors targeting several sites in the NS5B polymerase. Drugs in each class have now been approved for use by the U.S. Food and Drug Administration.

An overarching problem for DAAs is their ability to rapidly select for resistant viruses, which is due to the highly replicative nature of HCV infection¹⁴⁸ and the error-prone RNA synthesis that typifies positive-strand RNA viruses. However, this problem has been largely overcome by multi-drug regimens that combine two or more DAAs. Thus currently available, all-oral combination therapies taken for 12 weeks (and possibly less) provide SVR rates of ~95% for patients with genotype 1 infection. While currently available DAAs are limited in their coverage of other genotypes, particularly genotype 3 that is increasingly common in Europe, newer second generation DAAs now in development may ameliorate this issue.

Host targeting-antivirals represent an alternative approach to avoid resistance and achieve pan-genotypic coverage (see the figure, green boxes). This strategy is best exemplified by non-immunosuppressive inhibitors of cellular cyclophilins⁵², and an injected oligonucleotide (an ‘antagomir’) that binds to and sequesters miR-122^{85, 96}. Both cyclophilins and miR-122 are important host factors that participate in HCV replication. These host-targeting strategies have shown promise in clinical trials, but neither has achieved regulatory approval.

Approximately one in three acutely infected persons spontaneously clears HCV infection, but in most individuals HCV persists within the liver for years with minimal pathology and relatively constant levels of viremia¹⁸. Spontaneous clearance is closely associated with a polymorphism in the interferon lambda 4 (IFNL4) gene¹⁹. For reasons that are poorly understood but that may also involve cell intrinsic antiviral defenses, infection is restricted to a minority of hepatocytes with a patchy distribution within the liver^{20–22}. The

intracellular abundance of viral RNA and proteins is low, making the identification of these cells difficult. Pathology, exemplified primarily by increasing intrahepatic fibrosis, develops insidiously and is caused largely if not entirely by immune responses to the virus^{23, 24}. This ability of the virus to restrict infection to a minority of cells and to maintain a low intracellular abundance of viral RNA and proteins serves the virus well, but demands close regulation of the replicative machinery. For example, overly abundant production of new viral genomes and viral proteins could lead to apoptotic cell death, as observed in cell cultures infected with laboratory strains of HCV^{25, 26}. On the other hand, less efficient replication might allow the virus to be eradicated by a combination of innate and adaptive immune responses. Long-term persistence thus requires a balance between viral RNA synthesis and decay in infected cells.

In part, this balance is achieved by a unique capacity of the HCV replicase to be negatively regulated by virus-induced oxidative membrane damage²⁷. Whereas infection induces oxidative stress, the ability of HCV to replicate is restricted by peroxidation of lipids associated with its replication machinery. This may contribute to persistence by maintaining viral RNA and antigen synthesis at low levels, and preventing virus-induced cytopathology. Adding to this balancing act is an unusual adaptation by which the viral genome is stabilized and its degradation prevented by interactions with an abundant, liver-specific microRNA, miR-122 (hsa-miR-122-5p)²⁸. In this review, we describe the factors that control the balance between HCV RNA synthesis and decay and discuss how this balance affects viral pathogenesis and therapeutic strategies that aim at eliminating HCV infection.

THE YIN OF HEPATITIS C: HCV RNA SYNTHESIS

The HCV life cycle has been studied intensively, and how the virus enters cells, assembles, and is released from cells has been reviewed elsewhere^{5, 6}. Here, we focus on the organization of the viral genome and discuss the viral and cellular factors that are involved in HCV RNA synthesis.

HCV genome organization

HCV is a ‘positive-strand’ RNA virus, possessing a 9.7 kb single-stranded, messenger-sense RNA genome that is translated directly following its release into the cytoplasm. The RNA contains a lengthy open reading frame (ORF) flanked by highly structured terminal untranslated regions (UTRs) that are essential for RNA synthesis and viral protein translation^{7–11} (Figure 1). Unlike canonical cellular mRNAs, the genome lacks a 5′ terminal 7-methylguanylate cap (m⁷GpppN), terminating instead in a 5′ triphosphate¹². It also lacks a 3′ poly-A tail, terminating in a conserved, stable **stem-loop** structure¹⁰ (Figure 1). The ORF is translated under control of a type III **internal ribosome entry site** (IRES) located within the 5′UTR¹³. Translation results in the synthesis of a large polyprotein that is processed by cellular and viral proteases to yield 3 structural proteins (core, E1 and E2, which are components of the virion), and 7 nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), some of which possess distinct enzymatic activities¹⁴ (Figure 1). Self-replicating RNA replicons require only NS3, NS4A, NS4B, NS5A and NS5B for efficient viral RNA replication¹⁵. These nonstructural proteins assemble as a replicase complex in association with vesicle-like membranes called the “membranous web”, the presumed site of

viral RNA synthesis^{16, 17} (Figure 2). Thus, because viral RNA synthesis is directed by the membrane-bound replicase complex, it requires prior translation of the viral genome and expression of the nonstructural proteins.

Genome amplification commences with primer-independent initiation of the synthesis of a negative-strand complement ((-)RNA) of the positive-strand genome ((+)RNA)) catalyzed by NS5B, an RNA-dependent RNA polymerase. Recent crystallographic studies of the polymerase stalled at various stages of initiation provide a dynamic view of this process²⁹. A β -loop and C-terminal membrane-anchoring linker that restrict access to the active site in the apo state retract to accommodate and position the 3' end of the (+)RNA together with incoming nucleotides within the active site. This ensures that (-)RNA synthesis begins at the extreme 3' terminus of the genome. Following slow formation of a dinucleotide that acts as primer, additional conformational changes in NS5B allow egress of the elongating RNA chain with the polymerase proceeding in a 3' to 5' direction along the length of the (+)RNA genome (Figure 2). Since translating ribosomes move in the opposite, 5' to 3' direction, they present an insurmountable barrier to the replicase and translation must first be terminated before an RNA molecule can serve as template for RNA synthesis. However, the factors regulating this switch from translation to RNA synthesis are poorly understood.

Newly synthesized (-)RNA molecules then serve as template for the production of new (+)RNA molecules that have several possible fates, including: (i) moving to the ER to serve as messenger RNA (mRNA) for continuing viral protein synthesis; (ii) trafficking to **lipid droplets** that serve as a platform in assembly of progeny virions to be exported from the cell; (iii) remaining within the membranous web to template additional (-)RNA synthesis (although it is possible that an (+)RNA molecule must first be translated to become competent for templating (-)RNA synthesis); or (iv) undergoing degradation by host ribonucleases (Figure 2).

Architecture and composition of the HCV replicase

Cryoelectron microscopy of the membranous web reveals both single-membrane vesicles and double-membrane vesicles (DMVs) formed by invaginations of the ER membrane³⁰. The presence of double stranded RNA (dsRNA) and nonstructural proteins within DMVs suggest that they are the site of viral RNA synthesis^{30–32}, although conclusive proof for this is lacking. In addition to providing a platform for RNA synthesis and maintaining high local concentrations of essential nonstructural proteins, the DMVs may also contribute to viral immune evasion. For example, DMVs may sequester replication intermediates from cytosolic innate immune sensors of non-self RNAs such as **retinoic acid-inducible gene I** (RIG-I, or DDX58) and **protein kinase R** (PKR).

DMVs are enriched in cholesterol and sphingolipids³³, and dependent upon ongoing sphingolipid synthesis³⁴. The membranes of DMVs are relatively resistant to detergents and protect its contents from host ribonucleases and proteases³³. Notably, although ectopic expression of NS4B alone induces distinct membrane alterations, NS5A expression is required for formation of DMVs^{30, 35}. Thus, both NS4B and NS5A are likely to be involved in the biogenesis of the membranous web.

In addition to viral proteins, cellular factors are also involved in biogenesis of the membranous web. For example, phosphatidylinositol-4-phosphate catalyzed by phosphatidylinositol-4-kinase III α (PI4KIII α) regulates the formation and integrity of DMVs through a mechanism that is yet to be defined, while the activity of the kinase is stimulated by NS5A^{36, 37}. Consistent with this, DAAs targeting NS5A block assembly of the membranous web, but have little or no effect on ongoing RNA synthesis by previously assembled replicase complexes (Box 1)^{38–40}.

Although X-ray crystallography has provided model structures for the entire NS3 molecule (complexed with part of NS4A)⁴¹, the NS5B polymerase⁴², and the N-terminal domain of NS5A^{43, 44}, very little is known of how these key molecules associate with each other within the replicase. NS3 is anchored to membranes via its N-terminal domain, as well as interactions with its membrane-bound NS4A cofactor, while NS5B is anchored to membranes via its C-terminal domain¹⁴. NS5A is similarly anchored to membranes, most likely near a dimerization domain close to its N-terminus, while NS4B is an integral membrane protein. Each of these proteins is required for viral RNA synthesis, and several play multiple parts in this process. For example, DAAs targeting the NS3/4A protease not only block processing of the polyprotein (required for replicase assembly), but also directly inhibit RNA synthesis and viral assembly³⁹, suggesting a role for NS3 in each of these processes (Box 1). Therefore, the nonstructural proteins are multifunctional. Moreover, these proteins are expressed more abundantly than required for the formation of the membranous web⁴⁵. Substantial amounts of these proteins are present outside the membranous web where they may engage in functions other than RNA synthesis.

In addition to the role of PI4KIII α in the biogenesis of the membranous web, additional host proteins are required for HCV genome amplification. Cyclophilin A (PPIA) and vesicle-associated membrane proteins A and B (VAPA and VAPB) contribute directly to RNA synthesis and are associated with the membranous web^{46, 47}. PPIA, an abundant *cis-trans* peptidyl-prolyl isomerase, binds to NS5A and may facilitate recruitment of NS5B into the replicase complex by modifying proline side chains near the membrane anchor at the carboxyl terminus of the NS5B polymerase^{48, 49}. VAPA and VAPB are vesicle-associated membrane proteins that have roles in sphingolipid metabolism and vesicle transport; they form homodimers and heterodimers and are required for assembly of the replicase complex^{50, 51}. Like PPIA, VAPA and VAPB bind to NS5A and may also facilitate incorporation of NS5B into the membranous web. Importantly, cells that are depleted of these proteins do not support HCV replication, and cyclophilin inhibitors have shown therapeutic promise against hepatitis C in humans⁵².

Structure of HCV RNA

Highly structured, but variably conserved RNA elements throughout the HCV genome contribute to the efficiency of RNA synthesis⁵³ (Figure 1). While many are incompletely characterized, these RNA structures may interact with both viral and host proteins, including components of the translation machinery and replicase complex.

The structure of the 5'UTR is well established. It is highly conserved and contains a small stem-loop near the 5' terminus that is essential for RNA replication, most likely by

recruiting a viral or host cell protein^{7, 8}. Larger, complex stem-loops and a **pseudoknot** between nucleotides 45 and 355 form the IRES^{7, 54}, which binds the 40S ribosome subunit with high affinity in association with eukaryotic initiation factor 3 (EIF3) (Figure 1). Translation initiates internally on the RNA without **ribosome scanning**, and thus differs from the typical process in mammalian cells and superficially resembles events in prokaryotes. The 3'UTR, including a highly conserved 3' "X-tail" (the 3' terminal 98 nucleotides) is equivalently structured⁵⁵ (Figure 1). Also highly conserved, it contains several stem-loops and a U at the 3' terminal position that are important for replication, as is an upstream poly(U/UC) tract of variable length and sequence^{9, 56} (Figure 1). Both sequence and structure within these RNA elements are essential, but their exact roles in RNA replication are not known.

Recent studies using **selective 2'-hydroxyl acylation and primer extension (SHAPE)** have revealed additional, less conserved RNA structures within internal regions of the genome^{53, 57, 58}. These results extend earlier observations that support the functional importance of RNA structure within both the core and NS5B coding regions^{59, 60}. A stem-loop embedded within a cruciform structure in the NS5B region (5BSL3.2 or SL9266), identified previously as an essential *cis*-acting RNA element (CRE)⁶⁰, engages in medium-range interactions with nucleotides located upstream around nucleotides 9033⁶¹ or 9110⁵⁷, as well as a 'kissing' interaction with a stem-loop within the 3'UTR^{62, 63} (Figure 1). Other RNA elements within the NS5B-coding region upstream of 5BSL3.2 also appear essential for efficient replication^{53, 64}. Longer-range interactions between the CRE, the 3'UTR and the IRES have been identified, and may influence the structure of both the IRES and the 3' X-tail^{58, 65}. Thus, a network of variably conserved RNA-RNA interactions exists across the HCV RNA genome and between its 5' and 3' ends that may modulate various steps of the replication cycle. RNA-RNA interactions between the 5' and 3' ends may promote genome circularization, a common phenomenon among (+)RNA viruses that may either enhance translation by facilitating the recycling of 40S ribosome subunits, or seed the initiation of (–)RNA synthesis (Box 2).

Box 2

Circularization of positive-strand RNA viral genomes

Genome circularization is a crucial step in the initiation of RNA synthesis for some positive strand RNA viruses¹⁵¹, and can be mediated either by RNA-RNA interactions or RNA-protein(s)-RNA interactions at the 5' and 3' ends of the genome.

In replication of the 'classical' flaviviruses, including dengue virus and yellow fever virus, circularization is mediated by direct base-pairing between complementary sequences near the 5' and 3' ends of the genome. Mutations in these regions impair RNA synthesis without affecting translation of the viral RNA. The 5' end of the genome of these viruses contains a conserved Y-shaped stem-loop structure (5'SL) that is required for binding and activity of the RNA polymerase¹⁵². Thus, communication with the 5' end is required for initiation of negative-strand synthesis at the 3' end.

In contrast, circularization of the poliovirus genome is mediated by RNA-protein and protein-protein interactions between poly(A)-binding protein (PABP) bound to the 3'

poly(A) tail of the genome and poly(rC)-binding protein 2 (PCBP2) and the viral 3CD protein bound to a 5' cloverleaf-like structure¹⁵³. Following translation of the viral genome, 3CD together with PCBP2 interacts with the 5' cloverleaf to down-regulate translation, and induces circularization through interactions with PABP bound to the 3' end. This enables the viral 3D^{pol} RNA-dependent RNA polymerase to position itself at the 3' end of the genome and initiate negative-strand RNA synthesis. The long-range interaction with the ribonucleoprotein complex at the 5' end of the genome may also induce conformational changes in the 3' UTR required for RNA synthesis.

There is no conclusive proof for circularization of the HCV genome, but several lines of evidence suggest this occurs and is mediated by PCBP2, ILF3^{70, 72} and possibly other RNA-binding proteins that interact with both 5' and 3' UTRs (see Box 4).

Box 4

Does HCV genome circularization regulate its engagement in translation versus replication?

There is no conclusive proof for circularization of the HCV genome, but several lines of evidence suggest this occurs and is mediated by PCBP2, ILF3^{70, 72} and possibly other RNA-binding proteins that interact with both 5' and 3' UTRs. Hypothetically, circularization could regulate the balance between translation and RNA synthesis.

The figure displays a simplified model showing how miR-122 and PCBP2 could jointly regulate HCV genome circularization and thereby influence engagement of the RNA in the mutually incompatible processes of translation versus viral RNA synthesis. Binding of the IRES-transactivating factor PCBP2 to sequences near the 5' and 3' ends of the viral positive-strand (+)RNA provides a protein bridge that promotes genome circularization⁷² and facilitates IRES-initiated translation, in part by enhancing the recycling of 40S subunits disengaging from the RNA after completing a round of translation (see the figure). miR-122 competes with PCBP2 for binding to the 5'UTR⁹⁸, promoting an open, noncircular genome conformation (see the figure). miR-122 may also induce changes in the conformation of the IRES⁹⁵. This leads to a reduction in translation and an increase in initiation of negative-strand (–)RNA synthesis, possibly due to greater availability of the 3'UTR for interactions with the HCV replicase⁹⁸. miR-122 acts in association with AGO2 and also protects the 5' end of the genome from XRN1-mediated 5' to 3' decay¹². Interactions with multiple cellular proteins as well as stable HCV RNA structure likely protect the 3' end from exosome-mediated decay when the genome is in the open conformation (see the figure). Long-range RNA-RNA interactions within the viral genome may also be involved in the maintenance and/or induction of genome circularization (see Figure 1).

Host RNA-binding proteins

Numerous host RNA-binding proteins interact with the HCV genome and promote its replication. Those binding the 5'UTR include La protein (SSB), poly(rC)-binding protein 2 (PCBP2), polypyrimidine tract binding protein (PTBP1/2), heterogeneous nuclear ribonucleoprotein L (HNRNPL), insulin-like growth factor 2 binding protein 1 (IGF2BP1), interleukin enhancer binding factor 3 (ILF3, or NF90/NFAR-1), and U6 snRNA-associated Sm-like protein (LSM1-7), while synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP, or NSAP1) binds to an RNA sequence immediately downstream of the 5'UTR^{66–71}. In many cases, depletion of these proteins impairs HCV translation, suggesting they may act as IRES-transactivating factors (ITAFs) and enhance expression of the viral polyprotein.

Several different molecular mechanisms are likely to underpin ITAF activity, and these may differ depending on the experimental system employed. Most of these proteins bind both 5' UTRs and 3'UTRs, and as shown for PCBP2⁷², they may promote circularization of the genome, thus favoring translation (Box 2). Other possibilities include remodeling of ribonucleoprotein structure leading to enhanced recruitment of the translation initiation factor EIF3 which binds a stem-loop within the IRES and is essential for HCV translation⁷³. Other 5'UTR-binding proteins, such as HNRNPL, may interact with RNA within the replication complex and thus directly influence RNA synthesis⁷⁴. In most cases, a role for these proteins in protecting viral RNA against host nuclease-mediated degradation has not been studied and this possibility cannot be excluded (see below).

Additional host proteins bind the 3'UTR. Heterogeneous nuclear ribonucleoprotein C (HNRNPC), ELAV-like RNA-binding protein 1 (ELAVL1, or HuR), glyceraldehyde-3-

phosphate dehydrogenase (GAPDH), and PTBP1/2 bind to the poly(U/UC) tract^{75–79}. ELAVL1 and PTBP1/2 also bind the highly conserved 3' X-tail, and ELAVL1 also binds the 3' end of the HCV (–)RNA complement as well⁷⁶. While the functional importance of most of these specific protein-RNA interactions is not clear, experimental depletion of each of these proteins using **RNA interference (RNAi)** impairs viral replication⁸⁰. In the case of ELAVL1, this effect may be independent of the stabilizing actions that ELAVL1 possesses for cellular mRNAs⁷⁶.

In addition to proteins binding the 5' and 3'UTRs, the Ewing sarcoma breakpoint RNA-binding protein (EWSR1) promotes viral replication by binding to the CRE⁸¹. It does so preferentially in the absence of the kissing interaction between the CRE and the 3'UTR and is recruited into the membranous web, which suggests that EWSR1 has a direct role in RNA replication.

Interactions between the genome and host RNA-binding proteins may also impact HCV replication indirectly. For example, the binding of the multifunctional ATP-dependent DEAD box helicase 3 (DDX3X) to the 3'UTR activates the conserved helix-loop-helix ubiquitous kinase (CHUK, or iKK α) which increases sterol regulatory element binding proteins (SREBPs) through the CBP/p300 pathway, thereby increasing the formation of lipid droplets that serve as platforms for virus assembly⁸². HCV infection alters the subcellular distribution of DDX3X such that it co-localizes with viral core protein. Since DDX3X plays a supporting role in RIG-I-mediated induction of interferon- β ⁸³, its interaction with the viral core could also modulate innate immune responses to HCV. Thus, the viral genome has evolved a set of sophisticated mechanisms to manipulate the cellular environment in multiple ways that facilitate productive replication.

miR-122 and viral RNA synthesis

miR-122 is an abundant, liver-specific miRNA that regulates the expression of many genes, including those responsible for fatty acid and cholesterol metabolism^{84, 85}, and also has significant tumor suppressor activity⁸⁶. Whereas miRNAs typically regulate gene expression by binding to the 3'UTR of mRNAs in association with an **RNA-induced silencing complex (RISC)**, thereby down-regulating translation and enhancing mRNA decay, miR-122 binds to two tandem sites (S1 and S2) close to the 5' end of the HCV genome and promotes its replication^{87, 88} (Figure 1). Binding of miR-122 to S1 and S2 follows canonical miRNA binding patterns, with base-pair formation involving nucleotides 2–8 (the 'seed sequence') as well as downstream 3' bases^{89–91} (Box 3). This is consistent with the binding of miR-122 in association with argonaute 2 (AGO2) protein as a RISC-like complex that may also contain trinucleotide repeat containing 6A protein (TNRC6A)^{28, 92}. High-throughput sequencing and crosslinking immunoprecipitation (HITS-CLIP) using an antibody against human AGO proteins has confirmed binding of miR-122 to both S1 and S2⁹³. Other conserved potential seed sequence-binding sites, in the IRES and within the ORF, are also occupied by miR-122, but the dominant miR-122 interactions are at S1 and S2^{90, 93}. Although it is not the most abundant miRNA in hepatocytes, miR-122 is the dominant miRNA binding to the viral genome.

Box 3**Biogenesis of miR-122 and its interactions with the HCV genome**

Micro RNAs (miRNAs) are small duplex ~22–23 nucleotide noncoding RNAs with 2 base 3' overhangs that regulate the expression of eukaryotic genes by repressing translation and destabilizing mRNAs. They are produced by DICER-mediated processing of pre-miRNAs, which are short hairpin RNAs that are generated in the nucleus by DROSHA processing of primary RNA polymerase II transcripts (pri-miRNA) and transported to the cytoplasm by exportin 5 (XPO5). Duplex miRNAs associate with argonaute proteins (AGO1–4) with subsequent loss of one RNA strand (the 'passenger' strand) to form miRNA-induced silencing complexes (miRISC) in which the miRNA 'guide' strand is positioned with its 'seed sequence' (comprised of the nucleotides in positions 2 through 8) facing outward, poised for interactions with targeted mRNAs. miR-122 is one of ~1900 recognized human miRNAs. It is among the most abundant miRNAs in hepatocytes and is conserved from zebrafish to primates⁸⁴. miR-122 is derived from pri-miR-122, a 7.5 kb noncoding RNA transcribed from chromosome 18 under control of the hepatocyte-specific transcription factor HNF4 α that is processed by DROSHA to a ~70 nucleotide pre-miR-122 hairpin (see the figure). Two copies of the miR-122 guide strand bind to the 5' end of the positive-strand HCV RNA genome in association with AGO2^{28, 88}. miR-122 nucleotides 2–8 (seed sequence, in bold in the figure) base-pair with conserved tracts (S1 and S2) within the HCV sequence, while nucleotides 13–16 form additional accessory base pairs (see the figure)⁹¹. Poly r(C)-binding protein 2 (PCBP2) binds to sequence overlapping the S2 site, and competes with miR-122 for binding to the HCV 5'UTR⁹⁸

miR-122 binding to both S1 and S2 is important for viral replication, and recent work supports simultaneous occupancy at both sites^{90, 94, 95}. HCV RNAs with base substitutions within either S1 and S2 are impaired in replication, but can be rescued by complementary miR-122 mutants^{87, 94}. This indicates that miR-122 does not promote HCV replication by modulating host gene expression, but by direct interactions with the viral RNA. Highlighting the importance of miR-122 in the HCV life cycle, miravirsin, an anti-sense, locked nucleic

acid “**antagomir**” that binds and sequesters miR-122, demonstrated substantial antiviral activity in preclinical and clinical trials^{85, 96}.

While some studies suggest that miR-122 promotes HCV replication by stimulating translation^{94, 97}, more recent data support two distinct alternative mechanisms. First, miR-122 protects HCV RNA against degradation mediated by XRN1, a cellular 5′ exoribonuclease involved in host mRNA decay¹² (see below). Equally (if not more) important, miR-122 induces an increase in the rate of new viral RNA synthesis when transfected into infected cells⁹⁸. This increase in RNA synthesis can be measured by quantifying 5-ethynyl uridine incorporated into nascent HCV RNA, and is apparent within an hour of miR-122 transfection. Increases in RNA synthesis precede any measurable increase in the incorporation of labeled amino acids into newly synthesized viral proteins, consistent with a primary effect on the replicase⁹⁸. The ability of miR-122 to stimulate HCV RNA synthesis is dependent upon AGO2, and is not observed in AGO2-depleted cells⁹⁸. This is further evidence that miR-122 binds the RNA in association with AGO2, and is consistent with other studies demonstrating the dependence of HCV replication on AGO2^{28, 99, 100}. The miR-122/AGO2 complex could also mask the presence of a 5′ triphosphate and thereby limit recognition of HCV RNA by the immune sensor RIG-I¹⁰¹. However, this has not been experimentally demonstrated, nor is it likely the primary function of miR-122, as miR-122 promotes both HCV RNA stability and viral RNA synthesis in Huh-7.5 cells that lack functional RIG-I activity^{28, 98, 102}.

Despite the importance of miR-122 in the viral life cycle, some HCV mutants have reduced requirements for miR-122. The S1 site is ablated in a novel virus in which the 5′ stem-loop is replaced with cellular U3 **small nucleolar RNA (snoRNA)**, and although miR-122 continues to bind to S2, this virus replicates well in the absence of miR-122^{93, 103}. HCV variants with substitutions at nucleotide position 3 (C3U) or 28 (G28A) are also relatively resistant to miR-122 sequestration^{104, 105}. Low level HCV replication has also been reported in cells lacking detectable miR-122¹⁰⁶. Whether specific compensatory mechanisms support HCV replication under these conditions is unknown.

In addition to stabilizing HCV RNA and promoting its synthesis, the binding of miR-122 to the viral RNA exerts a “sponge” effect in cultured cells infected with robustly replicating laboratory strains of HCV, resulting in functional de-repression of host mRNAs that are normally targeted by miR-122⁹³. A meta-analysis of published array data provides some evidence for this effect in HCV-infected cirrhotic liver tissue⁹³, but it remains uncertain whether a biologically significant sponge effect occurs in vivo where miR-122 abundance is greater and HCV RNA abundance much lower than in infected cell cultures.

Balancing HCV translation versus RNA synthesis

Given the conflict inherent in 5′ to 3′ translocation of ribosomes on the HCV genome during protein synthesis versus the 3′ to 5′ movement of the polymerase required for (–)RNA synthesis, some mechanism must exist for balancing the engagement of the genome in translation versus RNA synthesis. Among other possibilities, miR-122 appears to have a critical role in this process. Increases in RNA synthesis mediated by miR-122 require active cellular protein synthesis and are blocked by treatment with cycloheximide or puromycin,

both potent general protein synthesis inhibitors⁹⁸. However, puromycin by itself enhances viral RNA synthesis in the short term, while cycloheximide does not⁹⁸. These results are best explained by the fact that puromycin induces premature release of translating ribosomes from RNA, while cycloheximide does not. Thus, it is likely that puromycin stimulates RNA synthesis by freeing up viral genomes that are engaged in translation, making them available for viral RNA synthesis. Under these conditions, miR-122 fails to further stimulate RNA synthesis, suggesting that miR-122 acts similarly and thus redundantly to puromycin, stimulating viral RNA synthesis by increasing the fraction of genomes engaged in this process⁹⁸. Consistent with this hypothesis, **polysome** analyses reveal a small but significant reduction in the fraction of HCV RNA associated with polysomes and engaged in translation following transfection of miR-122⁹⁸.

Several possible mechanisms may explain how miR-122 regulates the engagement of viral genomes in RNA synthesis versus translation. Electron microscopy and RNA pull-down experiments suggest that the HCV genome adopts a circularized structure through protein bridges forming between the 5' and 3' ends of the RNA in the presence of PCBP2⁷² or ILF3⁷⁰, which are both capable of binding the 5' and 3' ends of the RNA. As noted above, circularization of an RNA can enhance its translational efficiency by facilitating ribosome recycling (see Box 2). In addition, sequences within the HCV 3'UTR can bind the 40S ribosome subunit independently of the IRES, providing a second possible mechanism by which circularization could enhance translation¹⁰⁷. Since miR-122 competes with PCBP2 for binding to the extreme 5' end of the genome at the S2 site^{74, 98}, miR-122 might inhibit PCBP2-mediated circularization of the genome and thereby reduce its engagement in translation (see Box 4). Consistent with this hypothesis, miR-122 has no effect on viral RNA synthesis when transfected into cells previously depleted of PCBP2⁹⁸. Alternatively, both SHAPE and atomic force microscopy suggest that the binding of miR-122 may induce changes in the tertiary RNA structure of the IRES^{95, 108}, and this could also modulate engagement of HCV RNA with the 40S ribosomal subunit. These two hypotheses are not mutually exclusive.

In addition to PCBP2 and ILF3^{70, 72}, other host proteins that bind both ends of the viral genome, such as LSM1-7, PTB, IGF2BP1 and SSB (La) could facilitate genome circularization^{68, 69, 77, 109}. Mobility shift and SHAPE studies also suggest the existence of direct RNA-RNA interactions involving the 5' and 3'UTRs that may alter IRES conformation^{58, 110} (Figure 1). These RNA-RNA interactions are likely to be dynamic, and as in the case of EWSR1 binding to the CRE⁸¹, both influenced by and in turn influencing interactions of the genome with host RNA-binding proteins. Such interactions could regulate the translational efficiency of the RNA and thus the balance of its engagement in protein synthesis versus RNA synthesis.

RNA synthesis is also regulated by serine phosphorylation of the multifunctional NS5A protein. Several host kinases, including PI4K-III α and casein kinase I α (CKI α), and multiple viral nonstructural proteins, including NS2 and NS4A, have been implicated in the generation of basal (56 kDa) and hyperphosphorylated NS5A species^{37, 111}. Mechanistic details are lacking, but hyperphosphorylation appears to have a negative effect on the capacity of NS5A to support replicase formation and RNA synthesis, while enhancing its

movement to lipid droplets and promoting virion assembly^{112, 113}. Hyperphosphorylation of NS5A is thus likely to deplete the intracellular (+)RNA pool. However, the impact of hyperphosphorylation of NS5A on engagement of the residual RNA in translation versus RNA synthesis is unknown.

Lipid peroxidation and the HCV replicase

A unique feature of the membranous web is its sensitivity to oxidative membrane damage^{27, 114}. HCV infection disturbs cellular redox homeostasis and induces a state of oxidative stress that is likely potentiated by inflammatory immune responses¹¹⁵. The presence of reactive oxygen species results in the peroxidation of polyunsaturated fatty acids within membranes. This lipid peroxidation causes both qualitative and quantitative changes in the membranous web²⁷. While a detailed molecular understanding of these processes is lacking, peroxidation-resistant HCV variants selected in cell culture have mutations in membrane-proximal residues of NS4A and NS5B²⁷. Changes observed in the response to antivirals targeting the NS3/4A protease and NS5B polymerase in peroxidation-sensitive virus, but not in peroxidation-resistant virus, suggest that the conformations of these proteins are altered by lipid peroxidation²⁷, possibly through formation of protein adducts, and that this results in impaired viral RNA replication. Lipid peroxidation limits the ability of HCV to replicate in cultured primary hepatocytes, an effect that can be reversed with lipid-soluble antioxidants such as vitamin E²⁷. Notably, this phenotype is unique to HCV and is not observed in other (+)RNA viruses, including other members of the *Flaviviridae* family. The suppression of HCV RNA synthetic capacity by oxidative membrane damage provides a novel autoregulatory mechanism that likely acts to maintain HCV replication at low levels, a feature that may favor evasion of immune surveillance and survival of infected cells within the liver.

THE YANG OF HEPATITIS C: RNA DECAY

Eukaryotic cells are equipped with several distinct cytoplasmic RNA decay pathways that collectively act to maintain the proper abundance and integrity of mRNAs. These constitutively expressed decay pathways pose a barrier for the survival of RNA viruses, and can be supplemented by ribonucleases that are induced as part of cell-intrinsic innate immune responses. In response to these challenges, RNA viruses, including HCV, have evolved a variety of strategies to evade mRNA decay programs.

HCV RNA and host mRNA decay pathways

The degradation of host mRNAs takes place within the cytoplasm and begins with removal of the 3' poly(A) tail by deadenylases, followed by exonucleolytic decay of the RNA body in either a 5'-3' or 3'-5' direction¹¹⁶ (Figure 3). In the 5'-3' decay pathway, the m⁷GpppN cap is removed by decapping enzymes¹¹⁷, and the mRNA body then degraded by the cytoplasmic 5' exoribonuclease, XRN1¹¹⁸. Alternatively, in 3'-5' decay, the mRNA body is degraded by the **exosome complex**, a multi-protein complex with both 3'-5' exonuclease and endonuclease activities^{119, 120}, and the residual cap structure is degraded by the DCPS scavenger decapping enzyme¹²¹. Additional specialized mRNA decay machineries, including non-sense mediated decay (NMD), no-go decay, and non-stop decay, provide

surveillance over the integrity of cellular mRNAs^{116, 122}. Proteins involved in the NMD pathway restrict replication of some positive-strand RNA viruses¹²³, but none of these alternative RNA degradation pathways are known to contribute to HCV RNA decay.

In addition to lacking a 5' cap and a 3' poly(A) tail, HCV RNAs are not synthesized in the nucleus and thus do not associate with transcription or splicing factors. These features distinguish HCV RNA from cellular mRNA, and may thus target the viral RNA for degradation. However, the manner in which HCV RNA is degraded is dependent on its location in the cell and its functional status¹². For example, when synthetic HCV RNA transcripts are electroporated into cells they are degraded rapidly in both 5'-3' and 3'-5' directions by XRN1 and the 3' exosome complex, respectively, with a half-life ($t_{1/2}$) of ~1.5–2.0 hrs¹². In contrast, viral RNA is degraded much more slowly within infected cells after RNA synthesis is terminated with a specific inhibitor of the viral polymerase, decaying with a $t_{1/2}$ of 6–10 hrs^{12, 124}. This difference in kinetics of RNA degradation likely reflects protection afforded to the RNA by the membranous web, or by packaging into nascent virions, in the infected cells. In these cells, HCV RNA is degraded primarily by the 5'-3' XRN1 pathway, with little or no contribution from the 3' exosome complex¹². Consistent with this, only 5' degradation intermediates have been identified within infected Huh-7 cells¹². Notably, XRN1 exclusively degrades RNAs possessing a 5'-monophosphate, and since HCV RNA terminates in a 5'-triphosphate, a yet-to-be identified cellular pyrophosphatase must contribute to the XRN1-mediated decay of the viral genome in infected cells^{12, 125}. Recent studies in infected cell cultures suggest that XRN1 is stalled by secondary RNA structure within the HCV 5'UTR, and that this both represses its enzymatic activity and results in a global increase in host mRNA stability¹²⁶. However, like the putative miR-122 sponge effect described above⁹³, it is not clear that such an effect occurs in vivo where intracellular abundance of the viral genome is low.

The primary role of XRN1 in the decay of HCV RNA is consistent with its involvement in the decay of other positive-strand RNA virus genomes¹²⁷. However, a second 5' exoribonuclease, XRN2, has also been implicated in the decay of HCV RNA¹²⁸. XRN1 and XRN2 are very different proteins that share limited sequence homology only in their respective N-terminal ribonuclease domains. XRN1 is expressed in the cytoplasm where it is associated with **P-bodies** and functions in mRNA decay, whereas XRN2 predominantly locates in the nucleus, regulates termination of RNA polymerase II-mediated transcription and contributes to maturation of ribosomal RNA (rRNA) and snoRNA^{129, 130}. RNAi-mediated depletion of XRN2 can enhance HCV replication in cell culture, but this effect is limited to viruses such as JFH1 that replicate robustly and generate cytopathic effects^{124, 128}. In contrast, XRN1 depletion enhances replication of multiple HCV strains, making XRN1 a more general restriction factor^{12, 124, 131}. XRN1 depletion also results in greater increases in the $t_{1/2}$ of HCV RNA compared to XRN2, consistent with it playing the primary role in decay¹²⁴. Since HCV RNA does not localize to sites of XRN1 accumulation in P-bodies, it is likely to be degraded by XRN1 within the cytosol¹².

Antiviral defense and HCV RNA decay

In addition to degradation via constitutive mRNA decay pathways, several interferon-inducible ribonucleases constitute alternative mechanisms for degradation of HCV RNA^{132, 133} (Figure 3). For example, the 2',5'-oligoadenylate synthetase-dependent RNase L (RNASEL) is a pivotal interferon-inducible endoribonuclease that is important for intrinsic cellular defense against a variety of viruses. RNase L degrades single-strand RNAs of both viral and cellular origin, cleaving preferentially 3' of UU and UA dinucleotides¹³⁴. Although HCV induces little RNase L expression in infected cell cultures¹³⁵, RNase L is abundant in liver tissue from patients with chronic HCV infection and co-localizes with NS5A, a component of the replicase¹³⁶. Purified RNase L cleaves HCV RNA in vitro¹³⁷ and overexpression of RNase L in Huh-7 cells suppresses HCV replication¹³⁵. Reductions in the expected frequency of UA/UU dinucleotides, and their close proximity to stem-loop structures providing an unfavorable context for RNase L cleavage, suggest that the HCV genome has evolved under selective pressure from RNase L^{53, 137}. Importantly, small RNA fragments generated by RNase L cleavage may induce signaling via RIG-I that leads to activation of the interferon- β promoter¹³⁶, thereby amplifying cell intrinsic host defenses.

The interferon-stimulated gene 20 (ISG20) is a second interferon-stimulated ribonuclease¹³³ that possesses 3' exonuclease activity and restricts replication of a broad spectrum of RNA viruses¹³⁸. Overexpression of ISG20 suppresses HCV replication in cell culture, and this antiviral action is dependent upon its exonuclease activity^{138, 139}.

Similarly, zinc finger CCCH-type containing 12A protein (ZC3H12A, also known as MCP-induced protein 1, or MCP1P1) is an endoribonuclease that is induced by pro-inflammatory cytokines such as TNF- α , IL6 and IL-1 β arising in the context of viral infection¹⁴⁰. ZC3H12A degrades specific cytokine mRNAs, but also degrades HCV RNA and when overexpressed suppresses HCV replication¹⁴¹.

Zinc finger CCCH-type antiviral protein 1 (ZC3HAV1, otherwise known as ZAP) is induced by type I interferon, binds to a wide range of viral RNAs and both represses their translation and promotes their decay by recruiting host mRNA decay factors¹⁴². However, the role of ZC3HAV1 during HCV infection has yet to be studied.

In addition to interferon- and cytokine-induced ribonucleases, DROSHA, a nuclear type III endoribonuclease involved in miRNA biogenesis (Box 3), can act independently of interferon to restrict replication of RNA viruses¹⁴³. In such cases, infection induces translocation of DROSHA to the cytoplasm, where it cleaves viral RNA. However, DROSHA is required for biogenesis of miR-122, an important pro-virus host factor, and does not restrict HCV replication⁸⁰.

Viral antagonism of RNA decay

Viruses counteract cellular RNA decay mechanisms at several levels. For example, replicating HCV genomes are sequestered within the membranous web, which protects the RNA from decay machinery³⁰. As described above, this likely explains the slower degradation of replicating HCV genomes versus the rapid decay observed with electroporated synthetic RNA¹². Another viral evasion strategy involves active disruption of

constitutive cellular RNA decay pathways, either by targeting specific decay factors for cleavage by viral proteases or stimulating their degradation by the proteasome¹⁴⁵. However, there is no evidence that HCV relies on this strategy to avoid RNA degradation.

HCV may, however, restrict the effects of interferon-induced ribonucleases by actively constraining intrinsic antiviral cellular responses. Thus, although the interferon-regulated exonuclease ISG20 is transcriptionally induced within the infected liver¹⁴⁴, this response may be limited by the HCV NS3/4A protease that ablates signaling pathways involved in the induction of interferon responses by targeting specific adaptor proteins, MAVS and TRIF (TICAM-1), for cleavage¹. The interactions of HCV with the innate immune system are complex and beyond the scope of this review. Moreover, their impact in vivo is not well understood. Readers are referred elsewhere for a more complete discussion¹.

HCV exploits its interaction with miR-122 to protect its genome from 5' exonucleolytic degradation^{12, 28, 100}. This contrasts with conventional miRNA action, which typically promotes mRNA degradation. Transfection of miR-122 slows decay of electroporated synthetic HCV RNA, and also reduces the rate of decline in HCV RNA abundance when infected cells are treated with a viral polymerase inhibitor²⁸. miR-122 does not increase the stability of transfected synthetic RNAs to which a 5' cap has been added, indicating that it protects against 5' decay^{12, 28}. Consistent with the primary role of XRN1 in mediating viral RNA decay described above, miR-122 also has no effect on the rate of HCV RNA decay in cells depleted of XRN1¹². Furthermore, miR-122-mediated stabilization of the viral RNA requires transfection of duplex miR-122 that is capable of being loaded into AGO2 and forming a RISC complex, and was not observed in *Ago2*^{-/-} murine embryonic fibroblasts²⁸. Therefore, the viral RNA is stabilized by a miR-122/AGO2 complex (Box 3).

Interestingly, miR-122 stabilizes synthetic HCV RNA when incubated in HeLa cell lysates, conditions under which its degradation is mediated predominantly by the 3' exosome complex as described above¹². This remains unexplained mechanistically, but suggests that the binding of miR-122 may influence long-range RNA-RNA interactions that have been proposed to exist between the 5' and 3' ends of the genome^{58, 110} in a manner that provides protection against 3' exonucleolytic decay.

While the influence of miR-122 on HCV RNA stability has been studied in detail, there could well be other factors that stabilize the viral genome. Two proteins known to bind HCV RNA are well-documented host mRNA stability factors. ELAVL1, which binds to U-rich or AU-rich elements in cellular mRNAs to enhance their stability, also binds the HCV 3'UTR⁷⁶. Similarly, PCBP2 stabilizes α -globin mRNA in red blood cells¹⁴⁶, and binds both the 5' and 3' UTR of HCV RNA⁷². While depleting either PCBP2 or ELAVL1 impairs HCV replication^{72, 76, 80, 147}, their influence on HCV RNA stability has not been well studied.

YIN AND YANG: BALANCING RNA SYNTHESIS AND DECAY

Long-term, steady state persistence of HCV requires a balance between the synthesis and decay of viral RNA at the level of the infected host. Mathematical modeling suggests that virions are cleared from the circulation with a half-life ($t_{1/2}$) of ~45 minutes, and that $\sim 5 \times 10^{12}$ virions are released from the liver each day¹⁴⁸. Much less is known about the dynamics

of infection within single hepatocytes. As there are about $\sim 2 \times 10^{11}$ hepatocytes in the adult human liver¹⁴⁹, each hepatocyte can be estimated to release on average ~ 25 virions per day. However, since sensitive assays detect viral RNA or proteins in only a minority (1–50%) of hepatocytes, the number of virions released by each infected hepatocyte is likely to be substantially higher^{20–22}. Less than 50–100 RNA copies are present in each infected hepatocyte (much less than in commonly used cell culture systems)²¹, likely representing only a few functional replication complexes in most cells²². Infected cells are not dispersed within the hepatic parenchyma but are grouped in clusters of variable size. RNA abundance is highest near the center of the clusters, suggesting a dynamic in which cells in the periphery have been more recently infected^{20, 150}. However, a steady state interpretation is also possible, with replication in cells near the periphery of clusters constrained by interferon-stimulated gene expression²². Modeling studies suggest that the $t_{1/2}$ of infected cells may be in the range of only 4–7 days¹⁴⁸, but this is based on several assumptions and a paucity of hard data. Nonetheless, even with the uncertainties in these estimates, it is evident that a balance must exist at the single cell level between production of new HCV genomes and their loss through a combination of decay and virion assembly and export (Figure 2).

While the proportion of newly synthesized RNA genomes that are exported from hepatocytes in nascent virions versus those that decay within the cell is uncertain, the balance between RNA synthesis and decay is determined through interactions of the viral genome with multiple host RNA-binding proteins and miR-122, as discussed above. miR-122 plays a key part in this process, promoting replication by optimizing the balance of genomes engaged in RNA synthesis versus translation⁹⁸. miR-122 also prevents the 5' decay of (+)RNA¹², adding to the protective effects of the membranous web and the potential viral blockade of interferon-inducible ribonucleases. Host RNA-binding proteins (PCBP2 and ELAV1) could also contribute to the stability of the viral genome. Together, these actions favor viral replication, and enhance the production and cellular abundance of viral RNA. However, this increase in viral abundance is balanced by the unique sensitivity of HCV to oxidative membrane damage that limits the efficiency of the replicase complex and acts as a governor on replication²⁷. The net outcome of this conflict between synthesis and decay versus export of the viral genome is a very low abundance of viral proteins and RNA within individual infected hepatocytes^{20, 22}, which favors cell survival and long-term viral persistence.

OUTLOOK

Recent gains in understanding the functional impact of interactions between the HCV genome, host RNA-binding proteins, and miR-122 are beginning to provide insight into how HCV regulates the involvement of its genome in the competing processes of protein translation and viral RNA synthesis, and how this contributes to the overall balance of synthesis versus decay of the viral genome. miR-122 is now known to directly stimulate viral RNA synthesis, resulting in secondary increases in viral protein translation⁹⁸. miR-122 also stabilizes the viral genome by preventing XRN1-mediated 5' decay¹², thereby countering the restrictive effects of constitutive host RNA decay machinery. Notably, this action of miR-122 is completely contrary to the canonical destabilizing action of miRNAs on host mRNAs. The absence of 3' decay in the degradation of HCV RNA also distinguishes

it from cellular mRNAs¹². Thus, the ‘yin’ and ‘yang’ of HCV (+)RNA is unique. However, our understanding of both its synthesis and decay is far from complete, and we should anticipate that other host factors are likely to contribute to these processes.

Several important questions remain to be answered. For example, following shut-off of new RNA synthesis, a substantial rate of loss of viral RNAs remains that cannot be attributed to XRN1, XRN2, or the exosome complex^{12, 124}. How much of this loss is due to assembly and export of new virions? Or, do other RNA decay pathways contribute to this loss? Perhaps most important, can RNA decay pathways be manipulated pharmacologically to aid in therapy, not only for HCV but other viral infections as well?

The current goal with anti-HCV therapy (Box 1) is to suppress replication until the abundance of viral genomes reaches the point of extinction. In theory, this can be achieved either by inhibiting the synthesis of viral genomes, or by promoting their decay. A better understanding of this balancing act, and especially of the host and viral factors that regulate the rate of decay of the viral genome, will provide useful insight into therapeutic responses to direct-acting antivirals and could allow the design of optimized therapies requiring a shorter duration of treatment.

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GLOSSARY

Antagomir	A chemically synthesized, typically nuclease-resistant oligonucleotide that is complementary to the sequence of a miRNA and that acts to antagonize its activity by binding to and sequestering the miRNA from its mRNA target.
Exosome complex	A multi-protein complex with both 3'-5' exonuclease and endonuclease activities. Substrates of the exosome include deadenylated host cell mRNAs, ribosomal RNAs, and many species of small RNAs.
Flaviviridae	A family of enveloped, positive-strand RNA viruses comprised of four genera: flavivirus (dengue, yellow fever virus, and others), hepacivirus (HCV), pestivirus, and pegivirus.
Internal ribosome entry site (IRES)	A highly structured RNA sequence located in the 5' UTR of HCV that recruits ribosomes and initiates translation of the RNA independently of ribosome scanning or a 5' cap. A variety of IRES elements with different RNA structures are found in other viruses as well as in a subset of host mRNAs where they permit translation when cap-dependent translation is inhibited during mitosis, apoptosis or hypoxia.
Lipid droplet	A ubiquitous, dynamic cellular organelle that is rich in lipids and mainly composed of cholesteryl esters and triglyceride. It functions in energy storage by sequestering fatty acids in triglycerides, and as a

	platform for metabolism and transport of lipids as well as cellular signaling through generation of bioactive lipids.
P-body	Processing bodies are cytoplasmic aggregates of proteins engaged in the transport, storage and decay of host cell mRNA, and represent foci of accumulation of the 5' exonuclease XRN1.
Polysome	A large complex formed by multiple mature 80S ribosomes bound to an mRNA that is actively undergoing translation.
Protein kinase R (PKR)	An interferon-induced, double-stranded RNA-activated protein kinase that is central to cellular responses to a variety of stresses such as virus infection, cytokine exposure, nutrient depletion, irradiation, or endoplasmic reticulum (ER) stress. PKR-mediated phosphorylation of eukaryotic translation initiation factor 2A (EIF2A) results in global translational repression.
Pseudoknot	A higher-order RNA structure containing at least two stem-loops and in which bases in one half of one stem pair with those in the loop of the second stem-loop.
Retinoic acid-inducible gene I (RIG-I, DDX58)	A cytoplasmic RNA helicase that senses viral RNAs and initiates signaling leading to host interferon production. RIG-I preferentially binds short double-stranded and AU-rich RNAs with 5' di- or triphosphates and subsequently activates its downstream adapter, mitochondrial antiviral signaling protein (MAVS), through interactions between shared caspase recruitment domains (CARDs). RIG-I and a second RIG-I-like receptor, melanoma differentiation-associated protein 5 (MDA5, or IFIH1), contribute to innate immune sensing of distinct types of viruses.
Ribosome scanning	An essential component of eukaryotic cap-dependent translation initiation, this is the process by which a 40S ribosome subunit with associated initiation factors moves in a 3' direction along an mRNA molecule in search of a 'start codon' (typically AUG) following its initial interactions and binding to the 5' terminal m7G cap structure. Assembly of the mature 80S ribosome and the initiation of polypeptide synthesis commences at the start codon.
RNA-induced silencing complex (RISC)	A ribonucleoprotein complex comprised of a small noncoding RNA, typically a microRNA (miRNA) or silencing RNA (siRNA) 'guide strand' loaded into an argonaute protein (AGO1-4), that acts to suppress translation and in some cases directly cleave an mRNA target.
RNA interference (RNAi)	A mechanism initially discovered in plants by which small noncoding RNAs suppress gene expression by targeting specific mRNA transcripts in association with an RNA-induced silencing complex (RISC). Transfected synthetic silencing RNA (siRNA) is often used

	experimentally to deplete the abundance of a specific protein in cell cultures.
Selective 2'-hydroxyl acylation and primer extension (SHAPE)	An experimental method that assesses backbone flexibility in an RNA molecule at single-nucleotide resolution based on the reactivity of 2'-hydroxyl groups toward an electrophile. Nucleotides that are flexible (unpaired) are preferentially chemically modified and are recognized as stops in a subsequent primer extension reaction, allowing unpaired bases to be distinguished from those for which flexibility is constrained by base-pairing.
Small nucleolar RNA (snoRNA)	A class of small noncoding RNAs that act to guide specific post-transcriptional modifications, such as 2'-O methylation or uridine isomerization, and occasionally cleavage, of other RNAs (primarily rRNA and tRNA).
Stem-loop (or "hairpin")	An element of RNA secondary structure formed by two segments of an RNA molecule with complementary nucleotide sequence base-pairing to form a two-stranded helix with an intervening single-stranded loop segment.

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Online summary

- The number of HCV RNA copies present within infected hepatocytes is determined by the rate of viral RNA synthesis in relation to the rates of intracellular HCV RNA decay and the packaging and export of newly replicated genomes in virions. Both viral RNA and viral proteins are typically present at low abundance, and this may contribute to cell survival, immune escape, and long-term viral persistence.
- HCV RNA is synthesized by replicase complexes associated with the ‘membranous web’, a collection of cytoplasmic single- and double-membrane vesicles that are formed as protrusions from the endoplasmic reticulum in a process dependent upon phosphatidylinositol-4 kinase III α (PI4KIII α) and multiple nonstructural HCV proteins including NS4B and NS5A.
- Newly synthesized positive-strand HCV RNAs are subject to 4 possible fates, either (i) cycling to ribosomes to direct translation of more viral protein, (ii) translocating to lipid droplets for packaging into new virions for export from the cell, (iii) remaining within the membranous web to template additional RNA synthesis (negative strand), or (iv) being degraded by XRN1.
- A network of variably conserved RNA structures extends across the entire positive-sense HCV RNA genome, but structure is especially prominent within 3′ and 5′ untranslated RNA segments (UTRs) and an internal cis-acting replication element (CRE). RNA-RNA interactions within and between these structures, as well as RNA-protein interactions involving poly(rC)-binding protein 2 (PCBP2) and possibly other host proteins, modulate the structure of the genome, including its circularization, and thereby likely regulate genome engagement in different stages of the viral lifecycle.
- HCV infection induces oxidative stress. This leads to cellular lipid peroxidation that acts in a counter-regulatory manner on the replicase complex to reduce viral replication, thereby providing a negative-feedback loop that helps to maintain viral RNA and protein abundance at low levels within the liver.
- miR-122 is an abundant, evolutionarily conserved, liver-specific micro-RNA. It binds two sites near the 5′ end of the HCV genome in association with AGO2, and promotes replication of the virus by (i) stabilizing and protecting the genome against XRN1-mediated decay, and (ii) directly stimulating viral RNA synthesis by reducing the proportion of RNA genomes engaged in protein translation, thereby increasing those available to template RNA synthesis.

Figure 1. The HCV RNA genome and polyprotein

(a) Secondary RNA structure within the single-stranded 9.7 kb RNA genome of HCV ((+) RNA), showing the short, highly structured 5' and 3' untranslated regions (UTRs, black) that flank the ends of the internal long open reading frame (ORF) encoding the viral polyprotein (blue and orange, see panel b). The 5' UTR contains essential RNA replication signals, including the two miR-122 binding sites (S1 and S2, red arrows) and an internal ribosome entry site (IRES) containing multiple pseudoknots (PKs) that binds 40S ribosome subunits with high affinity. The 3' UTR contains a highly variable poly(U/C) tract followed by the near absolutely conserved 98 nucleotides that comprise the '3'X-tail' sequence at the 3' terminus; both of these sequences are essential for RNA replication. Initiator AUG and terminator UGA codons at the ends of the ORF delimit noncoding and coding RNA sequences. Also shown is stem-loop 5BSL3.2, the core of a conserved compound RNA structure located near the 3' end of the ORF that functions as a *cis*-acting replication element (CRE). Additional, less conserved secondary RNA structure is also present within the polyprotein-coding region. The colored lines represent a network of dynamic long-range RNA-RNA interactions both within and between highly structured RNA domains at the ends of the genome. Adapted with permission from REF. 58. (b) The ORF encodes a single large polyprotein that is processed by cellular and viral proteases into 10 mature viral proteins: 3 that are structural and present in the virion, and 7 nonstructural proteins. The structural proteins (blue) include core, and two glycosylated envelope proteins, E1 and E2, and are derived from the N-terminus of the polyprotein. Nonstructural proteins required for RNA synthesis (orange) include NS3 (protease/helicase), NS4A (protease co-factor), NS4B

(integral membrane protein), NS5A (replicase formation and virion assembly factor), and NS5B (RNA-dependent RNA polymerase, RdRp). p7 (ion channel) and NS2 (*cis*-acting protease and assembly factor) are essential for virion morphogenesis and egress but not RNA replication (colored in green). All of these proteins are likely to be multifunctional. NS, nonstructural. Arrowheads indicate sites of cleavage by viral proteases: NS2/3 (violet) and NS3/4A (orange).

Figure 2. The Yin of HCV: RNA synthesis

Upon hepatitis C virus (HCV) entry into the infected cell, the viral positive-stranded (+)RNA (red) is released into the cytoplasm. (+)RNA from entering virions must first interact with ribosomes in the ER to undergo translation in order to produce viral nonstructural proteins required for the assembly of replicase complexes, which are associated with membranes derived from the ER, and form the membranous web (MW). Within the membranous web, (+)RNA is copied to a complementary negative-strand (–)RNA intermediate (blue), which serves as the template for the synthesis of multiple new (+)RNA strands. These progeny RNA molecules have four possible fates: (i) they may cycle to rough ER for translation and production of additional viral proteins, (ii) they may be shuttled to lipid droplets (LD) for packaging into nascent virions, (iii) they may be retained

within the MW to template the synthesis of additional negative-strand RNA, or (iv) they may undergo intracellular decay (such as 5' XRN1-mediated decay). Factors regulating the movement of the viral RNA between compartments are poorly understood. The overall replicative cycle induces oxidative stress (ROS) with subsequent oxidative membrane damage that acts to restrict the efficiency of the MW (not shown). The inserts represent detailed schematics of viral translation and RNA synthesis. (+)RNA molecules can either engage in protein synthesis (left) or RNA synthesis (right), but not both simultaneously due to the conflicting direction of the movements of ribosomes (5' to 3') and the replicase (3' to 5') on (+)RNA. Adapted with permission from REF. 98.

Figure 3. The Yang of HCV: RNA decay

(a) Host mRNAs differ from HCV RNA with respect to the presence of a 5' m⁷GpppN cap and a 3' poly(A) tail, both of which help to stabilize host cell mRNA. General mRNA decay initiates with removal of the 3' poly(A) tail by a deadenylase complex and is followed by either 5'-to-3' or 3'-to-5' degradation. In the 3'-to-5' pathway, the RNA body is degraded by the 3' exosome complex. The residual cap-oligonucleotide is cleaved by the scavenger decapping enzyme, DCPS. In the 5'-to-3' pathway, the 5' cap is removed by decapping enzymes and the RNA body degraded by the cytoplasmic 5' exonuclease, XRN1. (b) Positive-strand HCV RNA possesses a 5' triphosphate and terminates in a stable 3' stem-loop. Its decay by constitutive mRNA decay pathways commences with removal of the 5' triphosphate by an unknown cellular pyrophosphatase, followed by XRN1-mediated degradation as in the host mRNA 5'-to-3' decay pathway. HCV RNAs are protected from decay by their location within the detergent-resistant membranes of the membranous web, and by miR-122 interactions with the 5'UTR. Whether miR-122 mediates protection against the putative pyrophosphatase or XRN1 (or both) is not known. Unlike decay of host mRNAs, the exosome complex plays no role in this process. Interferon and cytokine-inducible endoribonucleases and exoribonucleases represent alternative mechanisms for degradation of HCV RNA. Whereas these ribonucleases are actively expressed within the

HCV-infected liver, their level of expression may be moderated by the ability of the HCV NS3/4A protease to cleave key adaptor proteins involved in signaling from pathogen recognition receptors such as RIG-I and Toll-like receptors (TLRs) that are capable of sensing the presence of foreign RNA.

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