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MIR-122 AND DDX6 FACILITATE HEPATITIS C VIRUS REPLICATION VIA INDEPENDENT MECHANISMS

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MIR-122 AND DDX6 FACILITATE HEPATITIS C VIRUS REPLICATION VIA INDEPENDENT MECHANISMS

By

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Dissertation

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

August 2010

To my beloved family

ACKNOWLEDGEMENTS

I convey my heartfelt indebtedness and sincere thanks to my mentor and Chairman, Dissertation Committee, Dr. Stanley M. Lemon for his persistent support, painstaking supervision and affectionate attention in planning and execution of this study. His composure, compassionate attitude and encouraging words have guided me during this study and will continue to inspire me to achieve my goals in future. His ability to always look at the big picture will be with me as a life-long lesson. It has been a great learning experience with immense pleasure to grow professionally as well as personally under his exemplary guidance.

My sincere thanks are due to the respected members of my Dissertation Committee, Drs. Peter W. Mason, Craig E. Cameron, Shinji Makino and Steven A. Weinmann for their constructive suggestions, invaluable scientific input and ever-encouraging remarks on my research. Their commitment and guidance has a major role in taking this study to a successful finish.

I would also like to express my gratitude to Drs. Thomas K. Hughes, Robert A. Davey and Rolf Konig, Program Directors, Microbiology and Immunology Graduate Program for their support. Special thanks are due to Dr. Dorian Coppenhaver, Senior Associate Dean for Student Affairs, Graduate School of Biomedical Sciences for his support and guidance.

I sincerely thank the generous support of James W. McLaughlin Endowment Fund in the form of McLaughlin predoctoral fellowship during the course of this study.

I am highly indebted to Dr. MinKyung Yi for her unwavering support, technical guidance and scientific input on my project. Past and current members of the Lemon lab have provided a friendly lab setting that was full of fun and scientific discussions. I specially thank Drs. David R. McGivern, Meital Gal-Tanamy, Rodrigo A. Villanueva, Zongdi Feng, Tetsuro Shimakami, Seungtaek Kim, Jennifer Timpe and Lin Qu for very helpful discussions and suggestions during my stay in the lab. I express my thanks to Jeremy Yates, Yinghong Ma, Yuqiong Liang, Teri Chapa and Sher Singh for their help and joyous collegiality.

A special mention is required to express my gratitude to Drs. Kui Li, Michael R. Beard, Meital Gal-Tanamy and Rodrigo A Villanueva for giving me the opportunity to collaborate on various projects.

Relentless assistance, friendly atmosphere and unselfish support provided by Martha Lewis and Aneth Zertuche, Program Co-ordinators, Microbiology and Immunology Graduate Program; Deborah James and Mary Ives, Institute of Human Infections and Immunity; and Laura Teed and other staff at the office of the Graduate School of Biomedical Sciences, UTMB is greatly appreciated.

The company of great friends – Aditya Hindupur, Aditya Joshi, Trupti Godbole, Abhisek Mukherjee, Priyanka Chatterjee, Selvi Subramanian, Tania Garron,

Aishwarya Ravindran, Anasuya Roychowdhary, Keethi Gottipathi, Jeeba Kuriokose, Larry Bellot, Vinay Mamidala, Chandana Kotwal, Surendra Negi, Urmila Rawat, Truptesh Kothari, Krishna Narayanan, Lokesh Rao, Santhana Devraj, Nasib Singh, Kavita Singh, Payal Seth, Kunal Ashar, Akanksha Bahrani and Anurag Mishra – made my time at Galveston, Texas memorable. I specially thank all of them for their wonderful company and support.

I would not have been in a position to write this acknowledgement without the support, love and sacrifices of my mom and dad and all the family members, whose moral support and affectionate love keeps me going everyday.

I owe my heartfelt thanks to my sweet friend and wife, Sushma Bharrhan, for all her support and encouragement. Her deep love and unwavering support has motivated and driven me to successfully finish this study.

Last but not the least, my thanks to anyone and everyone who has directly or indirectly contributed to the success of this study and has not found a mention here.

MIR-122 AND DDX6 FACILITATE HEPATITIS C VIRUS REPLICATION VIA INDEPENDENT MECHANISMS

Publication No._____

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The University of Texas Medical Branch, August 2010

Supervisor: Stanley M. Lemon

Globally 130 million people are infected with hepatitis C virus (HCV) that leads to chronic hepatitis. Current therapy is limited in its efficacy; hence better interventions are required. A detailed understanding of the host factors involved in HCV replication such as miR-122 and DEAD-box RNA helicase, DDX6 may facilitate development of novel therapies. miR-122 is a liver-specific microRNA that is required for efficient replication of HCV in hepatocytes. Mutational analyses of the genotype 1a/2a chimeric HCV (HJ3-5) genomes indicated that miR-122 promotes replication by binding directly to the two miR-122 binding sites in the genomic RNA and, at least in part, by stimulating internal ribosome entry site-mediated translation. A comparison of mutants with substitutions in only one site revealed S1 to be dominant over S2 site for its role in viral replication. However, a comparison of the replication capacities of the double binding-site mutant and an IRES mutant with a quantitatively equivalent defect in translation suggests that the decrement in translation associated with loss of miR-122 binding is insufficient to explain the profound defect in virus production by the double mutant. miR-122 is thus likely to act at an additional step in the virus lifecycle. Overexpression and knockdown experiments indicated that DDX6, a miRISC component that is upregulated in liver during chronic hepatitis C, is another cellular factor supporting HCV replication. DDX6 forms a complex containing HCV core protein, and both viral and cellular RNAs via its helicase domain and its helicase activity is required to facilitate virus replication. However, the relevance of DDX6-core interaction is unclear as DDX6 abundance influenced the replication of subgenomic replicon RNAs lacking core sequence. Importantly, DDX6 knockdown caused minimal reductions in cellular

proliferation, generally stimulated cellular translation ([^{35}S]-Met incorporation), and did not impair translation directed by the HCV internal ribosome entry site. Thus, DDX6 helicase activity is essential for efficient HCV replication, reflecting essential roles for DDX6 in HCV genome amplification and/or maintenance of cellular homeostasis. Finally, DDX6 knockdown did not impair miR-122 biogenesis nor alter HCV responsiveness to miR-122 supplementation suggesting that DDX6 and miR-122 facilitate HCV replication via independent mechanisms.

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CHAPTER 1. INTRODUCTION

Globally, nearly 130 million people are infected with hepatitis C virus (HCV) and thus at risk for potentially lethal complications of chronic liver disease (115). Despite significant improvements in diagnostics and therapeutics, HCV-related liver fibrosis and hepatocellular carcinoma continue to be a major burden on public health with little change expected in the coming decades (23). Current standard-of-care anti-HCV therapies combining interferon (IFNα) and ribavirin are only effective in ~50% of cases, have severe side effects, and are poorly tolerated. Hence, development of effective, novel anti-HCV interventions is essential. To that end, various drugs targeting the key enzymes in viral replication, i.e., the viral protease (NS3/4A) and RNA-dependent RNA polymerase (NS5B) are being developed (184). However, virus-specific therapies are fraught with rapid development of resistance owing to very high replication levels of virus along with error-prone synthesis of the HCV genome. A better understanding of the host factors involved in HCV replication would facilitate the development of novel host-specific therapies that may be less subject to resistance development. The potential for such therapies was vividly demonstrated in a recent study involving knockdown of miR-122, an essential host factor in HCV replication, by an antisense oligonucleotide, which had a dramatic antiviral effect in HCV-infected chimpanzees without apparent adverse effects (112). The work presented in this thesis is focused on developing better understanding of the role of miR-122, a highly abundant, liver-specific microRNA, and a second potential cofactor, DDX6, a DEAD-box RNA helicase, in the HCV life cycle. Such knowledge may facilitate the development of improved therapies for this disease.

HEPATITIS C

Prevalence

HCV is a major public health problem, as infection, which is associated with chronic hepatitis, cirrhosis and hepatocellular carcinoma. In the United States alone, nearly 4 million people are HCV-seropositive. There are 10,000 – 20,000 deaths occurring due to HCV-related liver disease annually, and disease due to HCV infection is a major indication for liver transplantation (183). Acute HCV infection is generally asymptomatic and difficult to diagnose. However, more than 70% of infected persons develop persistent infection, many of whom go on to develop chronic hepatitis with progressive fibrosis leading to cirrhosis and potentially liver failure over a period of 20 years or more. Approximately 1-4% of patients with HCV-associated cirrhosis develop hepatocellular carcinoma annually (183).

Transmission of the virus primarily occurs percutaneously via contaminated needles used for injection of illicit drugs, blood transfusion (much less commonly today with universal screening of all blood products) and/or organ transplantation. Although new infection rates have been reduced significantly as a result of better diagnostics and other preventive measures, the burden of HCV on the public health system will remain high for the foreseeable future (23). The current standard-of-care anti-HCV regimen of pegylated-interferon (peg-IFN) and ribavirin is only effective in eliminating virus in ~50% patients with genotype 1 virus infections; moreover, it is expensive, contraindicated in many patients, and associated with severe side-effects (184). No vaccine is available for hepatitis C, nor is any on the near horizon. Development of newer and more effective therapeutic/prophylactic interventions is thus essential.

Hepatitis C Virus (HCV): Genome Organization

HCV, classified today in the genus Hepacivirus of the family Flaviviridae, is an enveloped, hepatotropic virus with a positive-sense, single-stranded RNA genome ~9.6 kb in length (115). The uncapped viral genome contains a highly conserved internal ribosomal entry site (IRES) within its 5'-end untranslated RNA (5'UTR) segment that directs cap-independent initiation of translation. A single large open reading frame encodes a ~3010 amino acid-long polyprotein that is co- and post-translationally processed by viral and cellular proteases into both structural (Core, E1, E2) and non-structural proteins (p7, NS2-NS5B). Figure 1.1 below summarizes well-known functions of the various viral proteins.

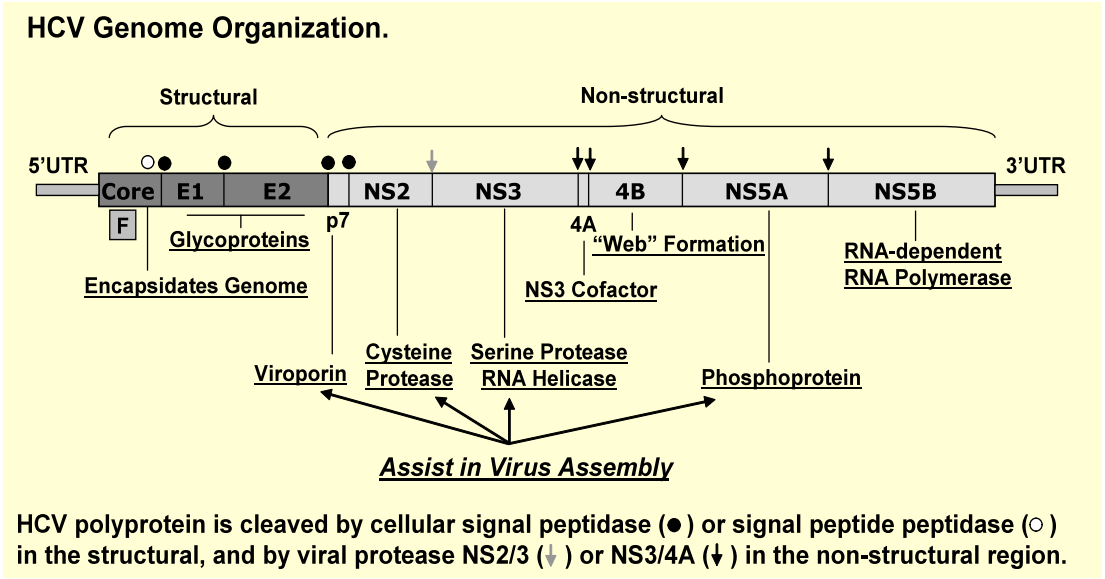

Figure 1.1 HCV genomic organization and proteins. Not drawn to scale. The HCV genome consists of an open reading frame flanked by 5' and 3'UTRs at each end. The amino terminal section of the polyprotein is processed into the structural proteins while the carboxy terminal two-third constitutes the nonstructural proteins.

Structural Proteins of HCV

The first HCV protein to be translated in the viral polyprotein is the core protein, a highly conserved, basic, proline-rich protein that, together with the genomic RNA, forms the viral nucleocapsid. As shown in Figure 1.1 above, the N-terminus of the polyprotein is proteolytically processed by the cellular ER-resident signal peptidase to produce a ~23 kd, 191 amino acid residue-long, core protein (77), which is further processed by another cellular membrane-resident protease, signal peptide peptidase, to yield the ~21 kd, approximately 173 residue-long, mature core protein (140, 176, 220). (22). Core protein is comprised of two domains – a highly basic, RNA-binding, N-terminal domain (I) of ~117 amino acids and a hydrophobic, C-terminal domain (II) of ~50 amino acids that helps in proper folding of domain I (22). Being hydrophobic in nature, domain II also interacts with cellular membranes and lipid droplets (80). This domain consists of two amphipathic alpha helices separated by an interhelical hydrophobic loop (21). On the other hand, domain I is hydrophilic, binds nonspecifically with RNA (105, 176), and has been implicated in a variety of protein-protein interactions with a wide array of host proteins, leading potentially to pleiotropic effects on the cell (139).

Important host factors that have been shown to interact with the HCV core protein include those involved in cell signaling, transcription, apoptosis, lipid metabolism, immunomodulation, oxidative stress, cell-cycle regulation and carcinogenesis [reviewed in (84, 110, 139, 169)]. Specifically, the core protein has been shown to interact with heterogeneous nuclear ribonucleoprotein K (hnRNP K, a protein involved in cellular transcription and signaling) (81); the lymphotoxin β receptor (LTβR) (29, 138) and related tumor necrosis factor (TNF) receptor 1 (TNF-

R1) (234), both of which are involved in TNF signaling; and the complement receptor gC1qR, potentially leading to impaired T cell function (208, 218, 219). Moreover, core protein as well as the NS5A protein (see below) has been implicated in apoptosis via interactions with multiple pro- and anti-apoptotic proteins [reviewed in (56)]. However, the exact mechanism and contribution of these potential interactions to HCV virus replication and/or pathogenesis are not clearly understood.

The HCV genome encodes for two envelope glycoproteins, namely E1 and E2, translated in that order in the viral polyprotein and processed from the polyprotein by cellular signal peptidase to generate their mature forms. The C-terminal domains of E1 and E2 contain ER retention signals, while the ectodomains are highly glycosylated (43). Both the glycoproteins are embedded in the viral envelope, which is derived from host membranes, as noncovalent heterodimers (39) due to charged residues within the transmembrane domains (33). They appear to interact with the core protein. The E2 glycoprotein plays a major role in viral entry as it binds to various HCV receptors and co-receptors, including CD81, a member of the tetraspanin family of proteins that is central to HCV entry and expressed on the surface of the hepatocytes [reviewed recently in (12, 32, 42)].

Nonstructural proteins of HCV

The viral ion channel protein, p7 is a membrane-embedded protein that is processed at its both ends by the cellular signal peptidase (70, 119). It is uncertain whether it is present in the virion, and is normally considered to be a nonstructural protein. It is presumably localized to the ER membrane (25). p7 is essential for HCV

infectivity in the chimpanzee model (175) and plays a crucial role in assembly and release of infectious virions from infected cells in available cell culture systems (192).

The other HCV nonstructural proteins, NS2-NS5B are involved in both viral RNA synthesis and viral assembly. NS2, in particular, plays a central role in the assembly of new virus particles; NS3 and NS5A are also involved in viral assembly, a process that appears to occur at the surface of cellular lipid droplets [reviewed in (92)]. NS2 is a membrane bound protein that is cleaved from the polyprotein at its N-terminus by signal peptidase (180). Its C-terminus is processed by the autocatalytic activity of the NS2-3 protease, an enzymatic activity residing in the C-terminal domain of NS2 and N-terminal domain of NS3 (157, 170). Although NS2 is not required for genome amplification (127), recent data suggest that NS2, but not its protease activity, plays a crucial role in viral assembly (90, 191, 226). Studies by Yi et al. suggest that NS2 plays a role in early particle assembly despite the fact that it does not colocalize with NS5A and core protein at the surface of the lipid droplet, and that the C-terminus of NS2 is required for a second step at a late stage of virus assembly that confers and/or enhances infectivity (227).

The nonstructural proteins NS3-5B are both necessary and sufficient for robust HCV replication in cell culture (127). The NS3 protein contains an amino terminal protease and a carboxy terminal helicase domain. The protease domain encompasses the first 180 amino acids of the protein, and together with its NS4A cofactor is responsible for catalyzing the cleavage of the polyprotein at the NS3-4A junction, and at subsequent junctions sites downstream leading to production of the mature forms of the remaining nonstructural proteins (10). NS3 is a chymotrypsin-

like serine protease (102) that requires a 4A peptide as a cofactor for its proper folding and protease activity (120). Since NS4A is a membrane bound protein, it has been suggested to mediate membrane association of NS3 (72). NS3 (the helicase domain) also plays an important but poorly characterized role in assembly of viral particles.

The NS3/4A protease, in addition to polyprotein processing, also cleaves at least two cellular proteins, mitochondrial antiviral protein (MAVS, a.k.a, IPS-1, Cardif, or VISA) (116) and TLR/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF, a.k.a., TICAM-1) (57, 116), and thus blocks retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3) signaling pathways in infected cells, respectively. RIG-I and TLR3 pathways form a first line of defense against the invading virus, as they sense "non-self" RNAs produced during viral replication and activate a cascade of signaling events culminating in production of interferons and interferon-inducible genes that confer an antiviral state to the host tissue [reviewed in (98)]. Inactivation of these two pathways may contribute, at least partly, to the ability of HCV to establish persistent infection in the host.

Although the role of the NS3 protease domain in the viral life cycle is well characterized, the role of the carboxy-terminal helicase domain of NS3 in viral replication is unclear. It is known that the helicase domain is required for proper folding of the NS3 protease domain, has both unwindase and NTPase activities (44, 108), and is indispensable for viral replication (111). More recent data indicate that the NS3 helicase domain plays a central role in virion assembly (73, 161), at a stage after NS5A and core interact with each other on lipid droplets and before the assembly of core-containing virus particles (226). Exactly how this happens and

whether it requires helicase activity is not known, however. Hence, both the protease as well as helicase domains of NS3 protein are essential for virus RNA replication and assembly.

NS4B is an integral membrane protein that induces "membranous web" formation in infected cells (45, 46), the putative site of HCV RNA replication (66). NS5A is an RNA-binding phosphoprotein (82) with multiple roles in virus replication, assembly and/or modulation of the cellular milieu [reviewed in (135)]. It exists in hypo- and hyperphosphorylated forms in infected cells. NS5A possesses 3 distinct domains: domain I, the amino-terminal zinc binding domain; domain II, a helix-rich central domain; and domain III, an unstructured carboxy terminal domain (202). Although its precise role in viral RNA synthesis is unclear, it localizes to active replicase complexes (66, 144) and interacts with the viral RNA-dependent RNA polymerase NS5B to negatively modulate its polymerase activity (41, 186). Multiple serine residues in NS5A are targets of various cellular kinases, including casein kinase I (164) and II (38, 101) and polo-like kinase-1 (PLK-1) (30), leading to hypophosphorylated (~56 kD) and hyperphosphorylated (~58 kD) isoforms (171). Various adaptive mutations in NS5A that lead to increase in levels of the hypophosphorylated form seem to increase RNA replication in cultured cells, suggesting that hypophosphorylated NS5A somehow promotes viral RNA synthesis (18, 52, 53, 83). While domain I and II of NS5A are particularly essential for viral RNA replication, domain I and III also appear to be involved in HCV virion assembly by acting as a liaison between lipid droplets and HCV core protein (8, 137, 201).

NS5B, the viral RNA-dependent RNA polymerase (RdRp) is the workhorse of viral replication as it provides the catalytic activity for synthesis of new RNA

genomes. It associates with cellular membranes via its carboxy terminal hydrophobic tail that anchors it to the ER membrane (216). Although NS5B can catalyze both primer-independent and primer-dependent RNA synthesis in vitro (132, 154, 167, 233), no evidence has been found so far for the HCV RdRp using a primer in vivo. More importantly, HCV RdRp lacks proof-reading ability. The resulting error-prone replication coupled with selective immune pressure has generated tremendous diversity in viral genomes circulating in different global regions (187). There are 6 well-established major genotypes of HCV, with ~30% difference in the nucleotide sequence between them (named genotypes 1-6), while viruses belonging to a putative 7th genotype have been recently found to be circulating in central Africa (147). Genotype 1 is the most common in the United States and is more frequently associated with severe liver disease than other genotypes (183). Patients infected with genotype 1 respond especially poorly to peg-IFN-ribavirin therapy, underscoring the need for alternative therapies (145).

HCV untranslated regions (UTRs)

The HCV open reading frame is flanked by 5' and 3'-UTRs that are involved in viral genome replication and/or translation. These sequences fold into specific secondary and tertiary structures that are important for recruitment of cellular and viral factors critical for efficient viral translation and RNA replication (11, 115). The 5'UTR contains an IRES that directs the cap-independent assembly of a 48S ribosomal complex on the viral RNA, directly positioning the AUG codon in or near the P site of the ribosome (130, 159). Phylogenetic, biochemical and mutational

analyses suggest that the HCV 5'UTR is composed of 4 highly conserved structural domains, namely domains I-IV (78, 131). The 5' 125 nucleotides encompass domains I and II and are essential for RNA replication, although the complete 5'UTR is required for maximally efficient RNA replication (59). The RNA replication signals in the 5'UTR thus partially overlap with the IRES, which constitutes domains II-IV. The IRES binds directly to the 40S ribosomal subunit and to the eukaryotic initiation factor, eIF-3, without a requirement for other canonical transcriptional factors (130, 159).

The 3'UTR is 200-235 nucleotides (nts) in length and consists of three distinct regions, viz. a variable region, a poly U/UC tract, and the 98-nt so-called "3'X-region" which is invariant in sequence (225). The 3'X-region folds into 3 stem-loop structures that are involved in RNA-RNA and RNA-protein interactions essential for efficient replication of viral RNA (11, 223, 225).

HCV Life Cycle

Most of the finer details of HCV life cycle have not been well characterized, mostly because efficient and reproducible cell culture systems for HCV propagation became available only recently. However, with the advent of the HCV replicon system, pseudoparticles, and more recently cell culture-based infectious virus systems, significant progress has been made recently in our understanding of the viral life cycle.

Following virus attachment and binding to a complex set of receptors on the cell surface, clathrin-mediated endocytosis (17) delivers infectious HCV to the

cytoplasm via a highly organized, step-by-step process [reviewed recently in (42, 174, 207)]. Initial interactions of the viral glycoproteins are probably mediated by glycosaminoglycans on the cell surface, leading to a concentration of virus on the cell surface (13, 14, 63). This is followed by the engagement of CD81 and Scavenger receptor-B1 (SR-B1) receptors, both of which have been shown to bind to the E2 glycoprotein and to mediate infectious virus entry (230). Recently, additional entry factors have been identified that are associated with tight junctions: claudin-1 (54) and occludin (163). These proteins appear to be involved in later stages of viral entry (Fig. 1.2).

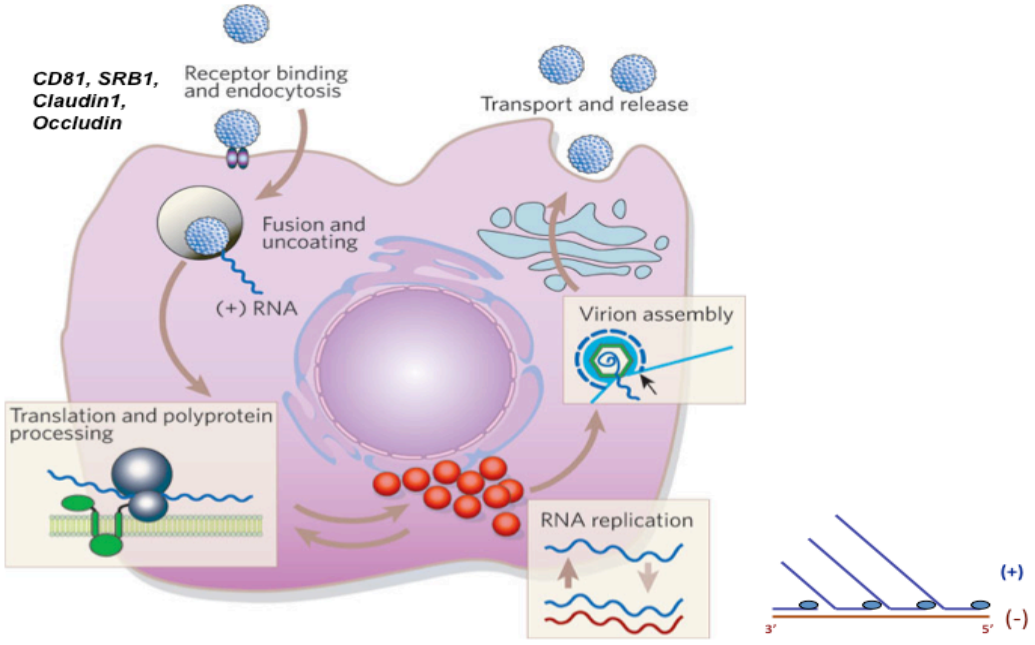

Figure 1.2 Simplified HCV life cycle. Following receptor-mediated entry and uncoating of the HCV genome, protein translation and viral RNA replication ensues. From a single initial copy of negative-strand RNA, multiple copies of positive-strand RNA are generated in an asymmetric fashion. Ultimately the viral glycoproteins and capsid protein are assembled with the viral genomes and released via budding along with an envelope derived from host cell membranes. Reprinted, after modifications, by permission from Macmillan Publishers Ltd: NATURE (123) © 2005.

Following entry and pH-mediated uncoating of the viral genome (204), the polyprotein is translated and processed by cellular and/or viral proteases to give rise to mature viral proteins that mediate viral replication in ER-derived membrane-associated replication complexes (115). As with other positive-strand RNA viruses, replication proceeds in an asymmetric fashion with a single copy of negative-strand RNA producing multiple copies of positive-strand genomes, leading to 10- to 100-fold excess of positive- over negative-strand molecules (3). Finally, newly synthesized viral genomes and structural proteins (core, E1 and E2) are assembled as virions, in a process that is facilitated by multiple nonstructural proteins as described above, in association with lipid droplets. The viral particles bud into the ER lumen, acquiring the lipid envelope in the process, and appear to be released from infected cells via the very-low density lipoprotein (VLDL) secretory pathway [reviewed in (92)].

Hepatitis C Virus Replication: In Vitro Models

Only human and chimpanzee are susceptible to HCV infection making in vivo experiments difficult. There are certain mouse models available for HCV studies, but these are highly inefficient, labor-intensive and technically very challenging to work with (106, 141). Most studies of HCV replication mechanisms have been carried out in vitro, and have used Huh7 cells harboring stable full-length genomic or subgenomic RNA replicons that were engineered to express various drug resistant markers (127). In these replicons, translation of the HCV nonstructural proteins (NS2-NS5B or NS3-NS5B) is directed by non-homologous IRES such as that from

the encephalomyocarditis virus (EMCV). In 2005, three different groups established complete replication cycle in cell culture leading to production of infectious virions using the HCV genotype 2a clone JFH1 (122, 209, 232). H77S, a modified prototype genotype 1a virus clone, can also produce infectious virions when transfected as RNA into Huh7 cells, though to a lesser level than JFH1 (228). However, a chimeric virus containing the structural region of the genotype 1a genome and nonstructural region of genotype 2a replicates as well as JFH1 in Huh7 cells (226). More recently, chimeric viruses containing structural regions derived from various HCV genotypes and the genotype 2a backbone have been successfully constructed and shown to undergo the complete life cycle in cell culture (68, 69, 89, 162, 177).

MICRORNAS

Biogenesis and mechanism of action

miRNAs are approximately 22 nucleotide long, single-stranded RNAs of endogenous origin that are generated by sequential processing of hairpin-shaped RNA polymerase II transcripts (103). After transcription, a 5'-capped and polyadenylated transcript, known as primary miRNA (pri-miRNA), is processed by the nuclear RNase-III type endonuclease complex of drosha-DGCR8 (DiGeorge syndrome critical region-8) to produce ~70-nt long pre-miRNA. Pre-miRNAs are transported to the cytoplasm by exportin5-RanGTP (Fig. 1.3) (104). Here, they are further processed by another RNase-III type endonuclease, dicer, to generate ~22-nt long mature miRNA that are assembled into the miRNA-ribonucleoprotein (miRNP) or miRNA-

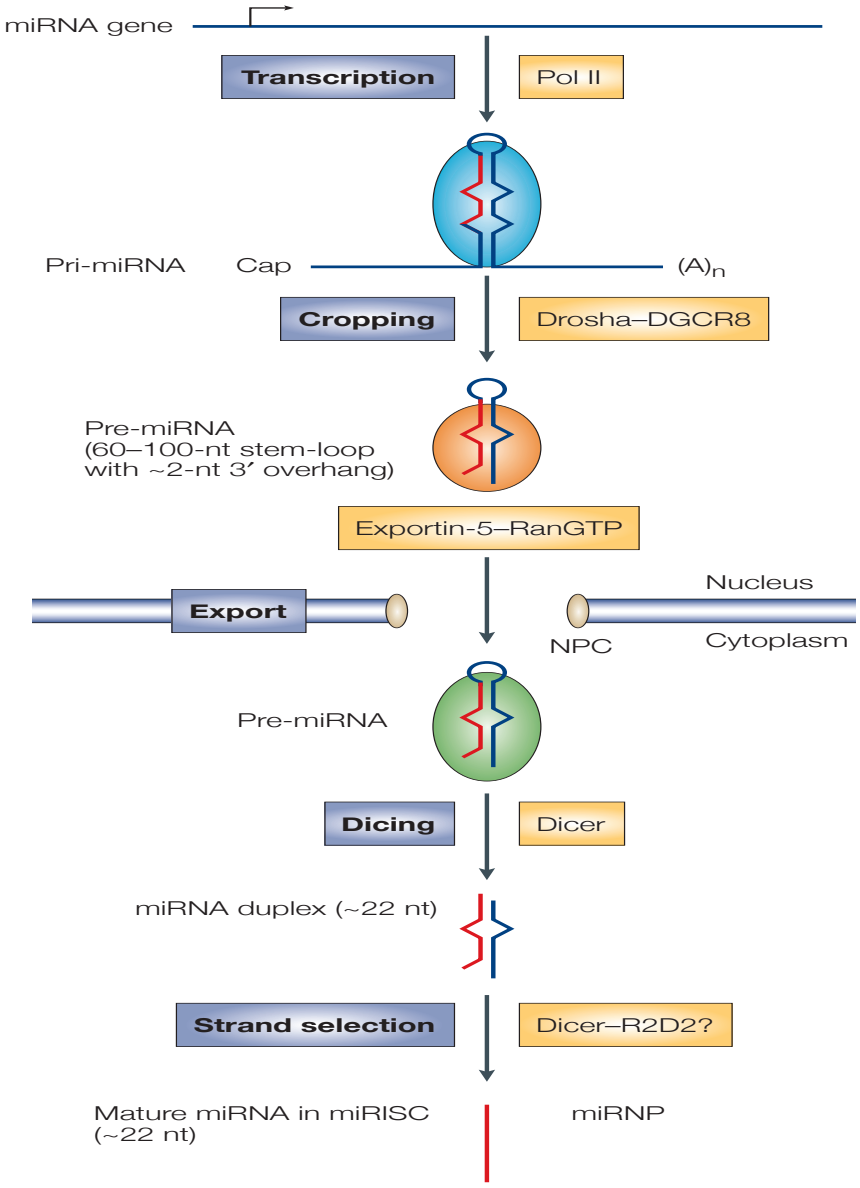

Figure 1.3 Biogenesis of miRNAs. miRNA processing starts in the nucleus usually with the production of a capped and polyadenylated primary miRNA (pri-miRNA) by RNA polymerase II. Following processing by the nuclear RNase type III Drosha-DGCR8 complex, the pre-miRNA hairpin is generated and transported out of the nucleus by the exportin5-RanGTP complex. Once in the cytoplasm, pre-miRNA is processed by another RNase type III enzyme, dicer, to produce the mature miRNA duplex, which is loaded onto the miRISC/miRNP. Usually one strand is degraded while the other one acts as a guide to direct the miRISC to its target mRNA based on sequence complementarity. Reprinted by permission from Macmillan Publishers Ltd: NAT REV MOL CELL BIOL (103) © 2005.

induced silencing complex (miRISC) (214), with subsequent loss of the sense strand, and retention of the antisense, "guide" strand of the duplex. The miRNA guide strand directs the miRNP/miRISC to its partially complementary target sites in cellular mRNAs, which are usually located in the 3'UTR, and thereby mediates translational blockade and/or mRNA degradation (55). Translationally-repressed miRNA-mRNA complexes localize to distinct cytoplasmic structures called processing bodies (P-bodies) where the mRNA is either degraded or stored temporarily (165). The ability of miRNAs to downregulate translation depends mostly on the sequence complementarity between nucleotides 2-8 of the miRNA and its target mRNA. This sequence is called the "seed" sequence and its corresponding sequence in target mRNA is called the "seed-match" (165). Recently, some miRNAs have been shown to enhance mRNA degradation by enhancing deadenylation of the target mRNA (215). Thus, miRNA can downregulate gene expression by mRNA degradation as well as by inducing translational repression, although the relative contribution of these two mechanisms probably depends on the individual miRNA-mRNA pair (85). Interestingly, some miRNAs appear to regulate gene expression at the level of transcription too (103).

Functions of cellular miRNAs

Bioinformatics-based predictions claim that human genome encodes for up to 1000 miRNAs that can potentially regulate one-third of human transcriptome (15). In fact, miRNAs have been implicated to play roles in development, differentiation, cell growth, cell metabolism, cancer (24), immunity (197), and virus infections (37, 194). More recently, various studies have also implicated an important role for miRNAs in

innate as well as adaptive immune responses, including control of T-cells, B-cells and T-regulatory cells as well as interferon and TLR signaling [reviewed in (129)]. They also play a role in antiviral immunity, as a host miRNA, miR-32, can inhibit translation of primate foamy virus-1 (PFV-1) transcripts in a sequence-specific manner (114). In addition, dicer and drosha, the workhorses of the miRNA-silencing machinery, are also involved in controlling HIV-1 replication (203). Both of these viruses actively suppress miRNA-mediated antiviral defense to ensure efficient replication (114, 203). miR-24 and miR-93 have been shown to suppress replication of vesicular stomatitis virus (VSV), a negative-strand RNA virus, in a mouse model, suggesting a direct antiviral effect of these miRNAs (155).

Viral miRNAs

In addition to cellular miRNAs, many DNA viruses, especially herpesviruses, have been shown to produce their own miRNAs, which can contribute to viral replication, pathogenesis or immune evasion [reviewed in (20, 37, 188)]. Among the first viral miRNAs with a well-characterized role is one produced by the polyoma virus, SV40 (195). This miRNA is generated from a transcript produced in the late stages of viral infection as an antisense to the large T antigen mRNA. The kinetics of this miRNA production inversely relates to large T antigen abundance in the cells. However, production of this miRNA does not have any deleterious effects on virus growth. In fact, it downregulates the expression of large T antigen, a major target of cytotoxic T cell mediated immune responses, in infected cells, and thus may facilitate immune evasion (195)! The number of miRNAs known to be expressed by DNA

viruses is growing on a regular basis. After the first viral miRNA discovery in Epstein Barr virus (EBV)-infected B cells by Pfeffer et al. (160), 25 miRNAs have been identified so far. Similarly, Kaposi's sarcoma herpesvirus (KSHV) encodes for 12 miRNAs, human cytomegalovirus (hCMV) 11 miRNAs, mouse cytomegalovirus (mCMV) 18, and HSV-1 and 2, 8 and 6 miRNAs, respectively [reviewed in (188)]. However, the biological role of most of these viral miRNAs remains unclear.

Recent studies suggest that herpesviral miRNAs may be important in latency and/or immune evasion. KSHV miRNA miR-K12-11 (67, 189) and the avian α-herpesvirus Marek's disease virus-1 encoded miRNA (231) are homologues of cellular miRNA, miR-155. Since miR-155 overexpression can lead to B cell lymphomas, it is possible that these viral miRNAs contribute to tumor formation in infected individuals. Another prime example of immune evasion mediated by viral miRNA is the targeting of major histocompatibility class I-related chain B (MICB) mRNA by the hCMV encoded miRNA miR-UL112. MICB is the ligand for NK cell activating receptor NKG2D and is required for efficient killing of virus-infected cells by activated NK cells. hCMV miRNA-mediated knockdown of MICB during virus infection facilitates immune evasion by reducing NK cell-mediated killing of infected cells (193). Thus, viral miRNAs can contribute to viral pathogenesis and/or immune evasion.

Unlike these DNA viruses, HCV does not encode any known miRNAs. However, as mentioned above, HCV interacts with a liver-specific miRNA, miR-122, in a sequence-specific manner. Instead of downregulating viral gene expression, this interaction leads to increases in HCV RNA abundance in Huh7 cells bearing HCV replicons (95)!

miR-122 – a liver specific miRNA

miRNAs can be expressed in both cell- and tissue-specific manners and might help facilitate the maintenance of cellular phenotypes The liver expression of miR-122 in mice is low at early embryonic stage, with its expression beginning at E12.5 days. Half-maximal levels are achieved at around E17.5 days during embryonic development. The expression levels approach 50,000-80,000 copies per cell within the liver just before birth and are maintained at this level throughout life. miR-122 is almost exclusively expressed in hepatocytes to very high levels (28).

miR-122 is transcribed from a noncoding gene called *hcr* that was originally identified as a fusion gene with c-myc in a unique woodchuck liver tumor, W64. This fusion occurs due to a rare chromosomal translocation that puts the c-myc gene under the control of the *hcr* promoter, thus leading to about a 50-fold increase in its expression (49, 146). Further evidence for miR-122 production by *hcr* transcripts was provided by Chang et al. (28) who demonstrated production of mature miR-122 after overexpression of 160 nts of the woodchuck *hcr* gene transcript corresponding to the related pre-miRNA. Mature miR-122 and the *hcr* gene sequences are conserved through evolution, from fish to humans, suggesting a physiologically important role for this miRNA. In the liver, miR-122 constitutes ~70% of the total miRNAs expressed. However, recent studies have suggested that even after a total ablation of all hepatic miRNAs, via tissue specific knockdown of dicer at a late gestation stage in mice, normal liver functions are maintained for 2 to 4 months after birth. Thereafter, the animals develop liver hypertrophy, progressive hepatocyte damage and apoptosis/proliferation. This suggests that even though miRNAs do not seem to

control normal liver functions, their long-term knockdown may not be well tolerated (74).

How miR-122 expression is controlled during development and in adult liver is not clear. Recent studies have suggested that miR-122 expression is controlled by genes that are also involved in regulation of circadian rhythm, and vice versa (62, 107). Gatfield et al. (62) showed that the levels of pre-miR-122 are controlled by the transcriptional repressor orphan nuclear receptor REV-ERBα in a circadian manner. However, only the levels of pre-miR-122 and pri-miR-122 oscillated with circadian rhythms and not that of the mature miR-122, presumably due to a longer than 24 hrs half-life of mature miR-122 (62). Conversely, miR-122 can potentially target some of the genes involved in circadian rhythms (62). One such target is a circadian deadenylase nocturnin which regulates post-transcriptional mRNA expression via poly(A) tail degradation (107). miR-122 can also downregulate expression of cationic amino acid transporter (CAT-1), a predicted natural target of miR-122, in reporter assays (28). It is also involved in cholesterol biosynthesis and fat metabolism (47, 48, 109). Knockdown of miR-122 by antisense oligos in mice reduced blood cholesterol levels and increased hepatic fatty acid oxidation (48).

miR-122 and HCV Replication

Early studies demonstrated that specific knockdown of miR-122 in Huh7 cells harboring HCV full-length genomic dicistronic replicons significantly decreased HCV RNA abundance (95). Conversely, supplementation of exogenous miR-122 by transfection increased the steady-state levels of HCV RNA. Moreover, HCV genomes

with point mutations in the miR-122-binding site located in 5'UTR were unable to replicate in Huh7 cells (95). However, replication of such mutants was rescued by transfection of miR-122 having corresponding complementary mutations, indicating that miR-122 and HCV RNA interact directly and in a sequence-specific fashion. More recently, Jopling et al. (94) reported that there are two well-conserved, tandem miR-122-binding sites (named S1and S2) within the HCV 5'UTR that work in concert with each other to up-regulate HCV RNA abundance. These authors reported that mutant HCV replicons with substitutions within these miR-122 binding sites did not differ significantly from wild-type in terms of RNA stability or translation, thus suggesting that miR- 122 may be specifically involved in HCV RNA replication (95). Importantly, however, HCV is not absolutely dependent on miR-122 for its replication as it can replicate in non-hepatic cells such as HEK293 cells that do not express detectable levels of miR-122; however, miR-122 supplementation enhances HCV replication in non-hepatic cells too (27). As mentioned above, miR-122 knockdown by an antisense oligonucleotide administration reduces cholesterol and fatty acid biosynthesis in mice and African green monkeys (47, 48, 109). It led to a reduction in mevalonate/cholesterol biosynthesis pathway activity, lower serum cholesterol levels and reduced fat accumulation in the liver (47, 48, 109). Even though HCV replication is intricately linked to cholesterol and fatty acid metabolism (196, 221), Norman and Sarnow (152) showed that metabolites of cholesterol biosynthesis/mevalonate pathway could not restore lost HCV RNA abundance due to miR-122 inhibition. Thus, miR-122 facilitates HCV independent of its role in cholesterol biosynthesis (151, 152). They also examined miR-122 effect on HCV RNA synthesis by incorporation of 4-thiouridine, over a short period of 1 hr, in HCV

replicon cells after miR-122 inhibition. However, no significant effect of miR-122 was seen on viral RNA synthesis (152).

Evolutionarily, an interesting question is why HCV would develop such a close dependence on miR-122 for its replication. Significantly, this is not observed among other members of the flavivirus family. Since miR-122 is conserved from fish to human (28), the HCV dependence on miR-122 does not define species specificity of HCV.

During viral RNA replication, positive-strand RNA is copied to negative-strand, which then templates multiple copies of positive-strand. To understand miR-122-facilitated HCV RNA replication better, it is important to characterize the stage at which miR-122 acts in viral RNA replication. The need for a mechanistic understanding of how miR-122 facilitates HCV replication has become even more pertinent, as Lanford et al. (112) demonstrated recently that specific knockdown of miR-122 in HCV-infected chimpanzees leads to long-term suppression of viremia without any significant adverse effects.

Henke et al. (76) reported recently that miR-122 positively modulates HCV translation via its interaction with the viral genome within its 5'UTR. miR-122 supplementation increased HCV IRES-mediated translation, by 3- to 4-fold, of a subgenomic reporter carrying firefly luciferase fused to HCV 5' and 3'UTRs. This effect was seen in both hepatic as well as nonhepatic (HeLa) cells and rabbit reticulocyte lysates. Assembly of a minigenome HCV RNA, containing HCV 5' and 3'UTR and a short open reading frame (ORF) of just 96 nucleotides, into the ribosomal 48S initiation complex was increased significantly in the presence of miR-122. However, the stimulatory effect on viral translation was modest, up to 2-fold, in

the context of full-length authentic HCV genomes (76, 150). However, how much this 2-fold increase in translation can actually contribute to the dramatic effect of miR-122 on HCV replication in the context of complete viral replication cycle is unknown. It is still possible that miR-122 is regulating some other step in viral replication cycle in addition to enhancing translation. Hence, a finer analysis is required of the effects of miR-122 on HCV translation, RNA stability and/or RNA replication in the context of authentic viral genomes.

DEAD-BOX RNA HELICASES

The first cellular RNA helicase to be identified was eukaryotic initiation factor eIF-4A (168). In the presence of ATP, eIF-4A could "melt" or unwind mRNA structure, increasing the sensitivity of the mRNA molecule to nuclease digestion (168). Since then, many putative RNA helicases have been discovered. The DEAD-box family of RNA helicases was established in 1989 based on the sequence Asp-Glu-Ala-Asp (DEAD) that is found in the motif II or Walker B motif of the protein (126). The DEAD-box and DEAH-box families, sometimes referred to as DExD/H helicase family, are part of the superfamily2 (SF2) of RNA helicases (65). Members of the SF2 family contain two α-β RecA like domains (26), referred to as domain 1 and 2 for simplicity. The DEAD-box family is the largest of these and includes more than 500 proteins, which in aggregate have been shown to be important for almost every aspect of RNA metabolism (35). However, despite their highly important roles in RNA processing, right from initial transcription to mRNA decay, their precise role,

mechanism of action, and means of regulation of these processes remain largely unknown (35, 124).

The membership of the DEAD-box RNA helicase family is based on phylogenetic alignment of the sequences. The members share 9 common motifs, although helicase enzymatic activity has not been demonstrated for all the members (198). The motifs are named Q, I, Ia, Ib, II, III, IV, V and VI (35, 199). A general structure and motif arrangement for a DEAD-box RNA helicase, Drosophila Vasa protein is shown in Fig. 1.4. Conserved motifs Q, I, II and VI are required for ATP binding, while residues in motifs Ia, Ib, II, IV and V mediate RNA binding (124).

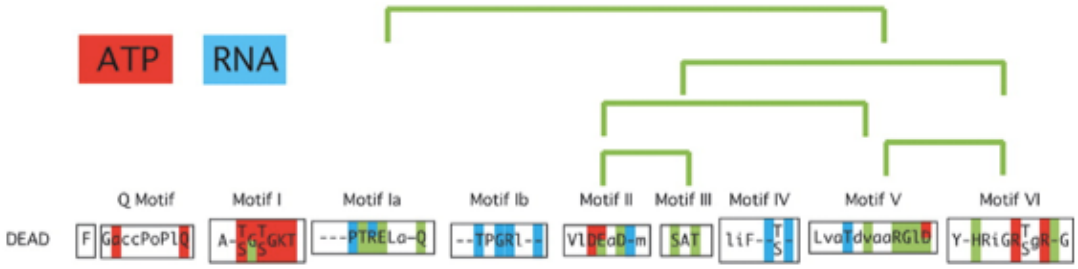

Figure. 1.4 Conserved motifs of DEAD-box RNA helicase. A schematic of Drosophila Vasa protein is shown here (181). Residues that are involved in binding with ATP (red), RNA (blue) and protein-protein interactions (green) are shown. Reprinted by permission from Oxford University Press: NUCLEIC ACIDS RES (124) © 2006.

Although hundreds of DEAD-box RNA helicase have been identified in various organisms, we know very little about their roles in biochemical processes or their structure, as only a few have been studied in detail. As expected from the presence of ATP binding domains, the DEAD-box helicases have ATPase activity, which is usually RNA-dependent or stimulated by RNA and not sequence-specific (35). In vitro studies suggest that DEAD-box RNA helicases unwind double-stranded RNA in an ATP-dependent manner and are not very processive in nature (35).

DEAD-box helicases act as modulators of RNP complexes by reorganizing them in an ATP-dependent fashion with their unwindase activity (124). Generally, there are two widely accepted models to explain the unwindase mechanism of DEAD-box RNA helicases (173, 199). According to the "Inchworm model", the helicase molecule couples ATP hydrolysis to the movement along a single strand of RNA, while opening and closing the cleft between domain 1 and 2 of the helicase, unwinding the dsRNA in the process. The "active rolling model", on the other hand, assumes that helicases operate as dimers that have different conformations and distinct binding affinities for ss- or dsRNA as well as ATP and ADP. Binding and hydrolysis of ATP leads to conformational changes in the helicase resulting in movement across the RNA, which unwinds the dsRNA in the process. However, a third "destabilization model" suggests that the helicase binds to a single strand of RNA first and then undergoes conformational changes in the relative position of domain 1 and 2 as a result of the ATP hydrolysis. This structural reorganization perturbs the local RNA structure sufficient enough to unwind a few base pairs at a time (35).

DDX6 – a DEAD-box RNA Helicase and a Component of miRNP

DDX6 (Rck/p54) is a DEAD-box RNA helicase that is upregulated in liver during chronic hepatitis C (143). DDX6 was first identified as the product of a gene located at a chromosomal breakpoint in the human cell line RC-K8 – hence named Rck/p54 – that was generated from a diffuse B cell lymphoma (128). Subsequently, it was recognized as a putative proto-oncogene that is overexpressed in colorectal

cancer (75). DDX6 is a general translational repressor (34) that directly interacts with Ago1 and Ago2 in miRNP/siRISC (siRNA-Induced Silencing Complex) and plays a role in miRNP-mediated translational repression specifically (31). It is an abundant protein that is localized in the cytoplasm, especially in P-bodies, which as explained above are dynamic perinuclear structures involved in mRNA storage, degradation and/or transport. Interestingly, other members of the DEAD-box RNA helicase family (e.g. DDX3 and DDX5/p68) have been implicated in HCV replication and HCV-associated hepatocellular carcinoma. DDX3 has been shown to interact with the HCV core protein (136, 156, 229) and its expression is required for efficient HCV replication (9), while HCV NS5B interacts with endogenous DDX5/p68 leading to its redistribution from nucleus to cytoplasm (64). In addition to its role in miRNP, human DDX6 can functionally replace its yeast homologue Dhh1p, which is required for brome-mosaic virus (BMV) genome replication and translation in a BMV-yeast model system that is an established model for the study of positive-sense RNA viruses (5). Thus, DDX6 may play a role in virus replication independent of miRNAs.

HYPOTHESIS AND AIMS OF THE STUDY

The role of miRNP components, including miR-122, in HCV replication is not fully understood. siRNA-mediated knockdown of the miRNA-processing machinery enzymes (drosha and dicer) and any one of the four argonautes (Ago1-4) reduces HCV replication significantly (166). Since miR-122 is required for efficient HCV replication and DDX6, a component of miRNPs, is overexpressed in the liver during HCV-related chronic hepatitis, I set out to study the roles of miR-122 and DDX6 in

the replication of HCV in the context of the complete virus life cycle. ***My hypothesis was that in addition to translation, miR-122 promotes an additional step in HCV life cycle and that DDX6 is required for efficient replication of HCV.***

The long-term goals of this study were to delineate the mechanism of miR-122-mediated HCV RNA replication and what role, if any, DDX6 plays in this. To understand the mechanism of action of miR-122, it is important to know whether miR-122 affects RNA replication, stability or protein translation. Chapter 2 describes my studies of the mechanism by which HCV genome amplification is promoted by miR-122, and in which we confirm that miR-122 promotes IRES-directed translation but also show that this effect is unlikely to account for the strong dependence of HCV replication on miR-122 expression. Chapter 3 describes the role of DDX6, one of the components of miRNP that is overexpressed in liver during HCV-related chronic hepatitis, in HCV replication.

As pointed out at the beginning of this introduction, most HCV-specific therapies are fraught with the rapid selection of resistant viruses. A conserved host target such as miR-122 provides an attractive alternative to virus-specific targets such as the NS3/4A protease. The aim of this study was to define the roles of miR-122 and DDX6, a miRNP component, in HCV replication with respect to infectious virus. My hope is that these studies delineating mechanistic details of miR-122-facilitation of HCV RNA replication will help speed the development of novel therapeutic and/or prophylactic interventions to therapeutically modulate HCV infection.

CHAPTER 2. MIR-122 REGULATION OF HEPATITIS C VIRUS TRANSLATION AND INFECTIOUS VIRUS PRODUCTION[1]

ABSTRACT

miR-122 is a liver-specific microRNA that positively regulates hepatitis C virus (HCV) RNA abundance and is essential for production of infectious HCV. Using a genetic approach, we show that its ability to enhance yields of infectious virus is dependent upon two miR-122-binding sites near the 5' end of the HCV genome, S1 and S2. Viral RNA with base substitutions in both S1 and S2 failed to produce infectious virus in transfected cells, while virus production was rescued to near wild-type levels in cells supplemented with a complementary miR-122 mutant. A comparison of mutants with substitutions in only one site revealed S1 to be dominant, as an S2 but not S1 mutant produced high virus yields in cells supplemented with wild-type miR-122. Translation of HCV RNA was reduced over 50% by mutations in either S1 or S2, and was partially rescued by transfection of the complementary miR-122 mutant. Unlike virus replication, however, both sites function equally in regulating translation. We conclude that miR-122 promotes replication by binding directly to both sites in the genomic RNA and, at least in part, by stimulating internal ribosome entry site-mediated translation. However, a comparison of the replication capacities of the double binding-site mutant and an

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IRES mutant with a quantitatively equivalent defect in translation suggests that the decrement in translation associated with loss of miR-122 binding is insufficient to explain the profound defect in virus production by the double mutant. miR-122 is thus likely to act at an additional step in the virus lifecycle.

INTRODUCTION

Hepatitis C virus (HCV) is an hepatotropic human virus classified within the family *Flaviviridae* and possessing a positive-sense, single-stranded RNA genome of about 9.6 kb that encodes a large polyprotein of approximately 3010 amino acids. It is an important human pathogen, with 1 in 50 persons chronically infected with HCV worldwide and at risk for developing cirrhosis and hepatocellular carcinoma (113). The polyprotein is co- and post-translationally processed by cellular and viral proteases into at least 10 individual proteins, including the nucleocapsid core protein, two glycoproteins, E1 and E2, a putative ion-channel, p7, and at least 6 nonstructural proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B [reviewed in (115)]. Standard-of-care therapy with pegylated interferon-α and ribavirin has only limited efficacy in eliminating the infection and is relatively toxic. There is thus an important need for new and potentially better therapeutic interventions (184). A greater understanding of the host factors involved in HCV replication may facilitate development of novel, host-specific therapies that are less likely to engender drug-resistance. One such host factor is miR-122, a well-conserved, highly abundant, liver-specific microRNA (miRNA) that facilitates HCV genome amplification by an uncertain mechanism that involves a direct interaction with positive-sense viral RNA (94, 95). Indeed, recent

studies have demonstrated a remarkable antiviral effect in chimpanzees following therapeutic silencing of miR-122 by administration of a locked nucleic acid (LNA) antisense oligonucleotide (112).

miRNAs are approximately 22 nucleotide-long, single-stranded RNAs of endogenous origin that post-transcriptionally regulate gene expression, typically by mediating mRNA degradation and/or translational blockade after binding to complementary sequences in the 3' nontranslated region (3'UTR) of the target mRNA (51). miR-122 is expressed at high abundance in adult human liver and also in Huh-7 cells, a human hepatoma cell line that is commonly used for propagation of HCV (28). miR-122 down-regulates the expression of cationic amino acid transporter (CAT-1) (28). It also modulates the expression of genes involved in hepatic lipid and cholesterol metabolism (47, 48, 109), processes intricately linked to HCV replication and infectious virus production (61, 222).

How miR-122 promotes the replication of HCV RNA is not understood. It binds to conserved sites within the 5'UTR of the HCV genome, which contains overlapping *cis*-acting signals involved in translation and RNA synthesis [reviewed in (115)]. Phylogenetic, biochemical and mutational analyses suggest that the 5'UTR is comprised of 4 highly conserved structural domains (78, 131). The extreme 5' 125 nucleotides, encompassing domains I and II, contain signals that are essential for RNA synthesis, with the remainder of the 5'UTR contributing in a less essential manner to the overall efficiency of RNA replication (59). These downstream 5'UTR sequences also contain an internal ribosomal entry site (IRES) that allows for cap-independent assembly of the 48S ribosomal complex on viral RNA with only a minimal requirement for canonical translation factors (130, 159). miR-122 interacts

with two well-conserved, tandem binding sites, each complementary to the "seed" sequence (nts 2-7) of miR-122 and located in close proximity to each other between stem-loops I and II of the 5'UTR (Fig. 2.1A) (94, 95). Mutational studies suggest that both sites are likely to be occupied by miR-122 in replicating viral RNAs (94). However, while both are involved in the promotion of genome amplification by miR-122, the relative contribution of each site to this process is unknown.

Although mutational analyses of the interactions of miR-122 with HCV RNA have shown their importance to viral RNA amplification, such studies have not been done in the context of systems producing fully infectious virus. Thus, although the ability of miR-122 to enhance the abundance of viral RNA in replicon systems is known to be largely if not entirely dependent upon its direct interaction with viral RNA (95), the same cannot be said for how miR-122 promotes the production of infectious virus. Previous studies do not exclude an additional indirect role for miR-122 in this process, as miR-122 could also regulate the expression of cellular genes required for viral entry, assembly or egress of infectious virus.

The mechanism by which the interaction of miR-122 with the HCV genome facilitates an increase in its abundance is also poorly understood and a subject of controversy. While Jopling et al. (94, 95) found no evidence that miR-122 specifically enhances HCV IRES-directed translation, Henke et al. (76) reported that it up-regulates the translation of short RNAs comprising a reporter protein sequence flanked by the viral UTRs. The relevance of these latter observations to the situation with the full-length viral genome is uncertain, however, as similar experiments with genome-length HCV RNA showed only minimal (<2-fold) up-regulation of translation by miR-122 (76). How much this translational effect might contribute to

the striking enhancement of genome amplification by miR-122 is unknown. Moreover, the need for direct binding of miR-122 to promote translation of the full-length viral genome, and the relative contributions of the two miR-122 binding sites within the HCV 5'UTR to viral translation, have yet to be determined.

We set out to study these issues using a laboratory strain of HCV (HJ3-5 virus) that replicates efficiently in cultured hepatoma cells (226) and is also infectious in the chimpanzee (M. Yi and S.M. Lemon, unpublished data). Our results show that the ability of miR-122 to promote the growth of infectious virus is dependent upon its direct interaction with both seed sequence-binding sites in the 5'UTR. However, binding to the 5' S1 site is more important for efficient replication than binding to the nearby S2 site. We also find that miR-122 positively regulates HCV translation, and that this is also dependent upon direct interactions of miR-122 with both the S1 and S2 sites.

MATERIALS AND METHODS

Cell cultures and virus

Huh-7, Huh-7.5 and FT3-7 cells (another clonal derivative of Huh7 cells that efficiently supports HCV replication) were maintained as described previously (226). Experiments with cell culture infectious virus were carried out with vH-NS2/NS3-J/Y361H/Q1251L (referred to here as HJ3-5) virus, an inter-genotypic chimeric virus in which sequence encoding core-NS2 of the genotype 1a H77c virus was placed within the background of the genotype 2a JFH-1 virus (226). Huh-7.5 cells (plated at

40% confluency 1-2 days previously) were infected with HJ3-5 virus at multiplicities of infection (m.o.i.) ranging from 1-2. Infectious virus yields were determined by a fluorescent focus assay, with results reported as focus-forming units (FFU)/ml, as described previously (228). For rescue of virus from in vitro transcribed RNAs, FT3-7 cells seeded into 6 well plates were transfected with 1.25 – 2.50 μg HJ3-5 RNA per well using the *Trans*-IT mRNA transfection reagent (Mirus Bio) for 6 hrs as per the manufacturer's protocol. After washing the cells with PBS, the cells were fed with fresh media, which was replaced at 24 hr intervals.

Plasmids and in vitro RNA transcription

HCV mutants were generated in the background of pHJ3-5 (226). miR-122-binding site and IRES subdomain mutants were constructed using an overlapping PCR strategy with primers containing the corresponding mutations. For translation studies, pHJ3-5 was modified using PCR-based mutagenesis to create an in-frame insertion of the *Renilla* luciferase (RLuc) sequence, fused to the foot-and-mouth disease virus (FMDV) 2A autoprotease, between the p7 and NS2 sequence of HJ3-5, similar to a luciferase reporter virus described by Jones et al. (91). HCV RNA was transcribed in vitro from plasmid DNA using reagents provided with the T7 MEGAscript kit (Applied Biosystems) and purified using the RNeasy Mini kit (Qiagen). The RNA products were analyzed by denaturing agarose gel electrophoresis to ensure their quality. A capped synthetic RNA transcript encoding firefly luciferase (FLuc) and containing a 30-nt long 3' poly(A) tail was produced by in vitro transcription from a PCR product containing a T7 promoter. FLuc RNA was

transfected along with HCV RNA as an internal control for transfection and translation.

Antibodies

Immunoblots were carried out using the following antibodies: mouse monoclonal antibody (mAb) to core protein (C7-50, Affinity BioReagents), rabbit polyclonal anti-NS5B (Cat # 266-A, Virogen), rabbit polyclonal anti-calnexin (Sigma) and mouse monoclonal anti-β-actin (AC-74, Sigma). Immunoblots in Fig. 2.1 and 2.2 were probed by horseradish peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (ECL-Plus kit, Amersham Pharmacia Biotech). IRDye 800CW goat anti-mouse and IRDye 680 goat anti-rabbit secondary antibodies (LI-COR Biosciences) were used to probe immunoblots in Fig. 2.5, followed by scanning with an Odyssey Infrared Imaging system (LI-COR Biosciences).

RNA oligonucleotides

RNA oligonucleotides and 2'-O-methylated oligonucleotides were synthesized by Dharmacon. The sequences for single-stranded RNA (ssRNA) oligonucleotides were: miR-122-ss (wild-type miR-122): 5'-UGG AGU GUG ACA AUG GUG UUU GU-3'; miR-122p34-ss: 5'-UGC UGU GUG ACA AUG GUG UUU GU-3'; miR-122p6-ss: 5'-UGG AGA GUG ACA AUG GUG UUU GU-3'; miR-122*-ss: 5'-AAA CGC CAU UAU CAC ACU AAA UA-3'; miR-124-ss: 5'-UUA AGG CAC GCG GUG AAU GCC A-3'; miR-124*-ss: 5'-CCG UGU UCA

CAG CGG ACC UUG A-3'; Anti-miR-122 (122-2'-O-Me): 5'-AGA CAC AAA CAC CAU UGU CAC ACU CCA CAG C-3'; and Anti-Random (Rand-2'-O-Me): 5'-CAC GUU AAA ACC AUA CGC ACU ACG AAA CCC C-3' (13). Mature miRNA duplexes were generated by annealing equimolar amounts of the ssRNA oligonucleotides as follows: wild-type miR-122, miR-122-ss and miR-122*-ss; miR-122p34, miR-122p34-ss and miR-122*; miR-122p6, miR-122p6-ss and miR-122*-ss; miR-124, miR-124-ss and miR-124*-ss. miRNAs were always transfected as duplexes, while the antisense Anti-miR-122 and Anti-Random were transfected as single-stranded oligonucleotides. A non-targeting pool of 4 siRNAs (ON-TARGETplus, Dharmacon), referred to here as Ctrl, has no known targets within mammalian genomes and was used as an additional negative control.

Northern blotting

Total RNA, isolated using RNeasy Mini Kit (Qiagen), was subjected to northern blotting with HCV- and β-actin-specific ^{32}P-labled RNA riboprobes (NorthernMax® Kit, Applied Biosystems). Briefly, 3-10 μg of total RNA was resolved on denaturing formaldehyde agarose (0.9%) gels followed by downward capillary transfer to a BrightStar-Plus® Nylon membrane (Applied Biosystems). Following UV cross-linking, membranes were hybridized with the riboprobes overnight at 68°C. The membranes were extensively washed and analyzed with a PhosphoImager (Storm 860, Molecular Dynamics). HCV RNA was detected with a riboprobe specific to the entire HCV 5'UTR.

miR-122 supplementation and HCV replication

FT3-7 cells in a 6-well culture plate were transfected with miRNA duplexes at 50 nM concentration using Lipofectamine™ 2000 (Invitrogen) as recommended by the manufacturer. At 24 hrs, the cells were re-transfected with in vitro transcribed HCV RNA (1.25 – 2.50 μg/well) for 6 hrs using the TransIT® mRNA transfection reagent (Mirus Bio) according to the manufacturer's protocol. The cells were transfected with miRNA once again 24 hrs later, fed with fresh medium every 24 hrs and supernatant fluid samples were collected on day 2 and 3 post-HCV RNA transfection for virus titration.

To assess the replication of HCV genomes expressing RLuc, FT3-7 cells transfected with miRNA duplexes the previous day (as described above, in 6-well plates) were cotransfected with HJ3-5/RLuc2A HCV RNA (1.25 μg/well) and FLuc mRNA (0.25 μg/well) and supplemented again with miRNAs 24 hrs later. RLuc and FLuc assays were carried out on cell lysates prepared at various points in time post-HCV RNA transfection using the Dual Luciferase assay kit (Promega). RLuc activity was normalized to FLuc activity and results presented as fold-increase over the RLuc/Fluc ratio at 8 hrs.

miR-122 supplementation and HCV translation

Huh-7.5 cells seeded into 6-well plates were transfected with RNA oligonucleotides at a 50 nM concentration as described above. Twenty hrs later, replication-deficient HJ3-5/RLuc2A-GND mutant RNA (1.25 μg/well) was cotransfected with capped and polyadenylated FLuc mRNA (0.25 μg/well) using the TransIT® mRNA transfection reagent (Mirus Bio). Cells were harvested for dual

luciferase assays, in duplicate, at the indicated points in time. Results are expressed as the ratio of RLuc/FLuc activity at each time point.

RESULTS

miR-122 enhances production of infectious virus through direct interactions with positive-sense RNA

Randall et al. (166) reported that silencing miR-122 by transfection of an antisense oligonucleotide reduces the yields of infectious virus. Consistent with this, we observed reduced expression of viral proteins (core and NS5B) in cells transfected with anti-miR-122, a 2'-O-methylated antisense oligonucleotide capable of sequestering and causing functional knockdown of endogenous miR-122, after infection with a chimeric genotype 1a/2a virus, HJ3-5 (Fig. 2.1B). We also noted a 65% reduction in foci of infected cells when we infected anti-miR-122-transfected cells with HJ3-5 virus at a low multiplicity of infection (Fig. 2.1C). Importantly, we did not note any enhancement in protein expression or infectious focus formation when cells were supplemented with additional synthetic miR-122, suggesting that the normal endogenous abundance of miR-122 is not always limiting for the replication of infectious virus in these cells (a modest increase in infectious virus yield was observed following miR-122 supplementation in subsequent experiments, as shown in

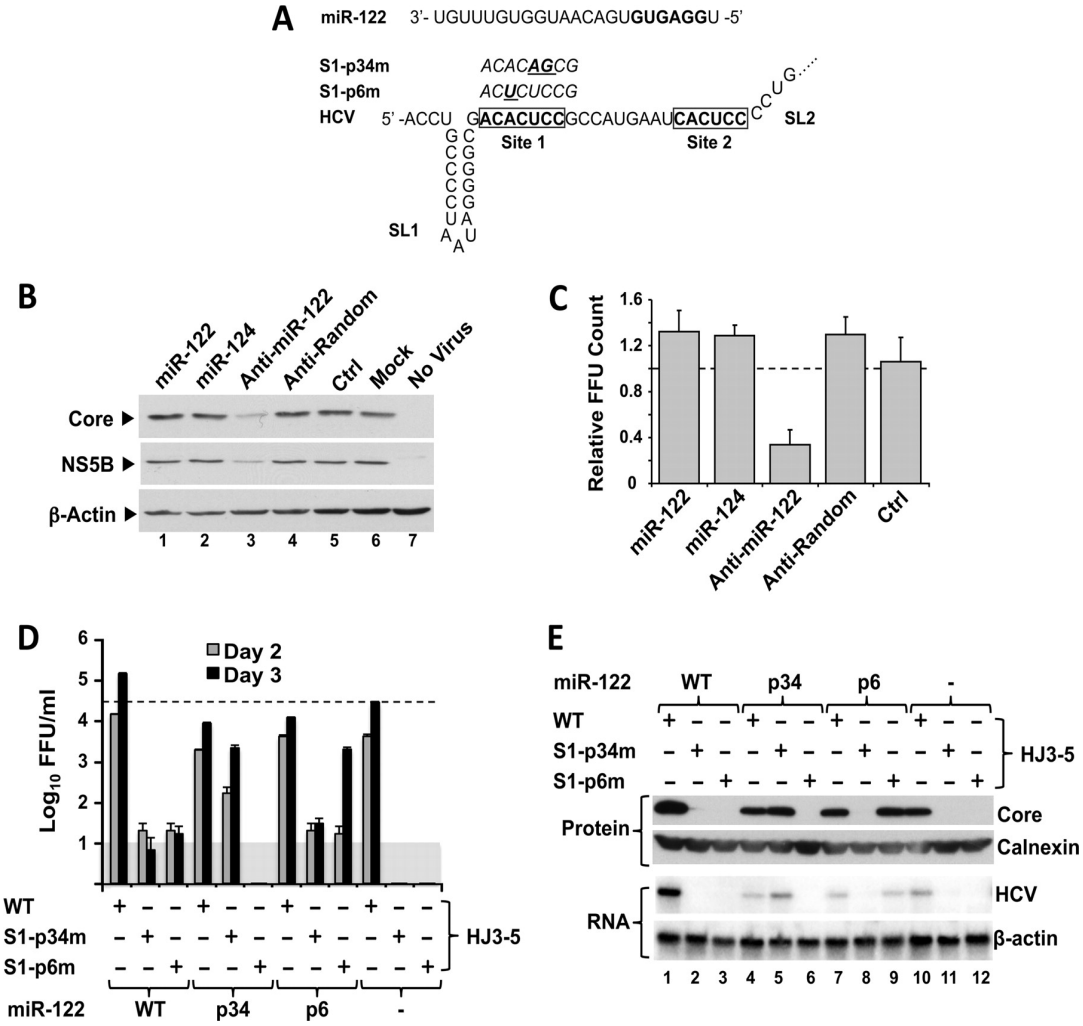

Figure 2.1. The replication of infectious HCV is dependent on direct interaction of miR-122 with the RNA genome. (A) Schematic representation of the two miR-122-binding sites, S1 and S2, located between stem-loop (SL) I and II in the 5'UTR of the HJ3-5 genome. At the top is shown the sequence of miR-122. The miR-122 seed sequence and miR-122 complementary sequences in HCV RNA (boxed) are highlighted in bold. The sequences of the S1-p34m and S1-p6m mutations are shown in italics with base substitutions underlined. (B) Huh-7.5 cells were transfected with 50 nM of the indicated RNA oligonucleotides, and 24 hrs later infected with HJ3-5 virus, an intergenotypic chimeric HCV, at an m.o.i. of 1. Seventy-two hrs post-infection, cell lysates were prepared and subjected to immunoblotting using β-actin, HCV core and NS5B-specific antibodies. (C) Huh-7.5 cells in 8-well chamber slides were transfected with the indicated RNA oligonucleotides at 50 nM concentrations, and 24 hrs later infected with 100-120 FFU of chimeric HJ3-5 virus. Seventy-two hrs later, cells were labeled for HCV core antigen, and individual foci of infected cells enumerated by immunofluorescence microscopy. Results shown represent the means from three independent

experiments, each performed in duplicate, ± S.D. (D) miR-122 regulates infectious virus production by HCV RNA-transfected cells. FT3-7 cells were transfected with wild-type (WT), p34, or p6 mutant forms of miR-122, or mock treated. Twenty-four hrs later, cells were transfected with wild-type (WT) or mutant HJ3-5 HCV RNAs, followed by another transfection of miR-122 and related mutants at 48 hrs. Supernatant fluids collected 2 and 3 days after HCV RNA transfection were assayed for infectious virus by FFU assay on naïve Huh-7.5 cells (average ± SD, n = 2). (E) Immunoblots (top panel) and northern blots (bottom panel) of extracts from the cells in panel D prepared 3 days after HCV RNA transfection.

Fig. 2.1D and described in the text below). We noted no differences in HJ3-5 replication in cells transfected with an unrelated miRNA, miR-124, a randomized antisense oligonucleotide, Anti-Random, or Ctrl siRNA (a pool of 4 non-targeting siRNAs with no known targets in mammalian genomes). Since miR-122 could potentially influence the expression of host cell genes required for virus entry, assembly or release, we studied the impact of miR-122 on the production of infectious virus by mutated HJ3-5 RNAs containing point mutations in the most 5' miR-122-binding site: S1-p34m and S1-p6m (Fig. 2.1A). RNA was transcribed in vitro from these constructs, and transfected into cells in parallel with wild-type HJ3-5 RNA and a replication-defective mutant with a GND substitution in the NS5B polymerase. Like the GND mutant (data not shown), no infectious virus could be detected in supernatant fluids from cells transfected with the S1-p34m or S1-p6m RNAs, while HJ3-5 RNA-transfected cells produced ~10^4 FFU/ml (Fig. 2.1D). In addition, no core protein expression was evident 3 days after transfection of either the S1-p34m or S1-p6m mutant, nor was any viral RNA detected by northern blotting (Fig. 2.1E). These data indicate that the integrity of the 5' S1 miR-122-binding site is critically important for replication of infectious HCV.

To confirm that mutations in the S1 site impair HCV production by ablating the binding of miR-122, we attempted to rescue replication of the mutants by prior

transfection of the cells with miR-122 mutants, p34 and p6, having sequences complementary to the modified binding site sequences in the S1-p34m and S1-p6m viral genomes, respectively. In both cases, transfection of the related miR-122 mutant specifically enhanced infectious virus yields over 100-fold by 3 days after transfection of the HCV RNA (Fig. 2.1D). Since the virus titrations were carried out in naïve Huh-7.5 cells that were not supplemented with the mutated miR-122s, the results shown in Fig. 2.1D are likely to underestimate the absolute level of production of infectious virus by the mutant RNAs (see below). Consistent with this, the size of foci of infected cells was significantly reduced for the mutant viruses, and infected cells also stained less brightly due to a lesser abundance of core protein when compared with cells infected by the wild-type virus (data not shown). Transfection of the mutant miR-122s also rescued core protein expression by the mutant RNAs and increased viral RNA abundance (Fig. 2.1E).

In these experiments, prior supplementation of cells with additional wild-type miR-122 increased the yield of infectious wild-type virus by about 4-fold, and also resulted in the production of minimal but detectable quantities of infectious p6m and p34m virus (Fig. 2.1D). Similarly, supplementation with the miR-122p6 mutant resulted in the production of small amounts of infectious S1-p34m virus. Importantly, however, viral RNA, protein expression and infectious virus yields from the mutated genomes were restored to levels approaching that of the wild-type virus after supplementation with the corresponding mutated forms of miR-122 (Fig. 2.1D-E). These results indicate that the production of infectious virus is dependent on direct interactions of miR-122 with the HCV genome, most likely due to the positive influence of miR-122 on viral genome amplification (95).

Relative importance of the two 5'UTR miR-122-binding sites in HCV replication

Jopling et al. (94) recently reported that both miR-122-binding sites (S1 and S2) are occupied by miR-122 in replicating viral RNAs, and suggested that both sites are equally important for viral RNA replication. To assess the importance of the S2 site in the viral life cycle, we created a series of HJ3-5 virus mutants with single base substitutions (p6m) in either or both miR-122 binding sites (Fig. 2.2A), and determined their ability to replicate following transfection as RNA into cells supplemented with wild-type miR-122, the complementary miR-122p6 mutant, or an unrelated miRNA, miR-124. Replication of the S1-p6m and S1-S2-p6m double mutant was severely impaired in either miR-122 or miR-124-supplemented cells, as no viral RNA or core protein could be detected by 3 days after transfection of the viral RNA (Fig. 2.2B). However, we did detect a small amount of infectious S1-p6m virus when we inoculated supernatant fluids from miR-122-supplemented cultures onto naïve Huh-7.5 cells (Fig. 2.2C). This confirms earlier results shown in Fig. 2.1D, and indicates that the integrity of the conserved S1 sequence is not absolutely essential for virus replication. To more accurately quantify the infectious virus produced from the mutant genomes, we supplemented Huh-7.5 cells used in the infectious virus titration (FFU) assay with the mutant miR-122p6. This resulted in nearly a 10-fold increase in the titer of the infectious S1-p6 virus detected in harvests from the miR-122-supplemented cells (to just over 10^2 FFU/ml), and also revealed a small quantity of infectious virus produced in cells supplemented with the control miR-124 (Fig. 2.2C). However, even under these conditions, we were unable to detect infectious virus produced by the double-mutant, S1-S2-p6m.

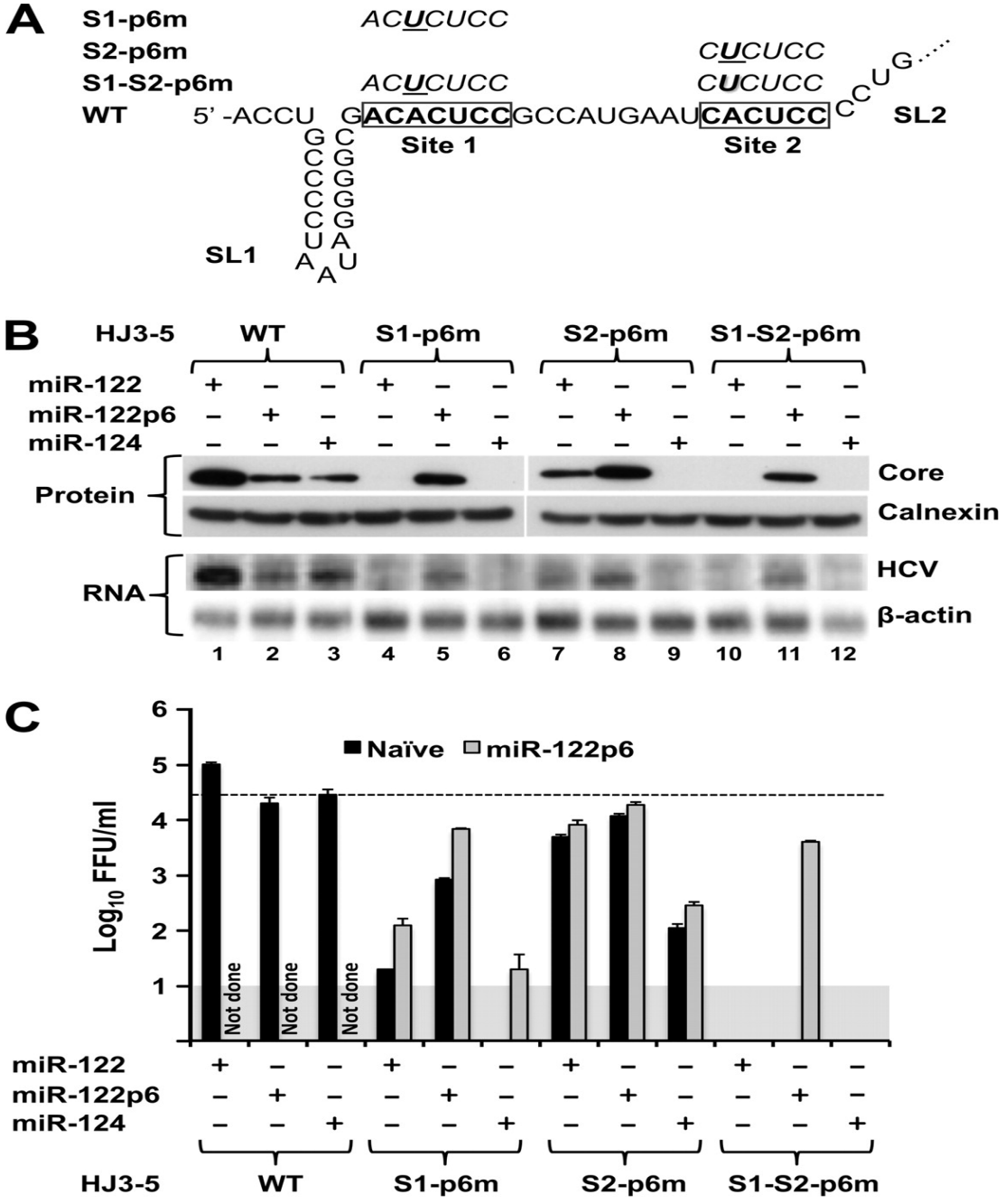

Figure. 2.2. Relative importance of the two 5'UTR miR-122-binding sites for production of infectious virus. (A) Schematic representation of the S1 and S2 miR-122 binding site mutant sequences. (B) FT3-7 cells were transfected with the indicated miRNAs, re-transfected with HJ3-5 RNAs 24 hrs later, and transfected once more with miRNAs 24 hrs after that. Samples prepared 3 days post-HCV RNA transfection were subjected to immunoblot analysis of HCV

core protein with calnexin run in parallel as a loading control (top panel), and northern blot for HCV RNA using b-actin mRNA as a loading control (bottom panel). (C) Infectious virus yields 3 days after HCV RNA transfection of the cells in panel B, as quantified by a fluorescent infectious focus assay using naïve or miR-122p6-supplemented Huh-7.5 cells transfected with miR-122p6 at 50 nM concentration 24 hrs prior to sample inoculation. Data shown are from a representative experiment.

In sharp contrast, viral RNA containing a similar one base substitution in the downstream miR-122 binding site (S2-p6m) replicated surprisingly well, producing detectable core protein and viral RNA (Fig. 2.2B) and infectious virus yields almost 1000-fold greater than the S1-p6m mutant in cells supplemented with wild-type miR-122 (Fig. 2.2C). Unlike the S1-p6m mutant, infectious S2-p6m virus was readily detected in supernatant fluids from cells supplemented with the control miR-124, even when the indicator cells used in the infectivity titration were not supplemented with the mutant miRNA, miR-122p6. These results demonstrate that the downstream S2 site, while important for viral replication, is significantly less essential than the upstream S1 miR-122 binding site. With the double mutant, S1-S2-p6m, core protein expression, viral RNA (Fig. 2.2B), and production of infectious virus (Fig. 2.2C) could only be demonstrated when the RNA was transfected into cells supplemented with the mutant miR-122-p6 (and infectious virus yields determined in infectivity assays using similarly supplemented indicator cells). Taken in aggregate, these results indicate that at least one binding site for miR-122 (or a mutated form of miR-122 if expressed in the cell) must exist for virus replication to occur, and that to some extent the two sites function redundantly albeit at lower efficiency when one or the other is mutated. A loss of the S2 site is readily compensated by supplementation of cells with additional miR-122, while this is not the case with the most 5' S1 site on which the replication of the virus is more dependent.

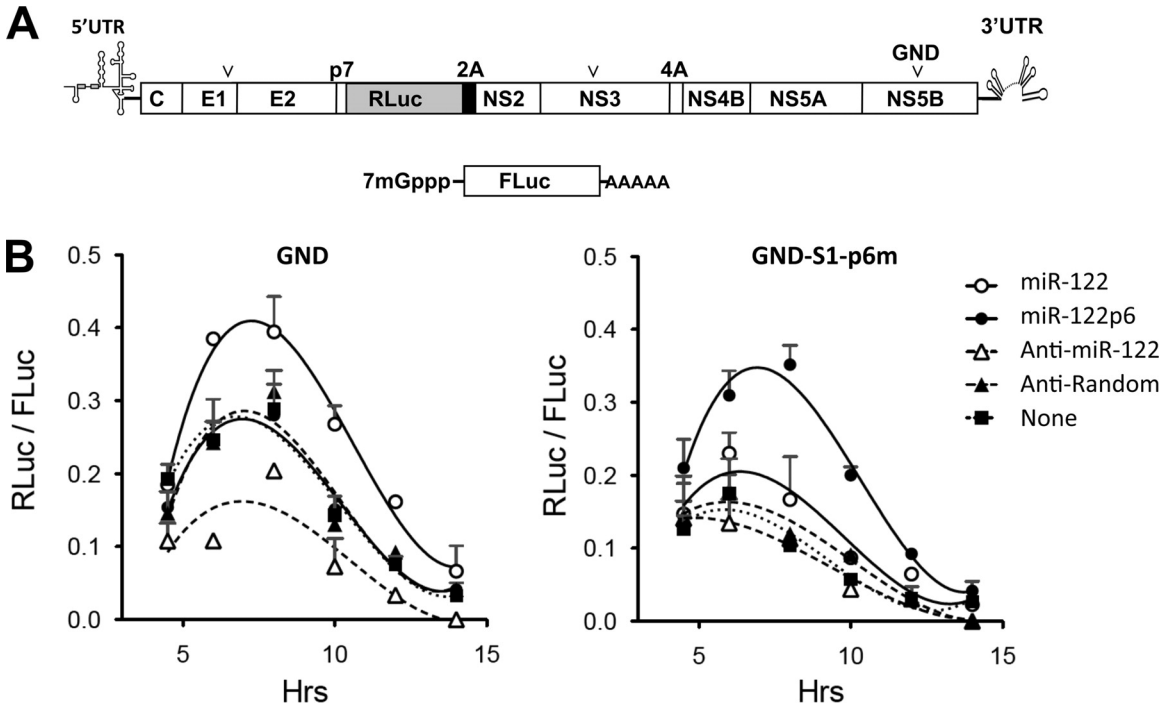

Figure 2.3. Binding of miR-122 to the S1 site promotes HCV translation. (A) Schematic representation of HJ3-5/RLuc2A-GND (referred to as GND in the text) that contains an in-frame insertion of the RLuc luciferase sequence fused to FMDV 2A (RLuc2A) between p7 and NS2 coding regions of HJ3-5, and a replication-lethal Asn substitution within the active site of the NS5B RNA-dependent RNA polymerase. (B) Duplicate cultures of Huh-7.5 cells in a 6-well plate were transfected with miR-122 to supplement endogenous miR-122 levels, or with a 2'-O-methyl antisense RNA, anti-miR-122, to functionally sequester endogenous miR-122. Twenty hours later, GND (left panel) or GND-S1-p6m (right panel) HCV RNA (1.25 µg/well) was transfected together with a capped and polyadenylated FLuc mRNA (0.25 µg/well). Dual luciferase assays were carried out on lysates prepared at the intervals noted following HCV RNA transfection, with results expressed as the mean RLuc/FLuc ratio (± S.D.) at each time point. Solid and dashed lines are best-fit third-order polynomial plots (R^2 between 0.78 and 0.99). The data shown are representative of multiple experiments.

Binding of miR-122 to the S1 site promotes HCV translation

Significant controversy exists concerning the potential effect of miR-122 on HCV translation. While Jopling et al. (94, 95) could not demonstrate any increase in HCV IRES-directed translation due to miR-122, Henke et al. (76) reported striking up-regulation of the translational activity of short reporter RNAs by miR-122, but

noted only small increases in translation of viral genomes and did not link the latter to a requirement for miR-122 binding. To better characterize the impact of miR-122 on translation of the viral genome, we introduced sequence encoding *Renilla* luciferase (RLuc, fused at its 3' end to the FMDV 2A autoprotease) between the p7 and NS2 sequences of HJ3-5 virus (Fig. 2.3A). To knock-out the capacity of this RNA to replicate, we engineered a GND mutation within the active site GDD motif of the NS5B polymerase to create HJ3-5-RLuc2A-GND (referred to here subsequently as "GND" for simplicity). We transfected this modified virus RNA into cells along with a capped and polyadenylated control mRNA encoding firefly luciferase (FLuc), and determined the ratio of RLuc to FLuc activities expressed at timed intervals as a measure of IRES-directed translation. As shown in Fig. 2.3B (left panel), IRES activity peaked between 6-8 hrs after transfection in the absence of prior supplementation of the cells with miR-122, and was increased by 80-90% if the cells were supplemented with miR-122 prior to transfection of the GND RNA. Conversely, sequestration of miR-122 by prior transfection of an anti-sense oligonucleotide reduced HCV IRES-directed translation by 60-80%. However, prior transfection of the cells with the mutant miR-122p6 or an antisense oligonucleotide targeting random sequence had no effect on the efficiency of HCV translation (Fig. 2.3B, left panel). These data confirm the results of Henke et al. (76), and suggest that miR-122 does indeed have a moderate positive effect on HCV translation.

To determine whether the promotion of HCV translation by miR-122 requires a direct interaction with the viral genome, we introduced an S1-p6m mutation (single base substitution, see Fig. 2.2A) into the GND construct shown in Fig. 2.3A and repeated these experiments as described above. In the absence of prior miR-122

supplementation, this GND-S1-6pm mutant RNA demonstrated reduced translational activity compared with GND RNA (Fig. 2.3B, compare right vs. left panel). Peak expression of RLuc from the GND-S1-p6m mutant also occurred somewhat earlier than from the related GND RNA, raising the possibility that the transfected mutant RNA might be less stable in the cells. Remarkably, however, the translational activity of the GND-S1-p6M mutant was significantly enhanced by prior supplementation with the related, mutant miRNA, miR-122p6, and only minimally influenced by supplementation with wild-type miR-122. Unlike the GND RNA containing a wild-type S1 site (Fig. 2.3B, left panel), sequestration of wild-type miR-122 also had only a minimal effect on translation of the GND-S1-p6m mutant. These results indicate that the ability of miR-122 to up-regulate the translation of HCV RNA is dependent on its interaction with the S1 miR-122 binding site within the 5'UTR. Interestingly, peak RLuc expression from the GND-S1-p6m mutant was delayed in cells supplemented with miR-122p6 (7-8 hrs vs. 5-6 hrs in non-supplemented cells, Fig. 2.3B, right panel), suggesting possible stabilization of the transfected RNA. We investigated this directly by monitoring the abundance of the transfected GND RNA by an RNase protection assay, but did not observe any differences related to miR-122 supplementation or sequestration (data not shown).

Contribution of S1 vs. S2 binding sites to miR-122 promotion of viral translation

To determine the relative importance of the two seed sequence-binding sites in the 5'UTR for miR-122 regulation of HCV translation, we created additional replication-deficient GND mutants with p6m mutations in either the S2 or both S1

and S2 binding sites (see Fig. 2.2A). We then transfected these along with the GND RNA (containing wild-type S1 and S2 sites) and the GND-S1-p6m mutant into cells supplemented with wild-type miR-122, miR-122p6 or miR-124. The translation of all three mutants was substantially reduced compared to GND RNA in miR-124-supplemented cells, and increased (but not fully restored to the level of the parental GND construct) in cells supplemented with the miR-122p6 mutant (Fig. 2.4A). In contrast to the marked differences in virus yield between HJ3-5 viruses containing mutations in S1 and S2 (Fig. 2.2C), the differences observed in the translational activities of GND-S1-p6m and GND-S2-p6m were minimal. The GND-S1-p6m mutant was only slightly less active than GND-S2-p6m in cells supplemented with wild-type miR-122, while the reverse was apparent in cells supplemented with miR-124. The double-mutant, GND-S1-S2-p6m, was only slightly more impaired in translation than either of the single mutants (Fig. 2.4A). These experiments were done using the TransIT mRNA transfection reagent (Mirus Bio), but similar results were obtained with electroporation of the RNAs (data not shown). We conclude that the two miR-122 seed sequence-binding sites function with roughly equal importance in miR-122 regulation of HCV translation.

We also assessed the impact of the S1 and S2 mutations on the replication capacity of HJ3-5-RLuc2A constructs lacking the GND mutation. While the insertion of the reporter protein sequence in HJ3-5-RLuc2A impaired replication of the viral RNA overall, exponential increases in RLuc activity were nonetheless evident by 76 hrs after RNA transfection and were significantly stimulated by supplementation of the cells with miR-122 (Fig. 2.4B, left panel). No such late increase in RLuc expression was observed following transfection of the cells with HJ3-5-RLuc-S1-p6m

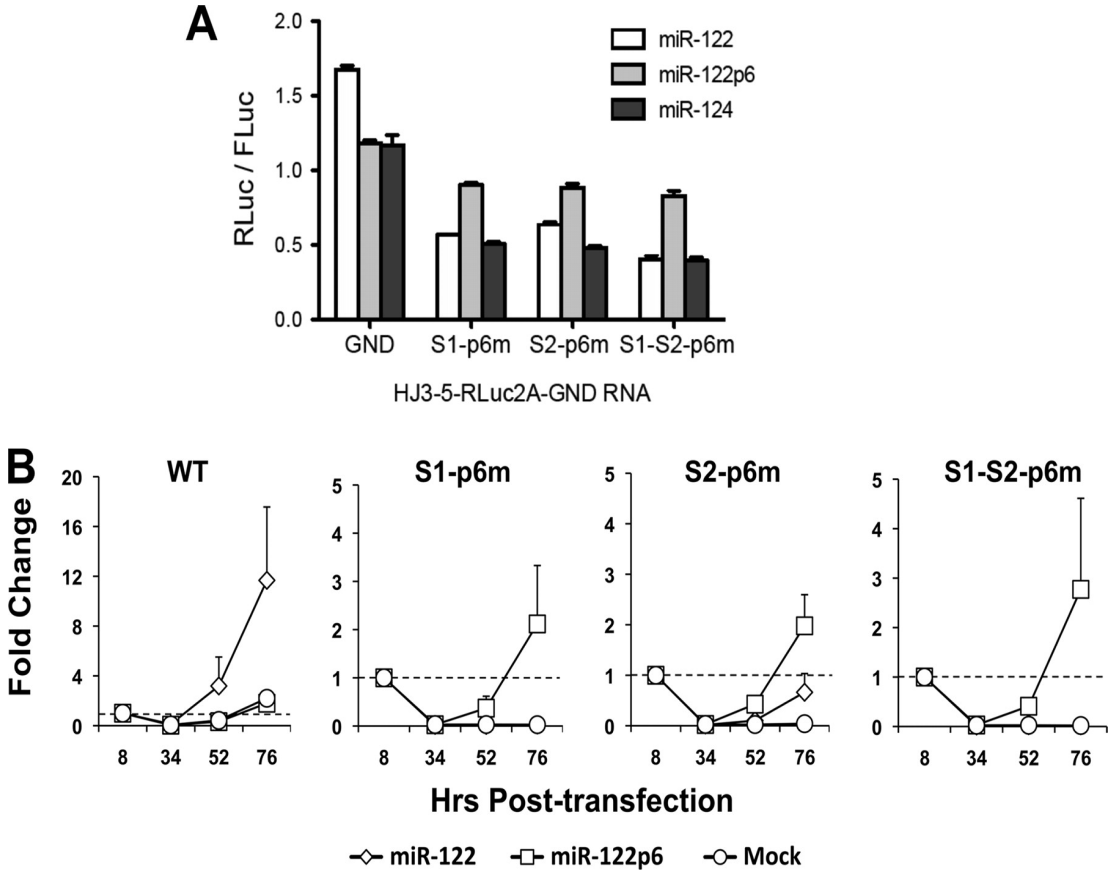

Figure 2.4. Contribution of S1 vs. S2 binding sites to miR-122 promotion of viral translation vs. RNA replication. (A) Huh-7.5 cells were transfected with miR-122, miR-122p6 or miR-124 and co-transfected 20 hrs later with the indicated GND (see Fig. 2.3A) and capped control FLuc RNAs. Results shown represent dual luciferase reporter assays of cell lysates prepared 8 hrs after HCV RNA transfection, presented as the ratio of RLuc/FLuc activity (mean ± S.D.). Similar results were obtained with 6 hr lysates. The data shown are representative of multiple experiments. (B) FT3-7 cells were transfected with miR-122, miR-122p6 or mock-treated, then re-transfected 24 hr later with HJ3-5-RLuc2A, or related -S1-p6m, -S2-p6m or -S1-S2-p6m mutant RNAs together with the capped control FLuc RNA. The cells were re-transfected with the miRNAs 24 hrs later. Dual luciferase assays were carried out on lysates prepared 8, 34, 52 and 76 hrs after transfection of the HCV RNA. RLuc results were normalized to the 8 hr FLuc value and shown as fold increase over RLuc activity at 8 hrs (mean ± SD, n = 3).

and HJ3-5-RLuc-S1-S2-p6m unless the cells were first supplemented with the mutant miR-122p6 (Fig. 2.4B). In contrast, the S2-p6m mutant remained partially responsive to the wild-type miR-122, consistent with the dominant role of the S1 binding site in miR-122 regulation of HCV replication shown in Fig. 2.3.

Is the influence of miR-122 on IRES-directed translation sufficient to account for its regulation of HCV replication?

Although the data shown in Fig. 2.4 demonstrate that miR-122 promotes translation directed by the HCV IRES, and that this is dependent upon its direct interaction with viral RNA, they leave unanswered the question of whether miR-122 positively regulates any other step in the viral life cycle. We asked if the 65-75% reduction in translation that we observed with the GND-S1-p6m and GND-S1-S2-p6m mutants (Fig. 2.4A) was sufficient to account for the 100- to 1000-fold decrease in yields of infectious virus when identical mutations were made in infectious RNAs (Fig. 2.2C). To answer this question, we generated a series of HJ3-5/RLuc2A constructs containing mutations in the IRES with a range of defects in translational activity. Domain III forms the core of the IRES and interacts with the 40S ribosomal subunit as well as eIF3 during internal entry of the ribosome on the RNA (130). Point mutations in the hairpin loop (–U264UGGGU269–) of stem-loop IIId impair 40S subunit binding and reduce IRES activity to various degrees (96, 99, 100). Since these mutations are located at a substantial distance downstream of cis-acting signals in the 5'UTR required for RNA replication (59), we reasoned that any effect

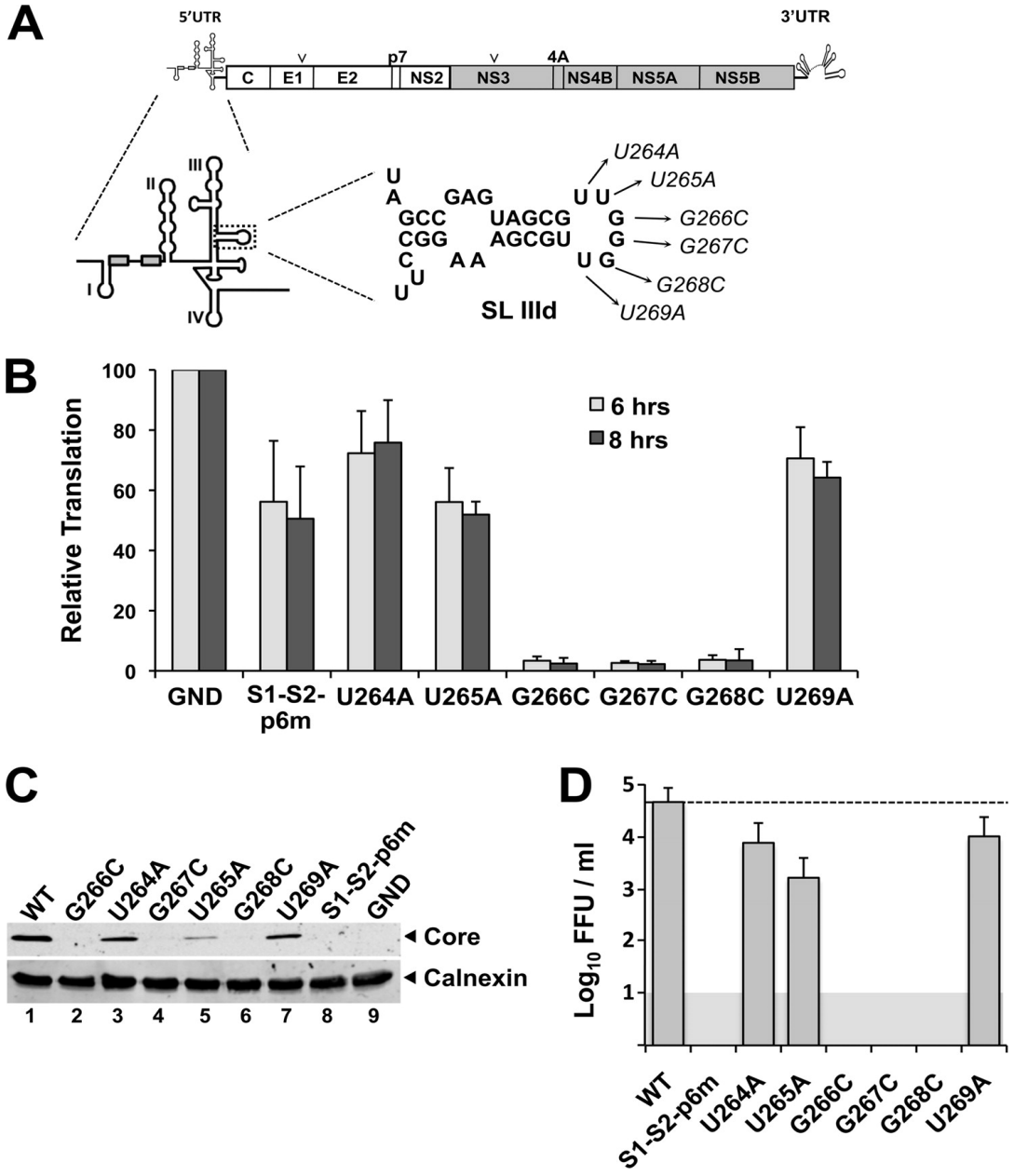

Figure 2.5. Comparison of infectious virus yields from the double binding site mutant and IRES mutants with quantitatively similar and different defects in translation. (A) Schematic representation of IRES mutations constructed in stem-loop (SL) IIId of the HJ3-5 5'UTR. The sequence of stem-loop IIId is depicted, with nucleotide positions numbered according to the prototype genotype 1a, H77 HCV sequence (GenBank Acc. No. NC_004102). (B) Translational activities of IRES mutants constructed in HJ3-5-RLuc2A-GND. Cells were co-transfected with GND or the indicated mutant GND RNAs with the capped, polyadenylated FLuc mRNA as an internal control for transfection and cellular translation efficiency. Dual

luciferase reporter assays were carried out at 6 or 8 hrs following transfection, with results expressed as the RLuc/FLuc ratio. The data shown represent the mean ± S.D. from five independent experiments. (C) Immunoblot assays for core protein expressed in FT3-7 cells 3 days after transfection with wild-type (WT) HJ3-5 RNA, or related S1-S2-p6m (double miR-122-binding site) and IRES mutants in the HJ3-5 background. (D) Infectious virus yield in supernatant fluids from HJ3-5 RNA-transfected cell cultures described in panel C. Infectivity titrations were done on fluids collected 3 days post-transfection, and are shown as mean ± range (n = 2).

they have on infectious virus yield would likely be due to reductions in translation. We constructed 6 mutants with nucleotide substitutions at highly conserved positions within the IIId loop sequence in HJ3-5-RLuc2A-GND (Fig. 2.5A), and assessed the translational activity of in vitro transcribed RNAs in comparison with the wild-type and the miR-122-binding site double mutant, GND-S1-S2-p6m, in transfected Huh-7.5 cells. As reported previously (96, 99), the GND-G266C, -G267C and -G268C mutants (numbered according to H77 sequence, GenBank NC_004102) were severely impaired for translation, producing RLuc/Fluc activities less than 5% that of the GND RNA (Fig. 2.5B). On the other hand, translation was only moderately affected in GND-U264A (75-80% of wild-type), -U265A (55-60%) and -U269A (70-75%) mutations. Importantly, the translational activities of GND-U265A and the double binding site mutant, GND-S1-S2-p6m, were comparable (approximately 55-60% that of the wild-type RNA in this series of experiments).

Next, we compared the effect of these IRES mutations with that of the S1-S2-p6m mutation on replication, when placed in the background of the HJ3-5 virus genome. When transfected into FT7-3 cells, wild-type HJ3-5 RNA replicated well as evidenced by core protein expression and virus yields of 4 x 10^4 FFU/ml by 72 hrs after transfection (Fig. 2.5C and 2.5D). In contrast, the three IRES mutants with

severe defects in translation (HJ3-5-G266C, -G267C and -G268C) produced neither detectable core protein (Fig. 2.5C) nor infectious virus (Fig. 2.5D). HJ3-5 RNA containing the U264A and U269A mutations, which caused only a 25-30% decrease in translation activity (Fig. 2.5B), produced minimally less core protein (Fig. 2.5C) and only 4- to 6-fold less infectious virus than the wild-type HJ3-5 RNA (Fig. 2.5D). Notably, the U265A mutation, which caused a reduction in translation comparable to that of the S1-S2-p6m mutation, resulted in a barely detectable abundance of core protein in transfected cells, and a reduction in infectious virus yields to approximately 28-fold less than wild-type RNA. These data thus indicate that a 40-45% decrease in translation is amplified into a much larger difference in infectious virus yield over the multiple rounds of replication occurring during the 72 hr period between RNA transfection and virus harvest in these experiments. Nonetheless, the S1-S2-p6m mutant demonstrated at least a 100-fold greater replication defect than U265A, generating neither detectable core protein nor any infectious virus. This was reproducible in multiple experiments, even when the yield of infectious virus was determined in cells supplemented with the miR-122p6 mutant (Fig. 2.2C).

We conclude from these experiments that the 2- to 3-fold decrement in IRES-directed translation resulting from loss of miR-122 binding in the S1-S2-p6m mutant (Fig. 2.3) contributes significantly to the severe defect in replication of this mutant. However, since the U265A mutant demonstrates a comparable defect in translation yet is still capable of producing infectious virus (up to 1500 FFU/ml in the experiment shown in Fig. 2.5D), miR-122 is likely to be essential for some other aspect of HCV genome amplification, either new RNA synthesis or the stability of viral RNA.

DISCUSSION

miR-122 is an evolutionarily conserved miRNA that is expressed at high abundance in adult human hepatocytes and in Huh-7 human hepatoma cells that are permissive for HCV replication. It is a key regulator of lipid and cholesterol biosynthesis in the liver (47, 48, 109). Interestingly, the HCV life cycle is also intimately linked to lipid and cholesterol metabolism, as products of the cholesterol/lipid biosynthetic pathway are essential host factors for HCV replication (210, 222). Cholesterol and lipoproteins are also integral components of virion structure and may play key roles in viral entry (6, 97, 149), while the apolipoprotein secretory pathway is important for egress of virus from infected cells (61). Despite these multiple associations, recent studies indicate that it is possible to uncouple the effects of miR-122 on cholesterol biosynthesis and replication of HCV RNA (153).

The genomic RNA of HCV contains 3 highly conserved, potential miR-122-binding sites that are complementary to the miR-122 seed sequence. These include the 5' proximal S1 site, located about 22 nts from the 5' end of the genomic RNA, and the nearby S2 site located only 16 nts downstream (Fig. 2.2A). miR-122 facilitates an increase in the abundance of HCV RNA by directly interacting with these tandem S1 and S2 binding sites (94, 95). Both of these sites in the 5'UTR are occupied by miR-122 in replicating RNAs (94). A third potential seed sequence-binding site exists within the 3'UTR, but it has yet to be linked functionally to miR-122. While Jopling et al. (95) first demonstrated that miR-122 was essential for amplification of HCV RNA in cell culture, Randall et al. (166) confirmed that miR-122 is also required for efficient viral replication as evidenced by a nearly 2-fold reduction in viral RNA levels and infectious virus production following transfection

of a miR-122-specific antisense 2'-O-methyl oligonucleotide. Our initial experiments confirmed this finding, demonstrating a 65% decrease in infectious focus formation in similarly treated cells (Fig. 2.1C). The therapeutic potential of silencing miR-122 has also been demonstrated recently in HCV-infected chimpanzees (112). However, the mechanisms underlying the dependence of HCV replication on miR-122 remain incompletely understood.

To determine whether direct interactions between miR-122 and the HCV 5'UTR are responsible for its positive effect on infectious virus production, we carried out a mutational analysis of the S1 and S2 binding sites in an infectious molecular clone. Mutations in the S1 or S2 binding sites, either singly or together, severely hampered the rescue of virus from this clone, reducing infectious virus yields by as much as 10^4 and suggesting that replication of infectious virus is highly dependent on direct interactions of the viral RNA with miR-122 (Fig. 2.1D and 2.2C). This was confirmed by demonstrating that the production of infectious virus by these mutants could be restored almost to the level of the parental virus by supplementation of cells with the complementary mutant forms of miR-122 (Figs. 2.1D and 2.2C). Thus most, if not all, of the effects of miR-122 on infectious virus production appear to be mediated via direct interactions of miR-122 with the S1 and S2 sites in the viral 5'UTR. While these data are not surprising, they fill an important gap in our understanding of the mechanism by which miR-122 facilitates growth of the virus.

Recent work by Jopling et al. (94) suggests that both miR-122 binding sites in the 5'UTR are important for the ability of miR-122 to upregulate RNA abundance. Our data, derived from an infectious virus system, confirm roles for both sites, but indicate that the most 5' miR-122 binding site plays a dominant role in replication.

Viral RNA with a nucleotide substitution in the S2 binding site (HJ3-5-S2-p6m) produced 10-fold more infectious virus than a mutant with a similarly altered S1 binding site (HJ3-5-S1-p6m) (Fig. 2.2C). Moreover, supplementation of cells with excess miR-122 drove the production of infectious virus by the S2 mutant almost to the level observed with the parental virus, and 100-fold more than that observed with the S1 mutant under similar conditions. It is difficult to predict whether there are differences in the affinity of the two sites for miR-122, but it seems unlikely that the S2 site would have greater affinity than S1 since the miR-122-complementary sequence at S2 is one nucleotide shorter (6 nts) than at S1 (7 nts) (Fig. 2.2A). Our data thus suggest that the S1 site plays a dominant role in HCV replication, and that high miR-122 occupancy at S1 may be capable of driving RNA replication and virus production despite loss of miR-122 binding at S2. The absence of any virus production from the double S1-S2-p6m mutant, even in cells supplemented with excess wild-type miR-122 (Fig. 2.2C), argues against the possibility that miR-122 might be able to exert a functional effect by binding with low affinity to either the mutated S1 or S2 sites.

Substantial controversy exists concerning the impact of miR-122 on translation directed by the HCV IRES. Jopling et al. (95) found that protein expression from monocistronic, full-length viral RNAs containing a mutation in the S1 site that ablated the miR-122 effect on RNA abundance was not measurably reduced from that produced by wild-type RNA in transfected cells. Other studies using various reporter RNAs also failed to demonstrate that miR-122 enhances HCV IRES-directed translation or the stability of HCV RNA (94, 95). Nonetheless, Henke et al. (76) reported that miR-122 is capable of modulating the translation of short

reporter RNAs containing both viral UTRs by as much as 3-4-fold from the norm. Nonetheless, miR-122 supplementation or sequestration was able to modulate the translation of a full-length, replication-defective RNA only by about 50% (76). Moreover, Henke and colleagues did not determine whether this minor change in translational activity was dependent upon direct interactions of miR-122 with the full-length viral genome. To resolve this controversy, we studied the role of miR-122 binding in translation of a monocistronic, full-length infectious RNA in which sequence encoding a reporter protein (RLuc) had been introduced into the polyprotein coding region. We ablated the ability of this RNA to replicate by introducing a lethal mutation into the NS5B polymerase and, as an internal control for transfection and cellular translation, co-transfected cells with a capped and polyadenylated mRNA expressing a second reporter (FLuc). Our results demonstrate that miR-122 does indeed positively regulate HCV translation by up to 2-fold, and that this is dependent upon the ability of miR-122 to bind to both the S1 and S2 sites which lie upstream of the IRES within the 5'UTR (Fig. 2.3B and 2.4A). Importantly, RNase protection assays did not demonstrate changes in the stability of the RNA in these experiments. Translation of a mutated viral RNA with nucleotide substitutions in both the S1 and S2 sites was partially restored by supplementing cells with a cognate miR-122 mutant (Fig. 2.4A). However, in contrast to the dominant role played by S1 in miR-122 regulation of RNA abundance and infectious virus production, both miR-122 binding sites appeared to be equally important for translation. While Norman and Sarnow (153) reported recently that miR-122 does not modulate the rate of new viral RNA synthesis in transfected cells, the difference we have documented here in the relative

roles of the S1 and S2 sites in translation versus replication indirectly suggests that miR-122 has HCV-specific function(s) beyond that of promoting viral translation.

Our analysis of an IRES mutant (U265A) with a defect in translation of comparable magnitude to that of the double miR-122 binding site mutant, S1-S2-p6m, indicated that relatively modest reductions in IRES-directed translation are magnified over multiple rounds of replication into large deficits in yields of infectious HCV (Fig. 2.5). However, it also demonstrated that the 2- to 3-fold decrease in translational activity observed with the S1-S2-p6m mutant is unlikely to be the only explanation for the inability of this RNA to produce detectable infectious virus. The U265A mutant was impaired only 28-fold in its ability to produce infectious virus, while the defect in virus production by the S1-S2-p6m miR-122 binding site mutant is on the order of 3000-fold or more (Fig. 2.4B and 2.2E). In carrying out this analysis, we assumed that the U265A mutation was unlikely to affect RNA replication based on its distance downstream of essential replication signals in the 5'UTR (58, 59). However, were this assumption to be incorrect and the U265A mutation were to impair RNA synthesis, it would only strengthen the argument that miR-122 must positively regulate some step in the lifecycle of the virus other than translation. There is no basis for suggesting that the U265A mutation might enhance RNA synthesis, which would be an alternative explanation for the discrepancy of the U265A and S1-S2-p6m mutant viruses. These experiments were repeated several times with freshly transcribed RNAs, making it very unlikely that early generation of revertant virus contributed to the replication success of the U265A mutant.

The ability of miR-122 to positively regulate translation was strictly dependent on complementarity between the miR-122 seed sequence and viral RNA

(Fig. 2.4A), indicating that this effect also requires miR-122 binding to the viral genome. Most miRNA-binding sites are located in the 3'UTR of their target mRNAs, and down-regulate translation upon recruiting the miRNA-associated silencing complex (miRISC). miRNA-binding sites placed artificially within the 5'UTR of reporter genes may still repress translation (133). However, the actions of miR-122 on the HCV genome appear to be dependent on the position of the binding site, since the HCV binding site suppresses translation when placed in the 3'UTR of a reporter RNA (94). Therefore, the context in which miR-122 binds to HCV RNA appears to be important to its stimulatory effect on translation. An interesting and yet to be answered question is whether miRISC components are required for miR-122 promotion of HCV translation. siRNA-mediated knockdown of miRISC components, including argonaute 1-4 (Ago1-4) and Rck/p54 (DDX6) impairs the replication of HCV (86, 166, 178), but we have found that Rck/p54 knockdown does not limit the ability of miR-122 supplementation to up-regulate HCV replication (86). Thus it is uncertain whether the miRISC functions in the context of the miR-122 interaction with HCV RNA to recruit viral and/or cellular factors that can promote translation. It is known, however, that under certain conditions, some miRNA interactions with mRNAs may activate rather than repress gene expression. For example, miR-369-3p recruits Ago2 and fragile X mental retardation-related protein 1 (FXR1) to the AU-rich element (ARE) of TNFα mRNA and activates TNFα translation in serum-starved cells (205, 206).

Alternatively, it is possible that miR-122 binding to the S1 and S2 sites may lead to conformational changes in the HCV IRES that promote translation. A recent study has proposed that miR-122 binding to the 5'UTR increases HCV translation by

inhibiting long-range interactions between nts 24-38 in the 5'UTR and nts 428-442 in the core-coding region (40). While this could change the IRES conformation from a less active "closed" conformation to a more active "open" conformation, miR-122 enhances the amplification of subgenomic replicons that lack the core-coding sequence involved in this long-range intramolecular RNA interaction. This putative conformational switch also cannot explain how miR-122 stimulates translation of reporter RNAs that do not contain the core-coding sequence (76).

In summary, we have shown that the ability of miR-122 to promote the production of infectious HCV is dependent on direct interactions with both of the miR-122 binding sites in the HCV 5'UTR, but that the most 5' S1 site plays a dominant role in this process. We have also shown that miR-122 positively regulates IRES-directed translation of genome-length RNAs by approximately two-fold, and that this is also dependent on its ability to bind to both sites in the 5'UTR. While this provides a partial explanation for the dependence of HCV replication on miR-122, experiments with IRES mutants suggest that the positive regulation of HCV translation by miR-122 is insufficient to fully explain the inability of HCV RNA deficient in miR-122 binding to replicate and produce infectious virus.

CHAPTER 3. DDX6 (RCK/P54) IS REQUIRED FOR EFFICIENT HEPATITIS C VIRUS REPLICATION BUT NOT IRES-DIRECTED TRANSLATION[1]

ABSTRACT

DDX6 (Rck/p54) is an evolutionarily conserved member of the SF2 DEAD-box RNA helicase family that contributes to the regulation of translation and storage and degradation of cellular mRNAs. It interacts with multiple proteins and is a component of the micro-RNA (miRNA)-induced silencing complex (miRISC). Since miRNA-122 (miR-122) is essential for efficient hepatitis C virus (HCV) replication, we investigated the requirement for DDX6 in HCV replication in cultured hepatoma cells. siRNA-mediated knockdown of DDX6 and rescue with an siRNA-resistant mutant demonstrated that DDX6 expression is indeed required for optimal HCV replication. However, DDX6 knockdown did not impair miR-122 biogenesis nor alter HCV responsiveness to miR-122 supplementation. Overexpression of DDX6 fused to EYFP (EYFP-DDX6) enhanced replication, while a helicase-deficient mutant with a substitution in the conserved DEAD-box motif II (DQAD) had a dominant negative effect, reducing HCV yields. Co-immunoprecipitation experiments revealed an intracellular complex containing DDX6, HCV core protein, and both viral and cellular RNAs, the formation of which was dependent upon the C-terminal domain of DDX6 but not DDX6 helicase activity. However, since DDX6 abundance influenced the replication of subgenomic HCV RNAs lacking core sequence, the relevance of this complex is uncertain. Importantly, DDX6 knockdown caused minimal reductions

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in cellular proliferation, generally stimulated cellular translation ([^{35}S]-Met incorporation), and did not impair translation directed by the HCV internal ribosome entry site. Thus, DDX6 helicase activity is essential for efficient HCV replication, reflecting essential roles for DDX6 in HCV genome amplification and/or maintenance of cellular homeostasis.

INTRODUCTION

Persistent hepacivirus infection is associated with progressive liver fibrosis and the development of hepatocellular carcinoma (115). Worldwide, over 130 million people are infected with hepatitis C virus (HCV), which is estimated to cause over 350,000 cirrhosis and cancer deaths annually (158). Chronic hepatitis C is thus a major threat to human health. Current interferon-based treatments are effective in less than 50% of patients infected with the most prevalent viral genotypes and there is a need for more effective therapies. While good progress is being made in the development of new antiviral compounds that target major enzymatic activities expressed by HCV (a serine protease and an RNA-dependent RNA polymerase), resistance emerges rapidly to small molecule inhibitors of these viral enzymes due to the highly replicative nature of the infection coupled with error-prone viral RNA synthesis (184). Unfortunately, efforts to develop more effective therapeutic measures are handicapped by the fact that many aspects of the biology and molecular virology of this pathogen remain poorly defined. A better understanding of its interaction with the host cell and, in particular, its dependence on host cell proteins and microRNAs (miRNAs) for replication, may point the way to alternative cellular targets for

antiviral therapies. Although far from certain, therapeutics targeting such host factors may be less likely to engender the development of resistance.

Several host cell proteins have been implicated in HCV genome replication, including in particular the SNARE-like vesicle-associated membrane protein-associated host protein, VAP-A (also known as hVAP-33) as well as VAP-B (60, 71); a geranylgeranylated F box protein, FBL2 (210), and cyclophilin B (CypB), the latter of which interacts with the NS5B polymerase and is targeted by several candidate antiviral compounds now in clinical development (212). In addition, recent studies show that a highly abundant, liver-specific miRNA, miR-122, facilitates replication of HCV, both positively regulating the abundance of autonomously replicating HCV RNAs in Huh-7 hepatoma cells (95) and enhancing replication of infectious virus (166). The requirement for miR-122 in HCV replication is strongly supported by a recent study demonstrating that pharmacologic sequestration of miR-122 has a dramatic antiviral effect in HCV-infected chimpanzees (112). How miR-122 promotes replication is incompletely understood, although some data suggest it does so by increasing the efficiency of translation of viral RNA, which is driven by a cap-independent process involving internal entry of 40S ribosomes directed by an internal ribosome entry site (IRES) in the 5' untranslated RNA (UTR) segment of the genome (76).

DDX6 (Rck/p54), is a cellular RNA helicase with ATP-dependent RNA-unwinding activity (213). It is a member of an evolutionarily conserved family of DExD/H helicases that are typically found as components of large messenger ribonucleoprotein (mRNP) complexes. DDX6 and its homologs are thought to function in remodeling mRNPs so as to facilitate multiple aspects of mRNA

metabolism, including transcription, splicing, translation, degradation and storage (35, 124). Human DDX6 was first identified as a 54 kD protein encoded by a gene located at a chromosomal breakpoint in a cell line, RC-K8, derived from a B cell lymphoma (hence its alternative name, Rck/p54) (128). It has been suggested to function as a proto-oncogene and is over-expressed in some colorectal cancers (75). Both it, and its closely related yeast homologue, Dhh1p, function as general translational repressors (34). DDX6 interacts with the initiation factor eIF-4E and other cellular protein partners to repress the translational activity of mRNPs. It is a component of stress granules, but localizes primarily to processing bodies (P bodies) in which its mRNP remodeling activities may contribute to mRNA storage, degradation, and recycling for translation (213). DDX6 directly interacts with Argonaute-1 (Ago1) and Ago2 in micro-RNA (miRNA)-induced silencing complexes (miRISC), and it plays a role in miRNA-mediated translational repression (31).

Since miR-122 is required for HCV replication, and DDX6 is a miRISC component, we considered the possibility that DDX6 could functionally regulate the replication of HCV. Viral interactions with two other members of the DExD-box RNA helicase family (e.g. DDX3 and DDX5/p68) have been implicated in the replication of HCV in cell culture. DDX3 interacts with the HCV core protein (136, 156, 229) and is required for efficient HCV replication (7, 9), while the viral polymerase, NS5B, interacts with DDX5, leading to its redistribution from the nucleus to the cytoplasm (64). DDX6 may also be relevant to hepatitis C since it is up-regulated within the liver during chronic infection (143). Our interest in studying its role in HCV replication was stimulated by the fact that its yeast homolog, Dhh1p, is required for efficient translation and replication of the plant brome mosaic virus

(BMV) in yeast, a well-established model for replication of positive-strand RNA viruses (5). Consistent with the strong evolutionary conservation of DDX6 function, DDX6 is capable of substituting for Dhh1p in supporting BMV replication in yeast cells.

Against this background, we set out to determine whether DDX6 is involved in HCV replication. While our studies were in progress, Scheller et al. (178) reported that RNAi-mediated silencing of Rck/p54 and several related proteins substantially impairs the replication of HCV in cell culture, ostensibly due to an impairment in HCV translation in the absence of DDX6 expression. Our results confirm that DDX6 expression is required for optimal replication of HCV RNA, but argue against this being due to a requirement for DDX6 during IRES-directed translation. We show that miR-122 facilitates HCV RNA replication independently of DDX6, and that mutations that ablate the ATPase and unwindase activities of DDX6 cause a relocalization of DDX6 within the cell and also negate the ability of DDX6 to support viral replication.

MATERIALS AND METHODS

Cell lines

Huh-7, FT3-7 (a clonal derivative of Huh-7 cells), Huh-7.5 cells (kindly provided by Dr. Charles Rice, Rockefeller University) were cultured as described previously (228). Several G418-resistant Huh-7-derived replicon cell lines were used in these studies, including: Htat2ANeo/NS3-5B/QR/VI/KR/KR5A/SI cells, which contain a subgenomic (NS3-NS5B), genotype 1a (H77) replicon (224); Htat2ANeo/C-5B/QR/KR/FV/SI, a related cell line in which the replicon contains the

complete HCV polyprotein-coding sequence (224); Ntat2ANeo/C-5B/2-3, which contains a similar full-length genotype 1b (HCV-N) replicon (83); and, Sg-2a cells, which contain a subgenomic genotype 2a (JFH-1) replicon. Replicon cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, 4 mM L-glutamine and 0.25 mg/mL of G418. Huh-7-191/20 cells conditionally express HCV core protein under tight regulation by the Tet-Off promoter, and were cultured as described previously (117). FRhK-4 cells, used for experiments with hepatitis A virus (HAV), were cultured as described previously (217).

Hepatitis C virus

Experiments with cell culture-infectious HCV were carried out with HJ3-5 virus (vH-NS2/NS3-J/Y361H/Q1251L) (226), an inter-genotypic chimeric virus produced in cell culture that is also infectious in chimpanzees (M. Yi and S.M. Lemon, unpublished data). The HJ3-5 genome is comprised of sequence encoding core-NS2 of the genotype 1a H77c virus placed within the background of the genotype 2a JFH-1 virus genome (226). It contains two adaptive mutations, Y361H and Q1251L, in E1 and NS3 respectively, that facilitate its replication (226). Virus infections and infectious virus titrations were carried out as described previously (228).

Hepatitis A virus

HAV infections were carried out using HM175/18f virus, a cytopathic, cell culture-adapted virus variant, as described previously (217). Infectious virus titers were determined using a modified infra-red fluorescence focus assay (36) as follows: 1.5 x 10^5 FRhK cells were plated in a 6-well plate. The following day, the cells were inoculated with serial dilutions of virus (600 µl) prepared in DMEM with 2% FBS, and virus was allowed to adsorb at 36°C for 1.5 hrs. Cells were then overlaid with DMEM containing 0.5% low melting point agarose, and incubated for 7 days at 36°C in 5% CO_2. The overlays were gently removed, and cells were fixed with 4% PFA in PBS at room temperature for 25 mins, washed twice with PBS for 5 mins each, and permeabilized with 0.2% Triton-X100 in PBS at room temperature for 12 mins. After further washing, the cells were blocked with 10% normal goat serum for 1 hr at room temperature, and incubated overnight with a murine monoclonal anti-HAV (6A5) antibody at 1:600 dilution (600 µl per well) in 3% milk at 4°C overnight. The cells were washed 4 times with PBS containing 0.1% Tween-20 (PBS-T) and incubated for 1 hr with 1:800 dilution (600 µl per well) of goat anti-mouse Alexa Fluor-680 antibody (Invitrogen) in 3% milk at room temperature. The plates were extensively washed, dried, and scanned with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NB). Foci of viral replication were counted, and results were calculated as focus-forming units (FFU)/ml.

Plasmids

pHJ3-5 is a T7 expression vector that contains the sequence of HJ3-5 virus, while pHJ3QL-ΔE1-p7 is a related vector containing an in-frame deletion of the E1-

p7 coding sequence (226). pHJ3-5-ΔC61-148 expresses HJ3-5 RNA with an in-frame deletion of amino acids 61-148 of core. pHJ-3-5-ΔC21-p7 contains an in-frame deletion from core to p7 but retains the first 20 amino acids of core. pHJ3-5-ΔC61-148 and pHJ-3-5-ΔC21-p7 were constructed from pHJ3-5 by standard PCR-based mutagenesis techniques. Similar methods were used to construct pHJ3-5/RLuc2A, which contains the *Renilla* luciferase sequence, fused at its 3' end to the foot-and-mouth disease virus (FMDV) 2A autoprotease sequence, inserted between the p7 and NS2 sequences of pHJ3-5 (91). To eliminate its ability to replicate, pHJ3-5/RLuc2A was further modified by creation of an Asp to Asn substitution within the GDD motif of the polymerase active site, resulting in pHJ3-5/RLuc2A-GND.

pEYFP-C1-Rck (referred to hereinafter as pEYFP-DDX6) was kindly provided by Dr. T.M. Rana, University of Massachusetts Medical School, Worcester, Massachusetts, USA, and encodes N-terminal EYFP-tagged, full-length DDX6 (31). pEYFP-DDX6-m6 contains 6 silent point mutations that ablate the binding of DDX6-1 siRNA (see below) but maintain the wild-type amino acid sequence of DDX6. It was generated using the QuickChange® Site-Directed Mutagenesis kit (Stratagene). pEYFP-DDX6-ΔC, generated by standard molecular biology techniques, contains a deletion of sequence encoding the C-terminal 183 amino acids of the DDX6 protein (C-terminal domain 2) (4). pEYFP-DDX6-EQ is an additional mutant that encodes Gln in lieu of Glu within the DEAD-box motif II of DDX6, and thus lacks helicase activity (213). pEYFP, encodes only the EYFP protein, and was used as a negative control in transfection experiments.

pRLHL expresses a dicistronic RNA containing *Renilla* luciferase sequence in the first cistron, and firefly luciferase sequence in its second cistron, separated by the

HCV IRES sequence (79). A PCR product carrying a T7 promoter at its 5'end and a poly(A) tail of 30 adenosines at its 3'end was used as a template for in vitro transcription of a capped and polyadenylated RNA encoding firefly luciferase.

Antibodies

Antibodies used in these studies included: mouse monoclonal antibody (mAb) to HCV core protein (C7-50, Affinity BioReagents); polyclonal rabbit anti-NS5A (gift from Craig E. Cameron, Pennsylvania State University, University Park, PA); mouse monoclonal 9E10 anti-NS5A antibody (a generous gift from Brett D. Lindenbach, Yale University School of Medicine, New Haven, CT), rabbit anti-HAV 2A protein (a gift from Verena Gauss-Muller, University of Lubeck, Lubeck, Germany), 6A5 anti-HAV mAb (a gift from J. Hughes, Merck Sharp and Dohme Research Laboratories, West Point, PA); anti-dsRNA mAb (J2, Scicons, Hungary); rabbit polyclonal anti-DDX6 (Bethyl Labs); mouse anti-GFP mAb (Clontech); rabbit polyclonal anti-GFP (Living Colors Full-length A. v. anti-GFP, Clontech); and rabbit polyclonal anti-calnexin (Sigma).

siRNA and miRNA oligonucleotides

RNA oligonucleotides and siRNAs were custom synthesized or purchased from Dharmacon. The sequences were: miR-122wt: 5'-UGGAGUGUGACAAUGGUGUUUGU-3'; miR-122*: 5'-AAACGCCAUUAUCACACUAAAUA-3' (95); miR-124: 5'-UUAAGGCACGCG GUGAAUGCCA-3'; miR-124*: 5'-CCGUGUUCACAGCGGACCUUGA-3'.

Mature miR-122 and miR-124 duplexes were generated by annealing equal molar amounts of miR-122wt and miR-122*, and miR-124 and miR-124*, respectively.

Two DDX6-specific siRNAs, DDX6-1 and DDX6-3, targeting different regions of DDX6 mRNA, were used to ensure specificity of RNAi experiments. Control siRNAs, used to monitor off-target effects of siRNA knockdown included DDX6-1m and DDX6-3m, sequence-related siRNAs each containing two mutations in the DDX6-1 and DDX6-3 siRNA sequences, respectively. The sequences of these siRNAs were: DDX6-1, 5'-GCAGAAACCCUAUGAGAUUUU-3' and 5'-AAUCUCAUAGGGUUUCUGCUU-3' (31); DDX6-1m, 5'-GCAGAAACCGAAUGAGAUUUU-3' and 5'-AAUCUCAUUCGGUUUCUGCUU-3'; DDX6-3, 5'-CCAAAGGAUCUAAGAAUCAdTdT-3' and 5'-UGAUUCUUAGAUCCUUUGGdTdT-3' (182); DDX6-3m, 5'-CCAAAGGAUGAAAGAAUCAdTdT-3' and 5'-UGAUUCUUUCAUCCUUUGGdTdT-3' (mutated sequences are underlined in the control siRNAs). Additional siRNA controls included the ON-TARGET*plus* non-targeting pool of 4 siRNAs (Dharmacon Cat # D001810-10-20; hereinafter referred to as Ctrl), which has no known targets within mammalian genomes.

Transfections

siRNAs (50 nM) and miRNAs (50 nM) were transfected into FT3-7 cells using Lipofectamine2000™ (Invitrogen) according to the manufacturer's recommended procedures. Lipfectamine2000™ (Invitrogen) was also used for transfection of plasmid DNA. HCV RNA and FLuc RNA transfections were carried

out using the TransIT® mRNA transfection kit (Mirus Bio) for 5 hrs as per the manufacturer's protocol.

siRNA-mediated knockdown of DDX6 and HCV replication

FT3-7 cells grown in 24-well plates were transfected with siRNA (50 nM) as above. At 48 hrs, the cells were infected with HJ3-5 virus at an MOI of 1.0. One day later, the cells were washed twice with PBS and refed with fresh media. Supernatant culture fluids were collected subsequently at 24 hr intervals (days 2 and 3 post-infection) and the infectious virus titers determined by FFU assay as described previously (228). On day 3 post-infection, cell lysates were prepared for immunoblot analysis. For experiments involving rescue of DDX6 knockdown, the cells were transfected with DDX6-1 or DDX6-1m siRNAs as described, and super-transfected, 24 hrs later, with pEYFP-DDX6-m6 (20, 100 and 500 ng/well) or pEYFP (500 ng/well) DNA using Lipofectamine2000^{TM} (Invitrogen). Twenty-four hrs later, the cells were infected with HJ3-5 virus (MOI = 0.5), and the infection was monitored as described above. Cell lysates, prepared three days after infection, were analyzed in immunoblots with DDX6, calnexin, HCV core and NS5A-specific antibodies.

DDX6 knockdown and HAV replication

FT3-7 cells were transfected with siRNAs as described in the preceding section, and infected 6 hrs later with HM175/18f virus at an MOI of 1.0. Virus was allowed to adsorb to cells for 1.5 hrs in antibiotic free DMEM with 2% FBS; the cells were then refed with fresh media and incubated at 36°C in a 5% CO_2 environment.

On day 5 post-infection, cell lysates were prepared for immunoblot analysis, while virus was harvested from supernatant fluids and by repetitive freeze-thawing of a parallel set of cultures. The cell lysate was sonicated, extracted with an equal volume of chloroform, and stored at -80°C for virus titration.

WST-1 assays

FT3-7 cells (1 x 10^4 cells/well of a 96 well plate) were transfected with DDX6 siRNAs as described above. At 0, 24, 48, 72 and 96 hrs post-transfection, 10 µl of WST-1 reagent (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulpho-phenyl]-2H-tetrazolium monosodium salt, Chemicon International, Inc.) was added to triplicate sets of wells, and the cells were incubated at 37°C for 1 hr prior to measurement of the absorbance at 450 nm.

Co-immunoprecipitation experiments

FT3-7 cells were infected with HJ3-5 HCV at an MOI of 0.2. When greater than 90% positive for HCV core expression as determined by immunostaining, the cells were transfected with EYFP-DDX6 or EYFP expression vectors as described above. Forty-eight hrs later, the cells were trypsinized, washed twice with ice-cold PBS, and resuspended in IP lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP40, 10% glycerol, and freshly added 2 mM DTT and Protease Inhibitor Cocktail (Roche)] on ice for 30 mins. The lysate was centrifuged at 14,000 rpm at 4°C for 10 min to remove debris and preclarified with Protein G Sepharose™ 4 Fast Flow beads (GE Healthcare) at 4°C overnight. The lysates were then subjected

to immunoprecipitation (500 μg protein in 500 μl of IP lysis buffer) with rabbit anti-GFP antibody (1:200 dilution) and Protein G Sepharose™ 4 Fast Flow beads. After extensive washing in IP lysis buffer (10 min each, 4 times at 4°C), the proteins were eluted by boiling in 2X SDS-loading buffer and subjected to immunoblotting. For reverse co-immunoprecipitation, HCV core protein was precipitated from HJ3-5-infected FT3-7 cell lysates using a murine anti-core mAb (1:100 dilution, C7-50, Affinity BioReagents) and immunoblotted with rabbit polyclonal anti-DDX6 (Bethyl labs). An anti-GFP mAb (Clontech) was used as an isotype control.

Confocal fluorescence microscopy.

HCV-infected FT3-7 cells grown in 8-well glass chamber slides were washed once with PBS and fixed with 4% paraformeldehyde (in PBS) for 25 min at room temperature. After washing with PBS-glycine (100 mM, for 15 min at room temperature), the cells were permeabilized with digitonin (50 μg/ml) for 5 min at room temperature. Following 3 washes of 5 min each with PBS, the cells were incubated with primary antibodies: anti-core (1:600 dilution in 3% BSA), anti dsRNA (1:500), or rabbit anti-DDX6 (1:200) overnight at 4°C. The cells were then washed 3 times with PBS-T and incubated with secondary antibodies: Alexa Fluor-488 goat anti-mouse IgG and Alexa Fluor-594 goat anti-rabbit IgG (Invitrogen, 1:200 each) for 1 hr at room temperature. Following 3 washes of 20 min each with PBS-T, the slides were counterstained with DAPI (1:1000 dilution) for 5 mins, washed with PBS-T, mounted in VectaShield mounting fluid (Vector Laboratories, Burlingame, CA), and examined with a Zeiss LSM510 Meta laser scanning confocal microscope.

For localization of various mutant forms of DDX6, FT3-7 cells were transfected with EYFP-DDX6 or EYFP expression vectors. At 48 hrs, cells were washed once with PBS, fixed with 4% paraformaldehyde and incubated with PBS-glycine (100 mM, for 15 min at room temperature) as above. After rinsing with PBS, slides were mounted in VectaShield mounting fluid, and examined as above.

Northern blots for HCV RNA

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen), and analyzed using reagents supplied with the NorthernMax® kit (Applied Biosystems). Briefly, 5 μg of each RNA sample was resolved on a 0.9% denaturing formaldehyde agarose gel, transferred to a BrightStar-Plus® nylon membrane (Applied Biosystems) by downward capillary transfer and hybridized overnight at 68°C with ^{32}P-labeled antisense riboprobes complementary to the genotype 2a HCV (JFH1) 5'UTR (340 nts) or β-actin (as a loading control). Following extensive washing, the membranes were scanned on a Personal Molecular Imager (Bio-Rad), and the band densities quantified with Quantity One software (Bio-Rad).

Detection of miR-122

Total cellular RNA (10 μg), extracted using TRIZOL Reagent (Invitrogen), was resolved on a 15% polyacrylamide/8M urea gel, and transferred to a positively-charged Nylon membrane (Applied Biosystems) by downward capillary transfer using NorthernMax® transfer buffer (Applied Biosystems) for 3 hours. After UV-crosslinking, the membrane was prehybridized in ULTRAhyb-Oligo hybridization

buffer (Applied Biosystems) for an hr at 42ºC, and hybridized with P^{32}-labeled riboprobes specific for miR-122 and 5S-rRNA at 42ºC overnight. The membranes were washed twice with wash buffer (2X SSC, 0.5% SDS) at 42ºC for 30 min each, and scanned on a STORM860 PhosphoImager (Molecular Dynamics).

HCV translation assays

Lysates of pRLHL-transfected cells were prepared 24- and 48-hrs following transfection and assayed for firefly and *Renilla* luciferase reporter activities using the Dual Luciferase Assay kit (Promega). Firefly luciferase activity (HCV IRES-mediated translation) was normalized to *Renilla* luciferase activity (cap-dependent translation). To study translation from authentic monocistronic HCV genomes, cells were cotransfected with HJ3-5/RLuc2A-GND HCV RNA (1.25 μg per well, 6-well plate) and polyadenyated firefly luciferase (FLuc) mRNA (0.25 μg per well) using the TransIT® mRNA transfection kit (Mirus Bio) for 5 hrs according to the manufacturer's protocol. Cell lysates were prepared 8 hrs later and assayed for *Renilla* (HCV IRES-mediated translation) and firefly luciferase (cap-dependent translation) activities as above.

[S^{35}]Methionine Incorporation

Cells were incubated in methionine- and cysteine-free DMEM containing 2% FBS for 1 hr before addition of 100 μCi/ml of Tran35S-Label™ (MP Biomedicals) for 0, 15, 30 and 60 mins. Cells were harvested in PBS containing 1% NP40, 0.1% SDS, 0.03% sodium deoxycholate, and protease inhibitor cocktail (Roche Applied

Sciences). S^{35} incorporation in TCA-precipitated cell lysates (25-50 μl) prepared from equivalent numbers of cells was quantified by scintillation counting (31) and presented as the mean ± SD from three independent experiments.

RESULTS

DDX6 knockdown impairs HCV replication.

We investigated the role of DDX6, an miRISC component that is upregulated in the liver during chronic hepatitis C, in the replication of HJ3-5 virus, an inter-genotypic, chimeric HCV that replicates efficiently in cultured human hepatoma cells (226). The structure of the genome of this chimeric virus is shown in Fig. 3.1A. To silence the expression of DDX6, we transfected cells with two different siRNAs [DDX6-1 (31) and DDX6-3 (182)] that target distinct sequences in human DDX6 mRNA. To control for the specificity of gene silencing, we designed two additional siRNAs, representing DDX6-1 and DDX6-3 with point mutations at nucleotides 9 and 10, respectively (DDX6-1m and DDX6-3m). As an additional control, cells were transfected with a pool of 4 other siRNAs (designated "Ctrl") that have no known targets in mammalian genomes (Dharmacon). FT3-7 cells, which are derived from Huh-7 human hepatoma cells and efficiently support HCV replication, were transfected with these siRNAs as described in the Methods, and 2 days later the cells were infected with HJ3-5 virus at an MOI of 1.0. Transfection of either the DDX6-1 or DDX6-3 siRNAs decreased the abundance of DDX6 protein by 5 days post-

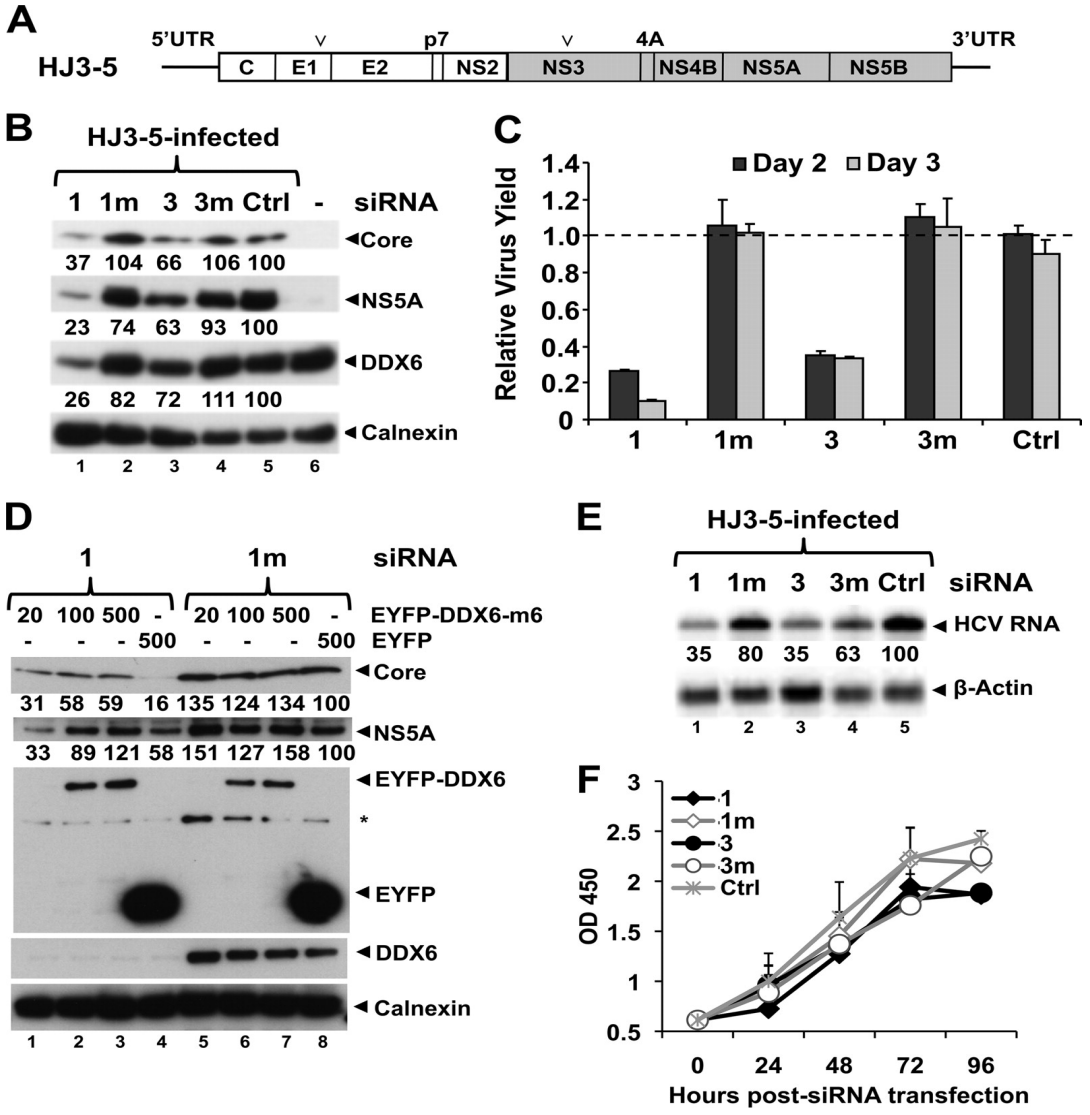

Figure 3.1. DDX6 knockdown impairs HCV replication. (A) Schematic diagram of genomic organization of chimeric HCV genotype 1a/2a (HJ3-5) RNA that contains core, E1, E2, p7 and NS2 sequence derived from H77s (genotype 1a, white box) and NS3-5B and both UTRs from JFH1 (genotype 2a, grey box) Arrowheads indicate position of two adaptive mutations, one each in E1 (Y361H) and NS3 (Q1251L) genes that enhance its replication. (B) FT3-7 cells were transfected with the indicated DDX6-specific or control siRNAs [1, DDX6-1; 1m, DDX6-1m; 3, DDX6-3; 3m, DDX6-3m and Ctrl, Ctrl]. At 48 hrs, cells were infected with HJ3-5 HCV at an MOI of 1 and cell lysates prepared 3 days after infection were subjected to immunoblotting. Various band intensities were measured using AlphaEaseFC™ Software version 4.0.0 (Alpha Innotech Corporation), normalized to calnexin levels and represented as percent of Ctrl siRNA (Ctrl) treated cells. (C) Virus supernatants collected 2 and 3 days post-

infection were titrated on naïve Huh-7.5 cells in triplicate. Results from two independent experiments are presented here as mean ± standard deviations. (D) FT3-7 cells were transfected with 1 [DDX6-1] or 1m [DDX6-1m] siRNA. At 24 hrs, cells were transfected with DNA encoding siRNA-resistant form of EYFP-DDX6 or pEYFP and were infected with HJ3-5 HCV (MOI = 0.5) on the next day. Cell lysates, prepared 3 days after infection, were immunoblotted. Band densities were quantitated as above and represented as percentages of that of DDX6-1m/pEYFP-transfected cells. (E) FT3-7 cells were transfected with indicated siRNAs and infected with HJ3-5 HCV at an MOI of 1.0 at 48 hrs post-transfection. Total RNA was isolated 3 days later and subjected to northern blotting. PhosphoImager quantitations are represented as percentage of that of Ctrl siRNA treated cells. (F) FT3-7 cells were transfected with the indicated siRNAs, followed by spectrophotometric measurements of WST-1 activity at 450 nm at indicated time points (mean O.D. 450nm ± SD, n = 2)

transfection (Fig. 3.1B, lanes 1 and 3). DDX6-1 siRNA was more efficient, and reduced DDX6 levels to 26% of Ctrl-transfected cells, while DDX6-3 reduced DDX6 expression only to 72% based on quantitation of immunoblots. As expected, the mutant siRNAs, DDX6-1m and DDX6-3m, and the Ctrl siRNA pool had little effect on DDX6 abundance (Fig. 3.1B, lanes 2, 4 and 5, 82-111% of normal), supporting the specificity of DDX6 knockdown, particularly by DDX6-1. Importantly, transfection of DDX6-1 also reduced the abundance of HCV core protein (37% that of Ctrl siRNA treated cells) as well as NS5A (23%) (Fig. 3.1B, lanes 1 and 3). The reduction in core and NS5A abundance correlated well with the efficiency of DDX6 knockdown. To assess the effect of DDX6 knockdown on production of infectious virus, we collected cell culture supernatant fluid at 24 hr intervals (replacing it completely with fresh media), and determined infectious virus yields using a standard infectious focus assay (228). This revealed that DDX6-1 transfection also reduced infectious virus yields by 8-fold on day 3 post-infection (Fig. 3.1C), which is concordant with the degree of DDX6 knockdown in these cells. DDX6-3, on the other hand, reduced virus yields by only 3-fold, consistent with its less efficient silencing of DDX6. Neither of the control siRNAs had any effect on virus yield, suggesting that the observed reductions

in virus replication were related specifically to reduced DDX6 expression. Northern blotting for HCV RNA indicated that DDX6 silencing also specifically reduced viral RNA abundance (Fig. 3.1E, lanes 1 and 3). Taken together, these results suggest that efficient HCV replication is closely linked to DDX6 expression in these hepatoma cells.

To rigorously test this conclusion, and show that impaired viral replication results specifically from DDX6 silencing and not an off-target effect of the siRNA, we constructed a mutated DDX6 expression vector (pEYFP-DDX6-m6) that contains 6 base mismatches within the site targeted by the DDX6-1 siRNA without changing the amino acid sequence of the protein. We silenced expression of endogenous DDX6 by transfection of the DDX6-1 siRNA as in Fig. 3.1B, and 24 hrs later re-transfected the cells with pEYFP-DDX6-m6 or a related empty vector (pEYFP, see Methods). The cells were then infected with HJ3-5 virus at an MOI of 0.5, and cell lysates prepared 3 days later for immunoblotting. As in the prior experiments, transfection of the DDX6-1 siRNA resulted in decreased expression of endogenous DDX6 as well as reduced abundance of core and NS5A proteins expressed by HCV, compared to cells transfected with the DDX6-1m siRNA (Fig. 3.1D, compare lanes 4 and 8). Ectopic expression of the siRNA-resistant EYFP-DDX6-m6 partially restored the levels of core as well as NS5A in the cells in which endogenous DDX6 had been silenced (compare lanes 1-3 with lane 4). In addition, EYFP-DDX6-m6 overexpression slightly increased the abundance of NS5A and core proteins in cells transfected with the inactive, control DDX6-1m siRNA (compare lane 5-7 with lane 8). These results confirm that the reduction in viral protein expression is caused specifically by DDX6 silencing, and not by any spurious off-target effects of siRNA transfection.

DDX6 knockdown and global changes in cellular metabolism and proliferation

DEAD-box RNA helicases, and in particular DDX6 and its various homologs in different vertebrate and invertebrate species, are involved in multiple aspects of RNA metabolism, including transcription, splicing, RNA processing, translation, storage and degradation (35, 124, 213). Therefore, DDX6 silencing could have pleiotropic effects on cellular metabolism and growth. Since HCV replication is linked to the proliferation status of cells (148, 179), we assessed the impact of DDX6 silencing on the growth and metabolism of FT3-7 cells in a WST-1 reduction assay. Transfection of the DDX6-specific siRNAs resulted in minor reductions in WST-1 reduction (20-25%) after 72-96 hrs, when compared to cells transfected with the siRNA controls (Fig. 3.1F). Similarly, DDX6 knockdown reduced cellular growth by 25% after 96 hrs, as determined by directly counting cells (see below). Thus, silencing DDX6 results in only a slight reduction in the growth of cells.

As an additional measure of the general health of FT3-7 cells and their ability to support viral replication following DDX6 silencing, we evaluated the replication of an unrelated hepatotropic virus, hepatitis A virus (HAV). Cells were transfected with DDX6-1 or DDX6-1m siRNA as described above, then infected with the HM175/18f strain of HAV at an MOI of 1.0. In contrast to the results we obtained with HCV, we observed no effect of DDX6 silencing on the cellular abundance of the HAV VP1-2A protein 5 days later (data not shown). Moreover, the yield of infectious HAV was not significantly reduced by DDX6 silencing (2.06×10^6 FFU/ml vs. 1.81×10^6 FFU/ml). Combined with the data shown in Fig. 3.1F, these results suggest that DDX6 is specifically required for efficient replication of HCV, and that the reductions in HCV

replication observed following DDX6 silencing are unlikely to result from a general defect in cellular metabolism.

Overexpression of DDX6 stimulates HCV replication

To determine whether overexpression of DDX6 enhances HCV replication, we monitored replication of virus in cells in which DDX6 was overexpressed as an N-terminal EYFP-fusion protein. There was little discernible difference in core, NS5A (data not shown), or NS3 expression levels in immunoblots (relative to a β-actin loading control, Fig. 3.2A), but overexpression of EYFP-DDX6 resulted in a two-fold increase in virus yields compared to cells transfected with a control EYFP vector

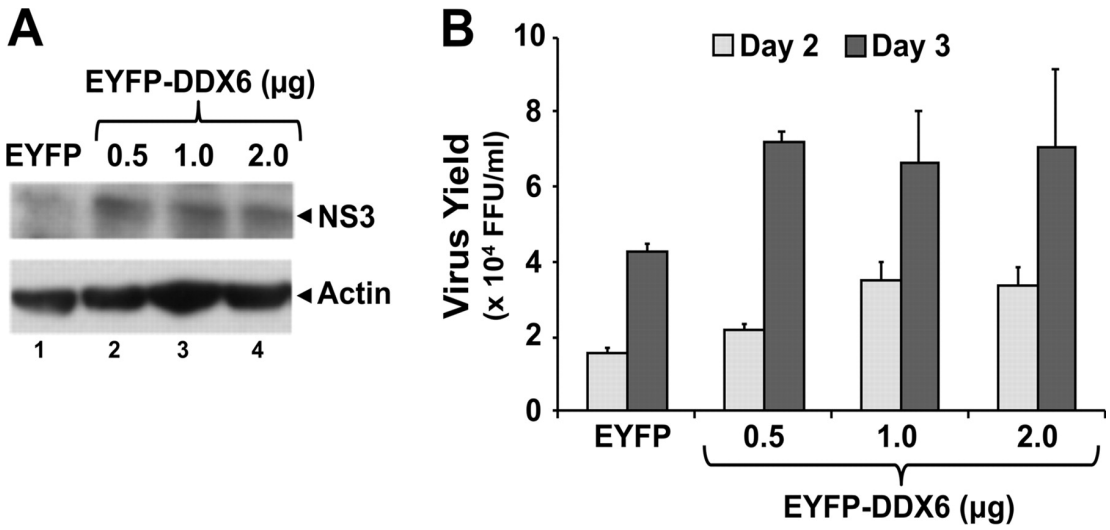

Figure 3.2. DDX6 overexpression stimulates HCV replication. FT3-7 cells were transfected with pEYFP-DDX6 or pEYFP plasmid DNA. Cells were then infected with HJ3-5 HCV at an MOI of 0.2 at 24 hrs post-transfection and were fed with fresh media every 24 hrs. (A) Cell lystates, prepared 3 days post-infection, were immunoblotted. (B) Virus supernatants collected 2 and 3 days post-infection were titrated on naïve Huh-7.5 cells, in triplicate. Results from two independent experiments are presented here as mean ± standard deviations.

(Fig. 3.2B). These results were reproducible in repeat experiments and support the findings in Fig. 3.1. While they confirm that DDX6 expression levels correlate with the efficiency of HCV replication, the small increases in virus yield observed with DDX6 overexpression suggest that the abundance of endogenous DDX6 is generally not limiting for viral replication in these cells.

DDX6 is not required for miR-122 facilitation of HCV replication

A direct interaction of miR-122, a liver-specific miRNA, with HCV RNA is required for efficient genome amplification (93-95). It is not known whether this also requires miR-122 recruitment of miRISC complexes, but silencing miRISC components such as the argonaute proteins (Ago1-4) inhibits HCV replication (166). Since DDX6 interacts with Ago1 and Ago2 (31), we considered the possibility that DDX6 might be required for miR-122 to promote replication. First, to determine if DDX6 abundance influences miR-122 biogenesis, we isolated total cellular RNA 4 days after transfection of the siRNAs and subjected it to northern blotting with P^{32}-labeled miR-122- and 5S rRNA-specific probes. As shown in Fig. 3.3A, we observed no changes in miR-122 abundance following transfection of the DDX6-specific siRNA (top panel), despite substantial reduction of DDX6 expression in these experiments (bottom panel). We next asked whether the ability of miR-122 to promote HCV RNA accumulation is sustained in cells in which DDX6 expression had been silenced. We transfected FT3-7 cells with the DDX6 siRNAs described above, and then supplemented the cells with miR-122, or miR-124 as a negative control (95), by transfecting synthetic miRNA duplexes 48 hrs later (see Methods).

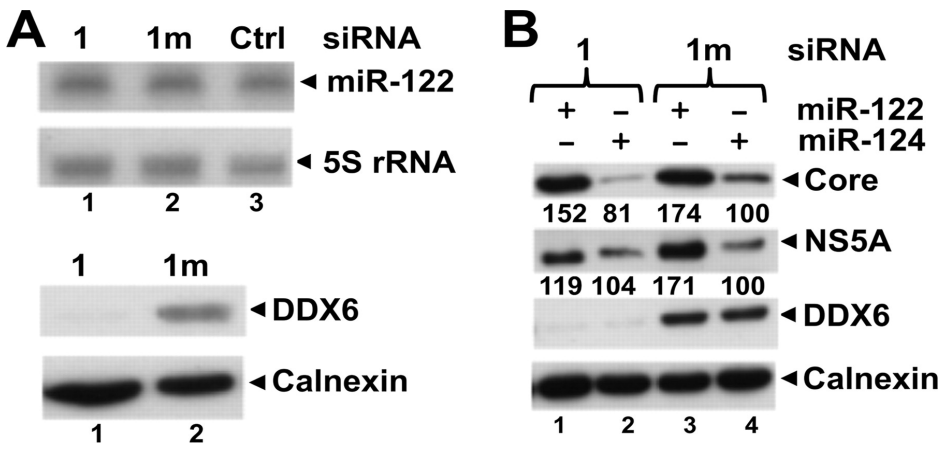

Figure 3.3. DDX6 is not required for miR-122 facilitation of HCV replication. (A) FT3-7 cells were transfected with 1 [DDX6-1] or 1m [DDX6-1m] siRNA and cell lysates were prepared 4 days later. Total RNA was subjected to northern blotting to detect miR-122 and 5S rRNA (used as a loading control) using ^{32}P-labeled riboprobes (top panel). Protein extracts were immunoblotted for DDX6 and calnexin (bottom panel) (C) FT3-7 cells were transfected with 1 [DDX6-1] or 1m [DDX6-1m] siRNA. After 48 hrs, cells were transfected with miR-122 or miR-124 (control miRNA) and infected with HJ3-5 HCV (MOI = 0.2) at 54 hrs. On day 4, cells were transfected with another dose of miR-122 or miR-124. Cell lysates were prepared on the next day and subjected to western blotting. Band intensities were measured as described in Fig. 3.1B and are represented as percentage of DDX6-1m/miR-124 transfected cells.

At 54 hrs, the cells were infected with virus at an MOI of 0.2, and supplemented with miR-122 or miR-124 again at 90 hrs. Cell lysates were prepared at 120 hrs and subjected to immunoblotting. As anticipated, miR-122 supplementation substantially enhanced the abundance of the core and NS5A proteins in cells transfected with the control siRNA, compared to similarly transfected cells supplemented with miR-124 (Fig. 3.3B, compare lanes 3 and 4). Core protein expression was reduced, as expected, in cells transfected with the DDX6-specific siRNA (Fig. 3.3B, compare lanes 2 and 4), but was nonetheless strongly upregulated by miR-122 supplementation (compare lanes 1 and 2) despite a high degree of DDX6 silencing. NS5A expression was also increased. These results indicate that the enhancement observed in viral

replication following supplementation with miR-122 is not dependent upon DDX6, and miR-122 and DDX6 facilitate replication via independent mechanisms. Importantly, miR-122 supplementation fully compensated for the loss of DDX6 in the knockdown cells, bringing both core and NS5A abundance to levels exceeding that in the control cells (Fig. 3.3B, compare lanes 1 vs. 4).

DDX6 forms a complex containing HCV RNA and core protein

To better understand the mechanism by which DDX6 facilitates the replication of HCV RNAs, we carried out co-immunoprecipitation experiments to assess whether DDX6 might interact with one or more viral proteins. FT3-7 cells were infected with HJ3-5 virus and cultured until more than 90% of the cells were positive for core protein expression as determined by immunostaining. The cells were then transfected with the pEYFP-DDX6-wt or pEYFP expression vectors and cell lysates prepared two days later for co-immunoprecipitation experiments. EYFP-DDX6 was efficiently immunoprecipitated with polyclonal rabbit anti-GFP antibody (Fig. 3.4A, left panel, lane 3). Immunoblotting of these precipitates with HCV-specific antibodies revealed the presence of core protein (Fig. 3.4A, bottom right panel, lane 3), but not NS3 (Fig. 3.4A, top right panel, lanes 3 and 4), NS5A, or NS5B (data not shown). Under the stringent conditions used for this co-immunoprecipitation experiment, only a small fraction of the total intracellular core protein co-immunoprecipitated with DDX6. However, the co-immunoprecipitation of core was specific to DDX6, since it was not observed in EYFP precipitates (Fig.

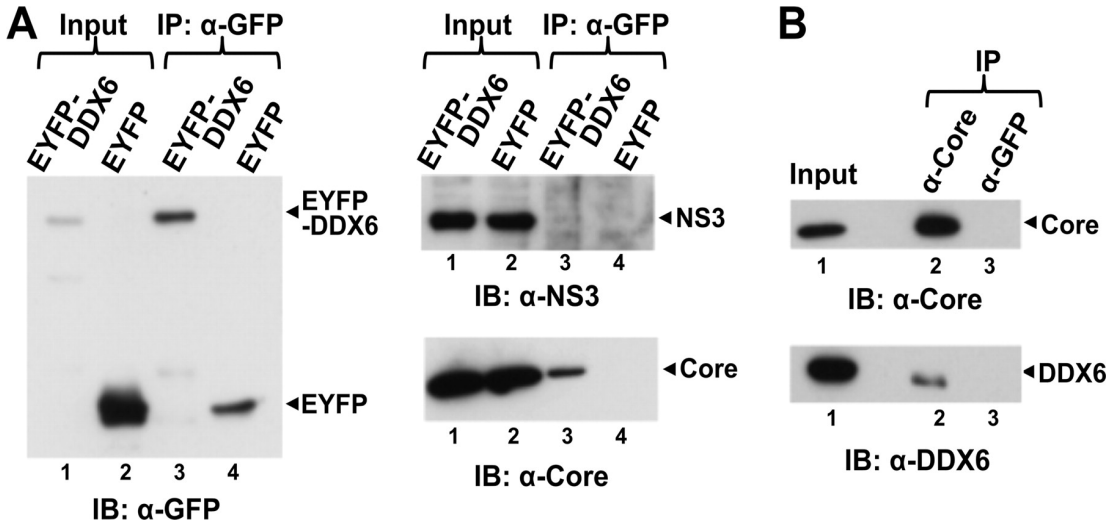

Figure 3.4. DDX6 forms a complex with the HCV core protein. (A) HJ3-5 HCV-infected FT3-7 cells were transfected with DNA vector expressing wild-type EYFP-DDX6 or EYFP alone. Cell lysates were prepared 2 days later and subjected to coimmunoprecipitation (Co-IP) with anti-GFP (rabbit polyclonal, Clontech) antibody. Co-IP samples were immunoblotted with GFP (mouse monoclonal, Clontech). Input represents 1/20th of the IP sample. Anti-GFP precipitates were probed with antibody to NS3 (top panel) or HCV core (bottom right panel) Input represents 1/20th of the IP for anti-GFP blot and 1/80th of the IP for NS3 and core blots. (B) Cell lysates prepared from HJ3-5 HCV-infected FT3-7 cells were subjected to Co-IP with HCV core (C7-50, Affinity BioReagents) or GFP-specific (mouse monoclonal, Clontech; used as isotype control) antibody and immunoblotted for HCV core (upper panel) or endogenous DDX6 (lower panel). Input represents 1/20th and 1/80th of the IP for the anti-HCV core and anti-DDX6 blots, respectively.

3.4A, right panel, compare lanes 3 and 4). We next confirmed the existence of a complex containing endogenous DDX6 and core protein by immunoprecipitating the core protein from lysates of virus-infected cells using a murine monoclonal antibody, and blotting these precipitates with antibody to DDX6. As expected, endogenous DDX6 co-immunoprecipitated with the core protein (Fig. 3.4B, bottom panel, lane 2), while an irrelevant isotype control antibody precipitated neither the core protein nor DDX6 (lane 3).

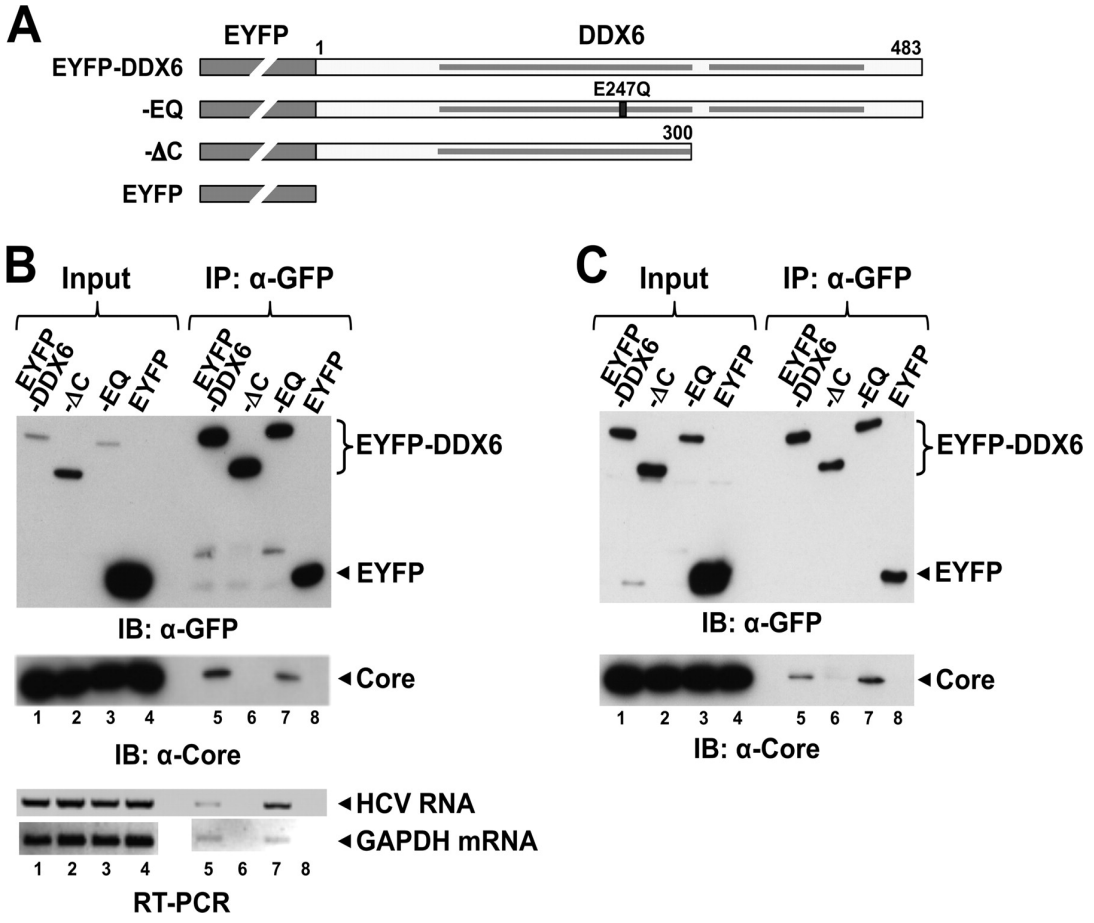

Figure 3.5. The interaction of DDX6 with HCV core and viral RNA is dependent on the C-terminal domain of DDX6. (A) Schematic representation of EYFP-DDX6 and related mutants, ΔC (C-terminal deletion mutant lacking 183 amino acids), EQ (mutation in the DEAD-box helicase motif II involving the substitution of Glu-247 by Gln) and empty vector expressing EYFP alone. The DC mutation removes the second of two conserved RecA-domains, identified by the shaded bars within the DDX6 sequence. (B) HJ3-5 HCV-infected FT3-7 cells were transfected with DNA vector expressing various forms of EYFP-DDX6 or just the EYFP. Cell lysates were prepared 2 days later and were subjected to coimmunoprecipitation (Co-IP) with anti-GFP (rabbit polyclonal, Clontech) antibody. Co-IP samples were immunoblotted with GFP (top panel) and core-specific (bottom panel) antibodies. Input represents $1/20^{th}$ of the IP for anti-GFP blot and $1/80^{th}$ of the IP for HCV core blot. Total RNA isolated from immunoprecipitates using the RNeasy Mini Kit (Qiagen) was subjected to RT-PCR for detection of genomic HCV RNA (primers targeting the NS3 region) and GAPDH mRNA. (C) Huh-7-191/20 cells were induced to express HCV core protein by removing tetracycline from the medium for 3 days. Cells were then transfected with various DNAs and subjected to Co-IP exactly as described for Fig. 3.5B.

To further characterize this complex, we generated an expression vector encoding a C-terminal DDX6 deletion mutant (pEYFP-DDX6-ΔC, or "ΔC") lacking the C-terminal 183 amino acids residues (4). This deletion removes the second of two RecA-like P-loop NTPase superfamily domains in DDX6, and based on studies with related DEAD-box family members would be expected to ablate ATPase and helicase activity (213). We also constructed a second mutant (pEYFP-DDX6-EQ, or "EQ") that lacks helicase activity due to a point mutation that replaces the Glu in the DExD-box motif (motif II) with Gln (213) (Fig. 3.5A). We transfected the wild-type (wt, pEYFP-DDX6-wt), ΔC, and EQ mutant expression vectors into virus-infected cells as described above, and prepared lysates two days later for co-immunoprecipitation studies. Similar to the results shown in Fig. 3.4A, wt DDX6 co-immunoprecipitated with core, while the ΔC mutant did not (Fig. 3.5B, lane 5 vs. 6), indicating that the C-terminal 183 amino acids are required for complex formation with the core protein. In contrast, the helicase active site mutant (EQ) was co-immunoprecipitated in a fashion similar to wt DDX6 (Fig. 3.5B, lane 7). To investigate whether other viral proteins are required for DDX6 to form a complex with core, we carried out a similar experiment using Huh-7/191-20 cells that conditionally express only the core protein (amino acids 1-191 of the HCV polyprotein) under control of the Tet-Off promoter (117). After inducing the cells to express core protein, they were transfected with the DDX6 expression vectors as above. Both the wt and EQ mutant were co-immunoprecipitated with the core protein, while the ΔC mutant was not (Fig. 3.5C, lanes 5 and 7 vs. 6). Thus, there is no requirement for other viral proteins to form complexes containing DDX6 and core, and (as indicated above), nonstructural viral

proteins known to be important for viral replication (NS3, NS5A, NS5B) do not appear to participate in this complex.

To determine if the DDX6-core complex also contains viral RNA, we extracted RNA from immunoprecipitates prepared from virus-infected and pEYFP-DDX6 transfected cells using anti-GFP, and subjected it to RT-PCR using HCV-specific primers targeting the NS3 region of the genome (226). While the amount varied between individual experiments, HCV RNA was consistently detected in precipitates of the wt and EQ mutant EYFP-DDX6 proteins, but not the ΔC mutant or EYFP (Fig. 3.5B, bottom panel). However, additional RT-PCR assays demonstrated that cellular (GAPDH) mRNA was also present in the wt and EQ mutant EYFP-DDX6 precipitates (Fig. 3.5B, bottom panel), indicating that the association of DDX6 with RNA is not specific to HCV RNA.

Taken together, the data shown in Figs. 3.4 and 3.5 indicate that DDX6 forms complexes containing core protein as well as viral and cellular RNAs in HCV-infected cells, and that the formation of these complexes is dependent on the C-terminal domain of DDX6, but independent of helicase activity. While this may reflect a direct interaction between core and DDX6, it is also possible that the co-immunoprecipitation of core and DDX6 may reflect binding to a common set of viral or cellular RNAs since the core protein has independent RNA-binding activity (185). To assess this possibility, we digested cell extracts with RNase prior to antibody precipitation. Unfortunately, the results of these experiments were inconclusive, as RNase digestion substantially enhanced non-specific interactions of core and resulted in its precipitation with irrelevant isotype control antibodies (data not shown). We also cannot exclude the possibility that core and DDX6 are bridged by a third, host-

encoded protein partner. This is not unlikely, since DDX6 and its closely related homologs interact with numerous cellular proteins (213).

DDX6 facilitation of viral replication is independent of core protein expression

To ascertain whether the influence of DDX6 expression on HCV replication might be functionally related to the DDX6-core protein complex identified above, we studied the effect of DDX6 silencing on replication of several subgenomic HCV RNAs that contain in-frame deletions removing all or part of the core-coding sequence. These included in-frame deletions of amino acid residues 61-148 within the core protein (ΔC61-148) (161), or sequence extending from amino acid residue 21 of core to the end of p7 (ΔC21-p7) (Fig. 3.6A). As a control, we also studied a mutant with an in-frame deletion spanning the E1 to p7 sequence but leaving the core sequence intact (ΔE1-p7) (226). Importantly, the core protein is not required for HCV genome amplification (127). Thus, we reasoned that we would observe no effect of DDX6 silencing on replication of viral RNAs that do not encode core if DDX6 facilitation of viral replication was mediated through the core-DDX6 complex described above. We transfected FT3-7 cells with the DDX6-1 and DDX6-1m siRNAs, and then super-transfected the cells 2 days later with the full-length or mutant subgenomic RNAs. As before, DDX6 knockdown reduced the abundance of core (34% of control siRNA-treated cells) as well as NS5A (23%) expressed by wt HJ3-5 RNA (Fig. 3.6B, compare lane 1 with lane 2). Surprisingly, the ΔC61-148 RNA, which contains a large internal deletion in the core gene, replicated very poorly even in the absence of DDX6 knockdown, as evidenced by very low level expression

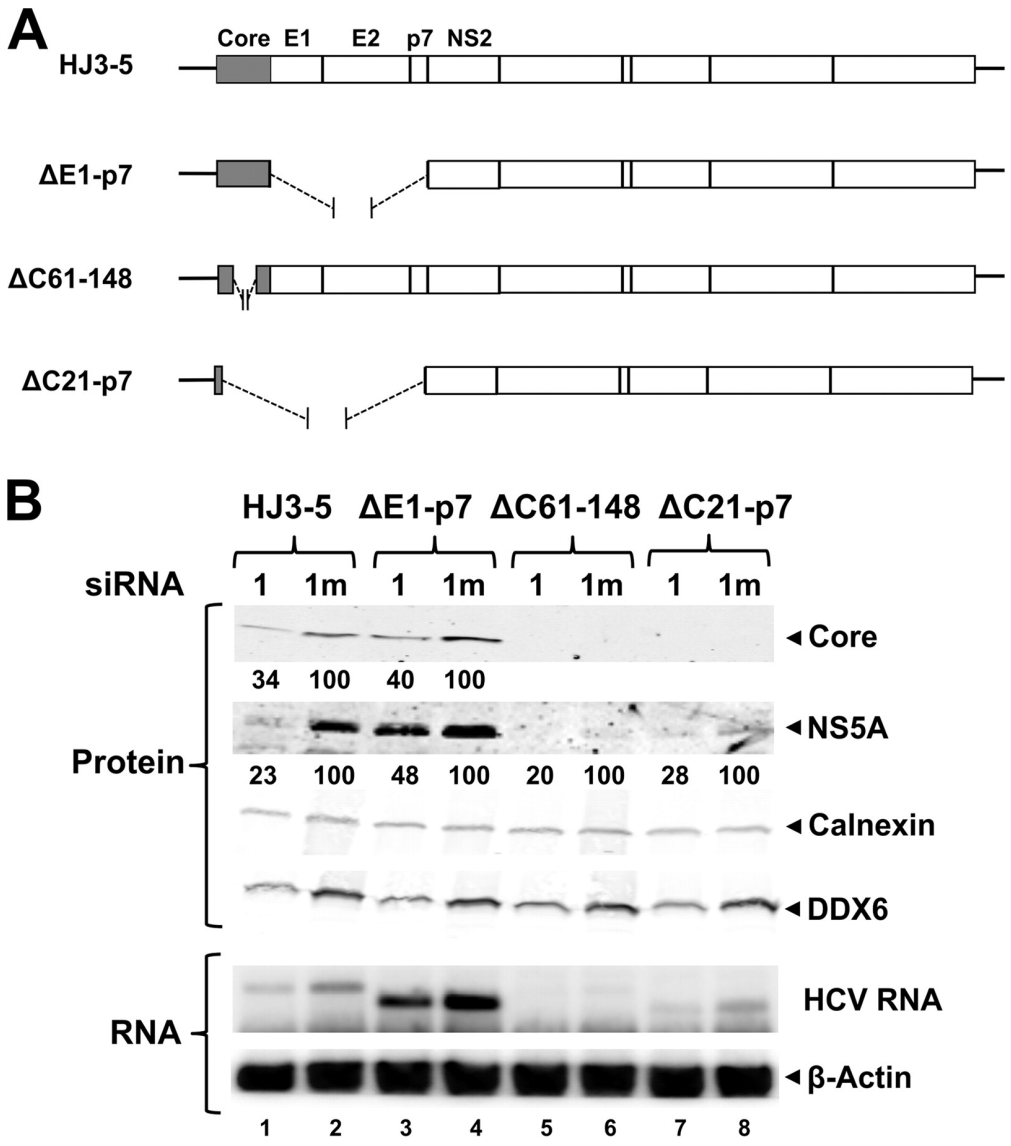

Figure 3.6. DDX6 promotes HCV replication by enhancing genome amplification. (A) Schematic representation of wildtype HJ3-5, HJ3-5-ΔE1-p7 (lacks E1, E2 and p7), HJ3-5-ΔC61-148 (lacks amino acids 61-148 of core protein) and ΔC21-p7 (containing first 20 amino acids of core fused in-frame with NS2 to 5B genes) (B) FT3-7 cells were transfected with 1 [DDX6-1] or 1m [DDX6-1m] siRNA. After 48 hrs, cells were transfected with the indicated HJ3-5 HCV RNA using TransIT® mRNA transfection reagent (Mirus Bio.). Immunoblotting of cell lysates prepared 3 days post-HCV RNA transfection is shown. HCV core and NS5A expression levels were quantitated on Odyssey Infrared Imaging system (LI-COR, Lincoln, NB) and are represented as the ratio of that in 1 [DDX6-1] siRNA treated and control, 1m [DDX6-1m] siRNA treated cells (set as 100) after normalization to calnexin levels.

of NS5A protein and little accumulation of viral RNA (Fig. 3.6B, compare lane 6 with lane 2). However, the levels of NS5A were reduced even further following DDX6 knockdown (8% of that of the DDX6-1m siRNA treated cells) (Fig. 3.6B, compare lanes 5 and 6), suggesting that DDX6 silencing further impairs the replication efficiency of this RNA despite the large internal deletion within core. The ΔC21-p7 RNA, which expresses only the first 20 amino acids of core, which were retained to facilitate IRES activity (211), replicated more efficiently than ΔC61-148 (Fig. 3.6B, compare lanes 6 and 8), but was also impaired by DDX6 silencing which reduced both viral protein as well as RNA levels (Fig. 3.6B, compare lane 7 with lane 8). These results suggest that the facilitation of HCV replication by DDX6 occurs independently of core expression, and is thus independent of the ability of DDX6 to form a complex with core.

The ΔE1-p7 RNA expresses the full-length core protein (Fig. 3.6A), but lacks the envelope protein sequence and cannot produce infectious virus (226). Its replication, judged by expression of core and NS5A, was also reduced by DDX6 silencing (Fig. 3.6B compare lane 3 with lane 4). Since neither this RNA, nor the other core-deletion variants described above, can produce infectious virus, these results also show that DDX6 functions primarily to enhance genome amplification, either by promoting translation, transcription, or stability of the viral RNA, and not by facilitating other steps in the viral life cycle (such as viral assembly, release, entry, etc.).

DDX6 helicase activity is essential to promote HCV replication

Although the EYFP-DDX6-EQ mutant, which lacks helicase activity (213), was capable of forming a complex with the core protein (Fig. 3.5), overexpression of this protein failed to enhance HCV replication (Fig. 3.7) as we had observed previously with wt EYFP-DDX6 (Fig. 3.2). In contrast, transient overexpression of EYFP-DDX6-EQ led to a reproducible decrease in HCV replication, as demonstrated by reduced levels of HCV core and NS5A protein expression (data not shown) and

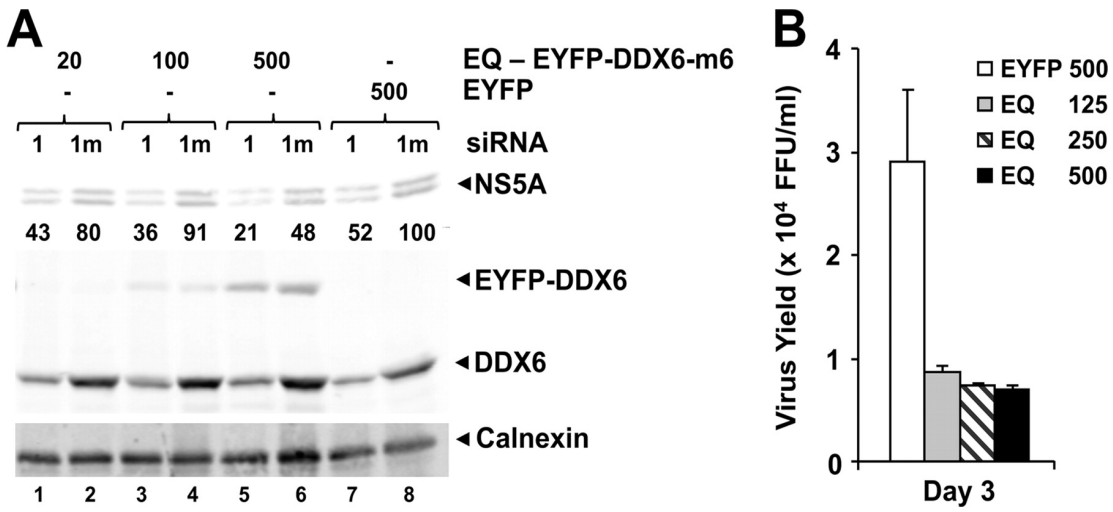

Figure 3.7. DDX6-EQ has a dominant negative effect on HCV replication. (A) FT3-7 cells were transfected with DDX6-1 (1) or DDX6-1m (1m) siRNA, and 48 hrs later re-transfected with increasing quantities of expression vectors encoding siRNA-resistant EYFP-DDX6-m6-EQ or EYFP. One day later, the cells were infected with HJ3-5 HCV (MOI = 0.5). Cell lysates prepared 3 days after infection were immunoblotted for HCV NS5A, calnexin and DDX6 proteins. NS5A band densities were quantified as described in fig 3.6A and are presented as the percent expression relative to DDX6-1m/pEYFP-transfected cells (B) FT3-7 cells were transfected with pEYFP-DDX6-EQ or pEYFP plasmid DNA. Cells were infected with HJ3-5 HCV at an MOI of 0.2 at 24 hrs post-transfection and fed with fresh media every 24 hrs. Virus supernatants collected 2 and 3 days post-infection were titrated on naïve Huh-7.5 cells, in triplicate. Representative results from one out of multiple experiments are presented here as mean ± standard deviation.

decreased virus yields (Fig. 3.7B). Reduced production of infectious virus was also observed in EYFP-DDX6-m6-EQ-expressing cells in which endogenous DDX6 had been silenced by transfection of DDX6-1 siRNA (Fig. 3.7A, compare lanes 1, 3, and 5 with 7). In aggregate, these data indicate that the helicase activity of DDX6 is essential to its ability to facilitate viral replication, and furthermore suggest that the DDX6-EQ mutant exerts a dominant-negative effect on viral replication. In contrast, overexpression of the EYFP-DDX6 ΔC mutant had no significant effect, positive or negative, on viral replication (data not shown).

The DDX6-EQ and ΔC mutants have altered cytoplasmic distribution

To better understand the inability of the EYFP-DDX6 mutants to facilitate virus replication, we utilized laser-scanning confocal microscopy to determine their subcellular localization in transfected FT3-7 cells. DDX6 is a component of P-bodies, cytoplasmic structures that are intimately involved in mRNA metabolism, degradation and storage (50). Consistent with this, we found that wt EYFP-DDX6 localized primarily to perinuclear, punctate P- body-like structures with only low level, diffuse background staining in the cytoplasm. We confirmed that the punctuate structures were P-bodies by demonstrating colocalization of EYFP-DDX6 with N-terminal ECFP-tagged Ago2 (ECFP-Ago2) in co-transfected FT3-7 cells (data not shown). In contrast, both the EQ as well as the ΔC EYFP-DDX6 mutants were distributed diffusely throughout the cytoplasm, with little if any localization to distinct P-body-like structures (EQ and ΔC panels, Fig. 3.8A). These results differ from a recent report (142), which suggested that the mutation of motif II (EQ) in the

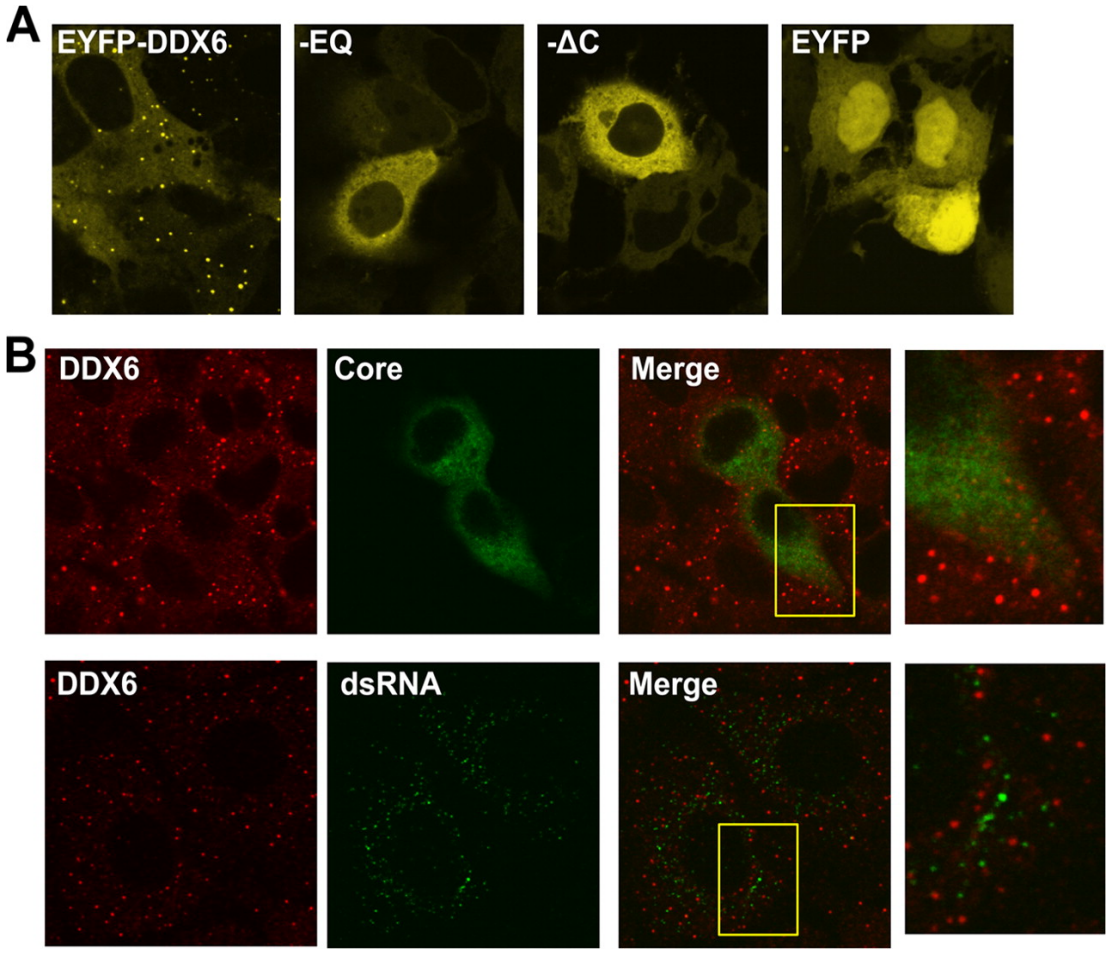

Figure 3.8. DDX6-EQ and DDX6-DC have altered subcellular distribution. (A) FT3-7 cells were transfected with pEYFP-DDX6, pEYFP-DDX6-EQ, pEYFP-DDX6-ΔC or pEYFP vector DNA. The cells were fixed with 4% paraformaldehyde 2 days later and imaged by laser-scanning confocal microscopy for EYFP fluorescence. Wild-type DDX6 was localized to punctate structures identified as P-bodies, while both mutants were distributed diffusely within the cytoplasm and EYFP alone entered nuclei. (B) FT3-7 cells were infected with HJ3-5 virus at an MOI of 0.2. Two days later, the cells were fixed with 4% paraformaldehyde, permeabilized with digitonin, stained with antibodies specific for DDX6 and HCV core (top panels), or DDX6 and dsRNA (bottom panels). In the top row of panels, HCV-infected cells are identified by cytoplasmic staining for core antigen (green) and demonstrated a moderate reduction in the number of P-bodies (identified by staining for endogenous DDX6). In the bottom row of panels, punctate staining for dsRNA (green) shows location of replicating RNA in infected cells (no such staining was observed in uninfected cells). Importantly, dsRNA did not localize to P-bodies. Expanded views of areas from the merged images (yellow boxes) are shown to the right.

Xenopus homolog of DDX6, Xp54, causes only a reduction, and not the complete loss of DDX6 localization to P-bodies that we observed. This discrepancy may reflect differences in the cell types studied and the dynamics of P-body turnover. Xp54-EQ did not support the assembly of new P-bodies, yet remained associated with previously assembled P bodies (142). Importantly, the lack of localization of DDX6-EQ to P-bodies in our studies indicates that the DDX6-core complex (which EYFP-DDX6-EQ retains the ability to form, as shown in Fig. 3.5) is not likely to be associated with P-bodies.

We next determined the intracellular localization of endogenous DDX6 in relation to viral proteins and replicating HCV dsRNA. Virus-infected cells were fixed and permeabilized, immunolabeled with antibodies to core and DDX6, and studied by laser-scanning confocal microscopy. As with ectopically expressed wt EYFP-DDX6 (Fig. 3.8A), these studies revealed endogenous DDX6 to be localized to intensely stained, punctuate perinuclear cytoplasmic structures consistent with P-bodies (Fig. 3.8B). However, low level, diffuse DDX6-specific labeling was also evident in the cytosol, again reflecting what was observed with EYFP-DDX6 (compare Fig. 3.8A and 3.8B). Consistent with previous studies, core was expressed with a granular, cytoplasmic distribution (Fig. 3.8B). Despite the evidence for formation of core-DDX6 complexes in co-immunoprecipitation experiments (Figs. 3.4 and 3.5), there was no significant co-localization of DDX6 with core in these images. This suggests that only a small fraction of the total core protein resides in complexes with DDX6 (consistent with the immunoprecipitation results in Fig. 3.4), and, as described in the preceding paragraph, the fact that the DDX6-core complex is unlikely to be associated with P bodies. As might be expected from the co-immunoprecipitation

experiments, we also observed no colocalization of DDX6 with the viral NS3 or NS5A proteins (data not shown).

Double-stranded RNA (dsRNA) is produced during replication of HCV. It can be detected by labeling infected cells with dsRNA-specific antibodies, and colocalizes with membrane-bound viral replicase complexes at the site of active viral RNA synthesis (200). HCV-specific dsRNA has also been demonstrated by microscopy within hepatocytes in frozen sections of liver biopsies from patients with chronic hepatitis C (118). We probed infected cells for the presence of dsRNA using a monoclonal antibody that recognizes dsRNA segments greater than 40 bp in length in a sequence-independent fashion (19), and that does not label any dsRNA in uninfected cells (data not shown). Dual staining of infected FT3-7 cells for DDX6 and dsRNA (Fig. 3.8B) revealed most dsRNA to be localized to punctate cytoplasmic foci which were distinct from the P bodies in which the DDX6 abundance was concentrated. These results suggest that HCV replication does not occur in close association with P-bodies, despite the localization of DDX6 to P bodies and the influence of DDX6 on HCV replication. However, these studies do not exclude the presence of small amounts of DDX6 at sites of new HCV RNA synthesis.

DDX6 does not promote HCV translation

The results described above indicate that the helicase activity of DDX6 facilitates HCV replication, but leave unresolved the mechanism by which this occurs. One possible explanation would be the specific promotion of viral translation

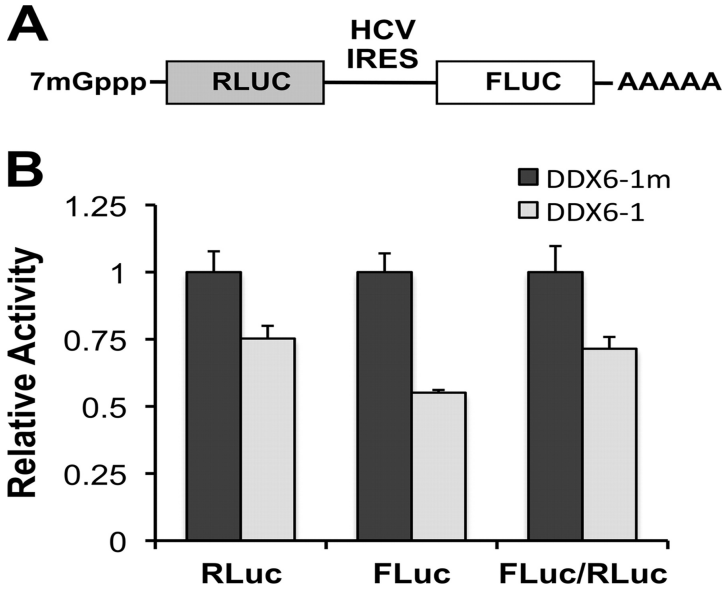

Figure 3.9. Influence of DDX6 knockdown on translation of a dicistronic RNA containing the HCV IRES. (A) Schematic representation of the pRLHL plasmid that expresses a dicistronic RNA containing *Renilla* luciferase sequence in the first cistron and firefly luciferase sequence in its second cistron, separated by the IRES of HCV. (B) pRLHL DNA was transfected into FT3-7 cells 64 hrs after transfection of the indicated siRNAs. Cells were harvested 48 hrs later for dual luciferase assays. Results shown represent RLuc (cap-dependent translation) and FLuc (IRES-directed translation) activities, and FLuc/RLuc ratio (relative IRES activity), normalized to that in DDX6-1m-transfected cells (n = 3, mean ± SD)

by DDX6 as suggested recently by Scheller et al. (178). HCV translation is directed by an IRES located within the 5'UTR that requires few canonical host translation initiation factors (130, 159). To assess its efficiency, we used a reporter plasmid (pRLHL) (79) that produces dicistronic transcripts under control of the CMV promoter in which the upstream cistron encodes *Renilla* luciferase (RLuc) and the downstream cistron firefly luciferase (FLuc), with the HCV IRES placed in the intercistronic space (Fig. 3.9A). The 3' end of the pRLHL transcript is polyadenylated. RLuc is thus translated by cap-dependent translation, while translation of FLuc is directed by the HCV IRES. We transfected FT3-7 cells with the

DDX6-1 or mutant DDX6-1m siRNAs, super-transfected the cells with the reporter plasmid, and prepared cell lysates for testing in a dual luciferase assay when silencing of DDX6 was maximal. We found that DDX6 knockdown resulted in a decrease in both RLuc and FLuc expression, with IRES-directed translation affected to a greater extent (Fig. 3.9B). In these experiments, the ratio of FLuc to RLuc reflects the activity of the HCV IRES relative to cap-dependent translation of the same transcript, and thus provides a measure of IRES activity that is independent of any differences in transfection efficiency. Concerns over possible changes in RNA stability are also eliminated since both FLuc and RLuc are expressed from the same RNA molecule. This analysis suggested that IRES activity was reduced by no more than 25-30% relative to cap-dependent translation following DDX6 silencing (Fig. 3.9B). We obtained similar results in Huh-7/191-20 cells that express core conditionally, and found no consistent effect of core protein expression on either HCV translation or the response to DDX6 knockdown (data not shown).

To further explore the effect of DDX6 silencing on HCV translation, we inserted the RLuc coding sequence (fused at its C-terminus to the foot-and-mouth disease virus 2A autoprotease) between the p7 and NS2 coding sequences of HJ3-5 to generate a replication competent, full-length HCV genome expressing the reporter protein as part of its polyprotein (HJ3-5/RLuc2A). We then constructed a replication-defective variant by creating an Asn substitution within the GDD motif of the NS5B polymerase (HJ3-5/RLuc2A-GND) (Fig. 3.10A). After silencing DDX6 expression by transfection of DDX6-specific siRNA (Fig. 3.10B), we transfected FT3-7 cells with this genome-length, replication-defective reporter RNA in combination with a capped, polyadenylated mRNA encoding FLuc as an internal control for transfection

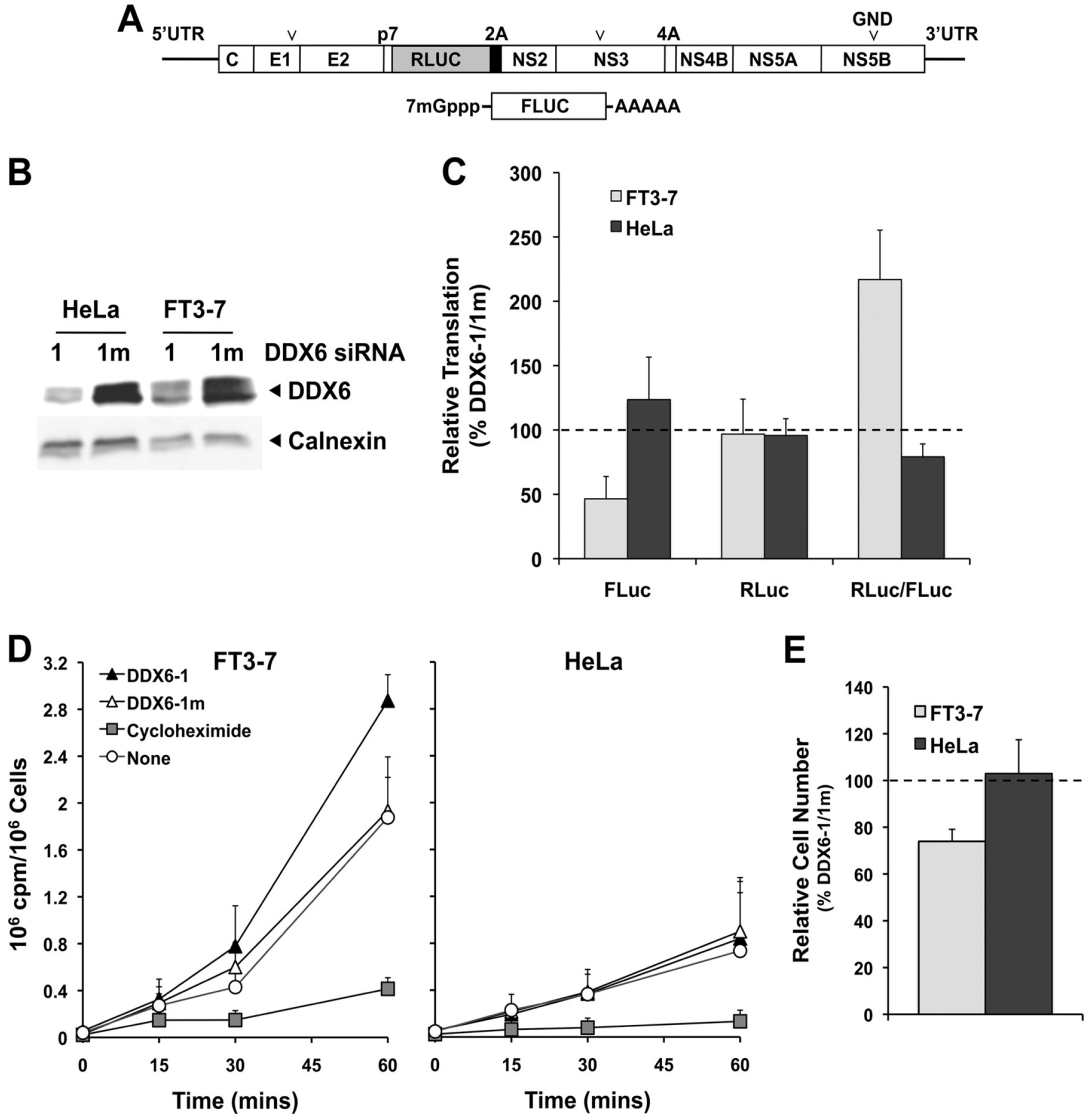

Figure 3.10. DDX6 regulation of HCV translation in FT3-7 and HeLa cells. (A) Schematic showing the genome organization of the HJ3-5/RLuc2A HCV which contains an in-frame insertion of *Renilla* luciferase (RLuc) sequence, fused at it C-terminus to the foot-and-mouth disease virus 2A autoprotease, between the p7 and NS2 coding sequences of HJ3-5 virus. The RNA also contains a replication-lethal mutation (GND) in the NS5B polymerase sequence. The 5' and 3' ends of this RNA are the authentic viral UTRs. Below is shown the capped FLuc mRNA that contains a 3' poly(A) tail of 30 adenosine residues that was used as an internal control for transfection and translation efficiencies. (B) Immunoblot showing efficient DDX6 knockdown following transfection of either FT3-7 or HeLa cells with the DDX6-1 siRNA. (C) Impact of DDX6 knockdown on HCV IRES-directed translation. FT3-7 or HeLa cells were transfected with DDX6 siRNAs, and 4 days later re-transfected with HJ3-

5/RLuc2A and FLuc mRNAs. Cells were harvested 8 hrs later and assayed for FLuc and RLuc activities. Results shown represent the mean percent ± range FLuc and RLuc activities, and RLuc/FLuc ratio, obtained in duplicate DDX6-1-transfected cultures, normalized to those obtained in DDX6-1m-transfected cells, and are representative of results from multiple experiments. (D) Metabolic labeling of FT3-7 (left panel) and HeLa (right panel) cells following DDX6 knockdown. Cells, transfected 4 days previously with siRNAs as described in panel D, were cultured for one hour in methionine- and cysteine-free medium, then incubated with 100 μCi/ml of Tran35S-Label™ (MP Biomedicals). Cells were harvested at the times indicated, and [S^{35}]-Met present in TCA precipitate measured in a scintillation counter. Results from three independent experiments are presented as cpm incorporated / 10^6 cells (mean ± S.D). (E) Impact of DDX6 knockdown on proliferation of FT3-7 and HeLa cells. Cells were enumerated 96 hrs after transfection with DDX6 siRNAs. Results are shown as the ratio of the number of cells in the DDX6-1-transfected cultures relative to those transfected with the mutant DDX6-1m siRNA at the end of this growth period (mean ± S.D., n=3).

and cap-dependent translation. Under these conditions, DDX6 silencing reduced FLuc activity expressed from the capped mRNA by about 50% (Fig. 3.10C). In contrast, RLuc expression from the HCV RNA was maintained at close to 100% that of cells transfected with the control DDX6-1m siRNA. When the RLuc activities were normalized to the FLuc values, to account for potential differences in transfection efficiency, the results suggested a two-fold increase in the relative translational efficiency of the HCV IRES following DDX6 knockdown (Fig. 3.10C). The explanation for the difference in the results shown in Fig. 3.9B and 3.10C (a small decrease vs. a 2-fold increase in IRES-directed translation relative to cap-dependent translation) is uncertain, but it may reflect differences in the 3'UTR sequences of the RNA transcripts used in these experiments. Similar experiments were done in HeLa cells, in which DDX6 knockdown was very efficient (Fig. 3.10B). We observed no significant changes in expression of either FLuc or RLuc in HeLa cells, and no apparent change in the relative efficiency of translation directed by the HCV IRES.

Because the decrease observed in translation of capped mRNAs in FT3-7 cells in both Fig. 3.9 and Fig. 3.10 is contrary to the notion that DDX6 is a general translational repressor (31, 34), we examined the overall effect of DDX6 knockdown on cellular translation during metabolic labeling with [^{35}S]-Met (31). Despite the reduction observed in translation of the transfected capped reporter RNAs, DDX6 knockdown resulted in a 50% increase in [^{35}S]-Met incorporation compared to FT3-7 cells transfected with the control siRNA, DDX6-1m (Fig. 3.10D). These results confirm that DDX6 exerts a general repressive effect on cellular translation in FT7-3 cells. The decreased FLuc activity observed with the transfected reporter RNAs is likely to reflect enhanced competition for ribosomes or translation factors by endogenous mRNAs following DDX6 knockdown. DDX6 knockdown had little effect on [^{35}S]-Met incorporation in HeLa cells (Fig. 3.10D), consistent with the absence of an effect on translation of the capped RNAs in these cells (Fig. 3.10C). In aggregate, however, the data shown in Fig. 3.10C indicate that translation directed by the HCV IRES, when placed naturally within the context of genomic RNA with authentic 5' and 3' UTR sequences, is less sensitive to changes in DDX6 abundance than cap-dependent translation of a polyadenylated transcript. Thus, the inhibition of virus replication and infectious virus yield that is observed in cells subjected to siRNA-mediated DDX6 knockdown (Fig. 3.1C) is not associated with a specific impairment of HCV RNA translation.

In addition to measuring [^{35}S]-Met incorporation, we assessed the impact of DDX6 knockdown on proliferation of FT3-7 and HeLa cells in these experiments. Interestingly, despite the increase observed in [^{35}S]-Met incorporation, FT3-7 cells were either slowed in their growth or had an increased rate of death, as we observed a

25% reduction in the number of cells present after a 96 hr incubation period compared to cells transfected with the control siRNA (Fig. 3.10E). This result is highly concordant with the WST reduction assay results described above (Fig. 3.1F). Interesting, DDX6 knockdown had no effect on the proliferation or survival of HeLa cells.

DISCUSSION

The studies we describe here provide evidence that DDX6, a member of the DEAD-box RNA helicase family, is required for efficient replication of HCV in cultured hepatoma cells. DDX6 is evolutionarily highly conserved from yeast to vertebrates and involved in multiple aspects of RNA metabolism. We employed RNA interference to knockdown DDX6 expression, and found that this substantially reduced HCV replication in Huh-7 cells (Fig. 3.1B-C and 3.1E). We confirmed a specific requirement for DDX6 by rescuing viral replication by ectopically expressing an siRNA-resistant DDX6 mutant in cells in which the expression of endogenous DDX6 had been silenced (Fig. 3.1D). We also found the requirement of DDX6 to be HCV-specific, as viral protein expression and infectious virus yields of another hepatotropic, positive-strand RNA virus, HAV (a picornavirus), were not reduced by DDX6 knockdown. DDX6 promotes genome amplification and not other aspects of the viral life cycle, since knockdown of DDX6 degrades the replication efficiency of viral RNAs with deletion mutations in structural proteins that preclude assembly of virus particles (Fig. 3.6).

DDX6 plays an important but incompletely understood role in cellular RNA metabolism, and an important concern in evaluating these results was that its knockdown by siRNA might have pleiotropic effects. This concern is magnified by the fact that HCV RNA synthesis is typically dependent on cellular proliferation (148, 179). Metabolic assays demonstrated that DDX6 knockdown has a relatively modest effect on the growth of Huh-7 cells, reducing both WST-1 reduction and cell proliferation over a 96 hr period by only ~25% (Fig. 3.1F and 3.10D). Whether this degree of cytotoxicity is sufficient to account for the 8-fold reduction in infectious virus yield observed in these cells (Fig. 3.1C) is difficult to determine, yet central to the interpretation of these results. To shed light on this question, we carried out further experiments aimed at determining whether DDX6 interacts specifically with any viral proteins, or is colocalized in cells with components of the viral replicase.

Co-immunoprecipitation experiments indicated that DDX6 forms an intracellular complex that involves the HCV core protein (Fig. 3.4), and that the C-terminal domain 2 (but not the helicase activity) of DDX6 is essential for this (Fig. 3.5). These complexes form in virus-infected cells, and also in cells expressing only core protein. They do not contain nonstructural proteins of the virus (NS3, NS5A, or NS5B), but are associated with both viral and cellular RNAs (Fig. 3.5). The existence of these complexes is of doubtful relevance to the requirement for DDX6 in HCV genome amplification, as subgenomic HCV RNAs that do not express core protein also demonstrate dependence on DDX6 expression in transient replication assays (Fig. 3.6) as well as in stable cell lines expressing HCV replicons (data not shown). This provides an interesting parallel to DDX3, another DEAD-box RNA helicase that also interacts with core (136, 156, 229). Although DDX3 is essential for efficient

HCV replication, recent studies indicate that this is not dependent on its interaction with core (7, 9). Importantly, core expression leads to the redistribution of DDX3 within the cytoplasm and co-localization with core. We did not see any significant change in the cellular distribution of DDX6 in cells expressing core, nor could we demonstrate co-localization of these proteins by confocal microscopy (Fig. 3.8). In both infected and uninfected cells, DDX6 was primarily localized to discrete, punctuate, perinuclear structures identified as P-bodies. The number and size of the P-bodies appeared to be decreased in cells infected with HCV (see Fig. 3.8B), but there was no association of core protein or viral dsRNA with these structures. On the other hand, both the EYFP-DDX6-EQ as well as EYFP-DDX6-ΔC mutants demonstrated a diffuse cytoplasmic distribution, and were only poorly localized to P-bodies (Fig. 3.8). This is consistent with a recent study showing that multiple conserved domains within the *Xenopus* homolog of DDX6, Xp54, are involved in recruitment of the protein to P-bodies (142).

Like the wild-type EYFP-DDX6, both viral and cellular RNAs co-immunoprecipitated from infected cell lysates with EYFP-DDX6-EQ, which lacks helicase activity, while this was not the case with EYFP-DDX6-ΔC (Fig. 3.5B). The RNA-binding activity of DEAD-box helicases is generally nonspecific, functionally localized to the C-terminal RecA homology domain, and not affected by mutations such as the EQ mutation in motif II of the protein (35). Since core is also a promiscuous RNA-binding protein (185), it seems likely that the complex we observed reflects binding to common RNAs rather than true protein-protein interactions. Our efforts to demonstrate an RNA-independent interaction between DDX6 and core were inconclusive, as the core protein precipitated in a nonspecific

fashion with several different antibodies following RNase A or T1 digestion of lysates (data not shown). Both wild-type DDX6 as well as the EQ mutant formed a complex with core (Fig. 3.5B-C), but only wild-type DDX6 up-regulated viral replication (Fig. 3.2). In contrast, EYFP-DDX6-EQ appeared to have a dominant negative effect on viral replication, as its overexpression resulted in reduced viral protein abundance and infectious virus yields (Fig. 3.7). This indicates that the helicase activity of DDX6 somehow facilitates replication of HCV RNA, but leaves unanswered the question whether this is a direct effect on the replicase or one that is exerted through one or more changes in cellular homeostasis resulting from loss of DDX6 helicase activity. Importantly, the capacity of DDX6 to facilitate HCV replication may be linked to its ability to localize to P bodies, since both the EQ and ΔC mutants failed to localize to P bodies.

How could DDX6 facilitate HCV replication? DDX6 is a part of the miRISC complex and miR-122, a liver-specific miRNA, is required for efficient replication of HCV (94, 95). However, DDX6 knockdown did not affect the abundance of miR-122 (Fig. 3.3A), and miR-122 supplementation reversed the defect in HCV replication observed after DDX6 knockdown (Fig. 3.3B). We conclude from these results that the ability of miR-122 to stimulate the accumulation of HCV RNA is not dependent upon DDX6, and that these two host factors promote HCV replication by independent and possibly redundant mechanisms.

While this work was on-going, Scheller et al. (178) reported that the knockdown of several proteins involved in regulating the fate of cellular mRNA, including PatL1, Lsm1-7, and Rck/p54 (DDX6), impaired the replication of HCV RNAs. In transient transfection experiments, DDX6 knockdown caused a 5-fold

reduction in replication of subgenomic RNAs and a ~30-fold decrease in infectious virus yield that these authors attributed to impaired translation directed by the HCV IRES (178). Our results do not support this conclusion. While we found that DDX6 knockdown decreased translation of a reporter protein under control of the HCV IRES placed in a dicistronic RNA, translation of the upstream cistron of this capped RNA was also decreased, resulting in only a modest net decrease in relative IRES activity (Fig. 3.9). More importantly, translation directed by the HCV IRES in its natural context in a genome-length RNA with authentic 5' and 3'UTR sequences, monitored by expression of an reporter protein embedded in the polyprotein, was not reduced by DDX6 knockdown in either Huh7 or HeLa cells (Fig. 3.10). To control for variation in transfection efficiency in this experiment, we co-transfected a capped and polyadenylated reporter mRNA with the viral RNA. In Huh-7 cells we consistently observed decreases in FLuc expression from this control RNA following DDX6 knockdown, but no changes in IRES-directed translation of RLuc embedded in the viral polyprotein, suggesting that IRES-directed translation may actually be favored in these cells following knockdown of DDX6. DDX6 knockdown caused little change in translation of either reporter in HeLa cells (Fig. 3.10). In aggregate, the results shown in Fig. 3.10 argue strongly against a specific requirement for DDX6 for HCV IRES-mediated translation. These results are consistent with the observation that DDX6 knockdown has no effect on translation directed by the classical swine fever virus (CSFV) IRES (142) which is closely related both structurally and functionally to the HCV IRES. We also found that DDX6 knockdown had no effect on the replication of HAV, a picornavirus in which translation is directed by an IRES that is heavily dependent upon cellular translation factors for its activity.

To determine the overall impact of DDX6 knockdown on cellular homeostasis, we monitored [^{35}S] incorporation as a measure of cellular translation and also determined the effect of gene knockdown on WST reduction and cellular proliferation. Interestingly, in Huh7 cells, DDX6 knockdown up-regulated translation generally but somewhat paradoxically slowed the rate of cellular proliferation (Figs. 3.1F, 3.10D and 3.10E). This is likely to reflect a general perturbation in cellular control of translation and perhaps P-body-related functions of mRNA storage and degradation. It is easy to envision how virus replication could become less efficient in this setting, making it possible that the impaired HCV replication observed both by us and by Scheller et al. (178) may stem from such general effects of DDX6 knockdown.

An alternative possibility is suggested by the fact that Dhh1p, the yeast homologue of DDX6, is required both for translation of BMV proteins as well as replication of the BMV genome in yeast cells (5). Human DDX6 can complement the loss of Dhh1p in yeast cells, but is more efficient in complementing the defect in replication than the defect in BMV translation in Dhhlp-deficient cells (5). This is consistent with our results, which suggest that DDX6 knockdown degrades RNA replication rather than IRES-directed HCV translation. Since DDX6 co-immunoprecipitates with HCV RNA (Fig. 3.5B), it remains possible that the helicase activity of DDX6 may promote viral replication by becoming engaged in remodeling of the viral RNA at some step in the HCV life cycle. This would explain not only the requirement that we have observed for DDX6 helicase activity in the promotion of HCV replication, but also the apparent dominant-negative activity of the helicase-defective DDX6-EQ mutant (Fig. 3.7). This latter observation is particularly difficult to reconcile with the hypothesis that DDX6 facilitates replication via indirect effects.

However, the extensive experimental data that we present in this communication do not allow a clear distinction to be drawn between these two possibilities, which are not mutually exclusive. Rather, they point to the challenges of determining whether host proteins act directly or indirectly in the HCV life cycle when modulation of their abundance is shown to affect viral replication.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

The liver-specific microRNA, miR-122 binds directly to the positive-strand HCV genome at two highly conserved, tandem, binding sites located within the 5'UTR between stem loops I and II (94, 95). This interaction leads to a dramatic increase in HCV RNA abundance as well as infectious virion production in human hepatoma Huh7 cells (87). Moreover, miR-122 supplementation in non-hepatic cells also leads to an increase in the replication of HCV subgenomic replicons (27, 121). Mutational studies suggest that the direct interaction of miR-122 with both miR-122 binding sites, S1 and S2, located within the 5'UTR of HCV genome also facilitates infectious virus production (Chapter 2) (87), as would be expected from the previous replicon based studies (94, 95). However, the exact mechanism by which miR-122 facilitates HCV replication is unclear. While initial studies ruled out a specific effect of miR-122 on HCV translation and/or RNA stability (94, 95) recent studies suggest that miR-122 significantly upregulates HCV translation by up to 2-fold (76). However, miR-122-mediated upregulation of viral translation by 2-fold is insufficient to fully account for the dramatic effect of miR-122 on HCV replication, suggesting that miR-122 may be involved in another step in viral replication cycle (Chapter 2) (87). Since the effects of miR-122 on HCV replication are mediated via direct binding to the viral genome, any secondary effects of miR-122 on HCV replication due to its influence on cellular metabolism and gene expression are minimal, if any (Chapter 2) (87, 94, 95, 152).

miRNAs are approximately 22 nucleotide long, single-stranded, non-coding RNAs of endogenous origin that regulate post-transcriptional gene expression by

binding to their target sequences, which are usually located in the 3'UTR of the mRNAs (103). Recruitment of the miRNA-induced silencing complex (miRISC) to the target site leads to the blockade of translation and/or enhanced degradation, in part via an increased rate of deadenylation (55, 85). Interestingly, the two miR-122-binding sites in the HCV genome are located within the 5'UTR of the viral genome (94). Insertion of the viral miR-122 binding sites in the 3'UTR of a reporter gene downregulates protein expression (94). However, artificial insertion of miRNA let-7 target sites within the 5'UTR, even upstream of the HCV IRES, leads to inhibition of gene expression, most likely by translational blockade (133). This suggests that the context in which the miR-122 binding sites are located in the viral genome is important for the "special" effect of miR-122 on HCV replication.

Since miR-122 binds to positive-strand HCV RNA, and not the negative-strand, it is logical to speculate that miR-122 might affect HCV RNA abundance in a strand-specific manner. Strand-specific northern blotting for HCV RNA in subgenomic replicon cells as well as HJ3-5 virus-infected cells suggests that miR-122 preferentially promotes the abundance of HCV positive-strand RNA (data not shown).

miR-122 can potentially affect three different steps in the viral life cycle: HCV IRES-mediated translation, RNA replication, or RNA degradation (that is, RNA stability). However, these are not mutually exclusive possibilities. There is a significant, but only 2-fold, effect of miR-122 on HCV translation (Chapter 2) (76, 87) which does not fully account for the dramatic effect of miR-122 on viral replication (Chapter 2) (87). As indicated above, this suggests that miR-122 must influence another step in viral replication. Although earlier studies suggested that

miR-122 may play a direct role in viral RNA replication based on indirect evidence (94, 95), direct evidence for a miR-122-mediated increase in RNA synthesis remains lacking. Recent studies by Norman and Sarnow (152) did not see a significant effect of miR-122 on HCV RNA synthesis in transient assays based on 4-thiouridine labeling of newly synthesized HCV RNA for 1 hr. In vitro assays with cell-free preparations of membrane-bound HCV replicase complexes further suggest that miR-122 does not affect the elongation stage of HCV RNA synthesis by preformed replicase complexes (Villanueva and Jangra et al. 2010, in press). However, these studies do not rule out a possible effect of miR-122 on initiation of RNA synthesis and/or assembly of the replicase complexes. Such an effect could ultimately be responsible for the increased viral RNA abundance. It is plausible that binding of miR-122, with or without miRISC components, to the HCV genome could lead to conformational changes that help in the recruitment of essential viral and/or cellular factors, thus facilitating RNA replication. However, future experiments to address these questions are limited by the lack of a system to study the assembly stage of HCV replicase complexes.

As a third possibility, miR-122 may affect HCV RNA stability, although the experimental data that is available thus far, derived from reporter assays after transient transfection of HCV RNAs, indicate no such effect (76, 87, 94, 95) (Chapter 2). Future experiments need to be carefully designed to look at viral RNA stability following miR-122 sequestration and/or supplementation in virus-infected cells in which replication has been blocked specifically. This could be accomplished using one of several available potent small molecule inhibitors of the HCV RNA-dependent RNA polymerase, NS5B. Such experiments would eliminate any confounding effects

of the overloading of cells with viral RNA in transfection experiments, as well as potential miR-122 enhancement of RNA synthesis in virus-infected cells, and would help to address the stability question in the context of authentic viral infection. Moreover, carefully designed experiments with proper controls to directly follow viral RNA stability via northern blotting of RNA, rather than reporter assays, following electroporation of RNA into cells instead of liposome-mediated transfection may also be useful.

What is the mechanism of miR-122 facilitated HCV translation? Most miRNA-binding sites are located in the 3'UTR of the target mRNAs that downregulate translation upon miRISC-mRNA interactions. However, miRNA binding sites artificially placed within the 5'UTR of reporter genes are still able to repress translation (133). As indicated above, the miR-122 binding sites are located within the 5'UTR of the HCV genome. However, the HCV miR-122 binding sites can still suppress translation when placed in the 3'UTR of a reporter gene (76, 94), suggesting that the context in which these sites are located is critically important for their stimulatory effect. To achieve this, miR-122 is likely to be working in concert with miRISC, as the classic miRISC components, argonaute 1-4 (Ago1-4) are also required for efficient replication of HCV (166). We now know that, at least under certain conditions, some miRNA-mRNA interactions do not always repress gene expression. For example, miR-369-3p recruits Ago2 and fragile X mental retardation-related protein 1 (FXR1) to the AU-rich element (ARE) of TNFα mRNA, and activates TNFα translation in quiescent cells but not in replicating cells (205, 206). Similarly, Embryonic Lethal Abnormal Vision (ELAV)-like protein, HuR is involved in the reversal of miR-122-mediated repression of cationic amino acid transporter 1

(CAT-1) mRNA, one of the cellular targets of miR-122, when Huh7 cells are subjected to stress (16). Interestingly, HuR has been shown to interact with the HCV 3'UTR (190) and to activate HCV IRES translation (172). Further experiments should be designed to address whether FXR1 and HuR play any role in miR-122-mediated HCV replication.

Alternatively, it is possible that miR-122 binding to the S1 and S2 sites leads to conformational changes in the HCV IRES that enhance translation and or RNA replication. Based on dsRNA-specific RNase digestion and gel-shift assays of synthetic RNA representing nts 1-570 of HCV, a recent study proposed that miR-122 binding to the 5'UTR increases HCV translation by inhibiting a previously documented long-range interaction between nts 24-38 in the 5'UTR and nts 428-442 in the core-coding region of the genome (40). This was postulated to change the IRES conformation from a less active "closed" conformation to a more active "open" conformation (40). However, this presumptive conformational switch fails to explain the miR-122-mediated increase in translation of HCV reporter RNAs that lack the core-coding sequence (76). Thus, more research will be required to fully understand the mechanism of miR-122 facilitated HCV translation.

Another important but unanswered question is the role of miRISC components, if any, in HCV replication. As alluded to above, Randall et al. (166) showed that siRNA-mediated knockdown of Ago1-4 as well as the miRNA processing enzymes drosha, DGCR-8 and dicer, reduces replication of HCV. However, whether Ago1-4 are working in concert with miR-122 and whether miR-122 requires Ago1-4 to facilitate HCV replication are unknown. Preliminary experiments using a chemically altered form of miR-122 that cannot recruit miRISC

suggests that the miR-122 effect on HCV replication is dependent on miRISC (data not shown). miR-122 supplementation experiments in cells where individual miRISC components have been already knocked down can help to address these questions. However, miR-122-mediated HCV replication is not dependent on the DEAD-box RNA helicase DDX6, an miRISC component, as miR-122 and DDX6 facilitate HCV replication via independent mechanisms (Chapter 3) (86).

DDX6 (Rck/p54) is required for efficient replication of HCV (Chapter 3) (86, 178). Experiments using subgenomic replicons suggested that DDX6 is involved in genome amplification at the level of viral translation and/or RNA synthesis, but not viral assembly or release (Chapter 3) (86, 178). Although Scheller et al. (178) reported a significant positive effect of DDX6 on HCV translation based on subgenomic and genome-length reporter RNA assays, we did not find any significant effect of DDX6 on HCV IRES-mediated translation in experiments in which we transfected cells with authentic full-length, monocistronic HCV genomes expressing a *Renilla* luciferase reporter together with a capped and polyadenylated mRNA encoding for firefly luciferase reporter as an internal control for translation and transfection (Chapter 3) (86). The explanation for the discrepancy between our results and those of Scheller et al. (178) is unclear, although it may be due to the way reporter activities were normalized in the Scheller study. While we normalized HCV IRES-directed translation to firefly luciferase reporter activity from a capped and polyadenylated mRNA that was cotransfected with the HCV RNA (Chapter 3) (86), Scheller et al. (178) used RNA copy numbers obtained from real-time RT-PCR assays performed 4 hrs post-transfection of HCV RNA. The RNA detected by such an assay may not necessarily be reflective of the abundance of translationally

competent RNA due to partial degradation and/or compartmentalization of the transfected RNA. It is possible that DDX6 might be promoting viral replication due to pleiotropic effects on cellular metabolism and/or growth, as its knockdown slowed cell growth while paradoxically increasing general cellular translation (Chapter 3) (86). This is consistent with its role as a general translational inhibitor (34). Such a disturbed cellular homeostasis might contribute to a reduction in HCV replication (Chapter 3) (86).

Alternatively, DDX6 might facilitate HCV genome amplification directly. The helicase activity of DDX6 is required for promotion of viral replication (Chapter 3) (86). Although highly speculative in nature, it is possible that DDX6 is recruited to the replicase complex where its helicase activity may somehow promote genome replication via remodeling of the replicase ribonucleoprotein (RNP) complex (124). Importantly, however, a specific, direct interaction of DDX6 with viral nonstructural proteins and/or replication complexes could not be demonstrated by coimmunoprecipitation and confocal microscopy experiments (Chapter 3) (86). Future experiments to address a direct role of DDX6 in RNP remodeling are complicated by the fact that most DEAD-box RNA helicases have redundant functions that are not well characterized (35). The HCV NS3 helicase is essential for viral replication, but its precise role in genome amplification is not clear. Whether HCV needs additional help from cellular helicases to complete its RNP-intensive replication cycle is an interesting but unanswered question. Various DEAD-box RNA helicases, e.g. RNA helicase A (RHA/Dhx9), RH116, DDX1, DDX3 and DDX24 (134), have been demonstrated to have multiple roles in HIV replication [reviewed in (88)]. DDX6 and its yeast homologues are also involved in the replication and

translation of brome-mosaic virus (BMV) – a positive-strand RNA virus (5). With BMV, DDX6 plays a greater role in replication than translation, similar to the influence of DDX6 on HCV replication. Further experiments will be required to determine whether it does indeed play a specific role in genome amplification.

Interestingly, other DEAD-box RNA helicases (DDX3 and DDX5/p68) have also been implicated in HCV replication. DDX5/p68 interacts with the viral RNA-dependent RNA polymerase, NS5B, leading to its redistribution from nucleus to cytoplasm (64). However, the relevance of this finding is unclear as DDX5/p68 knockdown affected HCV replication in transient transfection experiments only (64). DDX3 interacts with HCV core protein (136, 156, 229) and is required for viral replication (7, 9). However, the core-DDX3 interaction is not essential for its role in viral replication (7). DDX6 is also required for HCV replication and interacts with the viral core protein via its helicase domain (Chapter 3) (86). Similar to DDX3, its role in replication is also independent of the core-DDX6 interaction (Chapter 3) (86), suggesting a common theme. One possibility is that the core interaction with DDX6 and DDX3 contributes to viral pathogenesis, which may not observed in a cell culture system, and that these interactions may still be important in vivo in an infected individual. These questions can only be addressed in animal experiments. Mapping the DDX6 binding site in the HCV core protein could provide for an interesting comparison of DDX3-core and DDX6-core interactions. Both DDX6 and DDX3 have been implicated in a variety of processes such as carcinogenesis (1, 2), in addition to their role in virtually all aspects of RNA metabolism (124, 125, 173). HCV-related hepatocellular carcinoma is a complex condition that develops over a period of decades and it is possible that the interaction of core with these RNA helicases may

contribute to carcinogenesis. Understanding the role of DDX6 in particular and RNA helicases in general will further our knowledge of RNA biology in cellular physiology and pathology.

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PUBLICATIONS

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ABSTRACTS

Jangra, R.K, Yi, M. and Lemon, S.M. DEAD-box RNA helicase DDX6 is required for efficient replication of cell culture-infectious hepatitis C virus. Oral presentation at the 28th Annual Meeting of American Society for Virology (ASV), 11th – 16th July 2009, Vancouver BC, Canada.

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