CHARACTERIZATION OF INTERACTIONS BETWEEN HEPATITIS C VIRUS AND HOST PROTEINS AND THEIR EFFECTS ON APOPTOSIS

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NATIONAL UNIVERSITY OF SINGAPORE

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AUTHOR'S PUBLICATIONS

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TABLE OF CONTENTS

CHAPTER 3: HCV CORE PROTEIN CONTAINS A BH3 DOMAIN THAT REGULATES APOPTOSIS THROUGH SPECIFIC INTERACTION WITH HUMAN MCL-1

SUMMARY

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Although the molecular mechanisms of HCV pathogenesis remain unclear, HCV proteins are known to interact with various host factors and modulate diverse cellular pathways including apoptosis. Modulation of host cell apoptosis contributes to HCV-associated liver injury. However, the mechanisms underlying HCV-mediated modulation of apoptosis are poorly understood.

In this study, we have identified a Bcl-2 homology 3 (BH3) domain in the HCV core protein that is essential for its proapoptotic property and for specific interaction with the human myeloid cell factor 1 (Mcl-1), a prosurvival member of the Bcl-2 family. Moreover, the overexpression of Mcl-1 and the absence of proapoptotic Bax, protected cells against core protein-induced apoptosis. By using peptide mimetics as well as protein coexpression, the core protein was found to functionally complement the proapoptotic activity of Bad. Thus, the core protein is a bona fide BH3-only protein having properties similar to those of Noxa, a BH3-only member of the Bcl-2 family that binds preferentially to Mcl-1. The BH3 domain of the core protein contains three hydrophobic residues that are essential for its proapoptotic activity. Furthermore, the genotype 1b core protein is more effective than the genotype 2a core protein in inducing apoptosis due to a difference in one of these hydrophobic residues (residue 119). Substituting this residue in the J6/JFH-1 infectious clone (genotype 2a) with the corresponding amino acid in the genotype 1b core protein produced a mutant

virus, J6/JFH-1(V119L), which induced significantly higher levels of apoptosis in infected cells than the parental virus. In addition, the core protein of the mutant virus, but not that of the parental J6/JFH-1 virus, interacted with endogenous Mcl-1 in infected cells. Taken together, our findings show that the core protein is a novel BH3-only viral homologue that contributes to apoptosis induction during HCV infection.

Induction of apoptosis by the J6/JFH-1 virus, which lacks a functional BH3 domain in the core protein, indicates the presence of alternative pathways of HCV-induced apoptosis. Gene expression profiling of cells infected with a cell culture adapted variant of the J6/JFH-1 virus revealed upregulation of primarily proapoptotic genes (BIK, FAS, GADD45A and HRK) and downregulation of BNIP3 expression. Interestingly, the expression of Bcl-X_L, a prosurvival Bcl-2 protein, was also enhanced by HCV infection. When the level of Bcl-X_L in infected cells was further increased by stable transfection, there was a reduction in HCV-induced apoptosis, viral RNA replication and progeny virus release. When the upregulation of Bcl-X_L in infected cells was prevented by RNA interference, HCV-infected cells displayed higher levels of apoptosis and an initial spike in progeny virus release. However, enhanced apoptosis also resulted in a reduction in viable cell number and lower viral RNA replication was observed. Taken together, our results show that HCV can modulate host cell apoptosis via diverse pathways in order to maintain a fine balance between cell survival and apoptosis which maximises both viral replication and apoptosis-dependent virus release.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
AMC	7-amino-4-methyl coumarin
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
BSA	bovine serum albumin
CIU	cell-infecting unit
CPE	cytopathic effect
Ct	threshold cycle
CTL	cytotoxic T lymphocyte
cyt c	cytochrome c
d.p.i.	days post-infection
DAPI	4',6-diamidino-2-phenylindole
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
ffu	focus-forming unit
FITC	fluorescein isothiocyanate
G418	Geneticin
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione *S*-transferase
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus cell culture
HCVpp	hepatitis C virus pseudoparticle
hnRNP K	heterogeneous nuclear ribonucleoprotein K
HRP	horseradish peroxidase
HVR	hypervariable region
IFN	interferon
IgG	immunoglobulin G
IP	immunoprecipitation
IRES	internal ribosome entry site
ISDR	interferon-alpha sensitivity-determining region
JFH-1	Japanese fulminant hepatitis 1
LD	lipid droplet

LDL	low-density lipoprotein
Mcl-1	myeloid cell factor 1
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeability
mRNA	messenger ribonucleic acid
NANBH	non-A, non-B hepatitis
NF-κB	nuclear factor-kappa B
NS	non-structural
NTPase	nucleoside triphosphatase
ORF	open reading frame
p.i.	post-infection
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.05% Tween-20
PCR	polymerase chain reaction
Peg-IFNα-ribavirin	pegylated interferon-alpha and ribavirin
PI3K	phosphoinositide 3-kinase
PKR	RNA-activated protein kinase
qPCR	quantitative polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene I
RLU	relative light unit
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
STAT	signal transducers and activators of transcription
TGF-β	transforming growth factor-beta
TLR	Toll-like receptor
TMD	transmembrane domain
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
UTR	untranslated region
vBcl-2	viral Bcl-2 homologue
VLDL	very-low-density lipoprotein

CHAPTER 1: INTRODUCTION

1.1 Hepatitis C virus (HCV)

1.1.1 Epidemiology

HCV infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Approximately 2 – 3% of the world's population, or about 130 – 170 million people, are chronically infected with HCV and 3 – 4 million new infections are estimated to occur each year (WHO, 2011). Diagnosis of acute HCV infection is difficult as most of the cases are asymptomatic (McCaughan et al., 1992). Only 15 – 25% of infected individuals manage to clear the virus within 6 months after onset of acute infection while the rest progress to chronic active hepatitis (Alter and Seeff, 2000; Chen and Morgan, 2006; Thimme et al., 2001). Liver cirrhosis slowly develops in about 10 – 20% of individuals with chronic HCV infection and appears to be closely associated with the development of HCC in 1 – 5% of chronic HCV patients (Chen and Morgan, 2006; Colombo, 1999; Degos et al., 2000; NIH, 2002a).

HCV has been shown to have a worldwide distribution with high prevalence rates of chronic infection in Egypt, Pakistan and China (Lavanchy, 2011; WHO, 1997, 2000, 2011). In the United States and Europe, end-stage liver disease due to HCV infection is the most common indication for liver transplantation (Charlton, 2001). Hence, hepatitis C presents a major global health burden. To date, there is no vaccine available for the prevention of HCV infection and the current standard therapy, a combination of pegylated

interferon-α and ribavirin (Peg-IFNα-ribavirin), is often poorly tolerated and only effective in about 50% of patients (Feld and Hoofnagle, 2005; Houghton and Abrignani, 2005; Leroux-Roels, 2005; Zeuzem, 2004). Recent clinical trials have demonstrated that the use of viral protease inhibitors in combination with Peg-IFNα-ribavirin may have promising therapeutic applications against HCV (Kwo et al., 2010; McHutchison et al., 2010). Together with other direct acting antiviral drugs currently in development, they may offer major advances in HCV therapy (Ciesek and Manns, 2011).

1.1.2 Classification

Post-transfusion non-A, non-B hepatitis (NANBH) cases were first reported in the mid-1970s (Alter et al., 1975; Feinstone et al., 1975; Prince et al., 1974). However, the search for the causative agent of NANBH was greatly hampered by the lack of suitable cell culture or animal models for the propagation of the unidentified agent. A breakthrough was achieved through the successful transmission of the NANBH agent into chimpanzees and subsequent findings suggested that the etiological agent of NANBH would be a small, enveloped virus (Bradley et al., 1985; Feinstone et al., 1983; He et al., 1987; Tabor et al., 1978). In 1989, immunoscreening of an expression library derived from nucleic acids in the plasma of NANBH-infected chimpanzees led to the identification of the HCV genome (Choo et al., 1989).

Following its discovery, HCV was classified as the prototype member of the *Hepacivirus* genus within the *Flaviviridae* family (Choo et al., 1991). This virus family includes the classical flaviviruses (for example, dengue,

yellow fever and tick-borne encephalitis viruses), the animal pestivirus (for example, bovine viral diarrhoea virus) and GB viruses A (GBV-A), GBV-B and GBV-C (Thiel, 2005). HCV displays extensive sequence variability between different isolates, as well as a diverse population of quasispecies within each infected individual (Bukh et al., 1995; Kato et al., 1990; Simmonds, 2004). The high replication rate of HCV together with the lack of proofreading capacity by the viral RNA polymerase are responsible for this genetic diversity (Neumann et al., 1998).

There are six major HCV genotypes (numbered 1 through 6) and more than 50 subtypes (designated a, b, c and so on) within these genotypes (Simmonds et al., 2005; Simmonds et al., 1993). The sequence divergence of genotypes and subtypes are 30 – 35% and 20 – 25% respectively. The HCV genotypes exhibit differences in their geographical distribution, prevalence, pathobiology and response to therapy. Genotypes 1, 2 and 3 are globally distributed while genotypes 4, 5 and 6 are found in more distinct geographical locations (WHO, 2009). A close relationship between hepatic steatosis and HCV genotype 3 infection has been described and host genetic factors have also been shown to influence the degree of steatosis in patients infected with genotype 3 (Mihm et al., 1997; Rubbia-Brandt et al., 2000; Zampino et al., 2008). A clinically important difference among HCV genotypes is their susceptibility to IFNα-based therapy. The highly prevalent HCV genotype 1 has been shown to be more resistant to therapy compared to genotypes 2 and 3 (Kanai et al., 1992; NIH, 2002b).

1.1.3 Genome organization

The HCV genome is well characterized as a 9.6 kb single-stranded, positive-sense RNA molecule that encodes a large precursor polyprotein of approximately 3000 amino acids. It is composed of a single open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (UTRs) (Figure 1.1). Like other positive-stranded RNA viruses, the HCV genome serves as a template for both translation of viral proteins and RNA replication. The 5' UTR is highly conserved across HCV genotypes and possesses a complex secondary structure made up of four distinct domains (I – IV) (Honda et al., 1999). Domains II-IV forms an internal ribosome entry site (IRES) that mediates cap-independent translation of the viral RNA (Friebe et al., 2001). Although domain I is not required for IRES activity, domains I and II are critical for HCV RNA replication (Kim et al., 2002). Interaction of an abundant liver-specific microRNA (miRNA), miR-122, with binding sites in the 5' UTR has been shown to facilitate viral RNA replication (Jopling et al., 2006). HCV RNA replication is also regulated by the 3' UTR which is composed of a variable region, a poly(U/UC) tract and an extremely conserved 98 nucleotide X-tail at the 3' end of the HCV genome (Blight and Rice, 1997; Friebe and Bartenschlager, 2002; Tanaka et al., 1996). In addition, the X-tail interacts with a *cis*-acting replication element (CRE) in the NS5B-encoding region to form a tertiary RNA structure that is required for RNA replication (Friebe et al., 2005; You et al., 2004). Another long range RNA-RNA interaction involving the 5' and 3' UTRs is also essential for replication and strongly enhances IRES-mediated translation (Song et al., 2006).

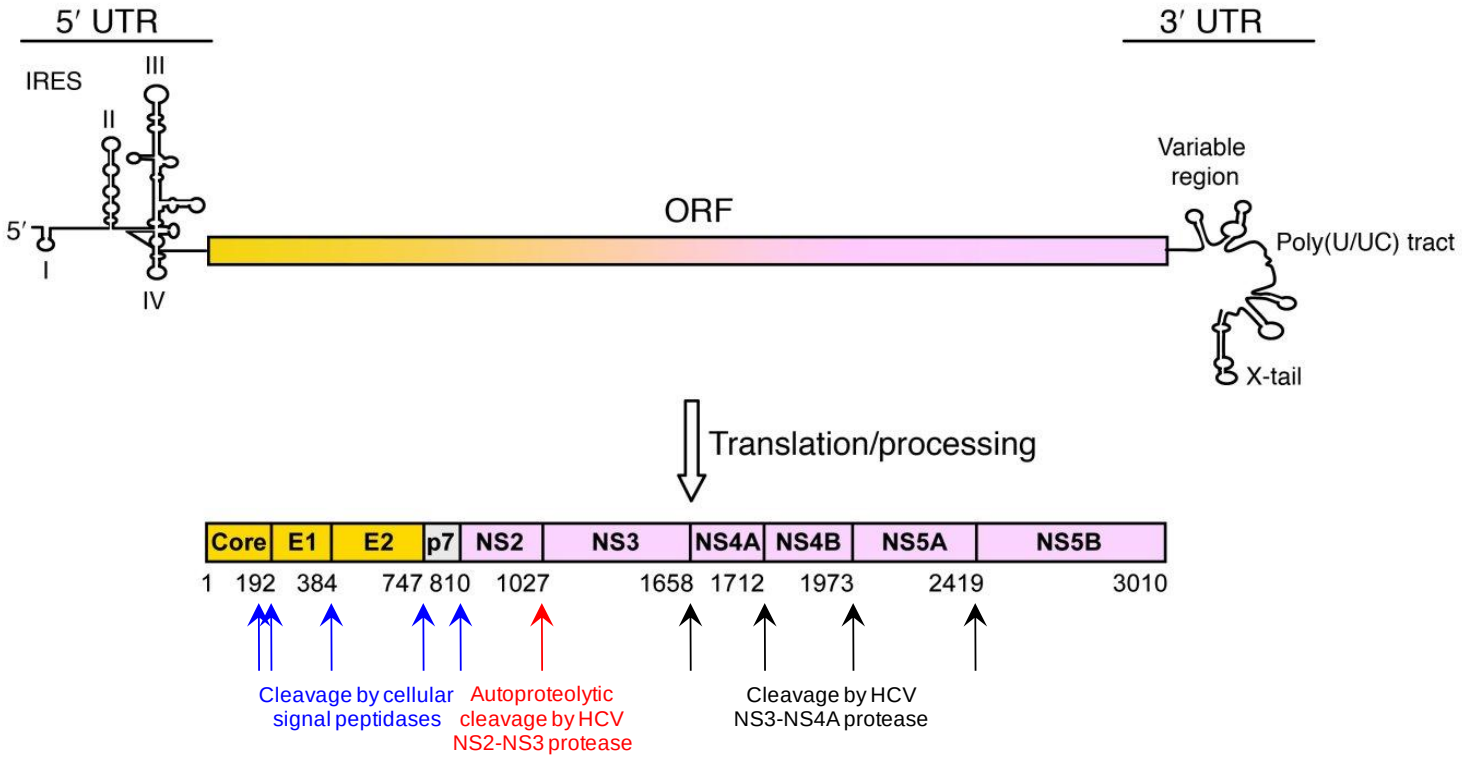

Fig. 1.1. Genome organization and polyprotein processing of HCV. The HCV genome is composed of a 5' untranslated region (UTR), a long open reading frame (ORF) and a 3' UTR (top). The conserved RNA elements in the 2 UTRs essential for replication and translation are shown. IRES, internal ribosome entry site; C, cytidine; U, uridine. The translated polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different products (bottom). The structural proteins are represented by yellow boxes while the non-structural (NS) proteins are represented by pink boxes. Amino acid numbers are shown below each protein and arrows indicate the cleavage sites on the HCV polyprotein. Figure adapted from Rehermann, B. *J. Clin. Invest.* **119**:1745 – 1754 (2009).

1.1.4 Viral proteins

The translated polyprotein is processed through a series of co- and post-translational cleavage events catalyzed by cellular and viral proteases (Figure 1.1). Cleavage by the endoplasmic reticulum (ER) signal peptidase liberates the N-terminal structural (core, E1 and E2) and p7 proteins (Hijikata et al., 1991). Maturation of the core protein is achieved through further processing by a signal peptide peptidase to remove the E1 signal peptide (McLauchlan et al., 2002). The C-terminus of the mature core protein is not precisely known but lies between residues 170 and 179 (Lai and Ware, 2000; McLauchlan, 2000; Ray and Ray, 2001). Using mass spectrometry analysis, the C-terminal end of the mature core protein expressed in Sf9 insect cells or 293T cells has been identified as Phe177 or Leu179 (Hussy et al., 1996; Ogino et al., 2004; Okamoto et al., 2008). The non-structural (NS) proteins are processed by two virally encoded enzymes, the NS2-3 cysteine protease and the NS3-4A serine protease. The NS2-3 protease cleaves at the junction of NS2 and NS3 while the NS3-4A protease processes the remaining proteins, namely NS4A, NS4B, NS5A and NS5B (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993). The HCV genome also contains a functional alternative reading frame that overlaps with the core protein gene and encodes an ARFP/F/core+1 protein (Walewski et al., 2001). To date, little is known of the biological role of the ARFP/F/core+1 protein (Vassilaki and Mavromara, 2009). The focus of our study is the nucleocapsid core protein and the properties and functions of this protein will be discussed in detail in section

1.1.4.2. The characteristics and roles of the other structural and non-structural proteins are outlined in Table 1.1.

1.1.4.1 Structural and non-structural proteins

Table 1.1 Summary of HCV structural and non-structural proteins

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
E1 (192 aa) and E2 (363 aa)	The E1 and E2 proteins are Type I transmembrane proteins with a large hydrophilic N-terminal ectodomain and a short C-terminal transmembrane domain (TMD) (Op De Beeck et al., 2004). The envelope proteins are mainly retained in the ER and ER retention signals have been mapped to the TMDs of E1 and E2 (Cocquerel et al., 1999; Cocquerel et al., 1998; Rouille et al., 2006). Virion-associated E1 and E2 proteins form large covalent heterodimers which are stabilized by disulfide bridges (Vieyres et al., 2010). The ectodomains of both envelope proteins undergo extensive post-translational N-linked glycosylation in the ER lumen. The E1 and E2 proteins harbour up to 6 and 11 putative glycosylation sites, respectively (Goffard and Dubuisson, 2003). Two hypervariable regions (HVR) have been identified in the E2 protein sequence. The first 27 residues of the E2 ectodomain represent HVR1 while amino acid residues 91 to 97 define HVR2 (Kato et al., 1992; Weiner et al., 1991).	The E1-E2 complex plays critical roles in virus adsorption, entry and assembly (Bartosch and Cosset, 2006). The E2 protein directly interacts with putative HCV receptors including CD81 and SR-BI (Pileri et al., 1998; Scarselli et al., 2002). The N-linked glycans and variable regions of the E2 protein have been shown to contribute to glycoprotein folding and virus entry (Callens et al., 2005; Goffard et al., 2005; McCaffrey et al., 2007). Based on sequence analysis and computational modelling, the E1 protein has been proposed to be a truncated class II fusion protein, possibly involved in the membrane fusion step (Garry and Dash, 2003).	The E2 protein elicits a neutralizing immune response and HVR1 has been shown to be a major target of neutralizing antibodies (Farci et al., 1996; Schofield et al., 2005). A recent study using antibodies against HVR1 revealed that the region plays an important role in virus entry at a post-attachment step (Vieyres et al., 2011). *In vitro* studies have shown that the E2 protein can interact with and inhibit the activity of interferon-inducible protein kinase PKR, thus possibly interfering with the antiviral effects of interferon (Pavio et al., 2002; Taylor et al., 1999). The envelope glycoproteins have been shown to induce ER stress and the unfolded protein response (Chan and Egan, 2005; Liberman et al., 1999). This may be related to the observation that E1 and E2 induce apoptosis when transiently expressed (Chiou et al., 2006; Ciccaglione et al., 2004). However, E2 displayed prosurvival activity when stably expressed and a recent study showed that E2-CD81 interaction protects B cells against apoptosis (Chen et al., 2011; Lee et al., 2005).

Table 1.1 − Continued

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
p7 (63 aa)	The p7 protein is an integral membrane protein consisting of two transmembrane segments linked by a short cytoplasmic loop with its N- and C-termini oriented towards the ER lumen (Carrere-Kremer et al., 2002). When expressed from a replication-competent genome, the full length p7 protein displayed ER localisation (Haqshenas et al., 2007).	The p7 protein is essential for infectivity of HCV *in vivo* but is not required for RNA replication, suggesting a role in virus particle assembly and release (Lohmann et al., 1999; Sakai et al., 2003). The interactions of p7 with HCV structural proteins and/or NS2 may be crucial for these processes (Steinmann et al., 2007; Yi et al., 2007). The p7 protein may function as a viroporin since it has been shown to form oligomers with ion channel activity (Montserret et al., 2010). The p7 protein may also be required to direct insertion of NS2 into the ER membrane (Tedbury et al., 2011).	It has been proposed that p7 may modulate host cell apoptosis by altering mitochondrial membrane permeability in a similar manner to other viroporins (D'Agostino et al., 2002; Jacotot et al., 2001; Rahmani et al., 2000). Moreover, p7 and other viroporins have been shown to induce caspase-dependent apoptosis in baby hamster kidney cells (Madan et al., 2008). Using a yeast two-hybrid system, p7 has been shown to bind many human liver proteins including tetraspanins and nucleoporin 214ku (NUP14) (Huang et al., 2005b). However, the significance of these interactions needs to be further investigated.
NS2 (217 aa)	The highly hydrophobic N-terminal domain of NS2 is proposed to contain multiple transmembrane segments that anchor the protein to the ER (Santolini et al., 1995; Yamaga and Ou, 2002). The C-terminal portion of NS2 contributes to the catalytic activity of the NS2-3 protease (Pallaoro et al., 2001). A crystal structure of the catalytic domain of the NS2-3 protease reveals that NS2 forms a dimeric cysteine protease with two composite active sites (Lorenz et al., 2006).	Together with the N-terminus of NS3, NS2 forms the NS2-3 protease responsible for the intramolecular cleavage of NS2 from NS3 (Grakoui et al., 1993). NS2 is dispensable for viral RNA replication in vitro but is essential for the production of infectious virus in vivo, possibly acting at an early stage of virion morphogenesis (Jones et al., 2007; Pietschmann et al., 2006).	NS2 potentially regulates cellular protein levels by interfering with transcription or translation. Two different studies have described inhibitory effects of NS2 on a variety of viral and cellular promoters including those involved in cytokine production (Dumoulin et al., 2003; Kaukinen et al., 2006). NS2 has been shown to inhibit CIDE-B-induced apoptosis through direct interaction and possibly by preventing CIDE-B dimerization or mitochondrial localization (Erdtmann et al., 2003). In addition, transient and stable expression of NS2 has been shown to inhibit cell proliferation (Yang et al., 2006).

Table 1.1 − Continued

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
NS3 (631 aa) and NS4A (54 aa)	The N-terminal domain of NS3 contains serine protease activity (Gallinari et al., 1998). The NS3 serine protease domain adopts a chymotrypsin-like fold with two β-barrel subdomains (Love et al., 1996). The C-terminal domain of NS3 contains RNA helicase/NTPase activity (Gallinari et al., 1998). The NS3 helicase couples unwinding of RNA to ATP hydrolysis and belongs to the DExH/D-box helicase superfamily 2 (Tai et al., 1996). Crystal structures of the helicase domain revealed that it is composed of three subdomains of similar size (Kim et al., 1998; Yao et al., 1997). The NS3 helicase can function as a monomer or oligomer depending on the concentrations of substrate and enzyme available (Jennings et al., 2009). The central portion of NS4A contributes one β-strand to the NS3 protease domain while the hydrophobic N-terminal portion of NS4A forms a transmembrane α-helix that targets the NS3-4A complex to the ER (Kim et al., 1996; Lin et al., 1995; Wolk et al., 2000).	The NS3-4A protease is responsible for the polyprotein processing of the non-structural proteins downstream of NS3 (Bartenschlager et al., 1995; Failla et al., 1994). NS4A functions as a cofactor for the protease and enhances the protease activity of the NS3-4A complex in polyprotein processing (Tanji et al., 1995). The NS3 helicase may function to unwind stem-loop structures and replicative double-stranded RNA products, eliminate stable RNA secondary structures and/or remove nucleic acid binding proteins during viral RNA replication. The NS3 helicase activity is positively regulated by interactions between the serine protease and helicase domains as well as by the presence of NS4A (Frick et al., 2004; Pang et al., 2002). Recently, modulation of NS3 helicase activity by NS5B has also been described (Jennings et al., 2008). The C-terminus of NS4A plays a role in viral RNA replication and virus assembly through interactions with other replicase components (Lindenbach et al., 2007; Phan et al., 2011).	The NS3-4A protease has been shown to interfere with innate immune sensing in the host cell. It disrupts the retinoic acid-inducible gene I (RIG-I) viral RNA-sensing pathway by cleaving and inactivating a crucial adaptor protein of the pathway, MAVS/Cardif/IPS-1/VISA (Bellecave et al., 2010; Li et al., 2005b; Meylan et al., 2005). In addition, it cleaves TRIF/TICAM-1, a key adaptor protein of the Toll-like receptor 3 (TLR3) double-stranded RNA-sensing pathway (Li et al., 2005a). Inhibition of RIG-I and TLR3 signalling blocks the cellular interferon antiviral response. The NS3-4A also cleaves T-cell protein tyrosine phosphatase which results in enhanced epithelial growth factor-induced signalling (Brenndorfer et al., 2009). Cleavage of MAVS by the NS3-4A protease also results in inhibition of MAVS-induced apoptosis (Lei et al., 2009). Moreover, NS3 may suppress host cell apoptosis by interacting with p53 and inhibiting p53-mediated transcriptional activation (Deng et al., 2006; Tanaka et al., 2006). On the other hand, several other studies have described proapoptotic activities for both NS3 and NS4A (Nomura-Takigawa et al., 2006; Prikhod'ko et al., 2004; Thoren et al., 2004).

Table 1.1 − Continued

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
NS4B (261 aa)	NS4B is a small hydrophobic protein which is predicted to contain four transmembrane segments, a cytosolic C-terminus and an N-terminus with dual topology (Lundin et al., 2003). Different studies have shown that the N- or C-terminus may be involved in targeting NS4B to the ER (Elazar et al., 2004; Gouttenoire et al., 2009; Liefhebber et al., 2009). NS4B has been reported to be palmitoylated at two C-terminal cysteine residues and to form oligomers (Yu et al., 2006). A nucleotide-binding motif has been identified in NS4B (Einav et al., 2004).	NS4B induces the formation of a membranous web that may serve as a scaffold for the replication complex (Egger et al., 2002). NS4B is essential for HCV RNA replication and may also have a role in virus assembly (Blight, 2007; Jones et al., 2009). In relation to its nucleotide-binding motif, NS4B has been shown to possess both adenylate kinase and nucleotide hydrolase activities (Einav et al., 2004; Thompson et al., 2009). However, the functions of these enzymatic activities in HCV replication are still unclear.	NS4B has been shown to induce ER stress and the unfolded protein response (Li et al., 2009; Zheng et al., 2005). Furthermore, direct interaction between NS4B and ATF6, an ER membrane transducer, has been reported (Tong et al., 2002). NS4B also enhances the expression of sterol regulatory element-binding proteins via the phosphatidylinositol-3 kinase (PI3K) pathway, leading to cellular lipid accumulation (Park et al., 2009). The nucleotide-binding motif of NS4B has been shown to contribute to cellular transformation (Einav et al., 2008). NS4B also has been implicated in the suppression of RIG-I-mediated interferon antiviral response (Tasaka et al., 2007; Xu et al., 2009).

Table 1.1 − Continued

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
NS5A (448 aa)	NS5A is predominantly hydrophilic and contains an N-terminal amphipathic helix which serves as an in-plane anchor in the cytosolic leaflet of the membrane bilayer (Penin et al., 2004). NS5A has been reported to localise to the perinuclear regions of ER and lipid droplets (Brass et al., 2002; Shi et al., 2002). Besides the N-terminal helix, NS5A can be defined by three distinct domains, of which domain I is best characterized (Tellinghuisen et al., 2004). A crystal structure of domain I revealed that it is composed of a basic N-terminal subdomain IA containing four conserved cysteine residues that coordinate a zinc ion, and an acidic C-terminal subdomain IB (Tellinghuisen et al., 2005). Dimerization of domain I results in the formation of a cytosolic-facing basic groove. NS5A exists in basally phosphorylated (p56) and hyperphosphorylated forms (p58). Hyperphosphorylation is dependent on the presence of other NS proteins (Kaneko et al., 1994; Koch and Bartenschlager, 1999; Liu et al., 1999; Neddermann et al., 1999).	NS5A is essential for HCV replication and forms part of the viral replicase complex (Mottola et al., 2002; Shimakami et al., 2004). The groove formed by the NS5A dimer is proposed to accommodate and protect the viral RNA during replication and consistent with this, NS5A has been shown to bind HCV RNA (Huang et al., 2005a). The phosphorylation state of NS5A modulates the efficiency of viral RNA replication and the hyperphosphorylated form correlates with reduced replication, probably through inhibition of NS5A-hVAP-A interaction (Appel et al., 2005; Evans et al., 2004; Neddermann et al., 2004).	The central portion of NS5A contains the interferon-α sensitivity-determining region (ISDR) which has been shown to mediate interaction with the cellular interferon-induced double-stranded RNA-activated protein kinase PKR (Enomoto et al., 1995; Gale et al., 1997). Thus, NS5A possibly modulates the host interferon response. Numerous studies have shown that NS5A can interact with various cellular proteins and modulate diverse cell signalling pathways (He et al., 2006). These include the MAPK mitogenic, Src kinase and PI3K-AKT cell survival pathways. In addition, NS5A has been reported to modulate cell proliferation and apoptosis through PKR and p53-dependent or -independent mechanisms (Gale et al., 1999; Lan et al., 2002; Siavoshian et al., 2004). Inhibition of TNF-induced apoptosis by NS5A is mediated by its interaction with adaptor proteins of the TNF receptor (Majumder et al., 2002; Park et al., 2003). NS5A may also function as a prosurvival Bcl-2 homologue that sequesters Bax and thus, inhibits apoptosis induction (Chung et al., 2003). A recent study showed that NS5A can activate a calpain cysteine protease that degrades Bid, leading to apoptosis inhibition (Simonin et al., 2009).

Table 1.1 − Continued

Protein	Protein characteristic(s)	Protein function(s)	Effect(s) on host cellular processes
NS5B (591 aa)	NS5B contains a C-terminal hydrophobic (21 aa) domain that mediates post-translational membrane association and ER localisation (Ivashkina et al., 2002; Schmidt-Mende et al., 2001). The catalytic domain of NS5B is located in the cytoplasm and forms a typical right-handed polymerase structure with fingers, palm and thumb subdomains and a fully enclosed active site (Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999). NS5B contains the hallmark GDD sequence motif that is essential for polymerase activity (Yamashita et al., 1998). Evidence suggests that NS5B functions as an oligomer with cooperative RNA synthesis activity (Qin et al., 2002; Wang et al., 2002).	NS5B serves as the RNA-dependent RNA polymerase (RdRp) of HCV (Behrens et al., 1996). Viral RNA replication proceeds via the synthesis of a complementary negative-sense RNA which then serves as the template for synthesis of genomic positive-sense RNA. NS5B is an error-prone enzyme and lacks proofreading capacity, thus contributing to a high rate of misincorporation and genetic diversity. The membrane anchor of NS5B is dispensable for polymerase activity in vitro but required for RNA replication in cells (Moradpour et al., 2004). In addition to modulation by other viral factors, the polymerase activity of NS5B has been shown to be enhanced by cyclophilin B interaction (Piccininni et al., 2002; Shirota et al., 2002; Watashi et al., 2005).	NS5B has been shown to interact with cellular IκB kinase and prevent TNF-α-induced activation of NF-κB. In addition, TNF-α-mediated JNK activity was observed to be synergistically enhanced by NS5B (Choi et al., 2006). It was reported that NS5B interacts with retinoblastoma tumour suppressor protein and targets it for degradation, resulting in activation of E2F-responsive promoters and cell proliferation (Munakata et al., 2005).

1.1.4.2 Core protein

The 21 kDa mature core protein is thought to constitute the HCV nucleocapsid and is the predominant form detected in virus particles purified from the sera of patients with chronic HCV infection (McLauchlan, 2000; Yasui et al., 1998). It has also been reported that the maturation of the core protein is required for the production of HCV using the Japanese fulminant hepatitis-1 (JFH-1) infectious clone (Targett-Adams et al., 2008). The highly conserved core protein is composed of two distinct domains of varying hydrophobicity (Bukh et al., 1994). The N-terminal two-third of the protein constitutes domain 1 (D1) which contains a high proportion of basic residues together with two short hydrophobic regions. The core protein has been shown to interact with viral RNA through the first 75 residues of D1 (Santolini et al., 1994). More recently, the *in vivo* interaction between the core protein and both the 5' and 3' UTRs has been described (Yu et al., 2009). D1 has also been implicated in the oligomerization of the core protein (Matsumoto et al., 1996). The ability to bind viral RNA and homo-oligomerization of the core protein are consistent with its perceived role as the viral nucleocapsid, as is its interaction with viral envelope glycoprotein E1 (Lo et al., 1996). Moreover, the core protein-E1 interaction has been shown to be dependent on the oligomerization of the core protein (Nakai et al., 2006). Recently, mutational analysis using the HCV cell culture (HCVcc) infection system has led to the identification of four basic residues (R50, K51, R59 and R62) in D1 that are essential for the production of infectious virus at a post-nucleocapsid assembly step (Alsaleh et al., 2010).

In contrast to D1, the C-terminal domain 2 (D2) of the core protein is less basic and more hydrophobic (Bukh et al., 1994). The core protein shows a cytoplasmic distribution through association with ER, lipid droplets and membranous webs possibly derived from ER membranes (Barba et al., 1997; Moradpour et al., 1996). Boulant and colleagues demonstrated that D2 mediates the core protein-lipid droplet interaction and that this interaction is critical for the production of infectious particles (Boulant et al., 2006; Boulant et al., 2007). It has been suggested that lipid droplets provide a platform for virus assembly following core protein-mediated recruitment of non-structural proteins and replication complexes to these sites (Miyanari et al., 2007). However, recent research using infectious clones of different infectivities suggested that p7- and NS2-mediated ER localization of the core protein is required for efficient virus assembly (Boson et al., 2011).

Besides its role in the encapsidation of viral RNA, the core protein has been shown to interact with numerous host proteins and interfere with various cellular pathways, including apoptosis, cell signalling, transcriptional activation, carcinogenesis, lipid metabolism and immune modulation. However, due to the lack of cell culture infection systems and suitable small animal models, most of the early studies on the core protein (and other viral proteins) relied heavily on heterologous expression systems that produce unphysiologically high levels of protein. Thus, in some cases, conflicting data have been reported and more importantly, it remains to be seen if these interactions occur in the course of a natural infection.

The core protein has been reported to inhibit as well as promote apoptosis via diverse pathways, depending on the death stimuli and types of cells used. For instance, several studies have demonstrated that the core protein acts as a positive regulator of Fas-mediated apoptosis in different cell lines (Hahn et al., 2000; Moorman et al., 2003; Ruggieri et al., 1997). Interestingly, the C-terminal of the core protein has been shown to induce Fas ligand-independent apoptosis in Jurkat cells by directly facilitating Fas receptor aggregation (Moorman et al., 2003). In addition to the Fas signalling pathway, the core protein also enhanced tumour necrosis factor (TNF)-induced apoptosis through direct interaction with the death domains of TNF receptor-1 and Fas-associated death domain (FADD) protein (Zhu et al., 1998; Zhu et al., 2001). Furthermore, interaction of the core protein with two other members of the TNF- and TNF receptor-like family, lymphotoxin-β receptor and TRAIL, have been shown to sensitize cells to apoptosis (Chen et al., 1997; Chou et al., 2005). However, contrary to these findings, several other studies have demonstrated core protein-mediated inhibition of Fas- and TNF-induced apoptosis via NF-κB activation, STAT3 activation or regulation of cellular FLICE inhibitory protein levels (Kawamura et al., 2006; Marusawa et al., 1999; Saito et al., 2006). Apoptotic signalling via the transforming growth factor-β (TGF-β) pathway was also inhibited through direct interaction of the core protein with Smad3, an important apoptosis modulator acting downstream of TGF-β-receptor-I/II (Pavio et al., 2005).

Modulation of the intrinsic or mitochondrial apoptotic pathway by the core protein has also been demonstrated. Expression of the core protein has

been reported to be associated with the induction of oxidative and ER stress, which then leads to apoptosis (Benali-Furet et al., 2005; Kang et al., 2009). For instance, binding of the core protein to Hsp60, a mitochondrial chaperonin, has been shown to trigger production of reactive oxygen species (ROS), which then enhanced sensitization to TNF-induced apoptosis (Kang et al., 2009). Interaction between the core protein and the 14-3-3ε protein also promoted apoptosis induction through indirect activation of Bax, a proapoptotic member of the Bcl-2 family (Lee et al., 2007). On the other hand, the core protein was shown to protect cells against apoptosis by upregulating the expression of prosurvival Bcl-X_L (Hara et al., 2006; Otsuka et al., 2002; Yoshida et al., 2002). Also, binding of the core protein to p53 or its interacting partners may lead to the induction or inhibition of p53-mediated apoptosis (Cao et al., 2004; Herzer et al., 2005; Kao et al., 2004; Otsuka et al., 2000). For example, the core protein was reported to bind to and interfere with the coactivator function of promyelocytic leukemia (PML)-IV, thus inhibiting the expression of downstream proapoptotic p53 target genes (Herzer et al., 2005). Overall, although the resultant effect of the core protein on host cell apoptosis is still unclear, these studies have highlighted several signalling pathways that are possibly regulated by the core protein.

The modulation of apoptosis by the core protein may contribute to hepatocarcinogenesis (Yoshida et al., 2002). Consistent with this, several studies proposed that the core protein may function as a cofactor in the development of HCC (Chang et al., 1998; Kamegaya et al., 2005; Ray et al., 1996). Furthermore, the core protein has been shown to induce HCC in

transgenic mice and promote immortalization and malignant transformation of primary human liver cells (Moriya et al., 1998; Ray et al., 2000; Shan et al., 2005). Aside from the inhibition of apoptosis, the core protein has been reported to promote cellular transformation through interaction with transcription factors and other proteins that regulate cell proliferation and growth arrest. Using a yeast two-hybrid system, heterogeneous nuclear ribonucleoprotein K (hnRNP K) was identified as a core protein-interacting partner (Hsieh et al., 1998). HnRNP K is a multifunctional protein that is involved in signal transduction, chromation remodelling, transcription, splicing and translation processes (Bomsztyk et al., 2004). It has been proposed that specific binding of the core protein to hnRNP K disrupts its many functions, including its suppressive effect on transcription, which may have implications for carcinogenesis (Hsieh et al., 1998). Another study demonstrated that the core protein inactivated transcription factor leucine-zipper protein (LZIP) through sequestration in the cytoplasm and the loss of LZIP function was associated with cellular transformation (Jin et al., 2000). The core protein has also been found to modulate the expression of cyclin-dependent kinase inhibitor p21, an important regulator of cell cycle progression (Kwun and Jang, 2003; Lee et al., 2002). Lee et. al. suggested that the core protein suppresses p21 transcription through inhibition of a TGF-β pathway, thus promoting cell proliferation (Lee et al., 2002). Consistent with this, a recent study demonstrated that the core protein may switch TGF-β growth inhibitory effects to tumour promoting responses in hepatocytes by decreasing Smad3 activation (Battaglia et al., 2009). Nuclear translocation of the core protein was observed through its interaction with isoforms of p73, a

member of the p53 tumour suppressor family (Alisi et al., 2003). This interaction prevents cell growth arrest mediated by p73α, but not by p73β, in a p53-dependent manner.

There is increasing evidence suggesting that HCV-related hepatic steatosis may contribute to the development of HCC (Moriya et al., 1998; Ohata et al., 2003). The close association of the core protein with lipid droplets suggests that this viral protein might induce changes in lipid metabolism that result in intrahepatic accumulation of triglycerides. In agreement with this, the core protein has been shown to induce steatosis in transgenic mice (Moriya et al., 1997). Gene expression analysis of core protein-expressing cells and mice revealed that the core protein downregulated lipid metabolism-associated gene expression and also reduced the expression of peroxisome proliferator-activated receptor α (PPARα), a major regulator of fatty acid degradation in hepatocytes (Yamaguchi et al., 2005). Furthermore, the direct interaction of the core protein with the nuclear proteasome activator PA28γ in transgenic mice has been shown to upregulate genes involved in fatty acid biosynthesis (Moriishi et al., 2007). The core protein also binds to and activates retinoid X receptor-α (RXR-α), a transcription factor that regulates many cellular functions, including lipid synthesis (Tsutsumi et al., 2002). As mentioned earlier, the core protein has been shown to induce oxidative stress and it was suggested that ROS production leads to the accumulation of intrahepatic lipid peroxidation products which impair very-low-density lipoprotein (VLDL) secretion (Lerat et al., 2002).

The core protein has been proposed to play an immunomodulatory role through its interaction with the complement receptor gC1qR (Kittlesen et al., 2000). Proliferation of T lymphocytes and the production of IL-2 and IFN-γ were shown to be inhibited by core protein-gC1qR interaction, thus leading to the suppression of T cell responsiveness which in turn ensures viral persistence (Soguero et al., 2002; Yao et al., 2001). Interestingly, the core protein has been reported to be secreted from cultured cells and free circulating core protein has also been detected in the plasma of HCV-infected individuals (Maillard et al., 2001; Sabile et al., 1999). This suggests that prior to the production of anti-core antibodies during the early acute phase of HCV infection, the circulating core protein could inhibit T cell responses by interacting with gC1qR on peripheral T lymphocytes.

The N-terminal of the core protein has been demonstrated to interact with a cellular DEAD-box RNA helicase DDX3 (Mamiya and Worman, 1999; Owsianka and Patel, 1999; You et al., 1999). Using the HCVcc infection system, DDX3 was shown to be essential for HCV replication (Ariumi et al., 2007). However, it was recently reported that the requirement of DDX3 for HCV replication is unrelated to its interaction with the core protein (Angus et al., 2010). Therefore, the biological function(s) of the core protein-DDX3 interaction remains to be determined.

1.1.5 HCV life cycle

HCV derived from patients, infected animals and tissue culture exists in various forms. This heterogeneity arises due to the association of HCV with low-density lipoproteins (LDL) and VLDL and the presence of immunocomplexed and free virions (Andre et al., 2002; Hijikata et al., 1993; Lindenbach et al., 2005; Thomssen et al., 1993). In both animal models and tissue culture systems, low density virion fractions were observed to be more infectious, suggesting a possible role for plasma lipoproteins in virus entry (Bradley et al., 1991; Lindenbach et al., 2005). HCV displays a distinct host species specificity and tissue tropism, naturally infecting only humans and chimpanzees with hepatocytes being the main target cells. The restricted tropism of HCV probably reflects a requirement for specific host factors at one or more stages of its life cycle, which comprises viral entry, translation and RNA replication, virion assembly and release of progeny virus (Figure 1.2).

HCV entry is a complex multistep process which is initiated by the adsorption of HCV to the target cell. The current model of HCV entry involves the initial interaction of HCV with the highly sulfated glycosaminoglycan (GAG) heparan sulfate and/or the LDL receptor on the surface of host cells (Barth et al., 2003; Molina et al., 2007). The latter may be mediated by HCV-associated LDL and VLDL (Thomssen et al., 1992). Inhibition of these interactions through enzymatic treatment, antibody blocking or host gene silencing resulted in reduced HCV attachment and/or infection (Germi et al., 2002; Owen et al., 2009). However, these two host factors have yet to be conclusively proven to be essential for HCV entry.

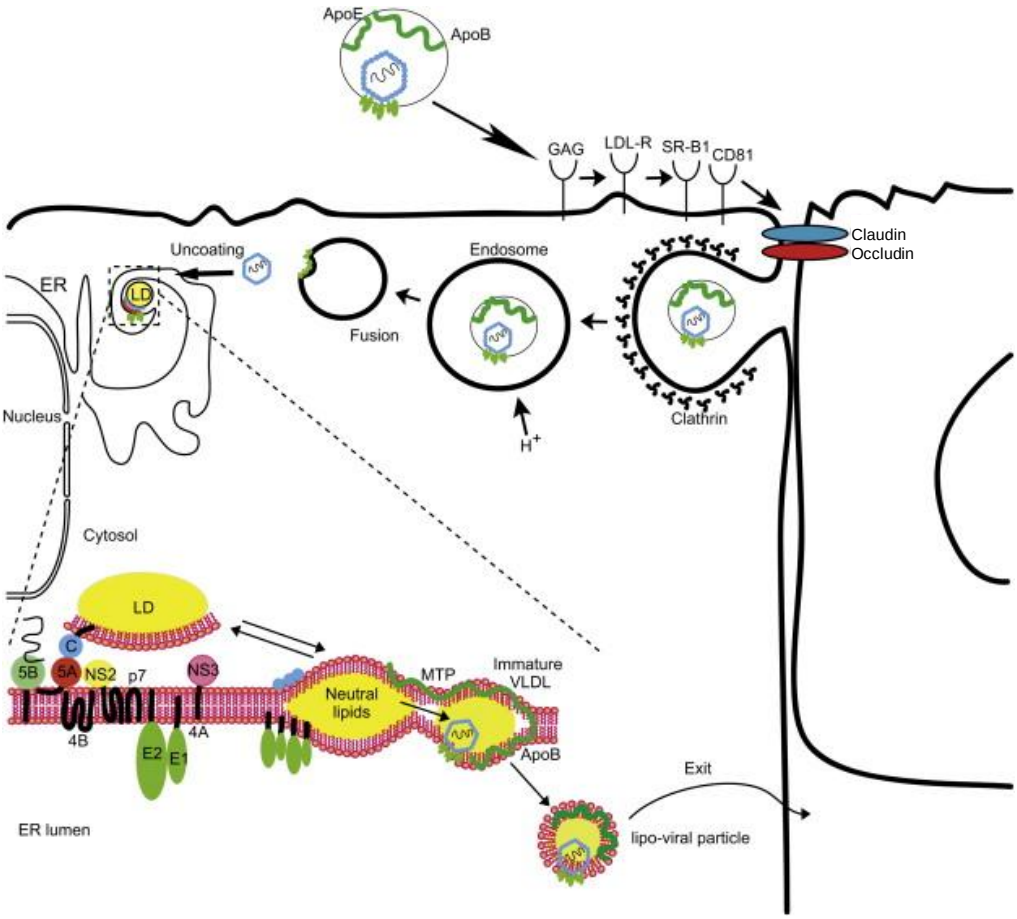

Fig. 1.2. Current model of the HCV life cycle.
Figure adapted from Joyce, M. A. *Microb. Infect.* **12**(4): 263 – 271 (2010).

Following initial attachment, the HCV E2 glycoprotein interacts with scavenger receptor B type 1 (SR-B1), which also acts as a receptor for LDL (Acton et al., 1994; Scarselli et al., 2002). Some studies have shown that this interaction is required for subsequent binding of HCV to tetraspanin CD81, the first putative HCV receptor identified (Kapadia et al., 2007; Pileri et al., 1998; Zeisel et al., 2007). Numerous studies have confirmed the relevance of CD81 in HCV entry as a co-receptor and it appears to function in a post-HCV binding step (Cormier et al., 2004; Evans et al., 2007). It has been proposed that the binding of HCV E2 to CD81 activates Rho GTPases and results in actin-dependent relocalization of the complex to tight junctions where the

virus encounters two other co-receptors, claudin-1 (CLDN1) and occludin (OCLN) (Evans et al., 2007; Ploss et al., 2009; Schwarz et al., 2009). The virus is then internalized via pH-dependent, clathrin-mediated endocytosis (Blanchard et al., 2006). Although the mechanisms underlying viral-host membrane fusion are still poorly understood, the acidic environment within the early endosome is thought to trigger E1-E2 glycoprotein-mediated fusion of virus envelope and endosomal membrane, thereby releasing the viral genome into the cytoplasm (Haid et al., 2009; Lavillette et al., 2007). In addition, CD81-independent cell-to-cell transmission of HCV has also been described and this poorly understood process may be mediated by tight junction proteins (Timpe et al., 2008; Valli et al., 2007).

As discussed earlier, translation of the viral RNA is initiated by ribosome-binding of the 5' UTR IRES (Friebe et al., 2001). This process is positively regulated by the 3' UTR and negatively regulated by NS4A (Bradrick et al., 2006; Kou et al., 2006; Song et al., 2006). In addition, viral protein synthesis has been shown to be regulated by several host cell factors. The liver-specific miRNA, miR-122, interacts with the 5' UTR and promotes initiation of translation by facilitating the binding of ribosomes to the viral RNA (Henke et al., 2008; Jopling et al., 2005). Recently, it was reported that autophagy proteins are required for the translation of HCV RNA (Dreux et al., 2009). Following precursor polyprotein processing, the E1 and E2 proteins are glycosylated and retained in the ER (Duvet et al., 2002).

Formation of the HCV replication complex induces extensive rearrangement of intracellular membranes. This includes generation of an ER-

derived membranous web, reorganization of cellular endosomal components and enhanced formation and localization of lipid droplets (Berger et al., 2009; Egger et al., 2002; Piodi et al., 2008). The N-terminal domain of NS4B appears to play a dominant role in membranous web formation and correct localization of the replication complex proteins while its C-terminal domain induces relocation of Rab-5, a component of early endosomes, to viral replication sites (Aligo et al., 2009; Elazar et al., 2004). As mentioned previously, the core protein alters cellular lipid metabolism and it is observed to be directly associated with lipid droplets in close proximity to the membranous web (Miyanari et al., 2007). The core protein and ER-associated NS5A are proposed to cooperatively recruit viral RNA and other replication components to the membranous web which possibly provides a platform for the replication complex and protects it from host antiviral responses.

Similar to other positive-stranded RNA viruses, HCV RNA replication by the RNA-dependent RNA polymerase (RdRp) NS5B occurs via a negative-stranded RNA intermediate (Behrens et al., 1996). Prior to this, the NS3 helicase may function to unwind secondary structures in the template RNA (Kim et al., 1995). It was recently reported that cyclophilin A (Cyp A) is an essential host factor for viral RNA replication while cellular Cyp B can enhance synthesis of viral RNA through its interaction with NS5B (Heck et al., 2009; Kaul et al., 2009; Yang et al., 2008). Subsequently, the sense viral RNA produced may be used for viral protein synthesis or packaged as genomic RNA of new virus particles. Although the precise mechanisms for the formation and release of infectious HCV particles are still unknown,

Gastaminza et. al. have shown that virus assembly takes place within the ER and that HCV possibly utilises the VLDL secretion pathway to exit the cell (Gastaminza et al., 2008). Consistent with this, inhibition of apolipoprotein B synthesis and microsomal triglyceride transfer protein activity, both of which are important factors for VLDL assembly and secretion, prevented production of infectious virus.

1.1.6 Model systems for studying HCV

Aside from the heterologous expression of viral proteins, several systems have been developed for studying the different stages of the HCV life cycle. For instance, the HCV tissue culture replicon system is suitable for studying viral replication. This system involves the transfection of bicistronic RNA molecules into the human hepatoma cell line Huh7 (Blight et al., 2002; Lohmann et al., 1999; Pietschmann et al., 2002). The first cistron contains the HCV 5' UTR that drives expression of the selectable marker neomycin phosphotransferase while the second cistron contains a portion of the HCV genome encoding nonstructural protein NS3 to NS5B or the full-length HCV genome under the control of the encephalomyocarditis virus (EMCV) IRES. The selectable marker allows for the selection of clones which support persistent HCV RNA replication. Enhanced replication was achieved through the introduction of cell culture adaptive mutations (Krieger et al., 2001; Lohmann et al., 2001). However, despite robust RNA replication, the full-length HCV replicons failed to produce infectious virus particles.

Retroviral or lentiviral pseudotypes bearing HCV glycoproteins E1 and E2, termed HCV pseudoparticle (HCVpp), can be used to study viral entry. HCVpp are generated through cotransfection of two expression plasmids encoding HCV E1 and E2 and the retroviral or lentiviral Gag-Pol proteins and a third plasmid containing a reporter gene into a packaging cell line. The HCVpp produced can be used to infect primary liver and hepatoma cells and HCV E1E2-mediated viral entry can then be assayed through the expression of the reporter gene (Bartosch et al., 2003; Hsu et al., 2003). An advantage of this method is the ability to examine the entry and antibody-mediated neutralization of various HCV genotypes (Pestka et al., 2007). However, it is important to note that the export pathway of HCVpp and native HCV may differ, thus resulting in particles with different characteristics.

The isolation of the genotype 2a JFH-1 strain from a patient with fulminant hepatitis, later found to be able to replicate efficiently without the need for adaptive mutations, provided the breakthrough for the development of a system capable of producing infectious HCV in cell culture (Kato et al., 2001; Wakita et al., 2005). Subsequently, more robust production of infectious particles was achieved using the intragenotypic chimeric J6/JFH-1 strain and Huh7-derived cell lines, Huh7.5 and Huh7.5.1, which are defective in the antiviral RIG-I pathway (Lindenbach et al., 2005; Zhong et al., 2005). Following electroporation of transcribed RNA into cultured cells, the J6/JFH-1 strain encodes the structural proteins, p7 and NS2 of the J6 isolate and the remaining non-structural proteins of the JFH-1 isolate and releases newly packaged virus particles into the culture medium. The virus produced in cell

culture, termed HCV cell culture (HCVcc), is also infectious in chimpanzees and humanised mice (Lindenbach et al., 2006). A limitation of this system is its inability to propagate HCV of other genotypes. Alternatively, infectious HCV particles of various genotypes can be produced in Huh7-derived cell lines by expressing full-length HCV-encoding DNA with self-cleaving ribozymes (Cai et al., 2005; Kato et al., 2007). Similar to HCVcc, the culture supernatants of these cells were infectious both *in vitro* and *in vivo*. Since these systems support viral entry, replication, assembly and egress, they offer the best *in vitro* HCV infection model currently available.

A suitable animal model for HCV research is still lacking. Although chimpanzees can be infected experimentally, they are far from ideal due to differences in HCV disease and therapy outcomes compared to humans (Major et al., 2004). Furthermore, there are ethical and economic issues relating to the use of non-human primates in research. Transgenic mouse models typically employ liver-specific promoters to drive constitutive or inducible expression of one or more HCV proteins (Chiyo et al., 2011; Ernst et al., 2007; Fimia et al., 2003). However, studies using these models have reported variable phenotypes, probably due to differences in mouse genetic backgrounds, transgene integration sites, promoters used to drive transgene expression and abundance of HCV protein expressed. Immunodeficient mice harbouring chimeric human-mouse liver which are susceptible to HCV infection have been successfully generated. Chimeric livers are produced by destroying mouse hepatocytes and transplanting human ones. Hepatotoxicity can be achieved through expression of the urokinase plasminogen activator

(uPA) or drug treatment of *Fah*$^{-/-}$, *Rag2*$^{-/-}$ and *Il2rγ*$^{-/-}$ mice (Bissig et al., 2010; Mercer et al., 2001). These humanised mice enable *in vivo* study of HCV infection and can be used for antiviral drug testing. However, the lack of a functional immune system limits the study of immune-mediated pathogenesis and immune-based therapies.

1.2 An overview of apoptosis

Apoptosis, or programmed cell death, plays a fundamental role in development, tissue homeostasis and aging through the controlled elimination of unwanted, damaged or aberrant cells. In addition, apoptosis serves as a defense strategy against microbial invasion and cytotoxic insults. However, apoptosis may also contribute to disease progression by causing tissue injury. Defective apoptotic signalling that results in excessive or insufficient cell death is the basis of many pathologic conditions including cancer, autoimmune disorders, neurodegenerative diseases and ischemic damage (Fadeel and Orrenius, 2005).

Apoptosis is generally characterized by a series of morphological and nuclear changes (Hacker, 2000). During the initial stages of apoptosis, cells undergo chromatin condensation and exhibit a striking decrease in cell volume (Kerr et al., 1972). Then, extensive plasma membrane blebbing occurs followed by nuclear fragmentation. The subsequent disintegration of dying cells into small apoptotic bodies containing dense cytoplasm with or without a nuclear fragment is a hallmark of apoptosis. These bodies are then engulfed by neighbouring phagocytes and degraded within phagolysosomes. This entire process is essentially non-inflammatory as cellular constituents are not released into the extracellular space and under normal conditions, apoptotic bodies are rapidly cleared by phagocytosis without any production of pro- or anti-inflammatory cytokines (Kurosaka et al., 2003).

Apoptosis can be triggered by a variety of external and intracellular stimuli which act via two main apoptotic signalling cascades, namely the

extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The two pathways will be discussed in sections 1.2.1 and 1.2.2. A common feature of these pathways is the sequential activation of cysteine-dependent aspartate-specific proteases, also known as caspases (Pop and Salvesen, 2009). Caspases are expressed in most cells as inactive zymogens and are themselves activated by proteolytic processing of conserved aspartate moieties. At least 14 members of the caspase family have been identified thus far and the ten major caspases can be generally categorized into initiator (caspase-2, 8, 9, 10), effector (caspase-3, 6, 7) and inflammatory caspases (caspase-1, 4, 5) (Elmore, 2007). Initiator procaspases undergo autoactivation in specialised protein complexes before catalysing the activation of other procaspases. This initiates a caspase cascade that culminates in the activation of caspase effectors.

Effector or "executioner" caspases induce the degradation of DNA and cytoskeletal and nuclear matrix proteins, resulting in the morphological and biochemical changes associated with apoptosis. For example, effector caspases cleave inhibitor of caspase-activated DNase (ICAD) and relieve the inhibition of CAD endonuclease activity (Sakahira et al., 1998). CAD then induces DNA fragmentation and chromatin condensation. In addition, effector caspases target many cytoskeletal proteins including components of actin microfilaments, microtubules and intermediate filaments (Kothakota et al., 1997; Lane et al., 2001; Morishima, 1999). Caspase-mediated destruction of the cytoskeleton probably contributes to cell shrinkage, membrane blebbing and "pinching off" of apoptotic bodies. Other substrates of effector caspases

include signal transduction proteins and chromatin-modifying and DNA repair enzymes (Timmer and Salvesen, 2007). Externalization of phosphatidylserine on the cell surface, another feature of apoptosis, may be regulated through caspase-dependent or -independent pathways (Ferraro-Peyret et al., 2002; Mandal et al., 2005). Surface exposure of phosphatidylserine facilitates recognition of apoptotic cells by phagocytes (Fadok et al., 2001). Biochemical changes such as effector caspase activation, substrate cleavage, DNA fragmentation and externalization of phosphatidylserine can be used as biomarkers for the detection of apoptosis.

1.2.1 Extrinsic apoptosis pathway

The extrinsic pathway of apoptosis is mediated by death receptors and their cognate ligands of the TNF receptor and ligand gene superfamily (Tansey and Szymkowski, 2009). Death receptors are transmembrane proteins with a cysteine-rich extracellular domain and a cytoplasmic "death domain" (Ashkenazi and Dixit, 1998). Some of the well-characterized death receptors and their ligands include Fas/FasL, TNF-R1/TNF-α and TRAIL-R1 or R2/TRAIL. The extrinsic signalling pathway is triggered by death receptor trimerization and binding of the corresponding trimeric ligand. Ligand binding induces recruitment of cytoplasmic death domain-containing adaptor proteins to the receptor through homotypic death domain interactions. Although death receptors may recruit different adaptors, the eventual association with Fas-associated death domain (FADD) protein and procaspase-8 appears to be common to all death receptors (Chinnaiyan et al., 1995; Harper et al., 2003; Sprick et al., 2000). This promotes the intracellular assembly of a

large death-inducing signalling complex (DISC) and the dimerization and autoactivation of caspase-8 (Kischkel et al., 1995; Oberst et al., 2010). Once activated, caspase-8 propagates the apoptotic signal by directly activating effector caspases or by cleaving the BH3-only protein Bid to initiate the intrinsic mitochondrial pathway (Li et al., 1998).

1.2.2 Intrinsic apoptosis pathway

In the intrinsic apoptosis pathway, members of the Bcl-2 family govern mitochondrial outer membrane permeability (MOMP) and thus, the subsequent activation of caspases (Chipuk and Green, 2008). Bcl-2 family proteins are defined by the presence of one or more Bcl-2 homology (BH) domains and can be functionally categorized as prosurvival or proapoptotic (Youle and Strasser, 2008). Major prosurvival Bcl-2 members include Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1 and they function by directly inhibiting the activity of their proapoptotic counterparts. The proapoptotic Bcl-2 members can be further subclassified into effector proteins (Bax and Bak) and BH3-only proteins (Chipuk et al., 2010).

Although the events leading to activation of Bax and/or Bak are still unclear, BH3-only proteins have been reported to play a central role in this process and two models of Bax/Bak activation have been proposed. In the indirect activation or neutralization model, Bax and Bak are sequestered by prosurvival Bcl-2 members until displaced by BH3-only proteins (Uren et al., 2007; Willis et al., 2007). Based on this model, some BH3-only members (Bim, Bid and Puma) are potent inducers of apoptosis due to their ability to

neutralize all the prosurvival Bcl-2 proteins while others (Bad, Noxa and Hrk) are weak inducers as they only bind to a subset of prosurvival proteins (Chen et al., 2005). In the direct activation model, certain BH3-only proteins (Bim and Bid) function as direct activators of Bax and Bak (Kim et al., 2009; Lovell et al., 2008; Wei et al., 2000). The remaining BH3-only proteins serve as sensitizers or de-repressors by binding to prosurvival Bcl-2 proteins, thus facilitating Bim/Bid activator function (Kuwana et al., 2005; Letai et al., 2002). Interestingly, recent research suggests that both pathways of Bax/Bak activation may function in vivo (Merino et al., 2009).

Upon activation, Bax and Bak perforate the mitochondrial outer membrane by forming large oligomeric channels (Nechushtan et al., 2001; Wei et al., 2001). This results in the release of proapoptotic factors such as cytochrome c and DIABLO/Smac from the intermembrane space into the cytosol. Cytochrome c recruits apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 to form the apoptosome, which in turn facilitates the autoactivation of caspase-9 and initiation of the caspase cascade (Bao and Shi, 2007; Riedl and Salvesen, 2007).

1.3 Apoptosis in HCV infection

Death receptors and ligands are ubiquitously expressed in hepatocytes, probably as a result of evolutionary pressure to protect the liver against hepatotropic viruses (Faubion and Gores, 1999). Thus, it is not surprising that liver diseases are often associated with activation of the extrinsic apoptosis pathway (Akazawa and Gores, 2007; Feldstein et al., 2003; Higuchi et al., 2002). In addition, hepatocytes are classified as so-called Type II cells in which the extrinsic apoptosis pathway requires amplification via the mitochondrial pathway for successful apoptosis induction (Scaffidi et al., 1998). This highlights the importance of both apoptosis pathways in liver cells. Massive apoptosis in the liver leads to acute liver failure while persistent hepatocyte apoptosis may contribute to chronic liver dysfunction, fibrogenesis and even hepatocarcinogenesis (Malhi and Gores, 2008). Apoptosis of hepatocytes during HCV infection has been proposed to be a direct cause of liver fibrosis (Canbay et al., 2004). *In vitro* and *in vivo* studies have shown that phagocytosis of apoptotic bodies by hepatic stellate cells induces a profibrogenic response (Canbay et al., 2003; Zhan et al., 2006).

Due to the lack of experimental animal models, most of the in vivo evidence for apoptosis in HCV infection comes from the analysis of liver biopsy samples obtained from patients with chronic hepatitis C. Early studies based on the detection of DNA fragmentation reported that apoptosis was slightly elevated (0.54%) in the HCV-infected liver compared to the healthy liver (Calabrese et al., 2000; Lau et al., 1998). However, the extent of apoptosis might have been underestimated as not all apoptotic cells display

DNA fragmentation and as DNA cleavage occurs at a late stage of apoptosis, rapid engulfment by phagocytes would have prevented visualization of this morphological change (Grasl-Kraupp et al., 1995; Schulze-Osthoff et al., 1994). Using immunohistochemical detection of activated caspases and cleaved caspase substrate PARP, another study showed caspase activation in 7 – 20% of the hepatocytes (Bantel et al., 2001). This group further compared apoptosis induction in HCV-infected patients and control individuals through specific detection of cleaved caspase substrate cytokeration-18 (CK-18), the major intermediate filament in hepatocytes, in serum (Bantel et al., 2004). Consistent with previous findings, the levels of CK-18 cleavage products were found to be significantly enhanced in the sera of HCV-infected patients, thus indicating caspase activation and apoptosis. Interestingly, these studies found a significant correlation between degree of apoptosis and disease grade, thus underlining the role of HCV-induced apoptosis in liver injury. However, the lack of correlation between apoptosis and viral load in these studies suggests an indirect mechanism of HCV-induced apoptosis.

There is increasing evidence suggesting that both immune-mediated processes and direct cytopathic effects of HCV might contribute to apoptosis in the HCV-infected liver. The observation that the onset of acute hepatitis coincides precisely with the activation of cytotoxic T lymphocytes (CTLs) suggests the involvement of cellular immune responses (Bowen and Walker, 2005; Lechner et al., 2000; Thimme et al., 2001). Furthermore, apoptotic hepatocytes in HCV-infected liver biopsy specimens were observed to be in close physical proximity to infiltrating lymphocytes (Calabrese et al., 2000;

Lau et al., 1998). Cytotoxic lymphocytes such as natural killer cells and virus-specific CTLs eliminate infected cells by engaging death receptors on the cell surface or by delivering toxic granzyme B to the cells (Waterhouse et al., 2004). However, virus-infected hepatocytes seem to be resistant to granzyme B-mediated cell killing, suggesting that CTLs may preferentially activate the extrinsic apoptosis pathway (Kafrouni et al., 2001). Consistent with this, immunohistochemical and gene expression studies have shown that HCV-infected hepatocytes express elevated levels of Fas while FasL expression is upregulated in infiltrating CTLs (Hiramatsu et al., 1994; Mita et al., 1994). Moreover, Fas-mediated hepatocyte apoptosis was found to be enhanced in the liver tissue of HCV-infected patients (Pianko et al., 2001).

The observation that liver injury progresses more rapidly in HCV-infected patients with defective or suppressed immune systems suggests that HCV may also exert direct cytopathic effects (Chen and Morgan, 2006). In addition, recent HCVcc infection studies using cultured hepatoma cells and chimeric *SCID/Alb-uPA* mice have reported induction of apoptosis by HCV in the absence of cellular immune responses (Deng et al., 2008; Joyce et al., 2009; Mateu et al., 2008). Viral proteins may modulate host cell apoptosis by interacting with cellular apoptotic signalling molecules or by inducing intracellular stress. Numerous studies have examined the role of individual HCV proteins in apoptosis using cell culture expression systems (Aweya and Tan, 2011). However, these findings may not provide a true reflection of the *in vivo* situation as the viral proteins were expressed at unnaturally high levels and the effects of possible viral-viral interactions were not taken into account.

Furthermore, studies using transgenic mice conditionally expressing the core, E1, E2 and NS2 proteins or only the HCV structural proteins in the liver have also shown contradicting results in terms of apoptosis induction (Machida et al., 2001; Tumurbaatar et al., 2007). Thus, the exact mechanisms underlying the direct cytopathic effects of HCV are still poorly understood. Future studies using the currently available HCVcc system may provide better understanding of HCV-induced apoptosis and its role in pathogenesis.

1.4 Project objectives

For a virus with only ten gene products, multiple interactions with various cellular proteins provide an efficient means of reorganizing host processes. Over the years, much effort have been put into characterizing and understanding the complex network of HCV-host interactions and their putative roles in the viral life cycle as well as HCV pathogenesis [recently reviewed by (Georgel et al., 2010)]. Although many aspects of these interactions have yet to be determined, they have been shown to modulate a variety of cellular processes including transcription, protein synthesis, membrane rearrangement, intracellular trafficking, lipid metabolism, immune response, carcinogenesis and apoptosis.

In this study, we aim to identify novel HCV-host interactions that possibly contribute to the modulation of host cell apoptosis. Numerous studies have reported the involvement of the HCV core protein in apoptosis induction and both proapoptotic and prosurvival activities have been described for the core protein (Aweya and Tan, 2011; McLauchlan, 2000). In Chapter 3, we used heterologous protein expression and the HCVcc system to investigate possible interactions between the core protein and members of the Bcl-2 family of apoptosis regulators and to identify specific region(s) within the viral protein that mediate this interaction. Following the experimental designs used in previous studies, the mature form of the core protein is assumed to be constituted by residues 1 to 173 of the HCV precursor polyprotein and this shall be referred to as the core protein in this study (Hope and McLauchlan, 2000; Liu et al., 1997; Yamanaka et al., 2002). This study may provide

further insight into the mechanisms underlying core protein-mediated apoptosis.

In the subsequent chapters, we focused on the ability of the whole virus to induce host cell death post-infection. Using the JFH-1-based HCVcc system, several groups have demonstrated induction of apoptosis in HCV-infected cells (Deng et al., 2008; Mateu et al., 2008; Walters et al., 2009). However, the exact mechanisms and specific host-virus interactions that contribute to HCV-induced apoptosis have yet to be identified. We performed protein expression analysis and pathway-focused transcriptional profiling to examine changes in the expression of apoptosis-related host factors during acute HCV infection. For two of the host prosurvival factors, Bcl-X_L and Mcl-1, the impact of overexpression and knockdown of these proteins on HCV-induced apoptosis and viral replication was also investigated. Our goal is to identify apoptosis pathways that may be regulated by HCV and the accompanying host-virus interactions, if any, in the context of a productive infection.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

The human hepatoma Huh7 cell line and the human embryonic kidney 293T cell line were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 0.1 mg/ml streptomycin and 100 U/ml penicillin. The Huh7.5 cell line (Blight et al., 2002), a highly HCV permissive subclone of Huh7 cells, was kindly provided by C. M. Rice, Center for the Study of Hepatitis C, The Rockefeller University, New York, NY, USA and cultured in complete DMEM supplemented with 0.1 mM non-essential amino acids. The human colorectal carcinoma HCT116 Bax wild-type ($Bax^{+/-}$) and null ($Bax^{-/-}$) cell lines (Zhang et al., 2000), a kind gift from B. Vogelstein, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Medical Institutions, Baltimore, MD, USA, were cultured in McCoy's 5A medium supplemented with 2 mM L-glutamine, 10% FBS, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Cells were grown at 37°C with 5% CO_2.

2.2 Antibodies

The primary antibodies used for Western blot analysis were anti-myc monoclonal, anti-myc and anti-Mcl-1 polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 and anti-Bax monoclonal (Calbiochem, La Jolla, CA), anti-actin monoclonal, anti-Hsp-60 monoclonal, anti-flag monoclonal and polyclonal (Sigma, St. Louis, MO), anti-poly (ADP-ribose) polymerase (PARP) and anti-Bax polyclonal (Cell Signaling Technology Inc., Beverly,

MA), anti-cytochrome c and anti-Bcl-x monoclonal (BD Biosciences, San Jose, CA), anti-Bak polyclonal (Upstate, Lake Placid, NY), anti-HRK polyclonal (AbD Serotec, Oxford, UK), anti-HCV NS3 monoclonal (Chemicon, Temecula, CA**)** and anti-HCV core monoclonal (Abcam plc, Cambridge, UK). Anti-core protein monoclonal antibody (clone 2H9; a kind gift from T. Wakita, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan) was used to detect the core protein of HCV (Wakita et al., 2005). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) and HRP-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) were used as secondary antibodies.

2.3 Construction of plasmids

Expression plasmids for the wild-type core protein and mutants were generated by polymerase chain reaction (PCR) using Titanium *Taq* DNA polymerase (Clontech Laboratories Inc., Palo Alto, CA). Two plasmids containing full-length HCV genomes were used as template. The first one is a 1b strain cloned in Singapore (Soo et al., 2002) and the second is the JFH-1 clone, which is a 2a strain (Wakita et al., 2005). The PCR products were cleaved with restriction enzymes BamHI and XhoI and spliced into the pXJ40flag vector. All sequences were confirmed by sequencing performed by the core facilities at the Institute of Molecular and Cell Biology, Singapore. The pXJ40flag vector is used so that a flag epitope is fused to the N terminus of the core protein and this allows the comparison of protein expression levels with an anti-flag antibody. Epitope-tagged HCV core protein has been shown

to be functional in terms of oligomerization and virus-like particle (VLP) formation (Acosta-Rivero et al., 2005; Strosberg et al., 2010).

2.4 Transient transfection of mammalian cells

Transient transfection of Huh7, 293T and HCT116 cells were performed in 6 cm dishes using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Typically, 1 to 3 µg of plasmid DNA was used per dish. Transfection was carried out in the absence of antibiotics and the cells were replenished with complete medium at 6 h post-transfection. Approximately 16 h after transfection, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 200 µl of immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholic acid, 0.005% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride). After six freeze-thaw cycles, cell debris was removed by centrifugation and the total protein concentration in the lysate was determined using the Coomassie Plus protein assay reagent from Pierce.

2.5 Western blot analysis

Equal amounts of proteins were prepared in Laemmli's SDS buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrotransferred onto a nitrocellulose Hybond-C (Amersham Pharmacia Biotech, Uppsala, Sweden) or polyvinylidene difluoride (Millipore, Bedford, MA) membrane. The membrane was blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 (PBST) for 30 min at room temperature and then incubated overnight with the primary antibody

at 4°C. After extensive washes with PBST, the membrane was incubated with an appropriate HRP-conjugated secondary antibody for 1 h at room temperature, followed by washing and detection by an enhanced chemiluminescence method (Pierce). An imaging densitometer (Bio-Rad, Hercules, CA) or the ImageJ software (http://rsbweb.nih.gov/ij/index.html) was used for quantification of the intensities of specific bands on autoradiographs.

2.6 Co-immunoprecipitation (Co-IP)

Anti-flag monoclonal antibody conjugated to sepharose beads (Sigma) were added to 150 µl of the cell lysates and the mixture was subjected to end-over-end mixing at 4°C for 6 h. Beads were washed four times with cold IP buffer, and then 15 µl of Laemmli's SDS buffer was added and the samples were boiled at 100°C for 5 min to release the immunocomplexes. Samples were separated by SDS-PAGE and subjected to Western blot analysis.

Alternatively, rabbit anti-Mcl-1 polyclonal antibody was used to immunoprecipitate endogenous Mcl-1 protein. In this case, 7 µg of antibody (either anti-Mcl-1 or anti-hemagglutinin (HA) polyclonal antibody (Santa Cruz Biotechnology)) were added to the lysates obtained from two 6 cm dishes of cells and allowed to mix for 1 h at room temperature. Protein A agarose beads (Roche, Indianapolis, IN) were added and the mixture was subjected to end-over-end mixing at 4°C overnight. The co-immunoprecipitated proteins were then detected as described above. Co-immunoprecipitation experiments with HCV-infected cells were performed in a similar manner.

2.7 Synthesis of peptides

A peptide that corresponds to residues 118 to 149 of the genotype 1b core protein (NLGKVIDTLTCGFADLMGYIPLVGAPLGGAAR) was synthesized and purified to 95% purity (Sigma Genosys, Japan). Peptides containing the BH3 domain of Bad (NLWAAQRYGRELRRMSDE FVDSFKK) or Noxa (VPADLKDECAQLRRIGDKVNLRQKL) also were synthesized and purified to 95% purity (Mimotopes, Clayton Victoria, Australia).

2.8 *In vitro* cytochrome c release assay

For use in the *in vitro* cytochrome c release assay, mitochondria were isolated from 293T cells as previously described (Fu et al., 2007). Briefly, 293T cells were suspended in isolation buffer (320 mM sucrose, 1 mM EDTA, 50 mM HEPES pH 7.5) and disrupted by 25 expulsions through a 27-gauge needle. The disrupted cells were centrifuged at 1000 × g for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 7000 × g for 10 min and the pellet was retained as the heavy membrane fraction containing the mitochondria. The mitochondrion-containing pellets were then resuspended in assay buffer (250 mM sucrose, 2 mM KH_2PO_4, 5 mM sodium succinate, 25 mM EGTA, and 10 mM HEPES pH 7.5) at 0.5 mg/ml. Equal amounts of mitochondria were treated with the indicated peptides for 30 min at room temperature, followed by centrifugation. Both the supernatant and pellet were then subjected to SDS-PAGE and Western blot analysis to determine the amount of cytochrome c released from the mitochondria. Hsp-60 was used as a loading control for the pellet.

2.9 Generation of stable cell lines

The Bcl-X_L and Mcl-1 open reading frames (ORFs) with a myc tag at the 5' end were cloned into the pXJ41neo vector (Zheng et al., 1992). 20 µg of plasmid DNA was transfected into approximately 1.0×10^7 Huh7.5 cells by means of electroporation (950 µF, 250 V) using a Gene Pulser (Bio-Rad). The transfected cells were plated onto 10 cm dishes and selection medium containing 1.5 mg/ml of Geneticin (G418; Invitrogen) was added 48 h post-transfection. After 3 to 5 days, single colonies were picked and transferred to individual wells in a flat-bottomed 96-well microplate. Confluent wells were expanded into 24-well plates and then 6 cm dishes for screening and freezing down. Stable overexpression of Bcl-X_L and Mcl-1 was confirmed by Western blot analysis. Control cells were generated by stably transfecting Huh7.5 cells with the empty vector and culturing under G418 selection.

2.10 Small interfering RNA (siRNA)-mediated gene silencing

A 21-nucleotide RNA duplex targeting the coding region of human Bcl-X_L gene (Zender et al., 2005) was synthesized using the Stealth RNA interference (RNAi) technology (Invitrogen). Pre-designed Silencer Select siRNA duplex directed against human Mcl-1 was purchased (Ambion, Austin, TX). The siRNA sequences were as follows:

siBcl-X_L

5'-GGA UAC AGC UGG AGU CAG U[dT][dT]-3' (sense)

5'-ACU GAC UCC AGC UGU AUC C[dT][dT]-3' (antisense)

siMcl-1

5’-CAA CUU CCG UAA UUA GGA A[dT][dT]-3’ (sense)

5’-UUC CUA AUU ACG GAA GUU G[dC][dA]-3’ (antisense)

The annealed and desalted siRNAs were reconstituted in nuclease-free water to a final concentration of 20 μM. Stealth RNAi negative control duplex (Invitrogen) and Silencer Select negative control #1 siRNA (Ambion) were transfected in parallel to serve as a control for sequence independent effects of siRNA transfection (siControl).

Huh7.5 cells were seeded without antibiotics in 6 cm dishes to achieve about 30% confluency the next day. Transfection of siRNA was performed using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s recommendations. Briefly, 50 nM of Bcl-X_L siRNA, 10 nM of Mcl-1 siRNA or their respective control siRNA was added to each dish. At 24 h post-transfection, the siRNA was removed and the cells were replenished with complete medium. Cells were harvested at 2, 4, 6 and 8 days post-transfection and the knockdown efficiency was examined by Western blot analysis.

2.11 Cell proliferation assay

Huh7.5 cells were seeded in flat-bottomed 96-well microplates at a density of 1.0×10^4 cells/well and allowed to attach overnight. The cells were then left untreated or infected with HCV or mock preparation and culture medium was replaced every two days. At specific time points after treatment, cell viabilities were determined using the cell proliferation reagent WST-1

(Roche), according to the manufacturer's protocol. Briefly, 10 µl of WST-1 reagent was added to each well containing cells in a final culture medium volume of 100 µl. After incubating at 37°C for 1 h, the absorbance of the samples was measured using a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 440 nm and a reference wavelength of 630 nm.

2.12 DNA fragmentation assay

This assay measures the degree of apoptosis based on the amount of histone-associated DNA fragments detected in the cytoplasm and was performed using a Cell Death Detection ELISA^{Plus} kit (Roche) as previously described (Deng et al., 2008). Briefly, Huh7.5 cells cultured in a 96-well microplate were spun at 200 × g for 10 min at 4°C to remove the culture medium. The cells were suspended in lysis buffer and centrifuged at 200 × g for 10 min. The supernatant was retained as the cytoplasmic fraction and 20 µl of supernatant was added to each well of a streptavidin-coated 96-well microplate. A mixture of biotin-labelled anti-histone antibody and peroxidase-labelled anti-DNA antibody was added to the wells and incubated for 2 h at room temperature. The wells were washed extensively before addition of 2,2'-azino-diethyl-benzthiazolin substrate and the peroxidase activities were measured spectrophotometrically using a microplate reader (Bio-Rad).

2.13 Detection of caspase enzymatic activities

The activation of caspase-3, a hallmark of apoptosis, was measured by using a CaspACE fluorometric assay system or a Caspase-Glo 3/7 luminescent

assay system (Promega, Madison, WI) according to the manufacturer's instructions. These assays involve the addition of fluorogenic or luminogenic substrates which contain the caspase-3 cleavage site tetrapeptide sequence DEVD. The amount of fluorescence or luminescence produced upon cleavage of these substrates is proportional to the amount of caspase activity present in the sample.

In the CaspACE fluorometric assay, 5 µl of cell lysate were added to individual wells of an opaque 96-well microplate and diluted in caspase assay buffer before incubation at 30°C for 30 min. After addition of the substrate which is labelled with the fluorochrome 7-amino-4-methyl coumarin (AMC), the plate was incubated at 30°C for 1 h. The yellow-green fluorescence of liberated AMC was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm using a microplate reader (Tecan).

The Caspase-Glo 3/7 assay employs a direct lysis method. An equal volume of Caspase-Glo reagent, which contains cell lysis buffer and substrate, was added to cells cultured in an opaque 96-well microplate. The plate was incubated at room temperature for 1 h. Caspase cleavage of the substrate releases aminoluciferin which is utilized in the luciferase reaction to produce light. Luminescence was measured in relative light units (RLU) using a microplate luminometer (Promega).

2.14 Viruses

The pFL-J6/JFH-1 plasmid encoding the entire viral genome of a chimeric strain of HCV genotype 2a, J6/JFH-1 (Lindenbach et al., 2005) , was

kindly provided by C. M. Rice. To generate mutant virus possessing a core protein mutation (V119L), a nucleotide substitution was introduced into pFL-J6/JFH-1 by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All PCR-amplified DNA fragments were verified extensively using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). Each of the plasmids was linearized by XbaI digestion and *in vitro* transcribed by using T7 RiboMAX (Promega) to generate the full-length viral genomic RNA. The *in vitro*-transcribed RNA (10 μg) was transfected into Huh7.5 cells by means of electroporation (975 μF, 270 V) using a Gene Pulser (Bio-Rad). The cells were then cultured in complete medium and the supernatant was propagated as a virus stock.

The J6/JFH-1-P47 virus (Bungyoku et al., 2009), an adapted strain of the J6/JFH-1 virus that possesses enhanced infectivity, was kindly provided by H. Hotta, Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo, Japan. The adapted virus was obtained by passaging the J6/JFH-1-infected cells 47 times and contains 10 amino acid mutations (K78E, T396A, T416A, N534H, A712V, Y852H, W879R, F2281L, M2876L and T2925A) and a single nucleotide mutation in the 5'-UTR (U146A).

2.15 Virus titration

Virus infectivity was measured by indirect immunofluorescence analysis as described (Deng et al., 2008). Briefly, culture supernatants containing HCV were serially diluted 10-fold in complete DMEM and used to infect 1.9×10^5 Huh7.5 cells seeded on glass coverslips in a 24-well plate.

The inoculum was incubated with cells for 5 h at 37°C and then replaced with fresh complete DMEM. At 24 h post-infection, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min and blocked with PBS containing 1% bovine serum albumin (BSA; Sigma) for 30 min. The cells were then incubated with the serum of an HCV-infected patient (1:200) for 1 h and washed with PBS three times before incubating with a fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody (1:200; Sigma) for 1h. After three washes with PBS, the coverslips were mounted on glass slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and examined under a fluorescence microscope (BX51; Olympus, Center Valley, PA). All incubations and washes were performed at room temperature. The viral titre was determined by the average number of HCV-positive foci observed at the highest dilutions according to a previously described method (Zhong et al., 2005) and expressed as cell-infecting units (CIU) or focus-forming units (ffu) per millilitre of supernatant.

2.16 HCV infection

For infection studies, Huh7.5 cells were seeded in either 96-well microplates at a density of 1.0×10^4 cells per well, 24-well plates at a density of 6.0×10^4 cells per well or 6-well plates at a density of 3.0×10^5 cells per well and cultured overnight. For infection with J6/JFH1 or J6/JFH-1(V119L), the cells were infected at a multiplicity of infection (MOI) of 0.1 ffu/cell. Infections with the J6/JFH-1-P47 virus were performed at an MOI of 2.0 ffu/cell. Control cells were treated with a mock preparation. The inoculum

was incubated with cells for 5 h at 37°C and then replaced with fresh complete DMEM. The culture medium was replenished every two days and the infected cells were assayed at different time points post-infection.

2.17 Isolation of total cellular RNA

Huh7.5 cells in 24-well plates were infected with HCV or mock preparation and total RNA was isolated at 2, 4 and 6 days post-infection (d.p.i.). Following removal of culture medium, the cells were rinsed twice in PBS and RNA was extracted using an RNeasy Mini Kit with an on-column DNase treatment step (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration and quality of RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). RNA samples with an $OD_{260\ nm}/OD_{280\ nm}$ absorbance ratio between 1.8 and 2.0 were used.

2.18 Reverse transcription

Total RNA was reverse transcribed into complementary DNA (cDNA) using the QuantiTect Reverse Transcription (RT) kit (Qiagen). Briefly, 400 ng of RNA was incubated with the gDNA Wipeout Buffer for 2 min at 42°C to eliminate any contaminating genomic DNA. The RT master mix was prepared by mixing the Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix containing an optimized blend of oligo-dT and random primers. Template RNA was added to the RT master mix and the RT reaction was performed at 42°C for 15 min. The reaction was then incubated at 95°C for 3 min to inactivate the reverse transcriptase.

For PCR array analysis, total RNA was reverse transcribed into cDNA using the RT^2 First Strand Kit (SABiosciences, Frederick, MD) according to the manufacturer's instructions. In brief, genomic DNA elimination buffer was added to 700 ng of RNA in 10 µl reactions and incubated at 42°C for 5 min. An equal volume of the reverse transcription cocktail containing primers (random hexamers and oligo-dT), nucleotides and reverse transcriptase was added to each reaction and incubated at 42°C for 15 min. The enzyme was then immediately inactivated by heating at 95°C for 5 min.

2.19 Viral RNA quantification

To measure intracellular HCV RNA replication levels, cDNA was subjected to quantitative real-time PCR analysis using the SYBR Green-based detection system. The cDNA was diluted in water and added in triplicates to individual wells of a MicroAmp optical 96-well reaction plate (Applied Biosystems) containing the RT^2 SYBR Green/ROX quantitative PCR (qPCR) master mix (SABiosciences) and gene-specific primers. One primer set amplifies a region in NS5A of the HCV genome while another probes the expression levels of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The primer sequences were as follows:

<u>HCV primers</u>

5'-AGA CGT ATT GAG GTC CAT GC-3' (sense)

5'-CCG CAG CGA CGG TGC TGA TAG-3' (antisense)

GAPDH primers

5’-CAT GAG AAG TAT GAC AAC AGC CT-3’ (sense)

5’-AGT CCT TCC ACG ATA CCA AAG T-3’ (antisense)

The plate was subjected to a two-step cycling program on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). HCV and GAPDH transcript levels were determined relative to standard curves derived from serial dilutions of plasmids containing either the HCV J6/JFH-1 cDNA or the human GAPDH gene.

2.20 Gene expression profiling using PCR arrays

Template cDNA was mixed with RT^2 SYBR Green/ROX qPCR master mix (SABiosciences) and 25 µl of this mixture was added to each well of the 96-well PCR array containing specific primer sets. The Human Apoptosis RT^2 Profiler PCR Array (SABiosciences) examines 84 genes involved in the apoptotic pathway. These genes include members of the caspase, Bcl-2, IAP, TRAF, CARD, CIDE, death domain, death effector domain, and TNF receptor and ligand families, as well as genes involved in the p53 and DNA damage pathways. The array also contains primer sets for five housekeeping genes and three RNA and PCR quality controls (Appendix 1).

The PCR cycling program was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Expression profiles of HCV-infected and mock-infected cells at the different time points were obtained from three independent experiments. The threshold cycle (Ct) values of each

gene were used to calculate the fold changes in gene expression using the RT^2 Profiler PCR Array Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

2.21 Quantitative real-time PCR

Gene expression changes observed with the PCR arrays were confirmed by quantitative real-time PCR using TaqMan chemistry. Probes and primers specific to the host genes that were identified by the PCR arrays to be up-regulated or down-regulated in response to HCV infection were obtained from Applied Biosystems. GAPDH was selected as the reference gene for normalization. Each reaction was performed in triplicates and no template controls were included for each primer/probe set. Amplification was monitored on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The difference in Ct values (ΔCt) between the gene of interest and GAPDH control was used to calculate the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) between HCV-infected and mock-infected Huh7.5 cells. Results were obtained from at least three independent experiments.

2.22 Statistical analysis

The two-tailed Student's *t* test or one-way analysis of variance was applied to evaluate the statistical significance of differences measured from the data sets. A *P* value of less than 0.05 was considered to be statistically significant.

CHAPTER 3: HCV CORE PROTEIN CONTAINS A BH3 DOMAIN THAT REGULATES APOPTOSIS THROUGH SPECIFIC INTERACTION WITH HUMAN MCL-1

3.1 A BH3-like domain is present in the core protein

The family of Bcl-2 proteins constitutes one of the biologically important gene products in the regulation of apoptosis [reviewed by (Adams and Cory, 2007; Danial, 2007; van Delft and Huang, 2006; Youle and Strasser, 2008)]. Members of the Bcl-2 protein family are defined by the presence of up to four Bcl-2 homology domains (BH1 to BH4) in their protein sequence. These Bcl-2 proteins may be classified broadly into three classes: prosurvival members containing multiple Bcl-2 homology domains, proapoptotic members containing multiple Bcl-2 homology domains and proapoptotic members containing only the BH3 domain.

The function of Bcl-2 family proteins in regulating cell survival is highly conserved in species ranging from nematodes and flies to humans (Colin et al., 2009). A number of viruses have also evolved to encode homologues of Bcl-2 family proteins so as to modulate the apoptotic pathways of the host in favour of viral propagation. Several large DNA viruses, including adenoviruses, herpesviruses and poxviruses, are known to encode prosurvival viral Bcl-2 proteins (vBcl-2s) [reviewed by (Cuconati and White, 2002; Galluzzi et al., 2008; Polster et al., 2004)]. It has been widely hypothesized that these vBcl-2s function to inhibit premature apoptosis of infected cells, thus allowing completion of the viral replication cycle. Inhibition of apoptosis may also contribute to the establishment of or

reactivation from latency and the development of a chronic, persistent infection. In contrast, only one viral homologue of proapoptotic BH3-only proteins has been identified thus far. Lu and colleagues demonstrated that HBSP, a spliced hepatitis B viral protein, contains a functional BH3 domain (Lu et al., 2006).

In the case of HCV, only NS5A has been previously identified as a potential vBcl-2 that inhibits apoptosis via direct interaction with Bax (Chung et al., 2003). Therefore, to determine if HCV encodes other homologues of Bcl-2 family proteins, we examined the amino acid sequence of other HCV proteins for the presence of BH domains. An examination of the amino acid sequence of the core protein revealed that there is a BH3-like domain near the C-terminus. An alignment of this domain with BH3 domains of the Bcl-2 family of proteins is shown in Figure 3.1A. The four hydrophobic amino acids that are responsible for making critical contacts with residues in the BH3 recognition grooves present on the surfaces of the prosurvival Bcl-2 family proteins are indicated as h1 to h4. The BH3-like domain of the core protein contains L (residue 119) and D (residue 124) separated by four residues, as observed in other known BH3 domains. This region within the core protein is highly conserved among the major HCV genotypes, with the exception of genotypes 2a and 6k, which have V and M residues at position 119, respectively (Figure 3.1B).

(A)

HCV 1b core (109-132)	P	I	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
hBim (82-105)	M	R	P	E	I	W	I	A	Q	E	L	R	R	I	G	D	E	F	N	A	Y	Y	A	R
hPuma (131-154)	E	Q	W	A	R	E	I	G	A	Q	L	R	R	M	A	D	D	L	N	A	Q	Y	E	R
hBad (104-127)	L	W	A	A	Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	V	D	S	F	K	K
hHrk (27-50)	S	S	A	A	Q	L	T	A	A	R	L	K	A	L	G	D	E	L	H	Q	R	T	M	W
hBik (51-74)	M	E	G	S	D	A	L	A	L	R	L	A	C	I	G	D	E	M	D	V	S	L	R	A
hBid (80-103)	E	D	I	I	R	N	I	A	R	H	L	A	Q	V	G	D	S	M	D	R	S	I	P	P
hNoxa (19-42)	A	E	L	E	V	E	C	A	T	Q	L	R	R	F	G	D	K	L	N	F	R	Q	K	L
mNoxaA (17-40)	A	E	L	P	P	E	F	A	A	Q	L	R	K	I	G	D	K	V	Y	C	T	W	S	A
mNoxaB (68-91)	P	A	D	L	K	D	E	C	A	O	L	R	R	I	G	D	K	V	N	L	R	O	K	L
hBcl-2 (87-110)	S	P	V	P	P	V	V	H	L	T	L	R	Q	A	G	D	D	F	S	R	R	Y	R	R
hBcl-xL (81-103)	I	P	M	A	A	-	V	K	Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R
hBcl-w (36-59)	G	P	A	A	D	P	L	H	Q	A	M	R	A	A	G	D	E	F	E	T	R	F	R	R
hMcl-1 (203-226)	G	A	T	S	R	K	A	L	E	T	L	R	R	V	G	D	G	V	Q	R	N	H	E	T
hBax (53-76)	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	N	M	E	L
hBak (68-91)	S	S	T	M	G	Q	V	G	R	Q	L	A	I	I	G	D	D	I	N	R	R	Y	D	S
hBok (60-83)	P	G	R	L	A	E	V	C	A	V	L	L	R	L	G	D	E	L	E	M	I	R	P	S

BH3-only

Pro-survival

Pro-apoptotic

h1 h2 h3 h4

(B)

Genotype

1a	P	T	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
1b	P	T	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
1c	P	S	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
2a	P	T	D	P	R	H	R	S	R	N	V	G	K	V	I	D	T	L	T	C	G	F	A	D
2b	P	T	D	P	R	H	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
2c	P	T	D	P	R	H	K	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
2k	P	T	D	P	R	H	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
3a	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
3b	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
3k	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
4a	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
5a	P	N	D	P	R	R	K	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
6a	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	F	A	D
6b	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	L	A	D
6g	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	L	A	D
6h	P	N	D	P	R	R	R	S	R	N	L	G	K	V	I	D	T	L	T	C	G	L	A	D
6k	P	N	D	P	R	R	R	S	R	N	M	G	K	V	I	D	S	L	T	C	G	F	A	D

Fig. 3.1. Identification of a BH3-like domain in HCV core protein. (A) Alignment of the BH3 domain of the genotype 1b core protein with the BH3 domains of members of the Bcl-2 family. Numbers in parentheses represent the positions of amino acid residues of the respective proteins. The four hydrophobic amino acids that make critical contacts with residues in the BH3 recognition grooves present on the surfaces of the prosurvival Bcl-2 family proteins are shaded and indicated as h1 to h4. (B) Alignment of the core protein (residues 109 to 132) of different HCV genotypes. The consensus sequences for these genotypes were obtained from http://hcv.lanl.gov/content/hcv-index. The highly conserved L and D residues found at positions 119 and 124 of the genotype 1b core protein are boxed.

3.2 Role of the BH3 domain in the HCV core protein

3.2.1 The BH3 domain of the core protein is essential for the induction of apoptosis

To characterize the putative BH3 domain, plasmid-liposome complex-mediated overexpression of the core protein was first performed. The expression of the core protein (with a flag epitope at the N terminus) in Huh7 cells was achieved through the transient transfection of a cDNA expression plasmid containing the genotype 1b core protein gene. Overexpression of the core protein induced significant levels of apoptosis as determined by the activation of caspase-3, which is a hallmark of apoptosis (Figure 3.2A). The deletion of the BH3 domain in the flag-tagged core protein (designated coreΔ115-128aa) abolished its proapoptotic property, indicating that this domain is essential for the induction of apoptosis. Consistently, the cleavage of endogenous PARP, a substrate of activated caspase-3, was clearly observed in Huh7 cells expressing the wild-type core protein but not in those expressing coreΔ115-128aa (Figure 3.2B). The activation of caspase-3 and cleavage of endogenous PARP caused by the overexpression of Bax, a classical potent inducer of apoptosis, are shown as positive controls.

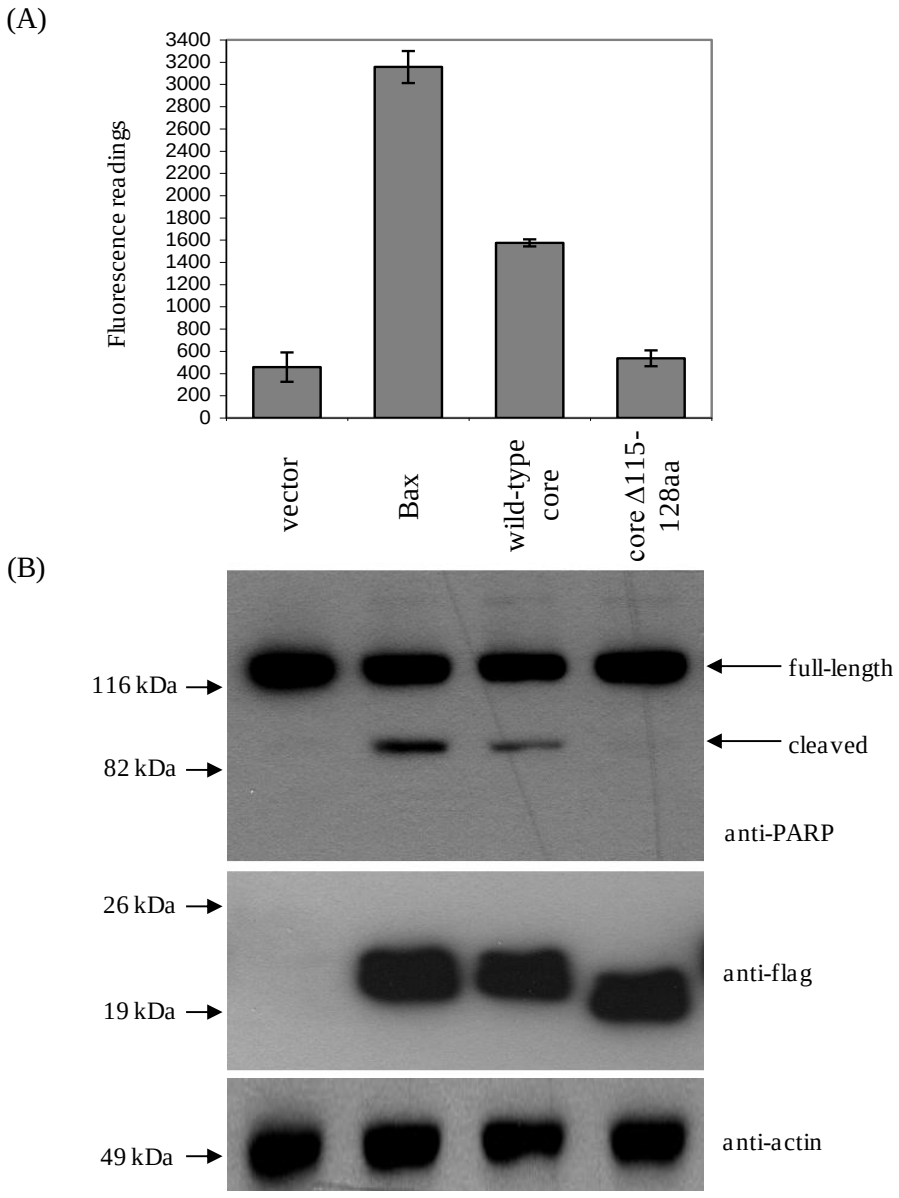

Fig. 3.2. Induction of apoptosis by the overexpression of core protein in Huh7 cells. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3, which is a hallmark of apoptosis, in Huh7 cells that were transfected with vector only, a classical apoptosis inducer (Bax), the wild-type core protein and a core protein mutant lacking the putative BH3 domain (coreΔ115-128aa). All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis was also performed to determine the cleavage of endogenous PARP, which is a substrate of activated caspase-3, from 116 to 83 kDa (top). Similarly, the expression levels of the different proteins were determined using anti-flag antibody (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

3.2.2 The BH3 domain of the core protein mediates specific interaction with human Mcl-1

To understand how the core protein modulates the function of the Bcl-2 family of proteins, coimmunoprecipitation experiments were performed in Huh7 cells to determine if the core protein can interact with representative prosurvival members of the Bcl-2 family. As shown in the top panel of Figure 3.3, Mcl-1 was specifically coimmunoprecipitated by the core protein (lane 8) but not by an irrelevant protein, glutathione *S*-transferase (GST) (lane 7). The BH3 domain of the core protein is essential for its interaction with Mcl-1, as the coreΔ115-128aa mutant failed to coimmunoprecipitate Mcl-1 (lane 9). In contrast, no significant interaction was observed between the core protein and two other prosurvival Bcl-2 family members, Bcl-X_L and Bcl-w (lanes 1 to 6). These results suggest that the core protein induces apoptosis by interfering directly and specifically with the prosurvival function of Mcl-1.

The interaction between the core protein and endogenous Mcl-1 was determined by overexpressing the core protein in Huh7 cells and performing coimmunoprecipitation experiments. As shown in Figure 3.4A, the wild-type core protein was coimmunoprecipitated with endogenous Mcl-1 (lane 3). In contrast, the core protein was not coimmunoprecipitated when an irrelevant antibody (anti-HA) (lane 4) was used for IP. Similar to the results in Figure 3.3, only a small amount of the coreΔ115-128aa mutant was coimmunoprecipitated with endogenous Mcl-1 (lane 5). CoreΔ115-128aa was also not coimmunoprecipitated when anti-HA was used for IP.

To compare the degree of binding between endogenous Mcl-1 and the wild-type core protein or the coreΔ115-128aa mutant, an imaging densitometer was used to quantify the intensities of specific bands on the autoradiographs obtained in three independent coimmunoprecipitation experiments (representative data is shown in Figure 3.4B). The ratios of signals for the expression of flag-tagged core proteins, endogenous Mcl-1 and actin (internal control) are all close to 1 (0.94 to 1.14), indicating that the expression levels of these proteins in the two sets of cells (either transfected with cDNA construct for expressing flag-core or flag-coreΔ115-128aa) were similar. From the three independent experiments, the average amount of coreΔ115-128aa coimmunoprecipitated specifically by the Mcl-1 antibody is 14.2% (± 10.7%) of the amount of wild-type core protein coimmunoprecipitated. This implies that the deletion of the BH3 domain does not completely abolish the interaction between the core protein and endogenous Mcl-1 but reduces this interaction greatly. Since binding of the coreΔ115-128aa mutant to Mcl-1 was not completely abrogated, this interaction may also be mediated by other minor interaction domains.

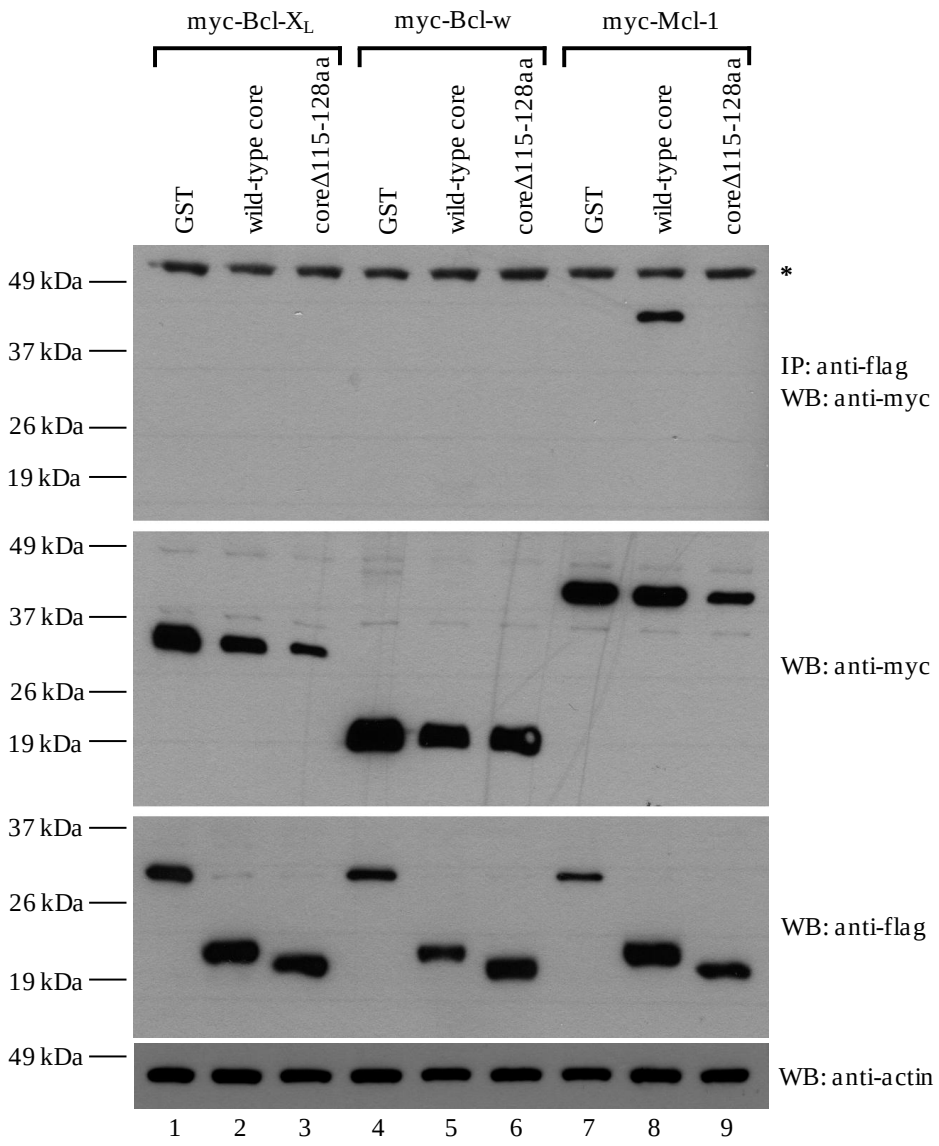

Fig. 3.3. Interaction of core protein with prosurvival members of the Bcl-2 family determined by coimmunoprecipitation experiments. Huh7 cells were transfected with cDNA constructs for expressing flag-GST (negative control), flag-core or flag-coreΔ115-128aa and myc-tagged prosurvival members of the Bcl-2 family (myc-Bcl-X_L [lanes 1 to 3], myc-Bcl-w [lanes 4 to 6] and myc-Mcl-1 [lanes 7 to 9]). The cells were harvested at ~16 h post-transfection, lysed and subjected to IP with anti-flag monoclonal antibody conjugated to sepharose beads. The amount of myc-tagged proteins that coimmunoprecipitated (IP) with the flag-tagged proteins was determined by Western blot analysis (WB) with an anti-myc rabbit polyclonal antibody (top). The amounts of myc-tagged and flag-tagged proteins in the lysates before IP were determined by subjecting aliquots of the lysates to Western blot analysis (middle). The protein marked with an asterisk represents the heavy chain of the antibody used for IP (top) and the amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

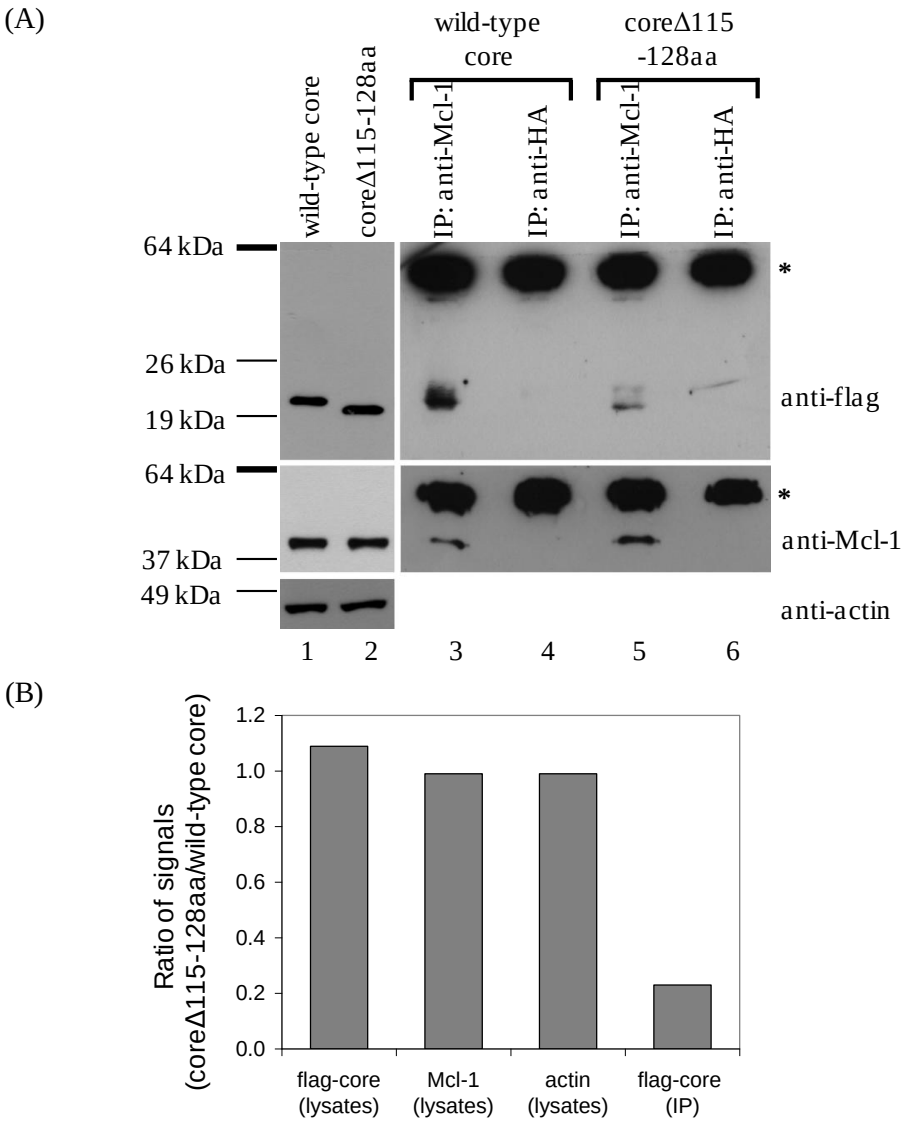

Fig. 3.4. Interaction of the wild-type core protein or the coreΔ115-128aa mutant with endogenous Mcl-1 in Huh7 cells. (A) Huh7 cells were transfected with cDNA constructs for expressing flag-core or flag-coreΔ115-128aa. Immunoprecipitation (IP) was then performed using anti-Mcl-1 or anti-HA rabbit polyclonal antibodies and protein A agarose beads. The amounts of flag-tagged core protein in the lysates before IP (lanes 1 and 2) or coimmunoprecipitated (lanes 3 to 6) were determined by Western blot analysis with an anti-flag monoclonal antibody (top). Similarly, the amounts of endogenous Mcl-1 in these samples were detected using an anti-Mcl-1 monoclonal antibody (middle). The protein marked with an asterisk represents the heavy chain of the antibody used for IP (top) and the amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (B) An imaging densitometer was used to quantify the intensity of specific bands on the autoradiographs. For the amount of proteins in the lysates before IP (lanes 1 and 2), the ratio of the signals for samples transfected with coreΔ115-128aa to that for samples transfected with wild-type core protein is plotted. For the amounts of flag-tagged core protein co-immunoprecipitated (lanes 3 to 6), the signal for samples co-IP by HA antibody (unspecific binding) was subtracted from the signal for samples co-IP by Mcl-1 antibody. Then, the ratio of subtracted signals for samples transfected with coreΔ115-128aa to that for samples transfected with wild-type core protein is plotted.

3.3 Overexpression of Mcl-1 or Bcl-X_L prevents core protein-induced apoptosis

Although the prosurvival Bcl-2 proteins show significant sequence and structural similarity, their functions and interaction profiles are not exactly the same (Certo et al., 2006; Chao and Korsmeyer, 1998). In particular, their abilities to protect cells against apoptosis can vary between different cell types and death stimulus (He et al., 2003; Memon et al., 1995; Panickar et al., 2005). Hence, we first overexpressed several prosurvival Bcl-2 proteins in Huh7 cells and measured the level of apoptosis (Figure 3.5A and B). The transient high-level expression of Bcl-2 caused apoptosis in Huh7 cells (lane 1), which is consistent with studies done using other cell lines (Cheng et al., 1997; Uhlmann et al., 1998). Interestingly, the overexpression of Bcl-w also induced a significant level of apoptosis in Huh7 cells (lane 3) and this phenomenon has not been reported previously. Cells overexpressing Bcl-X_L (lane 2), but not Mcl-1 (lane 4), also displayed a slightly higher level of apoptosis than that of the vector-transfected control cells (lane 5). However, the level of apoptosis in the cells overexpressing Bcl-X_L is much lower than those overexpressing Bcl-2 or Bcl-w. This suggests that Bcl-2 and Bcl-w are not likely to have prosurvival function in Huh7 cells, which is consistent with a previous report that described Bcl-X_L and Mcl-1 as the major prosurvival Bcl-2 proteins in the liver (Hikita et al., 2009).

Subsequently, the abilities of Bcl-X_L and Mcl-1 to protect against core-induced apoptosis in Huh7 cells were examined by cotransfection with plasmids for expressing myc-Mcl-1 and flag-core or myc-Bcl-X_L and flag-

core. As shown in Figures 3.5C and 3.5D, the level of apoptosis was significantly reduced in cells expressing both myc-Mcl-1 and flag-core (lane 3) compared to cells expressing the core protein only (lane 2). When the same experiment was repeated using Bcl-X_L, the level of apoptosis was reduced to a lesser extent (lane 5). This may be due to the low level of apoptosis induced solely by the overexpression of Bcl-X_L (lane 6). The level of the core protein expressed in the presence of Bcl-X_L also was decreased greatly but the smaller amount of the core protein present still induced a high level of apoptosis (lane 5). However, when a broad caspase inhibitor (z-VAD-fmk) was used, the core protein level in cells coexpressing Bcl-X_L increased (Figures 3.6A and 3.6B), indicating that the transfection efficiencies were similar in the different samples. To resolve this uncertainty, the coexpression experiment was repeated with a smaller amount of Bcl-X_L plasmid (0.5 µg). Under this condition, the overexpression of Bcl-X_L alone did not induce apoptosis (lane 8). The level of apoptosis was also reduced in cells expressing both Bcl-X_L and the core protein (lane 7) compared to those expressing the core protein only (lane 2). Thus, the results show that the overexpression of either Mcl-1 or Bcl-X_L protects against core protein-induced apoptosis.

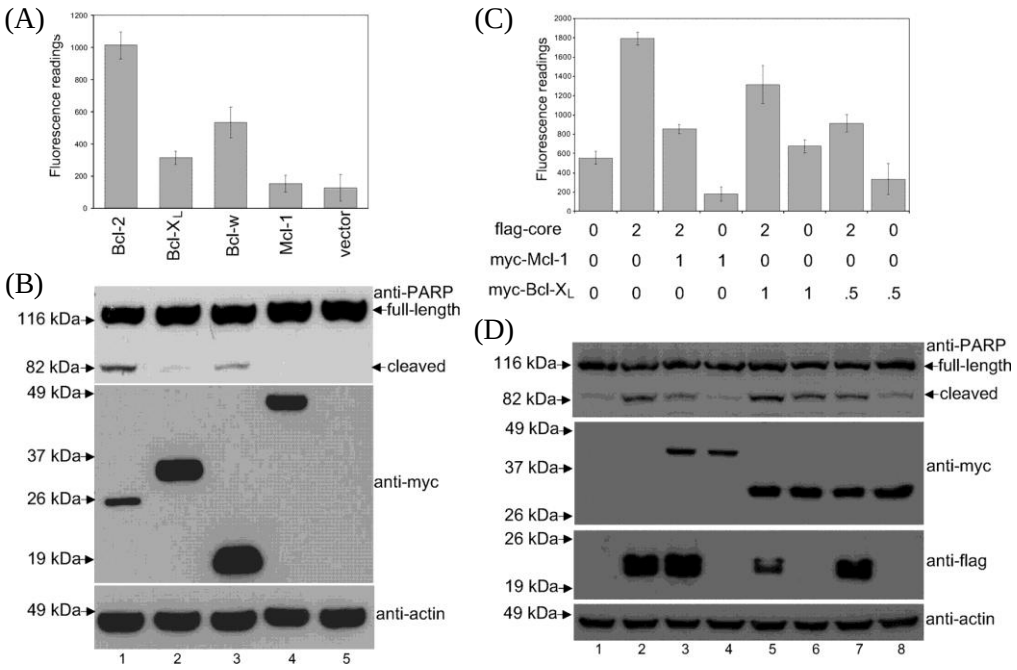

Fig. 3.5. Effects of Mcl-1 and Bcl-X_L overexpression on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were transfected with Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 or vector only. All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis was performed to determine the cleavage of endogenous PARP (top) and expression levels of the myc-tagged prosurvival members of the Bcl-2 family (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (C) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were singly transfected with vector, the wild-type core protein, Mcl-1 or Bcl-X_L, or that were cotransfected with wild-type core protein and Mcl-1 or Bcl-X_L. The amounts of flag-core and myc-Mcl-1 or myc-Bcl-X_L plasmid used in each transfection are indicated in micrograms. In each transfection, the total amount of DNA was normalised to 3 µg with the addition of empty vector if necessary. All experiments were performed in triplicate and the average values with standard deviations are plotted. (D) Western blot analysis was performed to determine the cleavage of endogenous PARP (top) and expression levels of myc-tagged Mcl-1 and Bcl-X_L and flag-tagged core protein (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

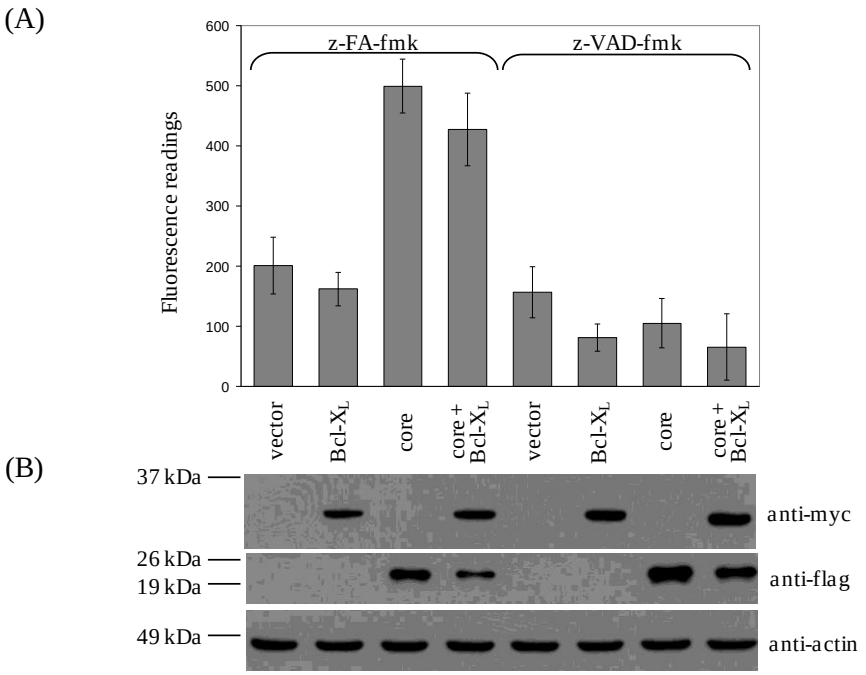

Fig. 3.6. Effects of apoptosis inhibition on the expression level of HCV core protein. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were singly transfected with vector, Bcl-X_L or wild-type core or co-transfected with wild-type core and Bcl-X_L in the presence of z-FA-fmk (an irrelevant peptide) or z-VAD-fmk (a pan-caspase inhibitor). All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis was performed to determine the expression levels of the myc-tagged Bcl-X_L and flag-tagged core protein (top and middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

3.4 Functional complementation between Bad and the core protein

3.4.1 Bad enhances the ability of the core protein to release cytochrome c from isolated mitochondria

Next, plasmid-liposome complex-mediated overexpression of the core protein was performed in 293T cells to determine if it also induces apoptosis in these cells. As shown in Figure 3.7, overexpression of the wild-type core protein in 293T cells induced significant levels of apoptosis in the same manner as that in Huh7 cells (lane 5). Consistent with the observations made in Huh7 cells, the coreΔ115-128aa mutant which lacks the BH3 domain of the core protein did not induce apoptosis in 293T cells (lane 6). The ability of Noxa, a known BH3-only protein that binds specifically to Mcl-1 and A1 but not to the other prosurvival members of the Bcl-2 family (Chen et al., 2005), to induce apoptosis in Huh7 and 293T cells is also demonstrated (lanes 3 and 7). Hence, the ability of a core protein peptide, which contains residues 118 to 149 of the genotype 1b core protein, to release cytochrome c from mitochondria was tested using 293T cells instead of Huh7 cells, as the method for the isolation of mitochondria from 293T cells is well established.

Isolated mitochondria from 293T cells were incubated with different concentrations of core protein, Bad or Noxa BH3 peptides and the amount of cytochrome c released into the supernatant of the treated mitochondria was determined by Western blot analysis. An alignment of the three peptide sequences shows that the core protein BH3 peptide lacks the h1 residue of the BH3 domain (Figure 3.8A). As shown in Figure 3.8B, both the Bad and core protein BH3 peptides alone were inefficient in inducing the release of

cytochrome c, as only a small amount of cytochrome c was detected in the supernatant when 200 µM of either peptide was used. However, when Bad and core protein BH3 peptides were used in combination, the release of cytochrome c was observed at a much lower total peptide concentration of 50 µM (consisting of 25 µM Bad BH3 peptide and 25 µM core protein BH3 peptide). Furthermore, the release of cytochrome c from isolated mitochondria increased in a dose-dependent manner. The amount of cytochrome c left in the treated mitochondria (i.e., pellet) also decreased correspondingly, while the amount of mitochondrial protein Hsp-60 (used as a loading control) was not affected.

The same experiment was repeated using the Noxa BH3 peptide instead of the core protein BH3 peptide (Figure 3.8C). Consistent with a previous study (Chen et al., 2005), the Noxa BH3 peptide alone was inefficient in inducing the release of cytochrome c from isolated mitochondria but when it was used in combination with the Bad BH3 peptide, the cytochrome c release was significantly enhanced. The total peptide dosage that induced the release of cytochrome c was similar to the amount required for the core protein and Bad BH3 peptides, indicating that the complementation between the core protein and Bad is similar to the complementation between Noxa and Bad. The results also suggest that the h1 residue in the BH3 domain of the core protein, a non-hydrophobic R residue, is not essential for the induction of mitochondrial cytochrome c release.

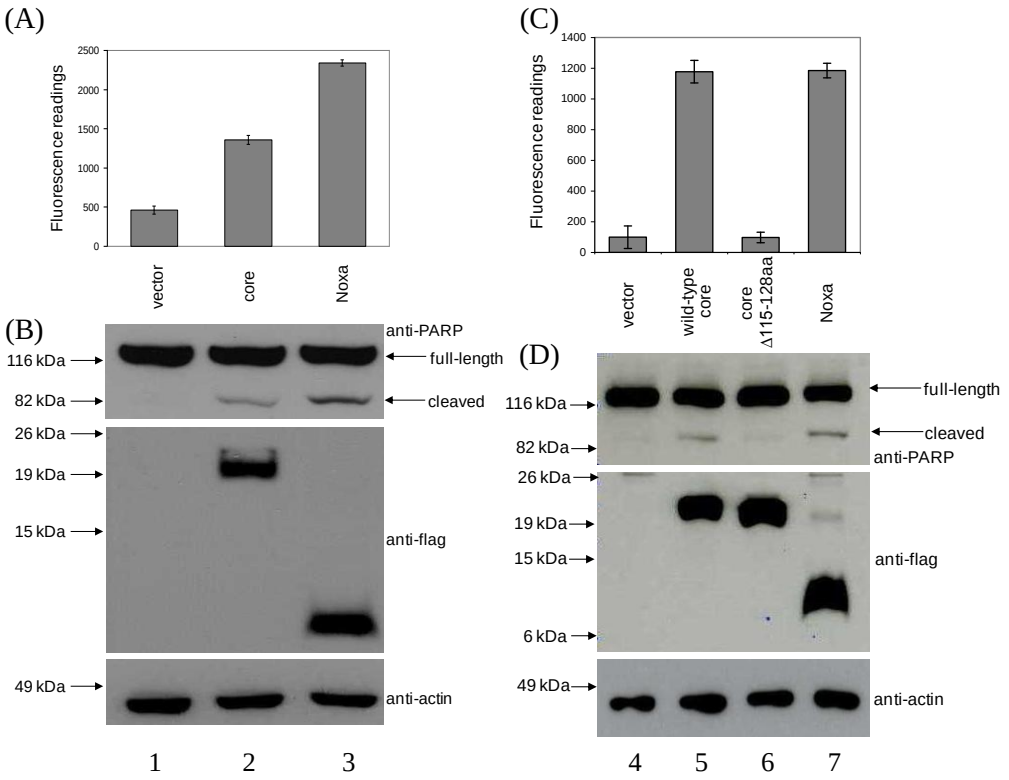

Fig. 3.7. Induction of apoptosis through the overexpression of core protein or Noxa in Huh7 and 293T cells. A CaspACE fluorometric assay system was used to measure the activation of caspase-3, which is a hallmark of apoptosis, in (A) Huh7 cells that were transfected with vector only, wild-type core protein and human Noxa or (C) 293T cells that were transfected with vector only, wild-type core protein, a core protein mutant lacking the putative BH3 domain (coreΔ115-128aa) and human Noxa. A flag epitope is fused to the N termini of the proteins as this allows comparison of protein expression levels with an anti-flag antibody. All experiments were performed in triplicate and the average values with standard deviations are plotted. Western blot analysis of the transfected Huh7 (B) and 293T cells (D) was also performed to determine the cleavage of endogenous PARP (top). Similarly, the expression levels of the different proteins were determined using an anti-flag antibody (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

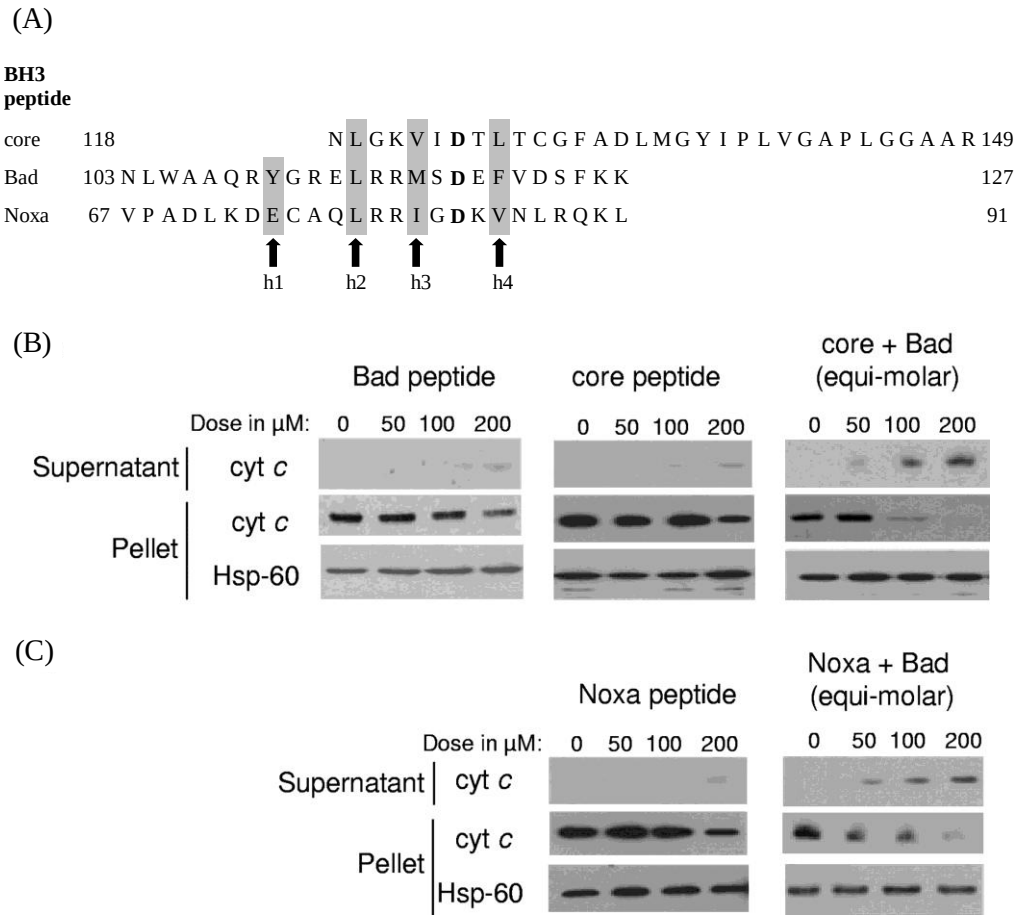

Fig. 3.8. Release of cytochrome c from isolated mitochondria by a combination of core-BH3 and Bad-BH3 peptides or Noxa-BH3 and Bad-BH3 peptides. (A) Protein sequence alignment of the core protein (residues 118 to 149), Bad (residues 103 to 127) and Noxa (residues 67 to 91) BH3 peptides. The four hydrophobic amino acids of the BH3 domain are shaded and indicated as h1 to h4. The highly conserved D residue between the h3 and h4 positions is highlighted in bold. (B) Mitochondria isolated from 293T cells were incubated with either Bad peptide, core protein peptide or a combination of the two peptides in equimolar concentrations. The total amount of peptide used in each experiment is indicated. Following centrifugation, the supernatants and pellets were subjected to Western blot analysis with anti-cytochrome c (cyt c) or anti-Hsp-60 antibodies. (C) The same experiment was repeated with Noxa peptide or a combination of Noxa and Bad peptides in equimolar concentrations.

3.4.2 Coexpression of the core protein and Bad resulted in enhanced apoptosis

Next, we investigated the synergistic proapoptotic activity of the core protein and Bad, a cellular BH3-only protein, using an overexpression system. This system allows us to examine apoptosis induction in intact transfected cells, instead of just isolated mitochondria. As shown in Figure 3.9, the overexpression of the core protein, Noxa or Bad singly induced similar levels of apoptosis in Huh7 cells as determined by the level of caspase-3 activation and the amount of PARP cleavage (lanes 2 to 4). However, when Bad was coexpressed with the core protein, the level of apoptosis was significantly enhanced (lane 5), indicating that Bad can potently cooperate with the core protein to induce high levels of apoptosis. A similar observation was made when Bad and Noxa were coexpressed (lane 6). These findings are consistent with the functional complementation between the core protein or Noxa BH3 peptides and the Bad BH3 peptide previously shown using the *in vitro* cytochrome c release assay (Figures 3.8B and 3.8C).

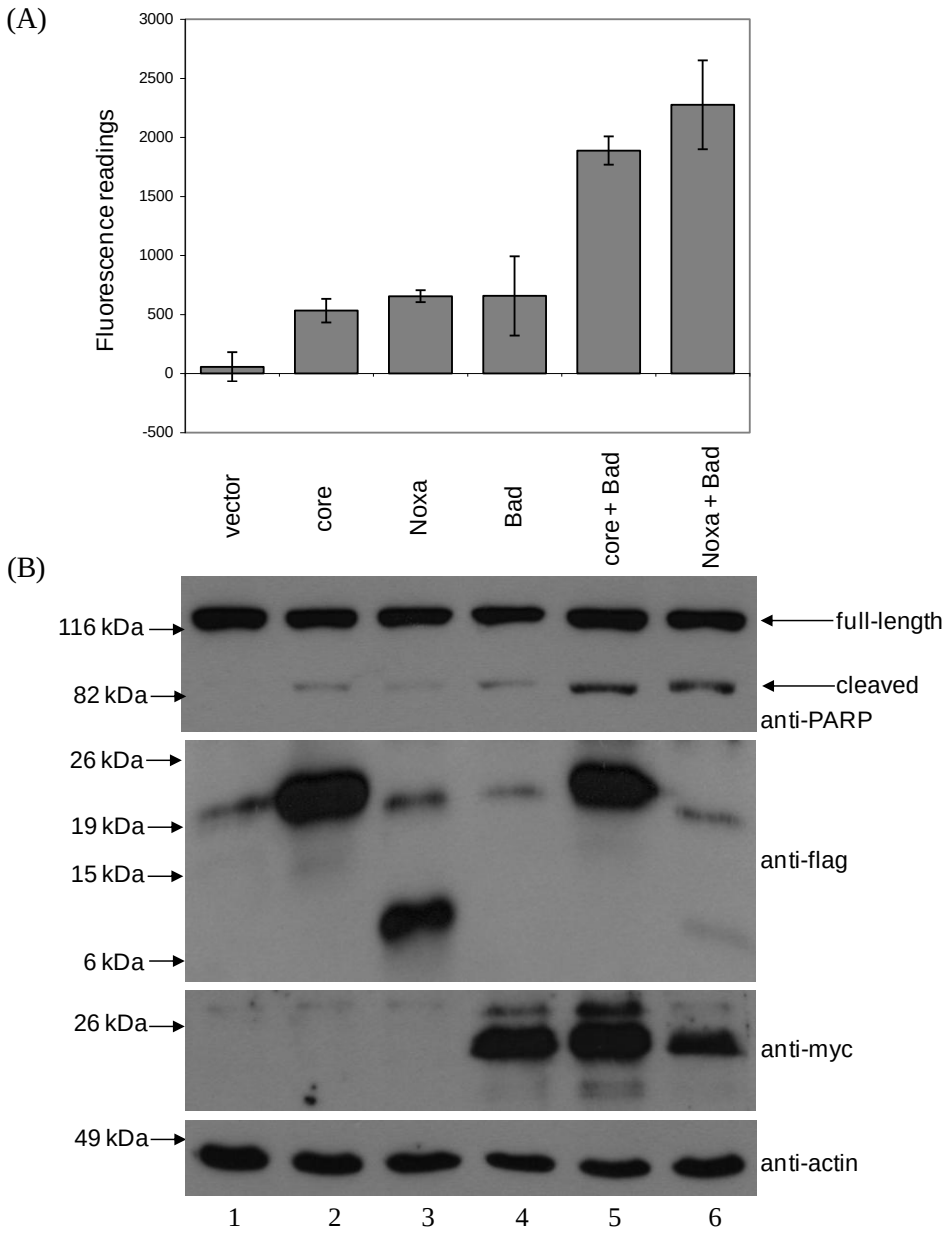

Fig. 3.9. Coexpression of core protein and Bad enhanced apoptosis in Huh7 cells. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, core protein, Noxa, Bad or a combination of core protein and Bad or Noxa and Bad. In each transfection, the total amount of DNA was normalised to 0.95 μg with the addition of empty vector if necessary. A flag epitope is fused to the N terminus of core and Noxa while a myc epitope is fused to that of Bad. All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis was also performed to determine the cleavage of endogenous PARP (top). Similarly, the expression levels of the different proteins were determined using anti-flag or anti-myc antibodies (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

3.5 Core protein-induced apoptosis is dependent on the expression of Bax

If the overexpression of the core protein affects the stoichiometry of the prosurvival and BH3-only Bcl-2 family members, this will lead to the activation of the proapoptotic multidomain Bcl-2 family members such as Bax and Bak, which are essential effectors of the mitochondrial outer membrane permeabilization (Chipuk and Green, 2008). Thus, the sensitivities of HCT116-Bax$^{+/-}$ and HCT116-Bax$^{-/-}$ cells to core protein-induced apoptosis were compared (Figures 3.10A and 3.10B). HCT116-Bax$^{+/-}$ cells are colorectal cancer cells containing one intact Bax allele while the HCT116-Bax$^{-/-}$ cells have two mutant alleles. Studies using these cells have provided direct evidence that Bax plays an essential role in mediating apoptosis induced by the BH3-only protein Bik and certain anticancer drugs (Gillissen et al., 2003; Prokop et al., 2003; Zhang et al., 2000).

As shown in Figure 3.10, the overexpression of the core protein induced apoptosis in HCT116-Bax$^{+/-}$ cells but not in HCT116-Bax$^{-/-}$ cells, indicating that core protein-induced apoptosis is dependent on Bax. Although the HCT116 cell lines express significant levels of Bak (Gillissen et al., 2003), they were insufficient to confer sensitivity to core protein-induced apoptosis in HCT116-Bax$^{-/-}$ cells. Bak has been reported to be sequestered by Bcl-X_L and Mcl-1 under normal conditions (Willis et al., 2005). We postulate that while the core protein can displace Bak from Mcl-1, Bak is still held in check by Bcl-X_L, which does not interact with the core protein (Figure 3.3). This is analogous to the Mcl-1-mediated sequestration of Bak that leads to the Bax

dependency of Bik-induced apoptosis (Gillissen et al., 2007). Thus, the data suggest that core protein-induced apoptosis is mediated via a primarily Bax-dependent mitochondrial pathway.

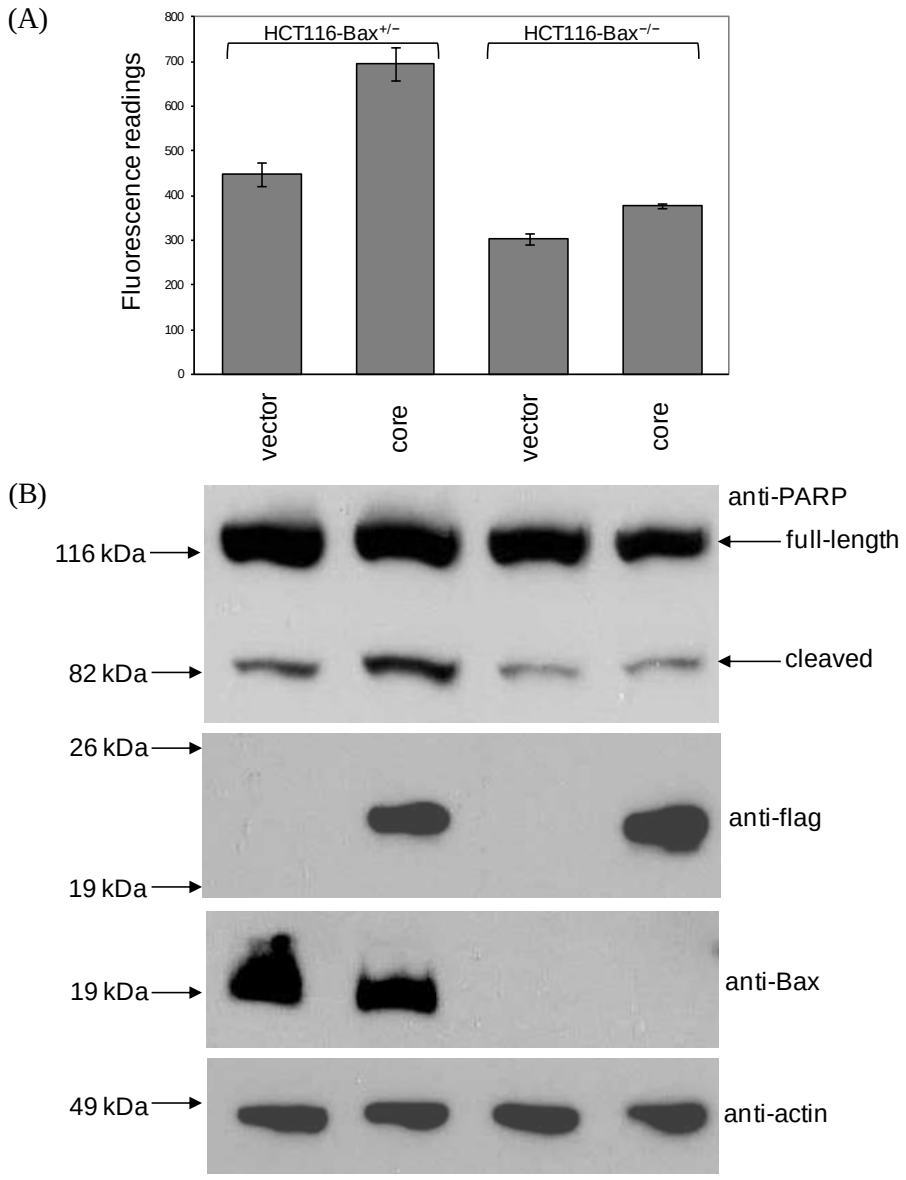

Fig. 3.10. Induction of apoptosis by the overexpression of core protein in HCT116-Bax$^{+/-}$ and HCT116-Bax$^{-/-}$ cells. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in HCT116-Bax$^{+/-}$ and HCT116-Bax$^{-/-}$ cells that were transfected with vector only or wild-type core protein. All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis was also performed to determine the cleavage of endogenous PARP (top) and expression level of flag-tagged core protein (middle). The differential expression of Bax in the two cell lines was demonstrated using an anti-Bax antibody (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

3.6 The three hydrophobic residues in the BH3 domain of the core protein are important for apoptosis induction

3.6.1 Effects of alanine substitutions on the proapoptotic property of the core protein

Site-directed mutagenesis and structural studies of the interactions between the prosurvival Bcl-2 proteins and BH3-only proteins have revealed the mechanism by which BH3 domains are bound to the hydrophobic grooves present on the surface of the prosurvival Bcl-2 proteins [see reviews by (Petros et al., 2004; Walensky, 2006)]. In particular, the BH3 domain usually contains four hydrophobic residues (h1 to h4) that make contacts with conserved hydrophobic residues at the base of the groove and these interactions are critical for the stability of the complex. Interestingly, the core protein also contains hydrophobic residues at the h2, h3, and h4 positions (Figure 3.1A). An alanine substitution experiment was performed to determine if these residues are essential for the proapoptotic property of the core protein (Figure 3.11A and 3.11B). The results showed that substitution of L119, V122 or L126 with A completely abolishes the proapoptotic property of the core protein.

In addition to the hydrophobic interactions, the invariant solvent-exposed D residue in the BH3 domain forms an important ionic interaction with a conserved R in the BH1 domain of the prosurvival protein (Hinds and Day, 2005). As such, it was surprising that replacement of the highly conserved D124 residue with A seems to increase the proapoptotic property of the core protein slightly. The levels of caspase-3 activation induced by the

wild-type core protein and the D124A substitution mutant in six independent experiments were compared using the two-tailed Student's *t* test and the difference was found to be statistically significant (Figure 3.11C). This phenomenon has not been reported for other BH3-only proteins and only a few known functional BH3 domains do not contain D at this position (Inohara et al., 1998; Tan et al., 2001).

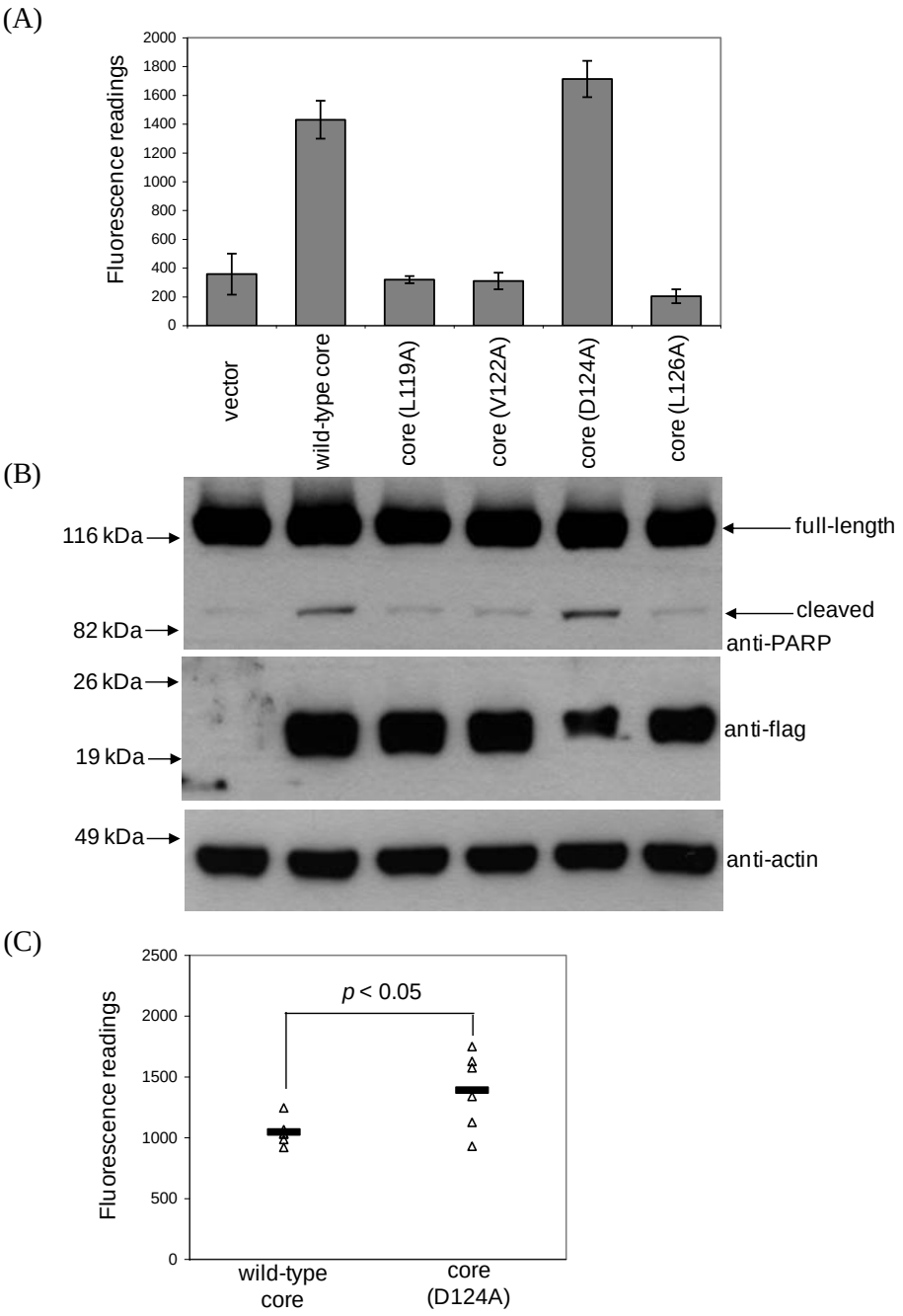

Fig. 3.11. Effects of alanine substitutions on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core protein or alanine-substituted core mutants. All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of the core proteins (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (C) The levels of activated caspase-3 induced by the wild-type core protein and the D124A mutant in six independent experiments were compared using the two-tailed Student's *t* test, and the difference was found to be statistically significant ($P < 0.05$). The values from each of the experiments are plotted as open triangles and the average values are plotted as solid lines.

3.6.2 Effects of alanine substitutions on the binding of the core protein to Mcl-1

Coimmunoprecipitation experiments were performed to test whether the alanine substituted core proteins can still bind Mcl-1. As shown in Figure 3.12A, the L119A, V122A and L126A substitution mutants displayed reduced binding to Mcl-1. Similar results were obtained in four independent experiments and the percentages of binding by the substitution mutants compared to that of the wild-type core protein were estimated by using an imaging densitometer to measure the intensity of the core protein signals after coimmunoprecipitation. For each experiment, three different autoradiographs (with different exposure times) were used for quantitation and the average values are shown in Table 3.1. The average percentages in binding of Mcl-1 to the L119A, V122A and L126A mutants are 33%, 62% and 9% of the binding to the wild-type core protein, respectively. For all three mutants, the reductions in interaction with Mcl-1 compared to the interaction between wild-type core protein and Mcl-1 are statistically significant (Table 3.1).

As the D124A substitution mutant induced a slightly higher level of apoptosis than the wild-type core protein (Figure 3.11), a coimmunoprecipitation experiment was also performed to determine if this mutant retains full ability to bind Mcl-1. Two different amounts of flag-tagged plasmids (0.5 and 1.0 μg) were used and the results showed that the D124A substitution mutant binds Mcl-1 to an extent similar to that of the wild-type core protein under both conditions (Figure 3.12B). When 1.0 μg of core (D124A) plasmid was used, the expression level of myc-tagged Mcl-1

was slightly reduced, probably due to increased apoptosis in this sample (Figure 3.11). As a result, lesser Mcl-1 was immunoprecipitated when more core (D124A) protein was expressed.

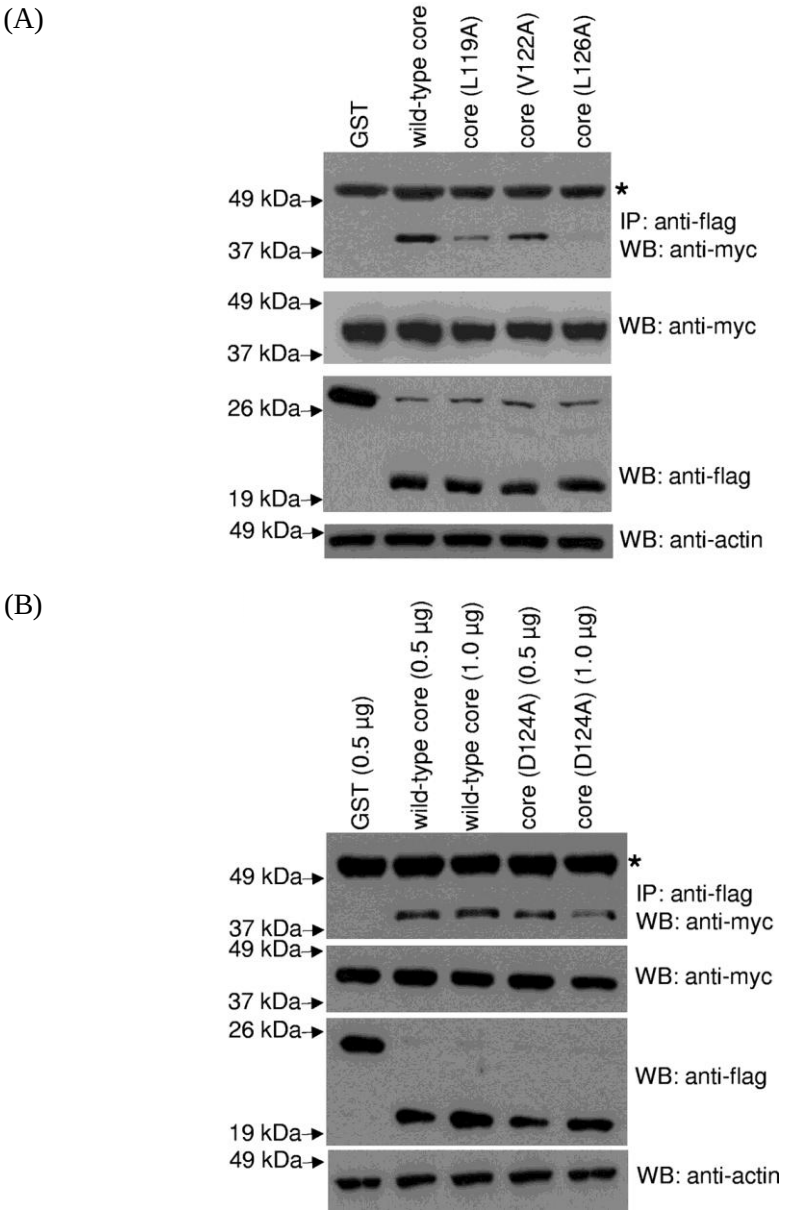

Fig. 3.12. Effects of alanine substitutions on the binding of the core protein to Mcl-1. (A) Huh7 cells were transfected with cDNA constructs (1.0 µg) for expressing flag-GST (negative control), flag-tagged wild-type core protein or single-alanine-substitution mutants (L119A, V122A and L126A). All cells were cotransfected with myc-tagged Mcl-1 (1.5 µg). (B) Huh7 cells were transfected with cDNA constructs for expressing flag-GST (negative control, 0.5 µg), flag-tagged wild-type core protein (0.5 or 1.0 µg) or single-alanine-substitution mutant D124A (0.5 or 1.0 µg). All cells were cotransfected with myc-tagged Mcl-1 (1.5 µg). Coimmunoprecipitation then was performed as described in the legend to Fig. 3.3. The amount of myc-tagged proteins that coimmunoprecipitated (IP) with the flag-tagged proteins was determined by Western blot analysis (WB) with an anti-myc rabbit polyclonal antibody (top). The amounts of myc-tagged and flag-tagged proteins in the lysates before IP were determined by subjecting aliquots of the lysates to Western blot analysis (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). The protein marked with an asterisk represents the heavy chain of the antibody used for IP (top). Similar results were obtained in four independent experiments and a representative set of data is presented.

Table 3.1. Effects of alanine substitutions on the binding of core protein to Mcl-1

Mutant	% binding[a]				Average[b]	*P*-value[c]
	Exp 1	Exp 2	Exp 3	Exp 4		
L119A	26	21	40	43	33 (± 11)	0.001
V122A	64	63	66	55	62 (± 5)	0.001
L126A	3	3	10	18	9 (± 7)	0.0001

[a]The percentage in binding to Mcl-1 for each of the mutants when compared to wild-type core protein was estimated by using an imaging densitometer to measure the intensity of core protein signals after co-immunoprecipitation (Fig. 3.13.). Four independent experiments were performed (Exp 1 to Exp 4). For each experiment, 3 different autoradiographs (with different exposure times) were used and the signals were normalised to the signal for wild-type core protein. Then, the average value was computed and shown.

[b]The mean value of the results from 4 independent experiments was computed and shown. The standard deviations are shown in parentheses.

[c]The difference between mutant and wild-type core in binding to Mcl-1 was compared using the 2-tailed Student's *t* test and the *P*-value is shown.

3.6.3 Proapoptotic properties of the core proteins of HCV genotypes 1b and 2a

Sequence comparison of the core proteins from different HCV genotypes revealed that while the V122 and L126 residues, at the h3 and h4 positions respectively, are highly conserved, the core proteins from genotype 2a strains typically have V instead of L at the h2 position (Figure 3.1B). In order to determine the impact of this variation on the apoptotic property of core, two substitution mutants were generated. For the first mutant, named core (L119V) (genotype 1b), the L119 in genotype 1b core was substituted with the corresponding V residue in genotype 2a. For the second mutant, named core (V119L) (genotype 2a), the V119 in genotype 2a core was substituted with the corresponding L residue in genotype 1b.

As shown in Figures 3.13A and 3.13B, substitution of the L119 of the genotype 1b core protein with V reduced the proapoptotic property of the core protein dramatically (lanes 2 and 3). This is similar to the reduction in the proapoptotic property of the genotype 1b core protein when the L119 residue was substituted with A, although the alanine substitution appeared to be more potent as it completely abolished core protein-induced apoptosis (Figure 3.11). Interestingly, in all known BH3-only proteins, this position is usually an L residue that is essential for the proapoptotic properties of these proteins (Figure 3.1A). The reverse experiment was performed by determining if the core protein of a genotype 2a strain (JFH-1 isolate) can induce apoptosis (Figure 3.13). The overexpression of the genotype 2a core protein induced a significantly lower level of apoptosis than the genotype 1b core protein (lane

4). However, replacing the V119 of the genotype 2a core protein with L resulted in a considerable increase in apoptosis induction, to a level similar to that induced by the genotype 1b core protein (lane 5). These results suggest that the dramatic difference in the apoptosis inducing ability of genotype 1b and 2a core proteins is due to the amino acid difference at position 119.

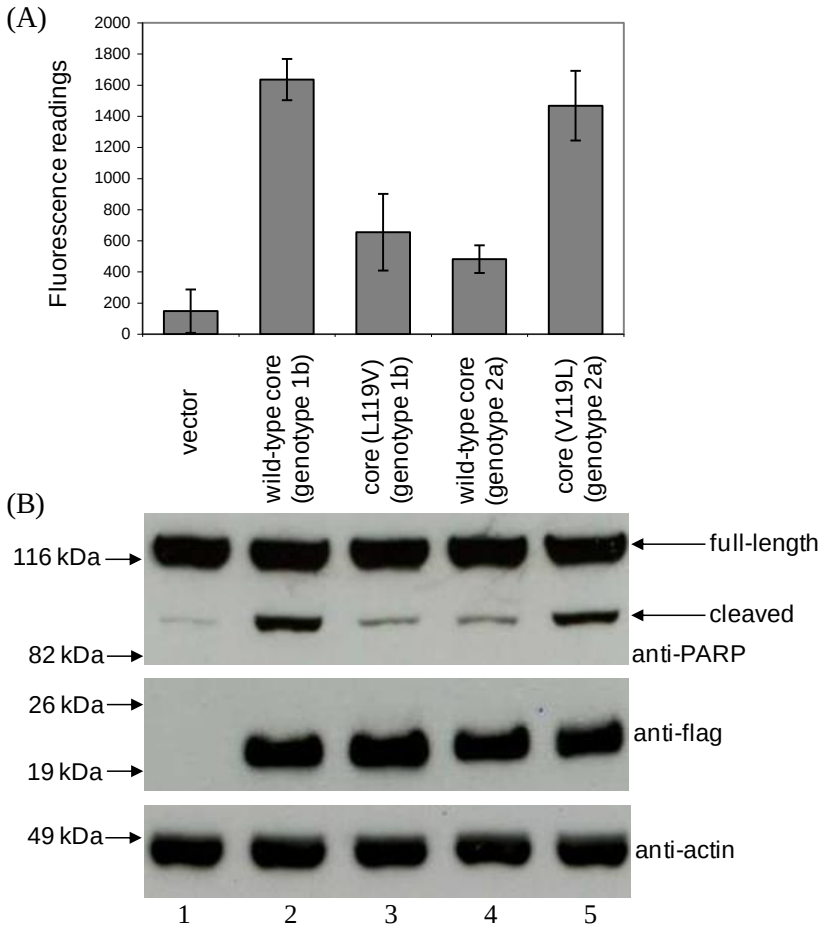

Fig. 3.13. Comparison of the proapoptotic properties of the core proteins of genotypes 1b and 2a. (A) A CaspACE fluorometric assay system was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core proteins of genotype 1b or 2a or their substitution mutants. All experiments were performed in triplicate and the average values with standard deviations are plotted. (B) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of the core proteins (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

3.7 A single substitution from V to L at residue 119 in the core protein of the HCV J6/JFH-1 strain is associated with increased abilities to induce apoptosis

Since the core proteins of HCV genotypes 1b and 2a displayed varying abilities to induce apoptosis in transfected cells due to an amino acid difference at residue 119, we sought to examine if this is also true in HCV-infected cells and the possible consequences of this difference. The pFL-J6/JFH-1 plasmid encoding the entire viral genome of an intragenotypic chimeric strain of HCV genotype 2a (J6/JFH-1) can be used to generate infectious HCV (Lindenbach et al., 2005). In the J6/JFH-1 clone, the core protein contains V at residue 119, just like the JFH-1 clone. A mutant virus, J6/JFH-1(V119L), was generated successfully by replacing the V119 residue with L so that its core protein contains a genotype 1b-like BH3 domain. At the time of this experiment, we have yet to complete a Material Transfer Agreement needed to acquire the J6/JFH-1 infectious clone. Hence, the infection experiments described in this chapter were performed by our collaborators from the Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo, Japan.

Parental J6/JFH-1 and mutant J6/JFH-1(V119L) viruses were used to infect naïve Huh7.5 cells and cell viabilities were measured at different time points after infection (Figure 3.14A). From day 2 post-infection (p.i.), cells infected by either virus have lower viabilities than mock-infected cells, indicating that the viruses have induced cytopathic effects (CPE). This is consistent with recent observations made by us and other researchers (Deng et

al., 2008; Mateu et al., 2008). Results from days 6 and 8 p.i. showed that the mutant J6/JFH-1(V119L) virus induced higher levels of CPE and thus, lower levels of cell viability compared to the parental J6/JFH-1 virus (Figure 3.14A), which is in agreement with the findings of the overexpression studies shown in Figure 3.13. The CPE is mediated primarily through apoptosis, as indicated by the levels of caspase-3 activation (Figure 3.14B) and DNA fragmentation (Figure 3.14C). The production of cell-free infectious virus particles by the J6/JFH-1(V119L) virus was also significantly higher than that produced by the parental J6/JFH-1 virus (Figure 3.14D). On the other hand, there was no significant difference in the percentage of HCV-infected cells in the cultures (Figure 3.14E) or the level of HCV RNA replication in the cells between the two viruses (Figure 3.14F).

We next analyzed the possible interaction between endogenous Mcl-1 and the core proteins of either J6/JFH-1 or J6/JFH-1(V119L) in virus-infected cells. As shown in Figure 3.14G, the core protein of J6/JFH-1(V119L) was coimmunoprecipitated with Mcl-1 (lane 6). In contrast, Mcl-1 interaction of the core protein of J6/JFH-1 was not detected under the same experimental conditions (lane 4). These results collectively imply the possibility that the V119L mutation of the core protein of the J6/JFH-1(V119L) virus promotes its interaction with Mcl-1 and is responsible for the increased ability of the virus to induce apoptosis, which favours a higher degree of infectious progeny virus release from the host cell at the late time points of infection compared to that of the parental J6/JFH-1 virus.

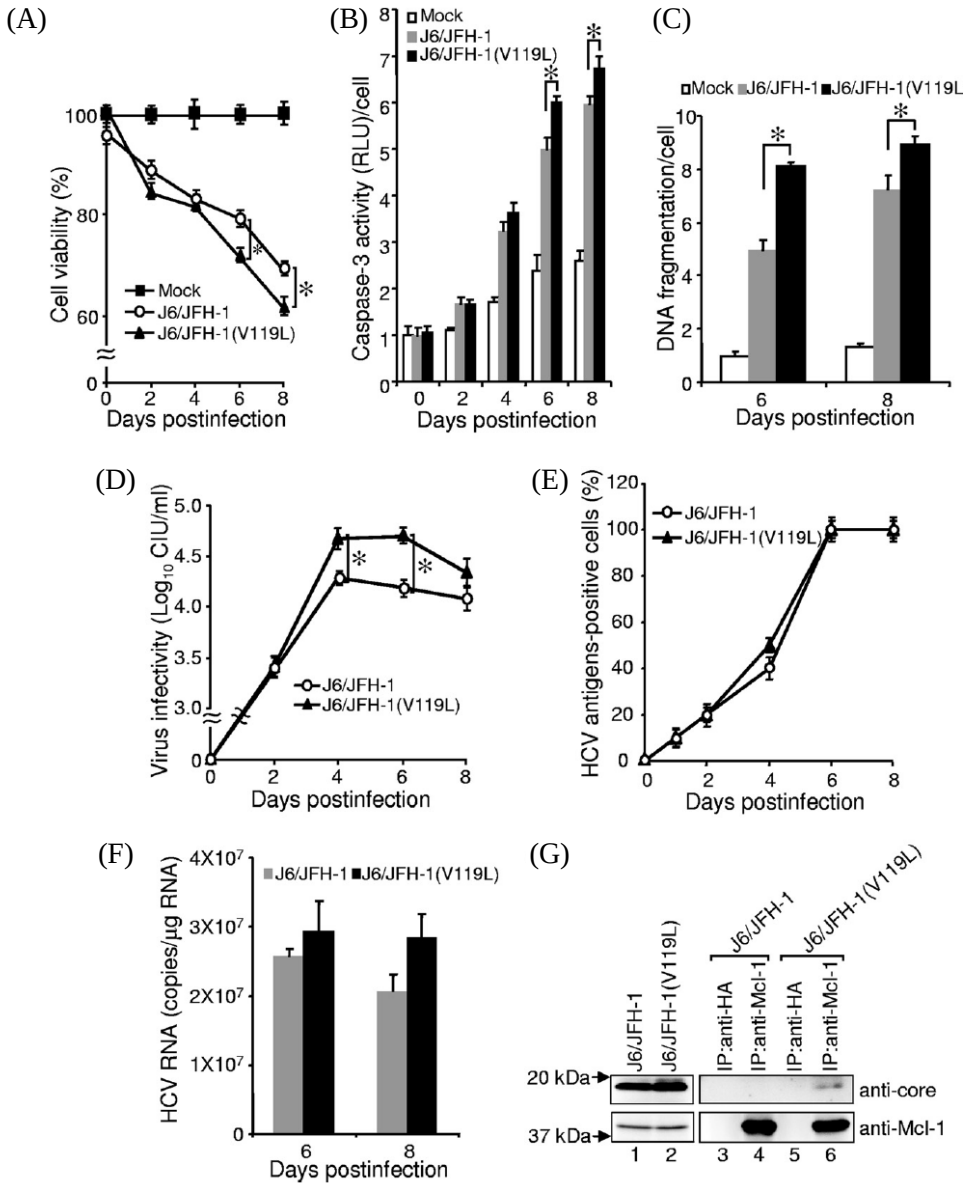

Fig. 3.14. Comparison of parental J6/JFH-1 and mutant J6/JFH-1(V119L) recombinant viruses. Huh7.5 cells were infected with recombinant HCV at MOI 0.1 or with a mock preparation and various assays were performed at different days post-infection (d.p.i.). (A) Cell viabilities were determined. (B) Caspase-3 activity per cell was determined. (C) The amount of DNA fragmentation per cell was determined. (D) The production of cell-free infectious virus particles was determined. (E) Virus spread in the culture was quantitated. (F) HCV RNA replication was determined by quantitative real-time PCR analysis. (G) Interaction of the core protein with Mcl-1 was determined by coimmunoprecipitation experiments at 3 d.p.i. IP was performed using anti-Mcl-1 or anti-HA rabbit polyclonal antibodies and protein A agarose beads. The amount of core protein in the lysates before (lanes 1 and 2) and after IP (lanes 3 to 6) was determined by Western blot analysis with an anti-core monoclonal antibody (top). Similarly, the amounts of endogenous Mcl-1 in the samples were determined using an anti-Mcl-1 monoclonal antibody (bottom). Statistical analysis was performed using the one-way analysis of variance and differences between parental and mutant viruses with P values of <0.05 (marked by asterisks) are considered to be statistically significant. Data were obtained from three independent experiments, each with triplicate cultures.

3.8 Discussion

Besides playing important roles in maintaining homeostasis in healthy cells through the regulation of apoptosis, members of the Bcl-2 family are also involved in viral infections. Indeed, several viruses have been shown to encode homologues of prosurvival Bcl-2 proteins and these viral proteins serve to inhibit apoptosis in infected cells and prevent the premature death of these cells [see reviews by (Cuconati and White, 2002; Hardwick and Bellows, 2003; Polster et al., 2004; White, 2006)]. Other viral proteins, which can be proapoptotic, prosurvival or both, do not share any sequence homology with members of the Bcl-2 family but can also modulate apoptosis in the host cells by interfering at different apoptotic checkpoints [see reviews by (Boya et al., 2004; Galluzzi et al., 2008; Hay and Kannourakis, 2002; McLean et al., 2008)].

Unlike the multi-BH domain members, the BH3-only members of the Bcl-2 family contain a single BH3 domain. Although all BH3-only proteins can bind to the hydrophobic groove on the surface of the prosurvival members, recent quantitative measurements have revealed that the affinities of association between different pairs of BH3-only and prosurvival members vary greatly (Chen et al., 2005; Kuwana et al., 2005). For example, Bim and Puma bind all prosurvival members tested, while Noxa binds strongly only to Mcl-1 and A1. On the other hand, Bad binds more strongly to Bcl-2, Bcl-X_L and Bcl-w than to Mcl-1. Taken together with results from successive studies, it becomes clear that the BH3-only members can be classified into subclasses

[see reviews by (Fletcher and Huang, 2008; Hacker and Weber, 2007; Shibue and Taniguchi, 2006; Willis and Adams, 2005)].

In this study, we demonstrate that the HCV core protein is a BH3-only viral homologue of the Bcl-2 family and its BH3 domain is essential for the induction of apoptosis (Figures 3.1 and 3.2). In coimmunoprecipitation experiments, the core protein interacts specifically with the prosurvival Mcl-1 protein but not with prosurvival proteins Bcl-X_L and Bcl-w (Figure 3.3), suggesting that its property is most similar to that of Noxa (Chen et al., 2005). Consistently, the overexpression of Mcl-1 protects against core protein-induced apoptosis (Figure 3.5). However, the overexpression of Bcl-X_L also protects against core protein-induced apoptosis (Figure 3.5). This may be due to the ability of a high level of Bcl-X_L to prevent the complementation between the core protein and endogenous Bad protein, which binds strongly to Bcl-X_L (Chen et al., 2005), as we have observed that a combination of the core protein and Bad peptide mimetics caused efficient cytochrome c release from the mitochondria (Figure 3.8). The complementation between Bad and the core protein is similar to that observed between Bad and Noxa, which act in combination to neutralize the two classes of prosurvival proteins, one containing Bcl-2, Bcl-X_L and Bcl-w and the other Mcl-1 and A1 (Chen et al., 2005). Similarly, in the overexpression studies, the core protein and Noxa induced comparably low levels of apoptosis when expressed individually and the induction of apoptosis increased substantially when they were coexpressed with Bad (Figures 3.7 and 3.9). Last but not least, core protein-induced apoptosis is dependent on the expression of Bax (Figure 3.10), which is one of

the proapoptotic effectors of the mitochondrial outer membrane permeabilization acting downstream of activated BH3-only proteins. Taken together, these findings suggest that the core protein can mimic Noxa and interfere directly with the prosurvival function of Mcl-1.

A comparison of the BH3 domain of the core protein with the corresponding domains of other BH3-containing proteins (Figure 3.1A) revealed that it contains three out of the four hydrophobic residues that can be accommodated within the hydrophobic pockets of previously described BH3 binding grooves [see reviews by (Petros et al., 2004; Walensky, 2006)]. Alanine substitution experiments revealed that all three hydrophobic residues in the BH3 domain of the core protein are essential for apoptosis induction (Figure 3.11). In coimmunoprecipitation experiments, these alanine substitution mutants bound Mcl-1 to a lesser extent than the wild-type core protein (Figure 3.12A). Although these alanine substitution mutants still bind Mcl-1, albeit at a lower level than that of the wild-type core protein, it appears that these interactions are not sufficient to induce apoptosis. In several mutagenesis studies, the interaction between Bcl-2 family members and apoptosis regulation have been observed to be discordant. For example, two mutants of the BH3-only protein Bik, Bik-(43-94) and Bik-(43-120), heterodimerized with prosurvival Bcl-2 and Bcl-X_L but were unable to induce efficient cell death (Elangovan and Chinnadurai, 1997). A Bad mutant containing an alteration of a critical residue within its BH3 domain, E113 to K, also was found to have significantly reduced apoptotic activity compared to that of wild-type Bad, despite binding to Bcl-2 and/or Bcl-X_L to the same

extent as wild-type Bad (Lee et al., 2004). Therefore, the induction of apoptosis by the core protein may be controlled by a critical threshold affinity of binding between the core protein and Mcl-1 or there are contributions from a yet-to-be characterized pathway(s).

Two of the hydrophobic residues (V122 and L126) in the BH3 domain of the core protein are conserved in the major genotypes of HCV but residue 119 is a V in genotype 2a instead of an L (Figure 3.1B). When L119 of the genotype 1b core protein was replaced with V, its ability to induce apoptosis was greatly reduced (Figure 3.13). Conversely, when V119 of the genotype 2a core protein was replaced with L, its ability to induce apoptosis was greatly enhanced. Thus, the results suggest that the genotype 1b core protein induces apoptosis efficiently via its BH3 domain, while the genotype 2a core protein is comparatively less efficient. Another highly conserved residue in the BH3 domain of the core protein is D124. However, the replacement of D124 with A did not reduce the proapoptotic function of the core protein (Figure 3.11). To date, there are only a few known functional BH3 domains that do not contain D at this position (Inohara et al., 1998; Tan et al., 2001). Unlike most BH3-only proteins, the core protein has a charged residue (R115) at the h1 position (Figure 3.1A). This residue does not appear to play a role in core protein-induced apoptosis as the core protein BH3 peptide lacking this residue is capable of complementing the Bad BH3 peptide to cause more efficient release of cytochrome c from isolated mitochondria (Figure 3.8). Interestingly, the second BH3 domain of mouse Noxa (mNoxaB) also has a charged residue (E74) at this position. Indeed, the nuclear magnetic resonance

structure of the complex between mouse Mcl-1 and a peptide mimetic of mNoxaB shows that E74 is tolerated at the h1 position because its charged carboxyl group is coordinated by another charged residue, K215, in mouse Mcl-1 (Czabotar et al., 2007). However, R115 of the core protein is basic instead of acidic, and how this residue can be accommodated in the hydrophobic groove of human Mcl-1 is unclear. Interestingly, replacing the residue at the h1 position (I58) of a novel BimBH3 variant, Bims2A, with A had little effect on its interaction with Mcl-1 (Lee et al., 2008). Thus, it appears that the residue in the h1 position is not always involved in the interaction between BH3-only proteins and Mcl-1 but further biophysical and biochemical studies are required to delineate the precise structure-function relationship for the interaction between the HCV core protein and Mcl-1.

To determine if the results from the overexpression studies are relevant to the modulation of apoptosis in host cells during HCV infection, the J6/JFH-1-based (genotype 2a) cell culture infection system was used to generate HCV carrying a substitution at residue 119 of the core protein. While both the parental wild-type and mutant viruses replicated efficiently in Huh7.5 cells, the J6/JFH-1(V119L) virus (which expresses the core protein with L at the h2 position of the BH3 domain) caused a significantly higher level of apoptosis in the infected cells than the parental J6/JFH-1 virus (which expresses the core protein with V at the h2 position of the BH3 domain) (Figures 3.14A to 3.14C). This is in good agreement with the overexpression studies and indicates that the BH3 domain of the core protein contributes to the induction of apoptosis in HCV-infected cells. Thus, it appears that core protein-

mediated apoptosis during infection by HCV of genotype 2a is less efficient than that of the other genotypes having L at residue 119 of the core protein (Figure 3.1B). Coimmunoprecipitation experiments revealed that the core protein of J6/JFH-1(V119L), but not that of J6/JFH-1, interacted with Mcl-l in virus-infected cells (Figure 3.14G). This result is consistent with the overexpression studies and suggests the possibility that the core protein induces apoptosis, at least in part, through interaction with Mcl-1 in HCV-infected cells. Interestingly, more progeny virus is released from cells infected with the J6/JFH-1(V119L) virus than by those infected with the parental J6/JFH-1 virus, while there is no difference in the efficiency of infection or amount of HCV replication inside the cells (Figures 3.14D to 3.14F).

We further examined the importance of residue 119 of the core protein in HCV replication. In multiple independent transfection experiments, we observed that the J6/JFH-1 mutant virus possessing A at position 119 [J6/JFH-1(V119A)] barely replicated in the cells and did not produce any infectious virus particles in the culture supernatants (data not shown). This result suggests the possibility that this single point mutation impairs the interaction of the core protein with other viral and/or cellular proteins that is required for HCV RNA replication and infectious virion production. Similarly, the J6/ JFH-1 mutants each possessing A at positions 122 [J6/JFH- 1(V122A)], 124 [J6/JFH-1(D124A)] or 126 [J6/JFH-1(L126A)] barely replicated in the cells and did not produce any infectious virion in the culture supernatants (data not shown), thus suggesting an important role for these residues as well as for

position 119. In this connection, the essential role for the HCV core protein in infectious virion production has been confirmed recently and numerous residues required for this role have been identified (Murray et al., 2007).

By using the JFH-1 infectious clone, recent studies have revealed that association of the core protein with lipid droplets (LDs) is critical for the production of infectious virus particles (Boulant et al., 2007; Miyanari et al., 2007). Boulant and co-workers reported that there are two amphipathic α-helices in the so-called D2 domain of the core protein (~118 to 179 aa) (Boulant et al., 2006; Boulant et al., 2005) and the hydrophobic residues within this domain are critical for the efficient attachment of the core protein to LDs (Boulant et al., 2006). Our results showed that residues L119, V122 and L126 of the core protein are essential for the induction of apoptosis and these residues are found on the hydrophobic face of the first α-helix of the D2 domain. Interestingly, the replacement of L119 with E did not affect LD association, while the replacement of L126 with E significantly reduced LD association (Boulant et al., 2006). The contribution of V122 to LD association was not investigated. Consistently, the J6/JFH-1(V119L) virus, but not the J6/JFH-1(L126A) virus, replicated efficiently to produce infectious virus particles. Since L119 of the genotype 1b core protein, which occupies the crucial h2 position in the BH3 domain, is essential for its proapoptotic property but not for its association with LDs, it is clear that the BH3 domain of the core protein is an independent motif that partially overlaps with the LD association domain.

Recently, Makes caterpillars floppy 1 (Mcf1), a bacterial toxin, was reported to contain a BH3-like domain (Dowling et al., 2007). In addition, HBSP, a spliced hepatitis B viral protein, also contains a BH3-like domain (Lu et al., 2006). Here, we show that the HCV core protein is another BH3-like viral homologue and it contributes directly to the induction of apoptosis during HCV infection. Our results also reveal that it is a bona fide BH3-only protein that appears to interfere with the prosurvival property of Mcl-1 in a manner similar to that of Noxa. As many viruses have adopted strategies to prevent apoptosis in infected cells so as to facilitate viral replication and the packaging of progeny genomes, our observation that the enhanced apoptotic activity of the J6/JFH-1(V119L) virus correlates with an increase in infectious progeny HCV release seems to be counterintuitive (Cuconati and White, 2002; Hardwick and Bellows, 2003; Polster et al., 2004; White, 2006). However, enhanced releases of virus from infected cells that are undergoing apoptosis have also been reported for other viruses, such as the infectious bursal disease virus, adenovirus and Aleutian mink disease parvovirus (Best et al., 2002; Mi et al., 2001; Yao and Vakharia, 2001), indicating that apoptosis can be advantageous for viral spreading at the late stages of infection.

Besides the genotype 1b core protein, the properties of the genotype 1a core protein have been examined in various studies (Hahn et al., 2000; Kang et al., 2009; Moorman et al., 2003; Saito et al., 2006). Although previous studies have attributed both prosurvival and proapoptotic properties to the genotype 1a core protein, these have yet to be verified using the JFH-1-based infectious clone system. Similar observations have also been described in

overexpression studies using the genotype 1b core protein and appear to be dependent on the death stimuli and types of cells used (Benali-Furet et al., 2005; Cao et al., 2004; Chang et al., 2008; Lee et al., 2007; Otsuka et al., 2002; Realdon et al., 2004; Sacco et al., 2003; Takamatsu et al., 2001; Zhu et al., 2001). Several studies have identified domains or regions within the core protein that interfere with specific apoptosis pathways. For instance, the N-terminal domain (residues 1 to 75) of the genotype 1a core protein interacts with Hsp60, leading to the production of reactive oxygen species and enhancement of tumour necrosis factor alpha-mediated apoptosis (Kang et al., 2009), while its C-terminal domain (residues 153 to 192) facilitates Fas oligomerization and is required for apoptosis induction in Jurkat cells (Moorman et al., 2003). The genotype 1b core protein (residues 1 to 153) binds to the death domain of FADD, resulting in enhanced apoptosis (Zhu et al., 2001). However, an overlapping domain spanning the first 46 aa of the core protein mediates interaction with a p53-binding protein, ASPP2, which leads to the inhibition of p53-mediated apoptosis (Cao et al., 2004). These findings suggest that multiple domains present in the core protein contribute to the modulation of apoptosis via diverse pathways. Therefore, the net apoptotic effect of the core protein may be dependent on the relative strength of its prosurvival and proapoptotic properties. Unlike the genotype 2a core protein, the BH3 domains of the genotype 1b core protein and the genotype 1a core protein share an identical sequence (Figure 3.1B) and are expected to function in a similar manner. However, we cannot rule out the possibility that there may be differences in the manner in which the core proteins of

genotypes 1a and 1b modulate apoptosis during infection. For example, they may be involved in different virus-virus or virus-host interactions.

It is apparent that the parental J6/JFH-1 virus also induces a high level of apoptosis in the infected cells and for the early time points, there was no significant difference in the levels of apoptosis induced by the parental J6/JFH-1 virus and the J6/JFH-1(V119L) mutant virus (Figures 3.14A to 3.14C). This implies that since the BH3 domain of the genotype 2a core protein is not a major contributing factor, there are other viral factors that contribute to the induction of apoptosis during J6/JFH-1 infection. For example, several non-structural HCV proteins such as NS3, NS4A, NS5A and NS5B have been shown to induce apoptosis when they are overexpressed in certain types of cells [see reviews by (Aweya and Tan, 2011; Fischer et al., 2007; Herzer et al., 2007)]. In addition, other domains in the core protein have been shown to bind host proteins and may contribute to apoptosis regulation by interfering with different cellular pathways [see reviews by (Lai and Ware, 2000; McLauchlan, 2000; Ray and Ray, 2001)]. However, the relative contribution of these various factors to apoptosis induction during HCV infection remains to be determined. Hence, using the J6/JFH-1 HCVcc system, we investigated other pathways that are modulated during HCV-induced apoptosis and this is discussed in the following chapters.

CHAPTER 4: APOPTOSIS IN HCV-INFECTED HUH7.5 CELLS

4.1 Efficient expression of HCV proteins in Huh7.5 cells infected with HCV J6/JFH-1-P47 strain

The recent development of an *in vitro* HCV cell culture (HCVcc) infection system facilitates further understanding of the HCV life cycle and pathogenesis (Lindenbach et al., 2005; Lindenbach et al., 2006; Wakita et al., 2005; Zhong et al., 2005). In Chapter 3, we demonstrated that the HCV J6/JFH-1 strain induces apoptosis in infected Huh7.5 cells, which is consistent with previous reports (Deng et al., 2008; Mateu et al., 2008; Walters et al., 2009). However, the mechanisms underlying this induction of apoptosis are poorly understood. Therefore, in order to further characterize HCV-induced apoptosis, we infected Huh7.5 cells with a tissue culture-adapted HCV strain, J6/JFH-1-P47, and investigated the possible effects of HCV infection on cellular apoptotic pathways.

Compared to the parental J6/JFH-1 virus, the J6/JFH-1-P47 strain exhibits higher expression of HCV proteins and produces a much higher titre of infectivity in Huh7.5 cell cultures. Growth curve analysis of cells transfected or infected with the wild-type and adapted viral RNA or virus showed that the adaptive mutant possesses advantages at the entry level, rather than the replication or translation level (Bungyoku et al., 2009). This is consistent with previous studies using the HCV replicon system which showed that replication-enhancing mutations impair virus release (Blight et al., 2003; Bukh et al., 2002). Furthermore, recent infection studies using JFH-1-based recombinants demonstrated that cell culture adaptive mutations largely

increase virus titres without affecting replication (Delgrange et al., 2007; Gottwein et al., 2009; Kaul et al., 2007; Pokrovskii et al., 2011; Yi et al., 2007). Owing to its high infectivity, the J6/JFH-1-P47 infection system is more convenient for our study which requires cells to be infected at a relatively high MOI of 2. Furthermore, since the J6/JFH-1-P47 strain was previously reported to exert a stronger cytopathic effect than the parental virus, the adapted virus may provide a more efficient system for studying HCV-induced apoptosis (Deng et al., 2008).

To confirm successful HCV infection, we first examined the expression of HCV proteins in infected and mock-treated Huh7.5 cells. Serum from a HCV-infected patient was used in indirect immunofluorescence experiments to visualize HCV-positive cells. At 2 days post-infection (d.p.i.), HCV antigens were detected in the infected cells, but not in the mock-treated cells, and displayed both diffuse and punctate cytoplasmic distribution (Figure 4.1A). More than 95% of the cells were infected with HCV by 2 d.p.i. Western blot analysis was also performed to determine the expression levels of two HCV proteins, NS3 and core, in HCV-infected cells. As shown in Figure 4.1B, expression of NS3 and core was only observed in the HCV-infected cells and high expression levels were detected over at least 8 days after infection. These data show that J6/JFH-1-P47 (henceforth referred to as "HCV") can successfully infect Huh7.5 cells in our system. Under these conditions, majority of the cells were infected 2 days after inoculation and this level of infection was maintained for the duration of the study.

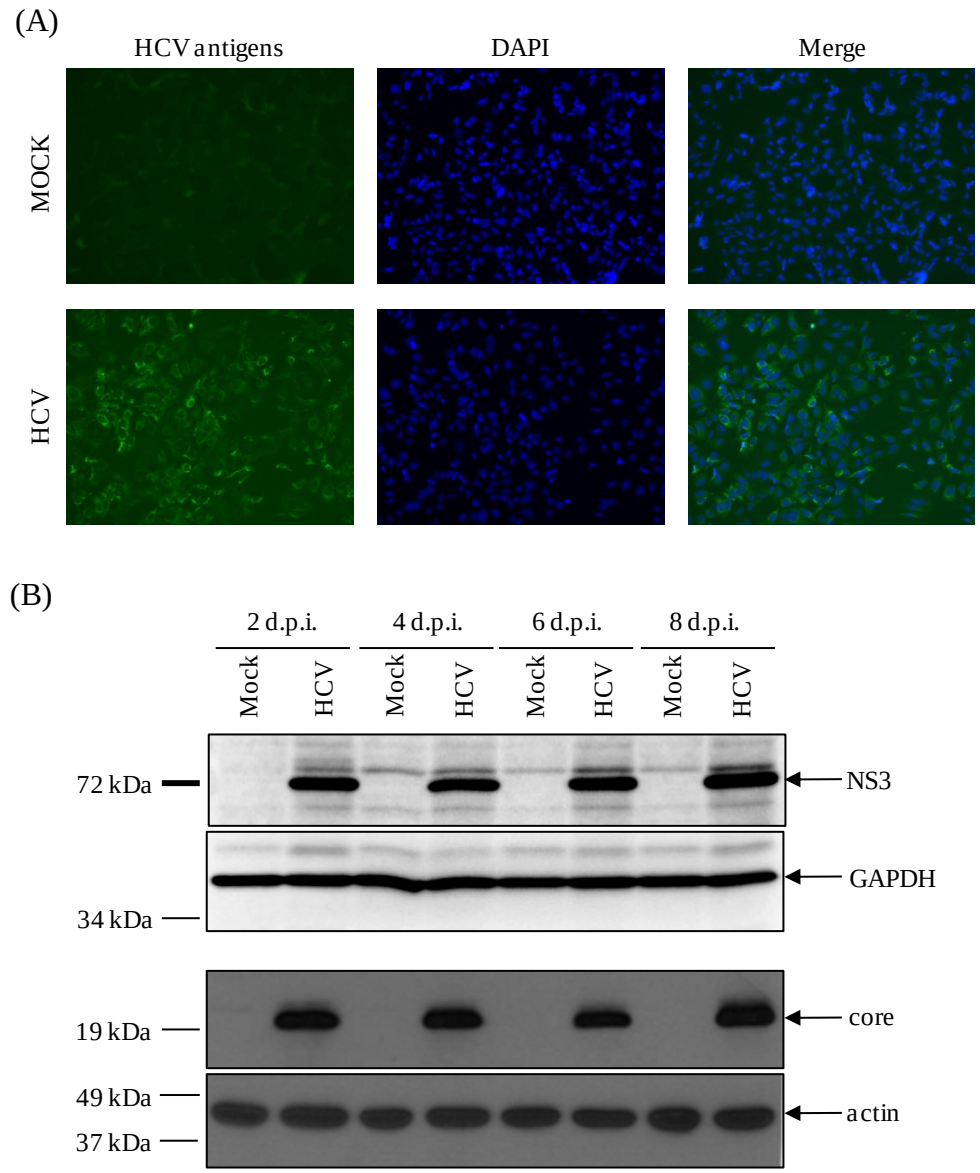

Fig. 4.1. Expression of HCV proteins in Huh7.5 cells infected with J6/JFH-1-P47 virus. (A) Huh7.5 cells infected with mock preparation (top row) or HCV (bottom row) at MOI 2 were subjected to indirect immunofluorescence analysis 48 h after infection. HCV antigens were detected using HCV-infected patient's serum and FITC-conjugated goat anti-human IgG (green staining) and cell nuclei were counterstained with DAPI (blue staining). (B) Whole cell lysates were prepared from mock-infected and HCV-infected Huh7.5 cells at 2, 4, 6 and 8 days post-infection (d.p.i.) and analyzed by Western blotting to determine the expression levels of HCV NS3 (first panel) and core (third panel). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous GAPDH (second panel) or actin (fourth panel). Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left.

4.2 Kinetics of viral RNA replication and virus production in HCV-infected Huh7.5 cells

In addition to viral protein expression, HCV RNA replication and the production of progeny virus were also monitored. The HCV RNA content in the infected cells was analyzed by quantitative real-time PCR and a steady-state level of viral RNA replication was observed from 2 to 6 d.p.i. (Figure 4.2A). The virus titres in the culture supernatant of infected cells peaked and reached a plateau at 4 d.p.i. (Figure 4.2B), as determined by indirect immunofluorescence analysis. These results confirm that HCV can replicate efficiently in the culture to produce high titres of infectious progeny virus. Since this system supports the complete HCV life cycle, it can be used to study the impact of a productive HCV infection on host cell function.

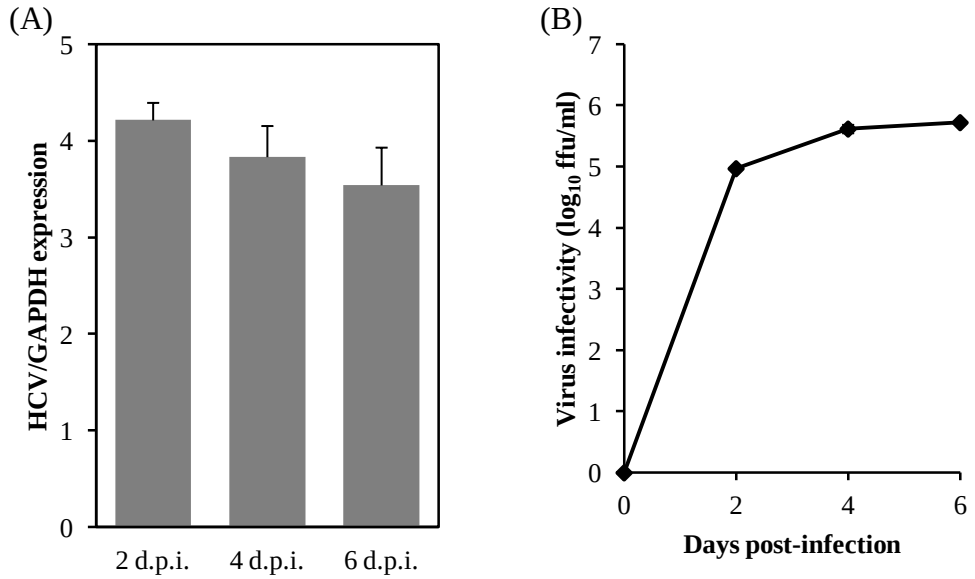

Fig. 4.2. HCV RNA replication and production of cell-free infectious virus particles. (A) Total RNA was isolated from HCV-infected Huh7.5 cells at 2, 4 and 6 days post-infection (d.p.i.) and reverse transcribed. HCV RNA replication levels were determined by quantitative real-time PCR analysis and normalised to endogenous GAPDH levels. (B) Culture supernatants of HCV-infected cells were collected at 2, 4 and 6 d.p.i. and used to inoculate naive Huh7.5 cells. Virus infectivity was determined by indirect immunofluorescence analysis at 24 h post-infection and expressed as focus forming units per ml (ffu/ml). Data shown are representative of three independent experiments.

4.3 HCV infection modulates cell viability and apoptosis in Huh7.5 cells

To investigate the effect of HCV infection on host cell growth, the viabilities of HCV-infected and mock-treated Huh7.5 cells were measured using WST-1 assay at 0, 2, 4 and 6 d.p.i. As shown in Figure 4.3A, HCV-infected cells showed significantly lower viabilities than mock-treated cells, indicating that the virus has induced cytopathic effects (CPE). To further characterize the CPE observed in HCV-infected cells, the activation of caspase-3, a hallmark of apoptosis, in these cells was determined. At 2 and 4 d.p.i., cells infected with HCV exhibited higher levels of caspase-3 activity than mock-treated cells, indicating that the CPE was mediated largely through apoptosis (Figure 4.3B). Interestingly, at 6 d.p.i., the level of caspase-3 activity in HCV-infected cells was significantly lower compared to that in mock-treated cells (Figure 4.3B). This decrease in apoptosis correlates with a higher percentage of viable HCV-infected cells at 6 d.p.i. (61%) than at 4 d.p.i. (49%) (Figure 4.3A). However, this was not observed in previous studies of apoptosis related to acute HCV infection (Deng et al., 2008; Mohd-Ismail et al., 2009), which showed increasingly higher levels of both CPE and apoptosis in HCV-infected cells than in mock-treated cells at 6 and 8 d.p.i. A possible reason for this disparity is the extensive cell death observed in the mock-treated samples due to overgrowth at the later time points in our infection system.

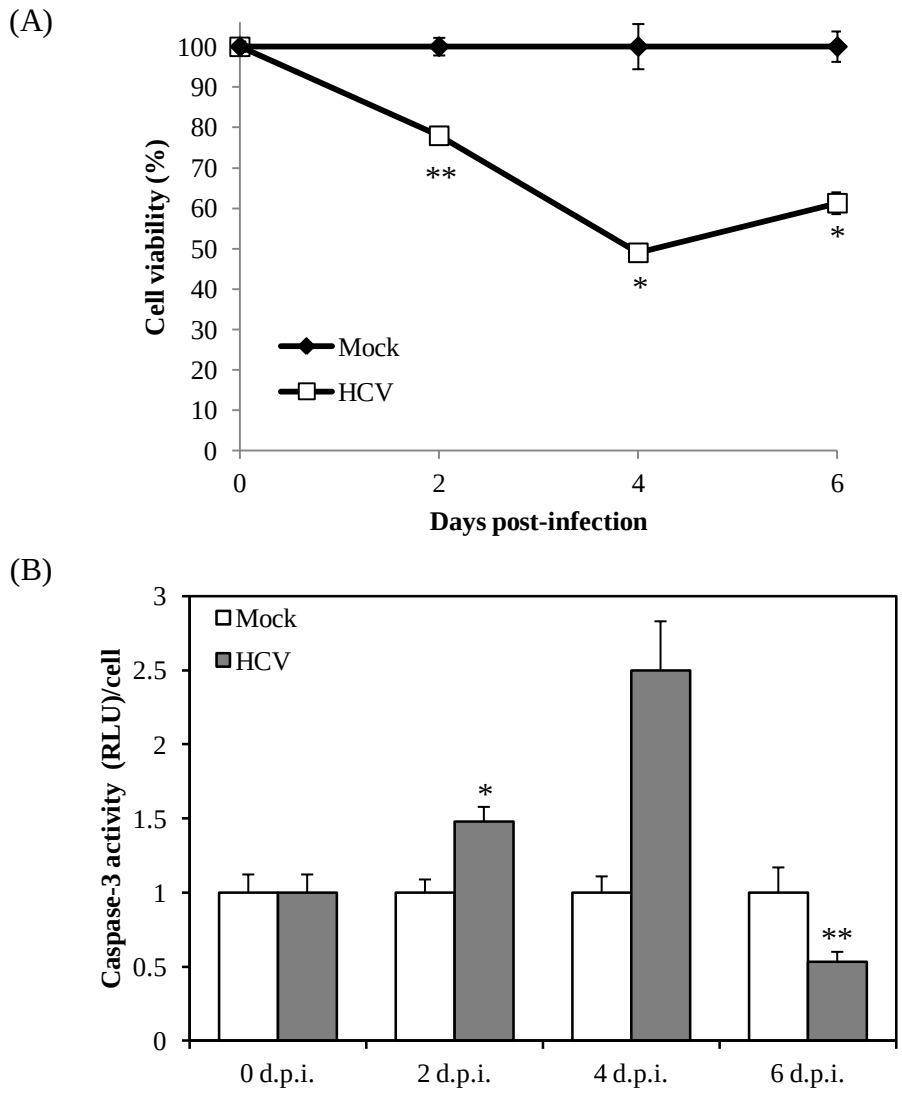

Fig. 4.3. Cell viability and caspase-3 activity of Huh7.5 cells infected with HCV. (A) Cell viability/proliferation was measured using WST-1 assay at 0, 2, 4 and 6 days post-infection (d.p.i.). The viability of HCV-infected cells (open symbols) is expressed as a percentage of the mock control cells (closed symbols) at each time point. (B) A Caspase-Glo 3/7 luminogenic assay system was used to measure the activation of caspase-3 in HCV-infected (filled columns) and mock-treated (empty columns) cells. The caspase-3 activity of mock control cells at each time point was arbitrarily expressed as 1.0. Data shown is representative of three independent experiments and statistically significant differences between mock and HCV-infected samples are marked by asterisks (*P <0.05 and **P <0.01).

4.4 Temporal expression of members of the Bcl-2 protein family in HCV-infected Huh7.5 cells

4.4.1 Western blot analysis of Bcl-2 family protein expression

The members of the B cell lymphoma-2 (Bcl-2) protein family play a central role in the mitochondrial pathway of apoptosis through the tight regulation of outer mitochondrial membrane integrity [see recent reviews by (Chipuk et al., 2010; Tait and Green, 2010)]. To investigate the possible involvement of these proteins in HCV-associated apoptosis, we first examined differences in the endogenous expression of prosurvival members, Bcl-X_L and Mcl-1, and proapoptotic members, Bax and Bak, in response to HCV infection. As shown in Figure 4.4A, HCV-infected Huh7.5 cells expressed significantly higher levels of Bcl-X_L protein from 2 to 8 days d.p.i. compared to the mock-treated control. The amount of Bcl-X_L protein detected in HCV-infected cells increased 1.5-fold within 2 days of infection and steadily increased to approximately 6-fold by 8 d.p.i. (Figure 4.4B). In contrast, the expression of Bak was slightly decreased in HCV-infected cells compared to the mock-treated control (Figure 4.4A). This small reduction in Bak protein level was consistently observed at later time points of infection (Figure 4.4C). On the other hand, the protein levels of Mcl-1 and Bax were not significantly altered by HCV infection of Huh7.5 cells (Figure 4.4A). The expression of the core protein indicated infection by HCV. These results suggest that HCV possibly disrupts the balance of Bcl-2 family members in the infected cells, which may in turn have an impact on apoptotic signalling.

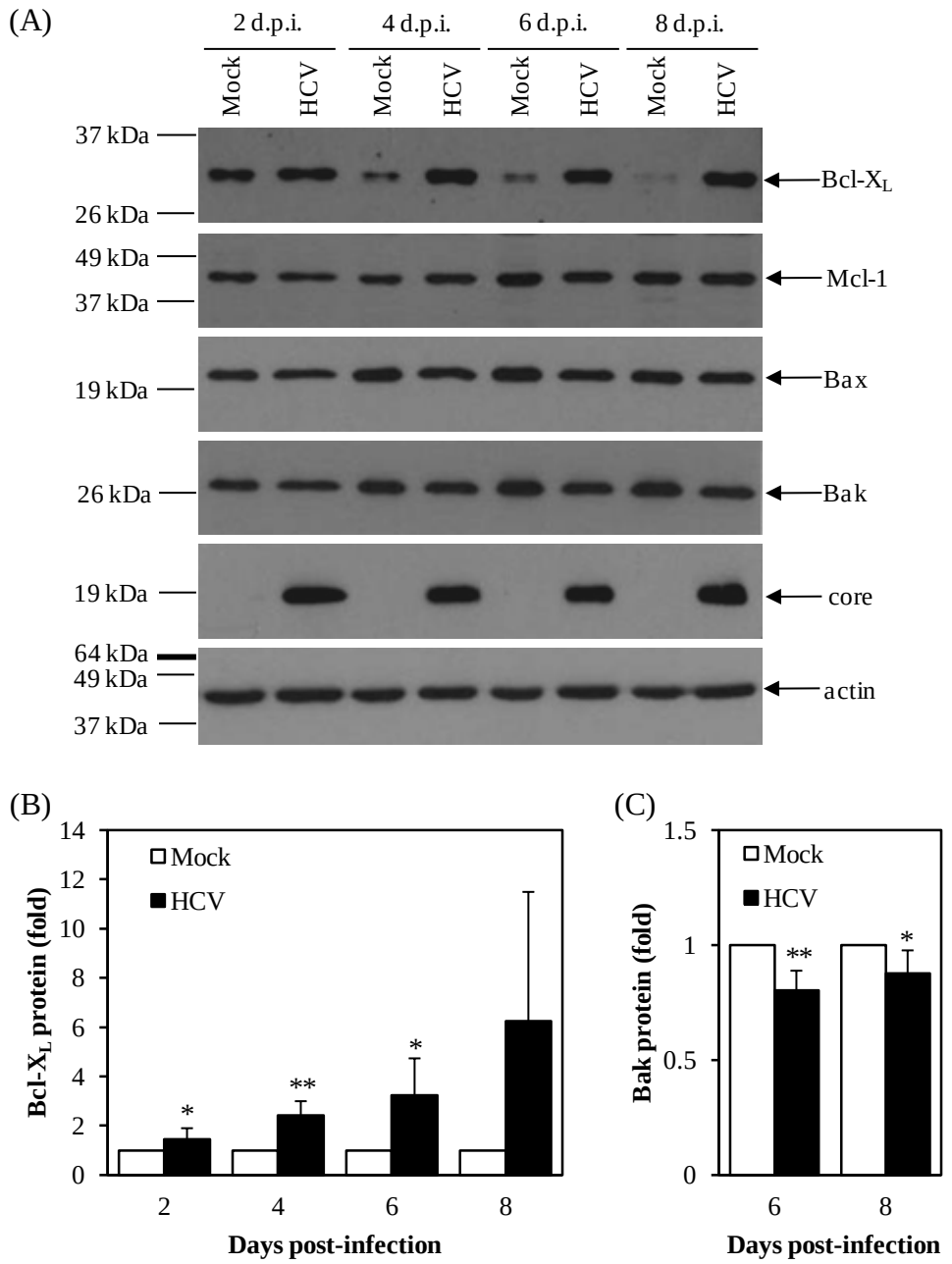

Fig. 4.4. Protein expression of Bcl-2 family members in HCV-infected Huh7.5 cells. (A) Whole cell lysates were prepared from HCV-infected cells and mock-treated control at 2, 4, 6 and 8 days post-infection (d.p.i.) and analyzed by Western blotting using antibodies against Bcl-X_L, Mcl-1, Bax, Bak, core and actin. The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin. Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. The intensity of Bcl-X_L (B) and Bak (C) protein bands in HCV-infected cells (filled column) and mock-treated control (empty column) was quantified using Image J. The intensity of the mock-treated control was arbitrarily expressed as 1.0. Data represent means ± SD of three independent experiments. **P* <0.05 and ***P* <0.01, compared to mock-treated control.

4.4.2 HCV infection upregulates Bcl-X_L mRNA levels in Huh7.5 cells

Since the Bcl-X_L protein levels dramatically increased in response to HCV infection, the mechanism underlying this upregulation was investigated. In order to determine if the elevated Bcl-X_L protein levels observed in HCV-infected cells were due to increased gene transcription, quantitative real-time PCR analysis was performed using total mRNA obtained from HCV-infected and mock-treated samples. As shown in Figure 4.5, Bcl-X_L mRNA levels were upregulated in HCV-infected cells by 1.7 to 4.5-fold from 2 to 6 d.p.i. The fold change in Bcl-X_L mRNA increased in a time-dependent manner and mirrored the increasing trend observed at the protein level (Figures 4.4A and 4.4B). Taken together, these results show that HCV upregulates the expression of Bcl-X_L by enhancing its gene transcription in infected Huh7.5 cells.

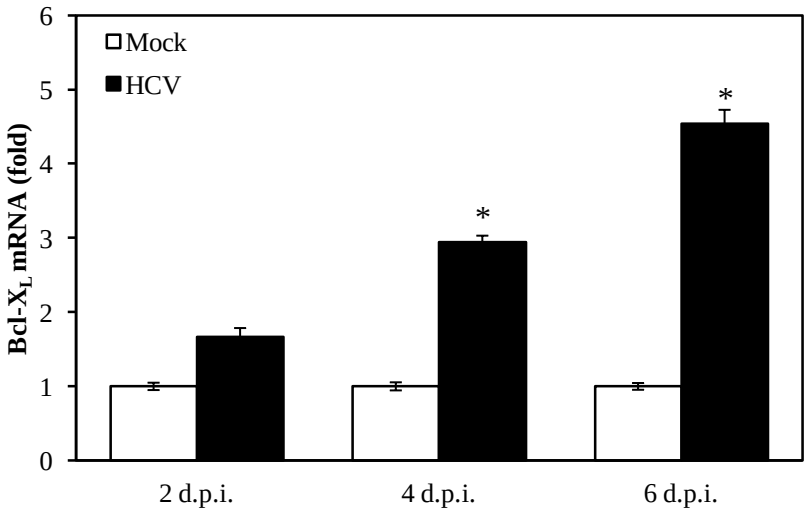

Fig. 4.5. HCV infection induces upregulation of Bcl-X_L mRNA in Huh7.5 cells. Cells were infected with HCV (MOI 2) and incubated for 2, 4 or 6 days post-infection (d.p.i.) before RNA extraction. Fold changes in Bcl-X_L gene expression were determined by quantitative real-time PCR. Data shown is representative of three independent experiments and statistically significant differences between mock-treated (empty columns) and HCV-infected (closed columns) cells are indicated by asterisks (*P <0.05).

4.4.3 Identifying HCV factors that upregulate Bcl-X_L expression

Previous studies have demonstrated upregulation of Bcl-X_L in cells expressing either HCV core protein or NS5A (Hara et al., 2006; Otsuka et al., 2002; Sarcar et al., 2004; Yoshida et al., 2002). It has been suggested that the core protein and NS5A enhance Bcl-X_L levels through the activation of the STAT3 signalling pathway. However, the core protein and NS5A examined in these studies were derived from HCV genotype 1b strains while our previous experiments were performed using the HCV genotype 2a infectious clone. Therefore, to determine whether the core protein and NS5A of the genotype 2a virus can induce Bcl-X_L upregulation in a similar manner, liposome-mediated transfection of cDNA expression plasmids containing the core protein gene or the NS5A gene was performed in Huh7.5 cells and the endogenous Bcl-X_L protein levels were analyzed by Western blotting.

As shown in Figure 4.6, cells expressing flag-tagged genotype 2a core protein or NS5A expressed comparable amounts of Bcl-X_L protein as the vector-transfected control cells (lanes 1 to 3). In contrast, the expression of flag-tagged genotype 1b core protein or NS5A enhanced Bcl-X_L protein levels slightly (lanes 4 and 5), which is consistent with the previous studies. These results suggest that in the case of HCV genotype 2a infection, other viral factors aside from the core protein and NS5A may contribute to the Bcl-X_L upregulation observed. Thus, further experiments are required to identify this viral factor(s) and delineate the mechanism(s) underlying this upregulation.

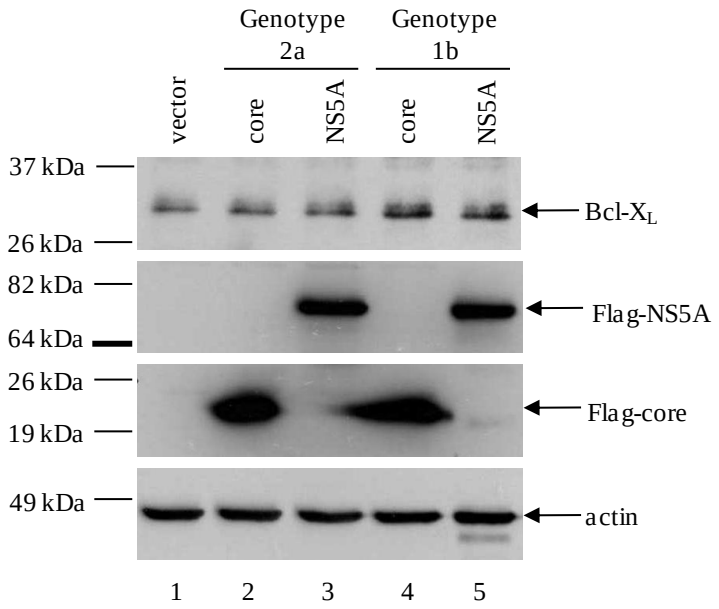

Fig. 4.6. Effects of HCV core protein and NS5A expression on the endogenous Bcl-X_L levels. Huh7.5 cells were transfected with vector only (lane 1) or cDNA constructs for expressing flag-core (lanes 2 and 4) or flag-NS5A (lanes 3 and 5) derived from HCV genotypes 2a and 1b. Western blot analysis using a specific antibody against Bcl-X_L was performed to determine the expression level of the endogenous protein (top). Similarly, the expression levels of the core protein and NS5A were determined using an anti-flag antibody (middle two). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

4.5 Apoptotic gene expression profiling of HCV-infected Huh7.5 cells

4.5.1 Real-time PCR array analysis of apoptosis-related genes in HCV-infected Huh7.5 cells

Gene expression profiling using pathway-focused real-time PCR arrays provides an alternative approach for the identification of host genes that may be involved in HCV-associated apoptosis. Here, the human apoptosis RT^2 Profiler PCR array was used to profile and compare the expression of 84 apoptotic genes in HCV-infected and mock-treated Huh7.5 cells. These genes include members of the caspase, Bcl-2, IAP, TRAF, CARD, CIDE, death domain, death effector domain and TNF receptor and ligand families, as well as genes involved in the p53 and DNA damage pathways. Previously, we demonstrated the induction of apoptosis in HCV-infected cells from 2 d.p.i. (Figure 4.3B). To further examine HCV-induced apoptosis during the early acute phase of infection, HCV-infected and mock control samples from 2 to 6 d.p.i. were subjected to PCR array analysis. Since each host gene is only represented by one well in the array, three independent experiments were performed to achieve reliability, thus providing three biological replicates.

In the three experiments, the Ct values of the five housekeeping genes were relatively similar in the HCV-infected and the mock-treated samples at all time points. This indicated that the expression of these genes were not altered by HCV infection and thus, can be used to normalize the expression of the assayed genes. As shown in Figure 4.7, the population size and intensity of differentially expressed apoptotic genes were relatively similar at 2 and 4 d.p.i. and then increased substantially at 6 d.p.i. Next, dissociation curve

analysis of the PCR products showed that 19 out of the 84 genes had multiple peaks in their melting curve, instead of a single sharp peak which indicates a specific PCR product (Appendix 2). This is rather surprising as the PCR array contains primer pairs that have been experimentally validated to ensure gene specificity. This could be due to non-specific binding of primers to the cDNA template or the presence of splice variants of the gene in liver cells, which might be different from the cells used for primer validation. As a result, the observed expression changes of these genes were likely to be inaccurate and thus, were excluded from subsequent analysis. The significance of the gene expression changes was determined using a Student's t-test and *P* values less than 0.05 were considered to be statistically significant. Out of the remaining 65 genes, the number of genes that showed statistically significant changes in expression of at least 2-fold or 3-fold at each time point is summarized in Table 4.1. Similar to the preliminary data shown in Figure 4.7, a higher proportion of the genes were differentially regulated at 6 d.p.i. than at 2 and 4 d.p.i., suggesting temporal regulation of host apoptotic genes by HCV.

Table 4.1. Summary of apoptotic gene expression changes observed in the PCR array analysis

Time point	**No. of differentially expressed genes**[a]	
	≥ 2-fold	**≥ 3-fold**
2 d.p.i.	2 (3.1%)	2 (3.1%)
4 d.p.i.	4 (6.2%)	1 (1.5%)
6 d.p.i.	10 (15.4%)	6 (9.2%)

[a] Only genes that gave rise to a specific PCR product and showed statistically significant expression changes ($P < 0.05$) were considered. The percentage of differentially expressed genes out of the 65 genes analyzed are indicated in parentheses.

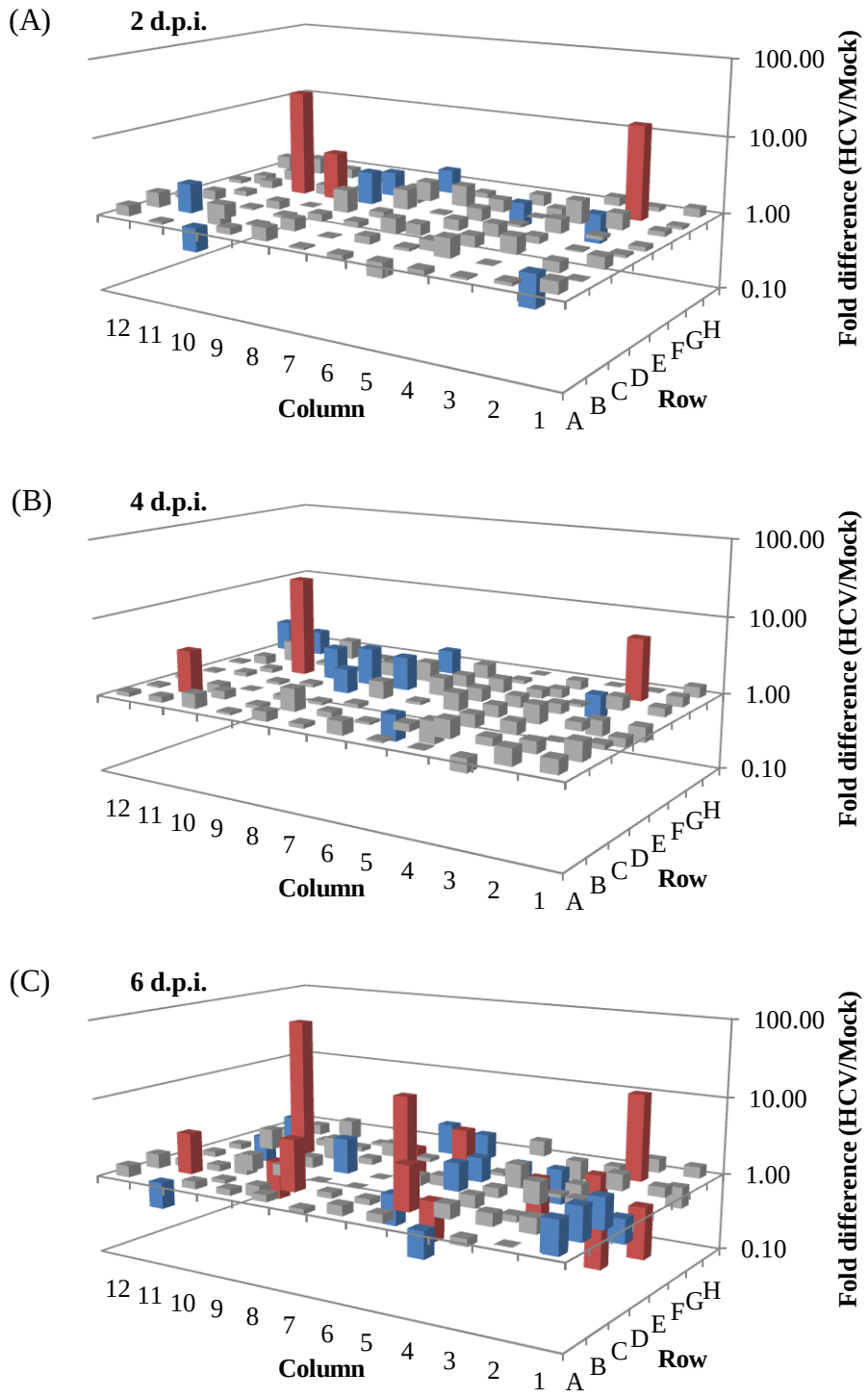

Fig. 4.7. 3D profiles of the gene expression changes in the PCR array analysis. Data obtained at (A) 2 d.p.i., (B) 4 d.p.i. and (C) 6 d.p.i. are presented in the 96-well format of the PCR array (x and y-axes) and the corresponding genes are listed in Appendix 1. Columns pointing up (with z-axis values > 1) indicate an upregulation of gene expression and columns pointing down (with z-axis values < 1) indicate a downregulation of gene expression in the HCV-infected sample relative to the mock control sample. Gene expression changes of between 2- and 3-fold are represented by blue columns and changes of more than 3-fold are represented by red columns. Data represent means of three independent experiments.

4.5.2 Quantitative real-time PCR validation of gene expression changes

Various selection criteria can be applied to the PCR array data in order to shortlist differentially expressed host apoptotic genes for further validation. Using one possible selection criteria, five genes were identified to be consistently up- or down-regulated by more than 3-fold at the same time point post-infection in all three experiments (Table 4.2). At 6 d.p.i., the expression of BIK and FAS was upregulated while that of BNIP3 was downregulated. In contrast, GADD45A mRNA levels displayed only an initial increase at 2 d.p.i. The expression of HRK exhibited the most significant change in response to HCV infection and was highly upregulated at all time points post-infection.

To validate the PCR array results, quantitative real-time PCR (qPCR) analysis was performed using pre-designed primers and probes based on TaqMan chemistry. BIK mRNA expression increased 5-fold within 2 days of infection and steadily increased 33-fold by 6 d.p.i. (Figure 4.8A). In contrast, BNIP3 mRNA expression decreased almost 3-fold by 4 d.p.i. (Figure 4.8B). An increase in the expression of FAS mRNA by 4-fold was only observed at 6 d.p.i. (Figure 4.8C) while GADD45A mRNA expression increased 3-fold by 2 d.p.i. and this upregulation was maintained until 6 d.p.i. (Figure 4.8D). Out of the five differentially expressed host genes that were selected for further validation, HRK mRNA expression was altered most dramatically, increasing 22-fold at 2 d.p.i. and almost 60-fold by 6 d.p.i. (Figure 4.8E).

In general, the results of the validation experiments were consistent with the expression changes observed in the PCR array analysis. As shown in Table 4.2 and Figure 4.8, the expression of BIK, FAS, GADD45A and HRK

were all upregulated at least 3-fold while the expression of BNIP3 was downregulated almost 3-fold in HCV-infected cells compared to the mock-treated control. However, several differences in trend were observed for the expression of BNIP3 and GADD45A. In the PCR array analysis, BNIP3 expression showed the biggest decrease at 6 d.p.i. while this was observed earlier at 4 d.p.i. in the validation experiment (Figure 4.8B). Similarly, the upregulation of GADD45A peaked at different times, 2 d.p.i. in the PCR array analysis and 6 d.p.i. in the validation experiment (Figure 4.8D). These variations may be due to the different sensitivities and specificities of SYBR and TaqMan chemistries or the efficiency of priming different target regions on the cDNA (Arikawa et al., 2008). The latter could not be addressed due to the lack of access to the proprietary information on the exact sequences of the primers and probes used in these two assays.

Table 4.2. Fold changes of apoptosis-related genes in HCV-infected Huh7.5 cells as identified by PCR array analysis

Gene Symbol[a]	**Fold Up- or Down-Regulation**[b]								
	2 d.p.i.			**4 d.p.i.**			**6 d.p.i.**		
	Set 1	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**
BIK	1.21	-1.09	2.56	1.36	1.16	4.48	**3.19**	**5.17**	**5.49**
BNIP3	-1.38	-1.86	1.02	-1.75	-1.60	-2.58	**-3.39**	**-3.07**	**-3.10**
FAS	2.51	1.09	2.15	2.91	2.04	3.15	**53.13**	**4.93**	**3.14**
GADD45A	**3.88**	**3.91**	**4.29**	2.49	2.72	2.52	1.94	2.21	1.63
HRK	**21.77**	**36.46**	**15.38**	**12.16**	**42.36**	**13.56**	**124.77**	**57.41**	**33.70**

[a] Only genes that were consistently up- or down-regulated by more than 3-fold are listed in the table.
[b] Fold changes of more than 3-fold that were observed at a specific time point in all three experiments (sets 1 to 3) are indicated in red (upregulated) or blue (downregulated).

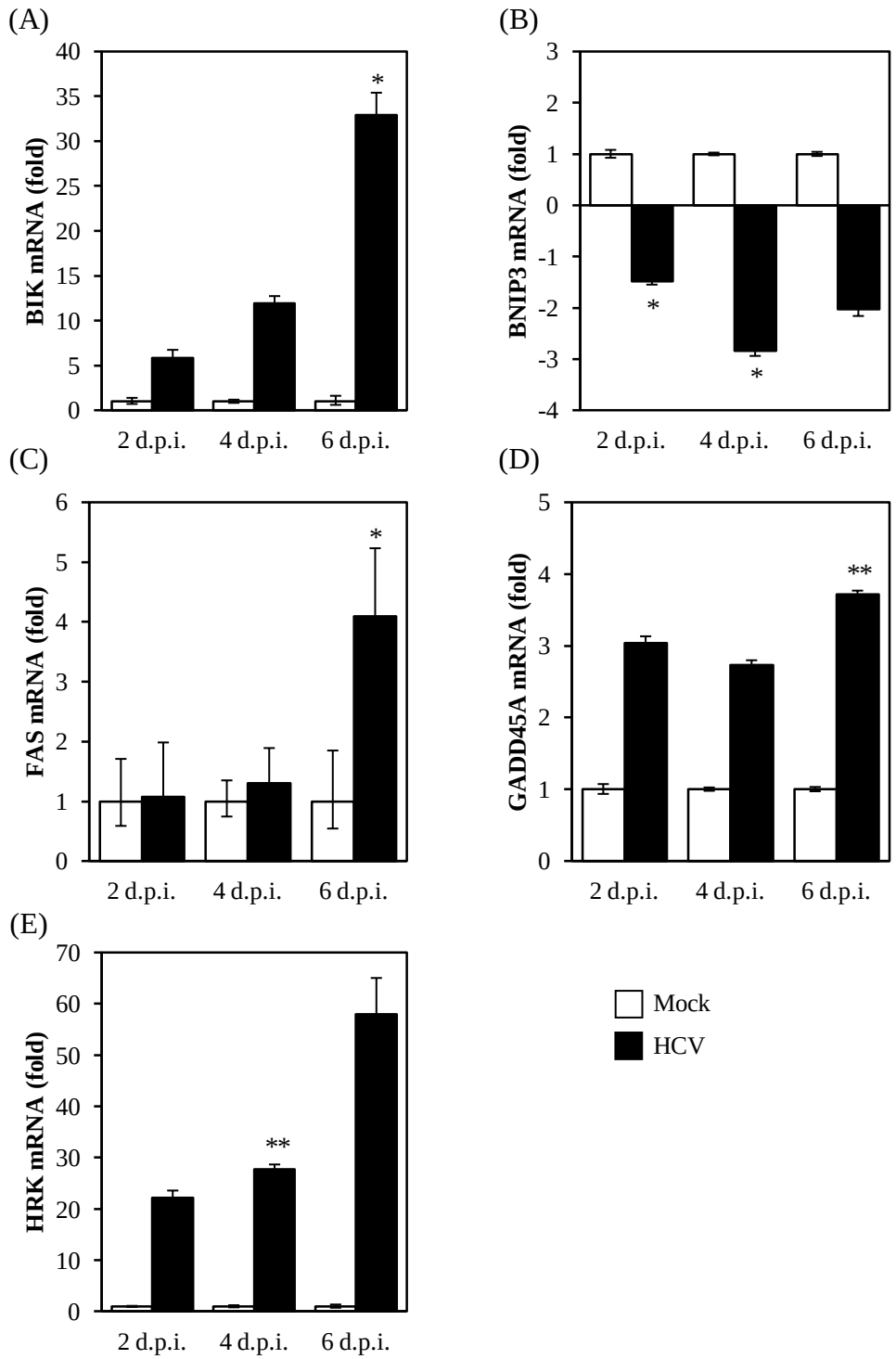

Fig. 4.8. Validation of mRNA fold changes in HCV-infected Huh7.5 cells by quantitative real-time PCR. Cells were infected with HCV (MOI 2) and incubated for 2, 4 and 6 days post-infection (d.p.i.). Fold changes in gene expression were determined by real-time PCR using specific primers to (A) BIK, (B) BNIP3, (C) FAS, (D) GADD45A and (E) HRK and normalised to GAPDH expression. Data shown is representative of three independent experiments and statistically significant differences between mock (empty columns) and HCV-infected (closed columns) cells are marked by asterisks (*P <0.05 and **P <0.01).

4.5.3 Expression of HRK in HCV-infected Huh7.5 cells

Since quantitative real-time PCR analysis using both SYBR-based and TaqMan-based detection showed that HRK is the most strongly upregulated gene at all time points post-infection, Western blot analysis was performed using a specific antibody against human HRK in order to determine if the upregulation of HRK mRNA expression by HCV infection translates to an increase in HRK protein level. Interestingly, the HRK protein level in HCV-infected cells was comparable to that in the mock-treated control during the first 6 days after infection (Figure 4.7A). At 8 d.p.i., the HRK protein level in HCV-infected cells increased by 1.2-fold (Figure 4.7B). However, this increase in HRK protein level is small compared to the extent of HRK mRNA upregulation (Figure 4.6E), suggesting that there may be other factors regulating the synthesis and/or stability of the HRK protein.

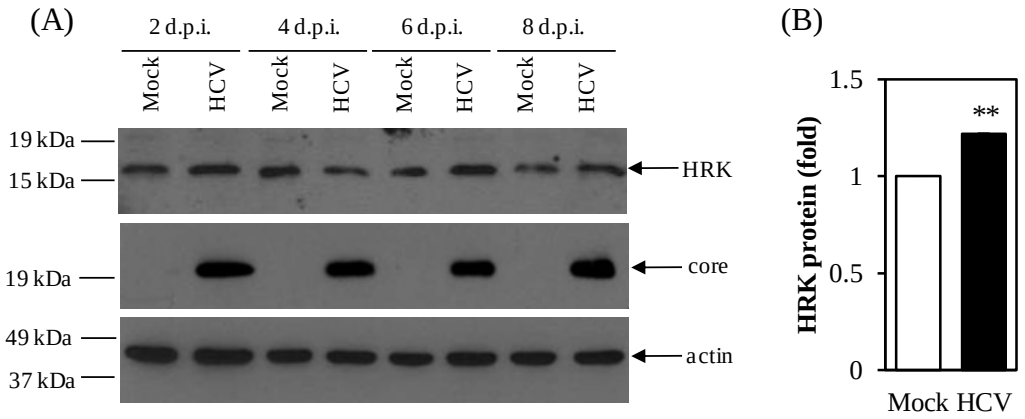

Fig. 4.9. Expression of HRK in HCV-infected Huh7.5 cells. (A) Whole cell lysates were prepared from HCV-infected cells and mock-treated control at 2, 4, 6 and 8 days post-infection (d.p.i.) and the expression of HRK, HCV core and actin was determined by Western blot analysis. The amounts of total cell lysate loaded were verified by measuring the levels of endogenous actin. Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. The intensity of HRK in HCV-infected cells (filled column) and mock-treated control (empty column) at 8 d.p.i. was quantified using Image J. The intensity of the mock-treated control was arbitrarily expressed as 1.0. Data represent means ± SD of two independent experiments. $^{**}P < 0.01$, compared to mock-treated control.

4.6 Discussion

HCV-induced liver injury is thought to be the result of both direct cytopathic effects of the virus and host immune responses [reviewed by (Mengshol et al., 2007)]. Apoptosis is a common feature of these processes and might be mediated directly by HCV replication and/or gene products or through the actions of cytotoxic lymphocytes (Bantel and Schulze-Osthoff, 2003; Fischer et al., 2007). Enhanced hepatocyte apoptosis has been observed in the liver of patients chronically infected with HCV and correlates with liver damage (Bantel et al., 2001; Calabrese et al., 2000). Apoptosis of hepatocytes may also contribute to liver injury through the induction of hepatic fibrogenesis (Bantel et al., 2004; Canbay et al., 2004). Given its central role in HCV-associated liver injury, it is important to understand the cellular mechanisms underlying HCV-mediated apoptosis. The HCVcc infection system provides a convenient tool for characterizing the direct cytopathic effects of HCV in cultured hepatocytes, without the complication of immune-mediated responses. In this study, by using the HCVcc system, we have identified several apoptotic genes that are differentially expressed in response to acute HCV infection, suggesting a role for them in HCV-associated apoptosis.

In common with other studies, we showed that acute *in vitro* HCV infection of cultured hepatocytes induced cytopathic effects, which were characterized by an increase in apoptosis induction (Figure 4.3). The intragenotypic J6/JFH-1 chimera has previously been shown to induce higher levels of apoptosis compared to the parental JFH-1 strain (Mateu et al., 2008)

while the tissue culture-adapted J6/JFH-1-P47 virus used in this study exerts an even stronger cytopathic effect than the J6/JFH-1 chimera (Deng et al., 2008). Increased abilities of the virus to induce apoptosis appeared to correlate with increased viral replication and virion production (Bungyoku et al., 2009; Mateu et al., 2008). The search for host factors that may be involved in HCV-associated apoptosis has thus far identified two members of the Bcl-2 protein family, Bax and Bcl-X_L, which act in the mitochondrial apoptosis pathway. HCV-induced activation of proapoptotic Bax triggered apoptosis in Huh7.5 cells infected with the J6/JFH-1-P47 virus (Deng et al., 2008). The genotype 1a infectious clone H77c sensitized hepatocytes to apoptosis in chimeric *SCID/Alb-uPA* mice by inducing Bax activation and downregulating prosurvival NF-κB and Bcl-X_L (Joyce et al., 2009). More recent studies have linked HCV-induced apoptosis to cell cycle perturbations. Consistent with previous reports (Kannan et al., 2011; Timpe et al., 2008), we observed that proliferation of HCV-infected cells was slower than naive cells (Figure 4.3A). This delay in cell cycle progression has been attributed to cell cycle arrest at the G_1 phase and/or at the interface of G_2 and mitosis, accompanied by differential expression of cell death genes and higher levels of apoptotic markers (Kannan et al., 2011; Marshall et al., 2005; Walters et al., 2009).

By examining the expression of a subset of the Bcl-2 protein family, we demonstrated that HCV infection significantly upregulates Bcl-X_L gene transcription (Figure 4.5) and protein expression (Figures 4.4A and 4.4B). Our finding is consistent with earlier studies that reported increased levels of

Bcl-X_L in the presence of HCV core or NS5A. Overexpression of the core protein enhanced the expression of Bcl-X_L mRNA and protein and resulted in the inhibition of apoptosis (Hara et al., 2006; Otsuka et al., 2002). Yoshida and colleagues demonstrated that the activation of STAT3 by the core protein upregulates Bcl-X_L expression and leads to cellular transformation (Yoshida et al., 2002). Similarly, activation of the STAT3 pathway by NS5A also enhanced Bcl-X_L expression (Sarcar et al., 2004). Conversely, inhibition of core protein expression in immortalized hepatocytes decreased Bcl-X_L protein levels (Meyer et al., 2005).

However, we showed that the expression of the core protein or NS5A derived from the JFH-1 clone (genotype 2a) did not significantly alter the endogenous Bcl-X_L levels in transfected Huh7.5 cells (Figure 4.6). This may be due to genotypic differences as the previous studies were done using HCV genotype 1b proteins and thus, implies that other HCV genotype 2a factors may contribute to enhanced Bcl-X_L expression in infected cells. In agreement with this, a very recent report showed that HCV E2-CD81 engagement is sufficient to induce the upregulation of Bcl-2 and Bcl-X_L expression in human B lymphocytes treated with purified genotype 1a E2 protein or infected with the J6/JFH-1 virus (Chen et al., 2011). Since this experiment was performed using B cells, further experiments are needed to determine if the E2 glycoprotein contributes to the Bcl-X_L upregulation observed in Huh7.5 cells infected with HCV genotype 2a. Alternatively, the differences observed between viral proteins derived from HCV genotypes 1b and 2a may be due to the low efficiency of liposome-mediated transfection. The core protein and

NS5A of genotype 2a may be less potent inducers of Bcl-X_L upregulation than their genotype 1b counterparts and thus, their effects on cellular Bcl-X_L protein levels may not be observable using this expression system. Hence, more efficient gene delivery systems such as viral vectors or more sensitive techniques such as promoter reporter assays may be used to examine the roles of the core protein and NS5A in HCV-induced Bcl-X_L upregulation.

It is intriguing that HCV-induced apoptosis occurs in this background of high Bcl-X_L expression. Bcl-X_L is one of two major prosurvival Bcl-2 proteins expressed in the liver and plays an important protective role against apoptotic stimuli (Hikita et al., 2009; Takehara et al., 2004). Nevertheless, it is possible that the level of Bcl-X_L is insufficient to completely inhibit apoptosis or that HCV-induced apoptosis is mediated by alternative pathways. Also, it is unclear at this point if the upregulation of Bcl-X_L is driven by the virus in order to moderate apoptosis levels in favour of viral replication or by the host in order to protect itself against massive viral-induced cell death. Joyce and colleagues reported that although the overall levels of NF-κB and Bcl-X_L in the liver of HCV-infected chimeric *SCID/Alb-uPA* mice were increased, both NF-κB and Bcl-X_L levels were lower in infected cells compared to adjacent uninfected cells (Joyce et al., 2009). This explains sensitization of infected hepatocytes to apoptosis and suggests that Bcl-X_L upregulation in the surrounding cells is a host response to HCV infection. However, this is unlikely to be the case in our system as the cultures which showed Bcl-X_L upregulation were inoculated at a sufficiently high MOI to infect all the cells (Figure 4.1A). Therefore, to further understand the

involvement of Bcl-X_L in HCV pathogenesis, we investigated the possible role(s) of Bcl-X_L during the early acute phase of HCV infection and this is discussed in Chapter 5.

Unlike Bcl-X_L, the expression of Mcl-1, the other major prosurvival Bcl-2 protein in the liver, was not significantly altered by HCV infection (Figure 4.4). Similarly, HCV infection did not modulate the levels of key proapoptotic mediators, Bax and Bak, to a large extent but their proapoptotic activity is also dependent on their conformation and/or subcellular localization (Westphal et al., 2011). In agreement with this, previous studies showed that HCV-induced apoptosis was accompanied by the activation of Bax, as characterized by its conformational change and mitochondrial accumulation, with minimal variation in its expression level (Deng et al., 2008; Joyce et al., 2009).

PCR array analysis of HCV-infected cells during the early acute phase of infection showed that host apoptotic genes were differentially regulated from 2 d.p.i., which corresponds with the HCV-induced apoptosis observed at this time point (Figures 4.3 and 4.7). Five host apoptotic genes were identified to be consistently differentially regulated by more than 3-fold in HCV-infected cells (Table 4.2). The changes in gene expression were independently verified by quantitative real-time PCR (Figure 4.8). These genes provide an insight as to which apoptotic pathways may be specifically modulated by HCV. Three out of the five shortlisted genes encode proapoptotic BH3-only members of the Bcl-2 protein family (BIK, BNIP3 and HRK) which generally act via the intrinsic, mitochondria-mediated apoptosis pathway. The remaining two

genes are Fas, a member of the tumour necrosis factor receptor (TNFR) gene superfamily that causes cell death via the extrinsic apoptosis pathway, and GADD45A, a protein that is involved in cell cycle arrest. These genes and their protein products are discussed in greater detail below.

One of the host genes that were upregulated by HCV infection is BCL-2 interacting killer (BIK) which is a proapoptotic BH3-only member of the Bcl-2 protein family (Figure 4.8A). In addition to binding cellular prosurvival proteins Bcl-2 and Bcl-X_L, BIK can also interact with viral anti-apoptosis proteins such as Epstein-Barr virus BHRH1 and adenovirus E1B-19K (Boyd et al., 1995; Han et al., 1996). Although it has not been extensively studied in relation to HCV infection, BIK has been shown to play an important role in adenovirus-induced apoptosis. $Bik^{-/-}$ mice infected with an apoptogenic adenovirus mutant did not display the apoptotic cytopathic effect (Shimazu et al., 2007). Furthermore, transcriptional activation of BIK was observed in adenovirus-infected human cells undergoing apoptosis (Subramanian et al., 2007). In a recent study, global transcriptional profiling of HCV J6/JFH-1-infected Huh7.5 cells by microarray analysis also detected BIK upregulation within the subset of cell death-related genes (Walters et al., 2009).

Out of the five shortlisted host genes, only the expression of Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) was downregulated (Figure 4.8B). BNIP3 is an atypical BH3-only protein that can induce apoptosis via Bax/Bak activation and/or necrosis via opening of the mitochondrial permeability transition pore (Kubli et al., 2007; Quinsay et al., 2010; Vande Velde et al., 2000). BNIP3 is also a potent inducer of autophagy,

which may confer protection against cell death or contribute to autophagic cell death depending on the experimental conditions (Azad et al., 2008; Hamacher-Brady et al., 2007; Kanzawa et al., 2005). Due to its multiple roles, it is difficult to predict if the HCV-induced downregulation of BNIP3 expression has protective or detrimental effects on host cells and further investigations are needed to address this. Interestingly, downregulation of BNIP3 has also been observed in astrocytes infected with the murine coronavirus mouse hepatitis virus and is postulated to promote viral persistence by inhibiting the host antiviral apoptotic response (Cai et al., 2003).

Fas, another cell-death related gene found to be upregulated by HCV infection (Figure 4.8C), is a death domain-containing cell surface receptor for Fas ligand (FasL). Upon FasL binding and recruitment of cellular adaptor proteins, a death-induced signalling complex is formed and apoptosis is initiated via the extrinsic apoptosis pathway (Zhang et al., 2005). Consistent with our finding, immunohistochemical studies have previously shown upregulation of hepatic Fas expression in chronic hepatitis C patients (Hiramatsu et al., 1994; Okazaki et al., 1996). Enhanced Fas expression in hepatocytes and FasL expression in infiltrating T lymphocytes may contribute to HCV-induced hepatocyte apoptosis. However, inhibition of the Fas/FasL system was observed in HCC, possibly as a mechanism to evade the host immune response (Bortolami et al., 2008). This suggests that HCV might regulate Fas expression differently during early and late stages of infection.

Upregulation of growth arrest and DNA damage-inducible alpha (GADD45A) was observed in HCV-infected cells (Figure 4.8D). GADD45A

plays a regulatory role in various cellular processes, including maintenance of genomic stability, DNA repair, cell cycle arrest and apoptosis (Hollander and Fornace, 2002; Tong et al., 2005). Several studies have reported cell cycle arrest in HCV-infected cells, which may be accompanied by apoptosis induction (Kannan et al., 2011; Marshall et al., 2005; Sarfraz et al., 2008; Walters et al., 2009). Furthermore, analysis of cell cycle arrest-related gene expression revealed that GADD45A is one of several upregulated genes in both HCV-infected hepatoma cells and chronic HCV infection, which is in agreement with our observation (Sarfraz et al., 2009; Walters et al., 2009). Therefore, GADD45A upregulation may contribute to the delayed cell proliferation and apoptosis observed in our infection system.

Harakiri (HRK), another proapoptotic BH3-only protein, was found to be significantly upregulated in response to HCV infection (Figure 4.8E). HRK regulates Bax-dependent apoptosis through specific interaction with Bcl-2 and Bcl-X_L (Harris and Johnson, 2001; Inohara et al., 1997). Enhanced HRK expression was also observed in the same genome-wide transcriptional profiling study that showed BIK and GADD45A upregulation in the early acute phase of HCV infection (Walters et al., 2009). Hence, enhanced HRK expression may be another contributing factor to HCV-induced apoptosis. Recently, apoptotic gene expression profiling of HepG2 cells infected with dengue virus, another member of the Flaviviridae virus family, showed a dramatic increase in HRK expression as well (Morchang et al., 2011).

Taken together, the PCR array data suggest that HCV infection generally promotes apoptosis induction via both the intrinsic and extrinsic

pathways by increasing the expression of proapoptotic genes involved in these pathways. However, since proteins, and not mRNA, are the major effectors of cellular function, the corresponding changes in protein level have to be confirmed before the contribution of these genes to HCV-induced apoptosis can be determined. For example, in the case of HRK, although there was a striking increase in its mRNA expression, this large increase was not observed at protein level (Figure 4.9). Translation of the HRK mRNA might have been blocked or the HRK protein may be too short-lived to be detectable by Western blot analysis. Thus, the enhanced expression of HRK mRNA is unlikely to have a profound impact in HCV infection.

In addition to these five genes, there may be several other differentially expressed genes that were unintentionally excluded from the list due to the strict selection criteria (at least 3-fold difference in gene expression observed in all three experiments). We showed in Table 4.1 and Figure 4.7 that increasing the stringency of selection resulted in fewer shortlisted genes. This also probably explains the absence of Bcl-X_L in the list, even though we have previously shown that its expression was significantly increased in response to HCV infection (Figures 4.4 and 4.5). The genes that were found to be differentially expressed to a lesser extent may also contribute to HCV-induced apoptosis. Six additional genes that showed at least a 2-fold difference in their expression at 6 d.p.i. are listed in Appendix 3. Among them, a homologue of BNIP3, BNIP3-like (BNIP3L), was also observed to be downregulated. Since BNIP3 and BNIP3L have similar functions including roles in cell death and autophagy (Zhang and Ney, 2009), the data suggest that concerted inhibition

of these functions may be important for HCV infection. Although CIDEB and TNFSF10 were not observed to be consistently regulated more than 3-fold in all three experiments, they showed an average downregulation of more than 3-fold at 6 d.p.i., which was nevertheless statistically significant. Interestingly, both TNFSF10 (also known as TRAIL or Apo-2L) and Fas, one of the five validated genes, belong to the superfamily of TNF- and TNF receptor-like molecules (Tansey and Szymkowski, 2009). In addition, two other receptors of the same family, namely TNFRSF21 and TNFRSF25, were also differentially regulated during HCV infection (Appendix 3). A subset of genes was also excluded from the analysis as they displayed multiple peaks in the dissociation curve and thus, did not give reliable results (Appendix 2). It might be worth to reanalyse the expression of these genes using different primers or other methods if they are known to be involved in specific apoptotic pathways that are modulated by HCV.

In this study, two different methods were employed to identify host factors that may be involved in HCV-induced apoptosis. Bcl-X_L was identified by Western blot analysis while BIK, BNIP3, FAS, GADD45A and HRK were identified by quantitative real-time PCR array analysis. There are advantages and disadvantages associated with both techniques. While Western blot analysis allows for direct visualization of changes in protein levels, screening a large number of proteins is a tedious process and the scope of candidate proteins that can be screened is dependent on the availability of good antibodies. In contrast, gene expression profiling by pathway-focused PCR arrays enables simultaneous screening of a large number of genes but the

changes in mRNA expression require further validation, especially on the protein level. Similar to the disparity observed between HRK mRNA and protein levels in this study, a poor correlation between selected mRNA and protein abundances in the liver has been previously demonstrated (Anderson and Seilhamer, 1997). Therefore, in this study, the modulation of Bcl-X_L expression by HCV infection is most convincing and the role of Bcl-X_L in HCV-induced apoptosis is further investigated in the next chapter.

CHAPTER 5: OVEREXPRESSION AND KNOCKDOWN OF BCL-X_L AND MCL-1 AND THEIR IMPACT ON HCV INFECTION

5.1 Stable overexpression of Bcl-X_L and Mcl-1 in Huh7.5 cells

Bcl-X_L and Mcl-1 are two major prosurvival Bcl-2 family members in the liver which are important for its development and homeostasis (Hikita et al., 2009). As discussed in Chapter 4, HCV may interfere with apoptotic signalling in infected cells by modulating the expression levels of host cell death proteins. We demonstrated that HCV infection induced the upregulation of Bcl-X_L in infected Huh7.5 cells but did not affect Mcl-1 levels (Figure 4.4A). The results suggest that Bcl-X_L may play a more significant role than Mcl-1 in regulating HCV infection. Therefore, to investigate the possible role(s) of Bcl-X_L in HCV infection, we examined the effects of Bcl-X_L overexpression and knockdown during the early acute phase of infection. Similarly, the effects of Mcl-1 overexpression and silencing on HCV infection were also examined and compared to that of Bcl-X_L.

Huh7.5 cells stably overexpressing Bcl-X_L or Mcl-1 were generated by electroporation of the expression plasmids followed by multiple rounds of G418 selection. For ease of detection, exogenously expressed proteins were tagged with a c-myc epitope at the N-terminus. Cells transfected with the empty vector were used as controls (clones V4 and V11) as they express only endogenous levels of Bcl-X_L and Mcl-1 (Figures 5.1A and 5.2A). Since these cells were selected with the same amount of antibiotics as the Bcl-X_L and Mcl-1 stable cells, cellular changes due to the selection process may be ruled out when these cells are used as the control cells. For the Bcl-X_L stable cells,

clones B60 and B69, expressing approximately 2.8-fold and 3.7-fold more Bcl-X_L than the control cells respectively, were selected for use in subsequent experiments (Figures 5.1A and 5.1B). Similarly for the Mcl-1 stable cells, clone M34, which expresses about 1.7-fold more Mcl-1 than the V4 control cells, was selected for the infection experiments (Figures 5.2A and 5.2B).

The random integration of the transfected DNA into the cell's chromosomes and the high levels of Bcl-X_L or Mcl-1 expression may have an effect on cellular proliferation. Thus, the proliferative abilities of the stable clones were determined by WST-1 assay and compared to determine if there is substantial difference in their growth rates. As shown in Figure 5.1C, the proliferation rates of the Bcl-X_L stable and vector control cells varied slightly. However, statistical analysis revealed that these differences were insignificant (data not shown). Likewise, the proliferation rates of M34 and V4 cells were comparable (Figure 5.2C).

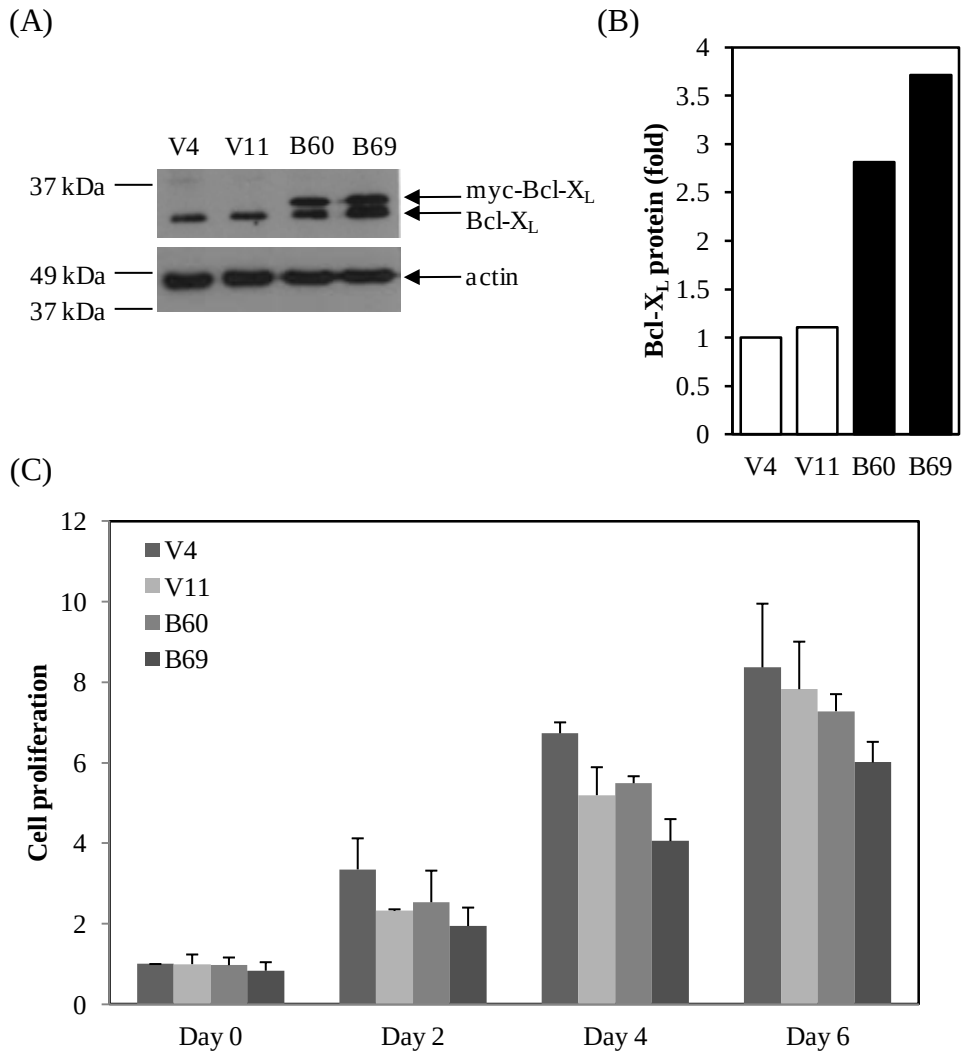

Fig. 5.1. Stable overexpression of Bcl-X_L in Huh7.5 cells. (A) Cells were electroporated with pXJ41neo-myc-Bcl-X_L plasmid or empty vector and cultured under G418 selection. Whole cell lysates were prepared from clones stably overexpressing Bcl-X_L (B60 and B69) and vector control cells (V4 and V11). Protein expression levels were confirmed by Western blot analysis using a primary antibody against Bcl-X_L (top). The amounts of total cell lysates loaded were verified by measuring the level of endogenous actin (bottom). Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. (B) The intensity of total Bcl-X_L in stable clones B60 and B69 (filled columns) and control cells V4 and V11 (empty columns) was quantified using Image J. The intensity of Bcl-X_L in V4 cells was arbitrarily expressed as 1.0. (C) Cell proliferation of the stable clones was measured using WST-1 assay at days 0, 2, 4 and 6 after cell seeding. Proliferation of V4 at day 0 was arbitrarily expressed as 1.0. Data represent means ± SD of two independent experiments.

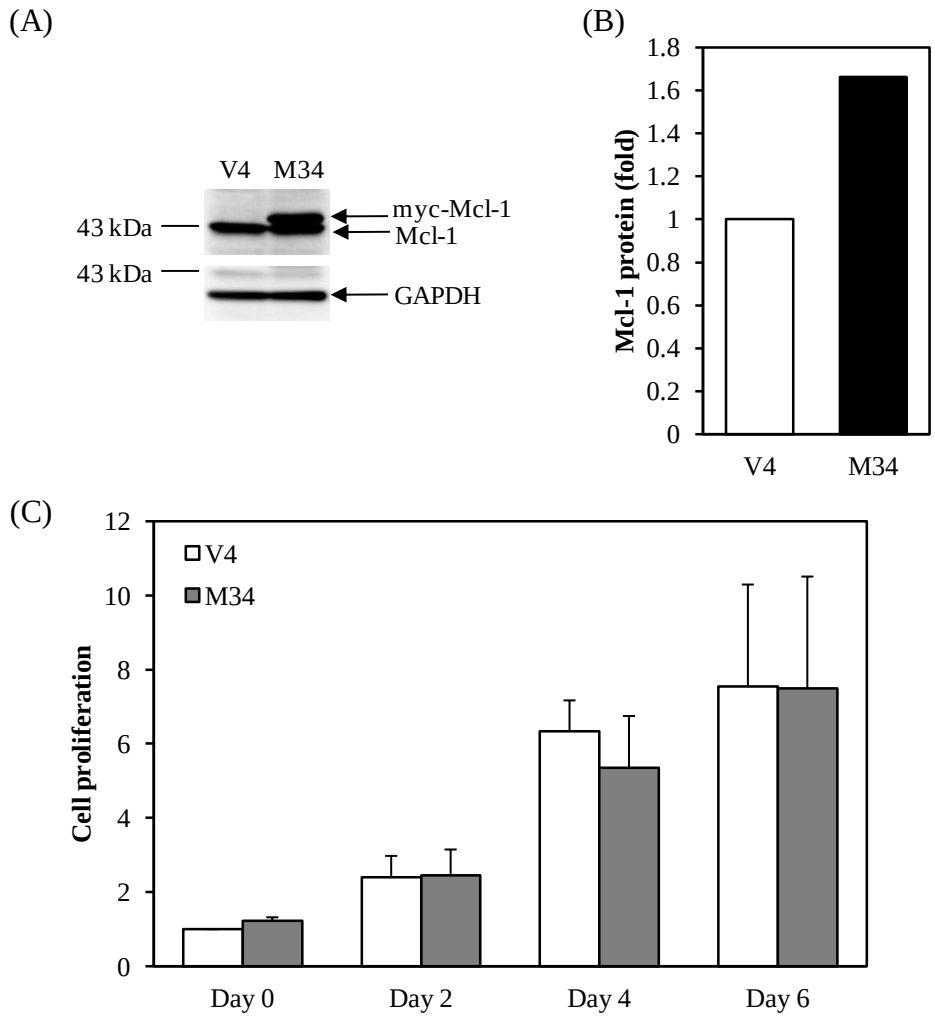

Fig. 5.2. Stable overexpression of Mcl-1 in Huh7.5 cells. (A) Cells were electroporated with pXJ41neo-myc-Mcl-1 plasmid or empty vector and cultured under G418 selection. Whole cell lysates were prepared from cells stably overexpressing Mcl-1 (M34) and vector control cells (V4). Protein expression levels were confirmed by Western blot analysis using a primary antibody against Mcl-1 (top). The amounts of total cell lysates loaded were verified by measuring the level of endogenous GAPDH (bottom). Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. (B) The intensity of total Mcl-1 in stable clone M34 (filled column) and control cells V4 (empty column) was quantified using Image J. The intensity of Mcl-1 in V4 cells was arbitrarily expressed as 1.0. (C) Cell proliferation of M34 and V4 was measured using WST-1 assay at days 0, 2, 4 and 6 after cell seeding. Proliferation of V4 at day 0 was arbitrarily expressed as 1.0. Data represent means ± SD of two independent experiments.

5.2 Silencing of Bcl-X_L and Mcl-1 expression in Huh7.5 cells by RNA interference

To achieve efficient knockdown of gene expression in Huh7.5 cells, transient transfection of small interfering RNA (siRNA) specifically targeting Bcl-X_L or Mcl-1 mRNA was performed. Cells transfected with the corresponding negative control siRNA (siControl) were used for comparison. Preliminary experiments were performed to determine the optimal concentration of siRNA for knockdown (data not shown) and subsequently, 50 nM of siBcl-X_L and 10 nM of siMcl-1 were used in all experiments. The efficiency of knockdown was analyzed by Western blotting every 48 h after transfection, up to 8 days post-transfection. As shown in Figure 5.3A, the expression of Bcl-X_L was completely silenced by 2 days post-transfection and this level of knockdown was maintained for the duration of the experiment. As for the siMcl-1-treated cells, substantial reduction in Mcl-1 expression was also observed by 2 days post-transfection (Figure 5.3B). Although the Mcl-1 level in the knockdown cells appeared to recover slightly 8 days after transfection, the level of Mcl-1 was considerably lower in siMcl-1-treated cells compared to the control throughout the experiment. The expression of prosurvival Bcl-X_L and Mcl-1 in the siControl-treated samples gradually decreased probably due to cell overgrowth and the subsequent onset of confluence-induced cell death (Chang et al., 2007).

Proliferation of the knockdown cells was determined as previously described. As shown in Figures 5.3C and 5.3D, the growth rates of the Bcl-X_L and Mcl-1 knockdown cells did not differ significantly from that of the control

cells. Collectively, the stable clones and knockdown cells are suitable for examining the role of Bcl-X_L and Mcl-1 in HCV-infected cells.

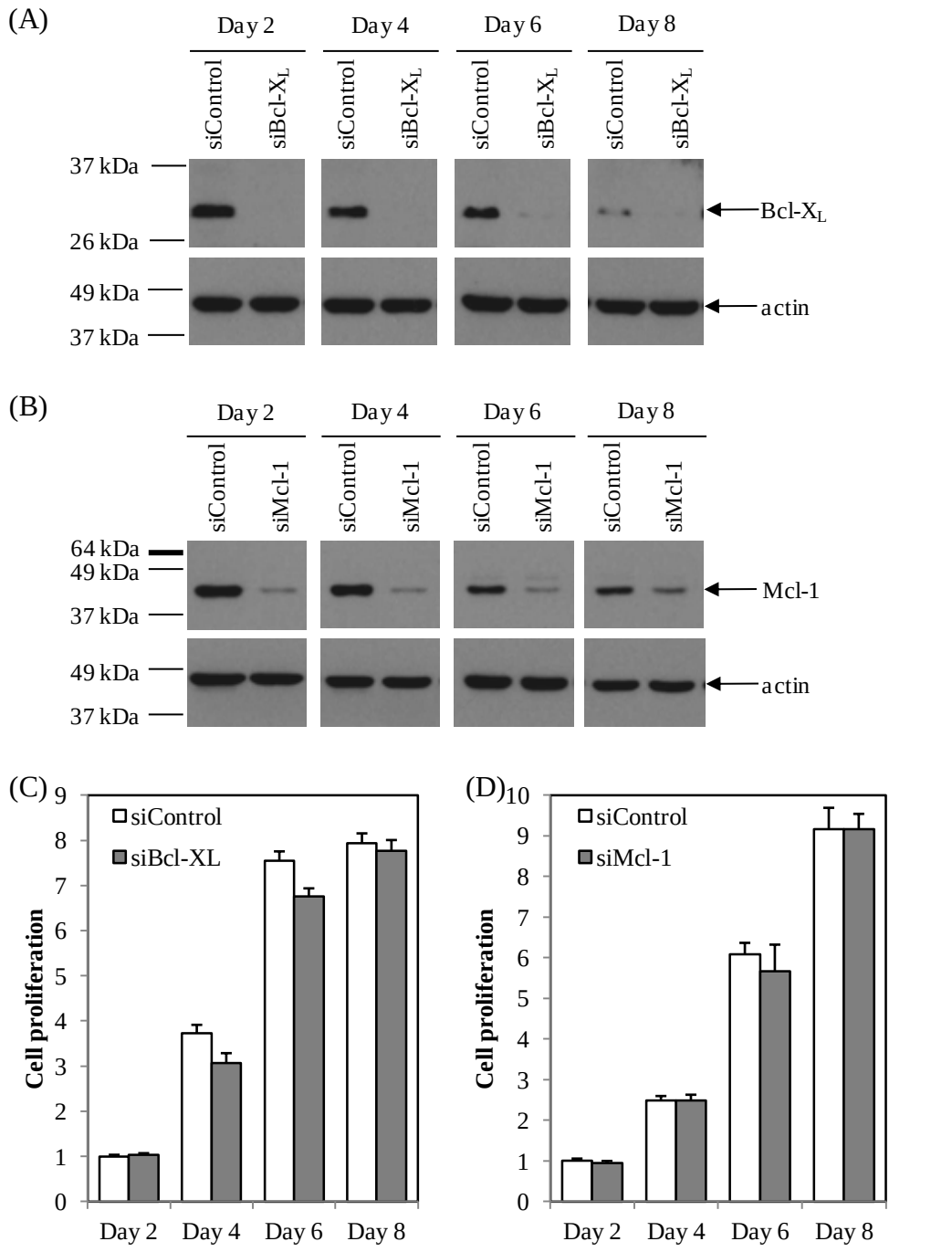

Fig. 5.3. Efficient siRNA-mediated knockdown of Bcl-X_L and Mcl-1 in Huh7.5 cells. The siRNAs directed against Bcl-X_L (siBcl-X_L) or Mcl-1 (siMcl-1) or their respective negative control siRNA (siControl) were transfected into Huh7.5 cells using Lipofectamine RNAiMAX reagent. Cells were harvested at 2, 4, 6 and 8 days post-transfection and the knockdown efficiency was examined by Western blot analysis using antibodies against (A) Bcl-X_L and (B) Mcl-1. The amounts of total cell lysates loaded were verified by measuring the level of endogenous actin. Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. Proliferation of the Bcl-X_L (C) and Mcl-1 (D) knockdown cells (filled columns) was measured using WST-1 assay at 2, 4, 6 and 8 days post-transfection. Proliferation of siControl cells (empty columns) at day 2 was arbitrarily expressed as 1.0. Data represent means ± SD of at least two independent experiments.

5.3 Role of Bcl-X_L in HCV infection of Huh7.5 cells

5.3.1 Overexpression of Bcl-X_L confers protection against HCV-induced apoptosis and inhibits virus replication and production

Cells stably overexpressing Bcl-X_L and control cells were infected with HCV and the proliferation and apoptosis status of the cells were examined up to 6 days post-infection (d.p.i.). As shown in Figure 5.4A, the Bcl-X_L stable cells exhibited more proliferation than the controls at 4 and 6 d.p.i. This increase in cell proliferation was preceded by a decrease in caspase-3 activation in the Bcl-X_L stable cells compared to the control samples at 2 and 4 d.p.i. (Figure 5.4B). Taken together, the data suggest that increased Bcl-X_L expression can protect cells against HCV-induced apoptosis, at least in the very early stages of an acute HCV infection, and this led to increased cell proliferation/viability.

The effects of Bcl-X_L overexpression on viral replication and virus production were also examined. Quantitative real-time PCR analysis showed that the HCV RNA content was significantly reduced in the Bcl-X_L stable cells compared to the controls from 2 to 6 d.p.i. (Figure 5.5A). This suggests that Bcl-X_L may regulate HCV RNA replication in a negative manner. The Bcl-X_L stable cells also produced considerably less progeny virus than the control cells in the same time frame post-infection (Figure 5.5B). The decrease in virus production is probably due to reduced viral replication in the Bcl-X_L stable cells. However, it is tempting to speculate that the inhibition of HCV-induced apoptosis by Bcl-X_L overexpression may also result in less

progeny virus since apoptosis has been postulated to be a mechanism for virus release.

To rule out the possibility of clonal variation, two different Bcl-X_L stable clones (B60 and B69) and two control clones (V4 and V11) were used in these experiments. As shown in Figures 5.4 and 5.5, B60 and B69 cells behaved in a similar manner in response to HCV infection, as did the V4 and V11 cells. This implies that the observed differences between the Bcl-X_L stable cells and the control cells were most likely due to the differential expression of Bcl-X_L in these cells.

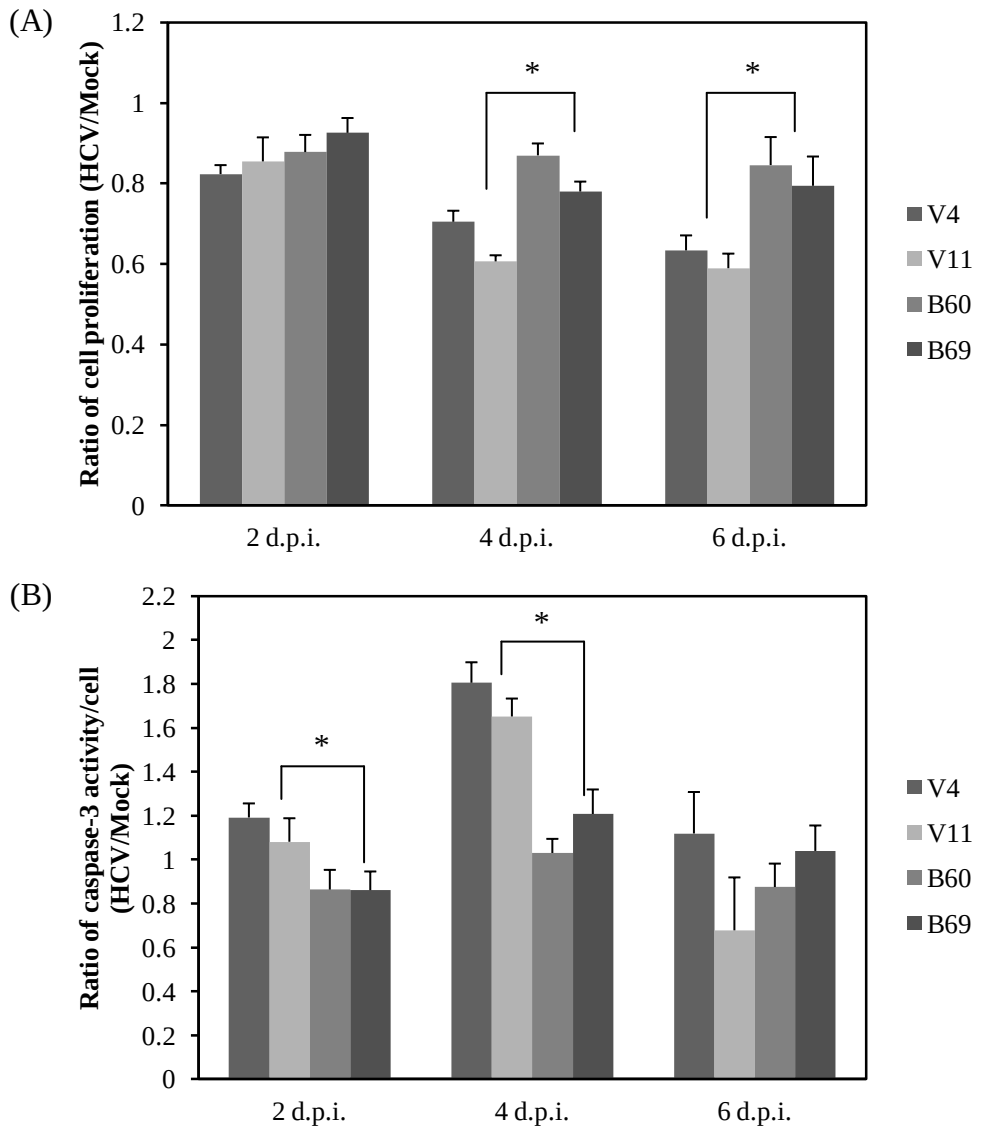

Fig. 5.4. Cell proliferation and caspase-3 activity of Bcl-X_L stable cells infected with HCV. (A) Cell viability/proliferation was measured using WST-1 assay at 0, 2, 4 and 6 days post-infection (d.p.i.). (B) A Caspase-Glo 3/7 luminogenic assay system was used to measure the activation of caspase-3 in HCV-infected and mock-treated cells. Luminescence readings were normalised to cell numbers, as determined by WST-1 assay. Proliferation/caspase-3 activity of HCV-infected cells is expressed relative to the proliferation/caspase-3 activity of mock-treated cells (HCV/Mock) at each time point. The proliferation/caspase-3 activity of V4 cells at 0 d.p.i. was arbitrarily expressed as 1.0 (data not shown). Data shown are representative of three independent experiments and statistically significant differences between Bcl-X_L stable clones (B60 and B69) and vector control cells (V4 and V11) at each time point are marked by asterisks (*P <0.05).

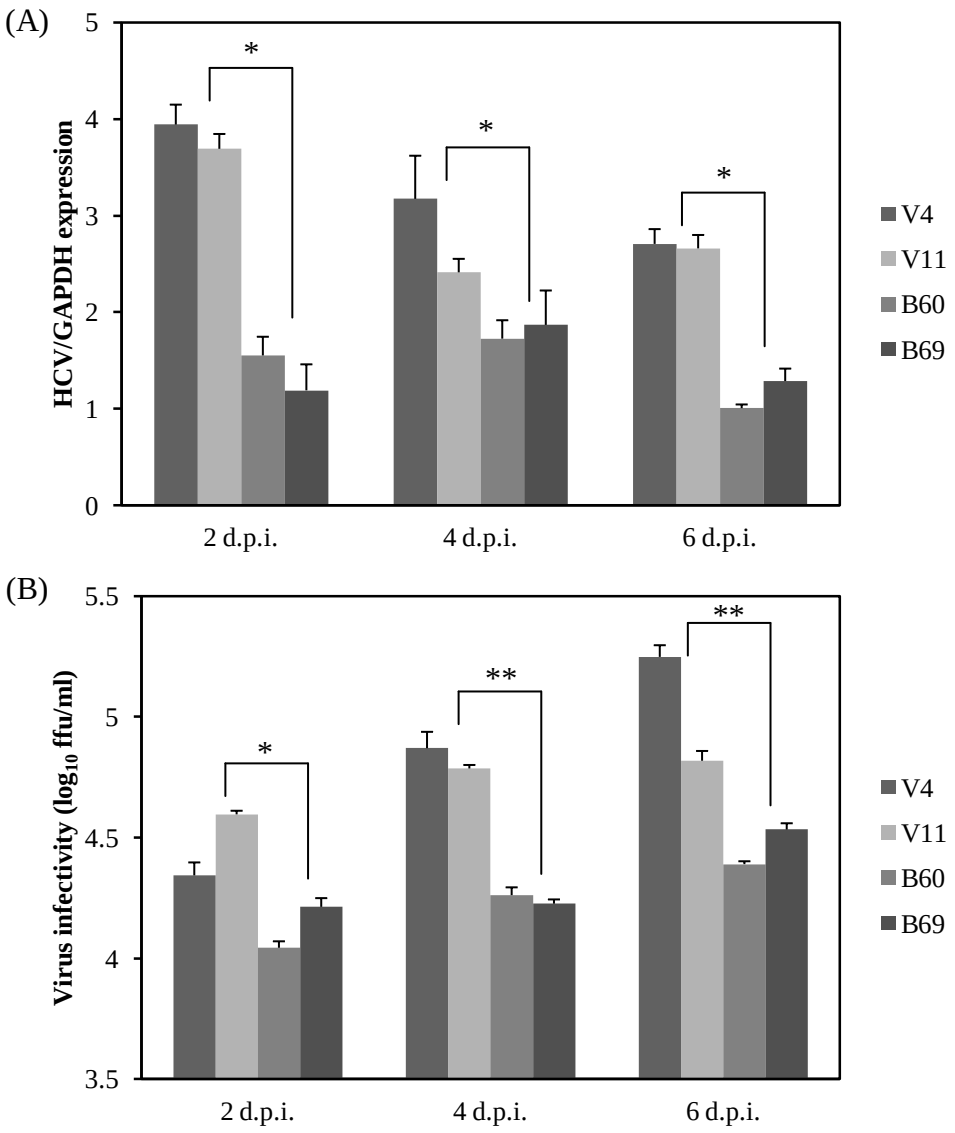

Fig. 5.5. Viral RNA replication and virus production in HCV-infected Bcl-X_L stable cells. (A) Total RNA was isolated from HCV-infected cells at 2, 4 and 6 days post-infection (d.p.i.) and reverse transcribed. HCV RNA replication levels were determined by quantitative real-time PCR analysis and normalised to endogenous GAPDH levels. (B) Culture supernatants of HCV-infected cells were collected at 2, 4 and 6 d.p.i. and used to inoculate naive Huh7.5 cells. Virus infectivity was determined at 24 h post-infection by indirect immunofluorescence analysis to detect HCV antigens using HCV-infected patient's serum and FITC-conjugated goat anti-human IgG. Data shown are representative of three independent experiments and statistically significant differences between Bcl-X_L stable clones (B60 and B69) and vector control cells (V4 and V11) at each time point are marked by asterisks (**P <0.01).

5.3.2 Depletion of Bcl-X_L sensitizes cells to HCV-induced apoptosis and modulates virus replication and production

The expression of Bcl-X_L in siBcl-X_L-treated cells was monitored by Western blot analysis up to 6 d.p.i. (Figure 5.6A). Consistent with our findings in Chapter 4.4, Bcl-X_L expression was increased in HCV-infected cells compared to mock-treated cells. This was true for both siBcl-X_L and siControl-treated cells and was apparent at 4 and 6 d.p.i. Despite the HCV-induced upregulation of Bcl-X_L, the level of Bcl-X_L in the knockdown cells was still considerably lower than that in the control cells. Hence, RNA interference can effectively suppress the HCV-induced upregulation of endogenous Bcl-X_L. Following HCV infection, the proliferation of the Bcl-X_L knockdown cells was significantly lower than the control cells from 2 to 6 d.p.i. (Figure 5.6B). A corresponding increase in apoptosis induction was also observed in the Bcl-X_L knockdown cells (Figure 5.6C). In agreement with the findings of the Bcl-X_L overexpression experiments, Bcl-X_L appears to play a protective role against apoptosis in HCV-infected cells and thus, HCV-induced apoptosis was enhanced in the absence of Bcl-X_L.

Depletion of Bcl-X_L reduced HCV RNA content slightly in Bcl-X_L knockdown cells compared to the control (Figure 5.7A). This inhibition of viral replication might be related to the high levels of apoptosis observed in Bcl-X_L knockdown cells (Figure 5.6C). Apoptosis is known to induce changes in cellular membranes and this possibly affected HCV RNA replication which is mainly localized to membranous webs in infected cells (Elazar et al., 2004). An increase in progeny virus production was observed

at 2 d.p.i. followed by slightly reduced virus production at 4 and 6 d.p.i. in Bcl-X_L knockdown cells compared to control cells (Figure 5.7B). This is consistent with our earlier observation that the enhanced apoptotic activity of the J6/JFH-1(V119L) mutant virus is correlated with an increase in infectious progeny HCV release (see Chapter 3). As viral-induced apoptosis of the host cell may function to facilitate viral egress (Blaho, 2004), we postulate that the initial spike in virus production was due to enhanced apoptosis-dependent virus release. Subsequently, the amount of virus released by the Bcl-X_L knockdown sample was probably limited by the number of viable cells left and the reduced level of HCV RNA replication in these cells.

To address the difference in viable cell numbers in the Bcl-X_L knockdown and control samples, the amount of virus produced per cell was determined by normalising the total virus infectivity titres to cell proliferation levels. As shown in Figure 5.7C, each Bcl-X_L knockdown cell produced more progeny virus than the control sample at all time points post-infection, albeit to a smaller degree at 4 and 6 d.p.i. than at 2 d.p.i. These results show that the slight decrease in total virus production observed previously at 4 and 6 d.p.i. was indeed due to the smaller number of cells left in the Bcl-X_L knockdown sample following extensive apoptosis (Figure 5.7B). However despite the possibility of enhanced apoptosis-dependent virus release, the Bcl-X_L knockdown sample only showed a small increase in virus infectivity per cell at 4 and 6 d.p.i., indicating that viral replication is probably impaired in these cells. This correlates with the lower level of viral RNA replication observed in the Bcl-X_L knockdown cells (Figure 5.7A).

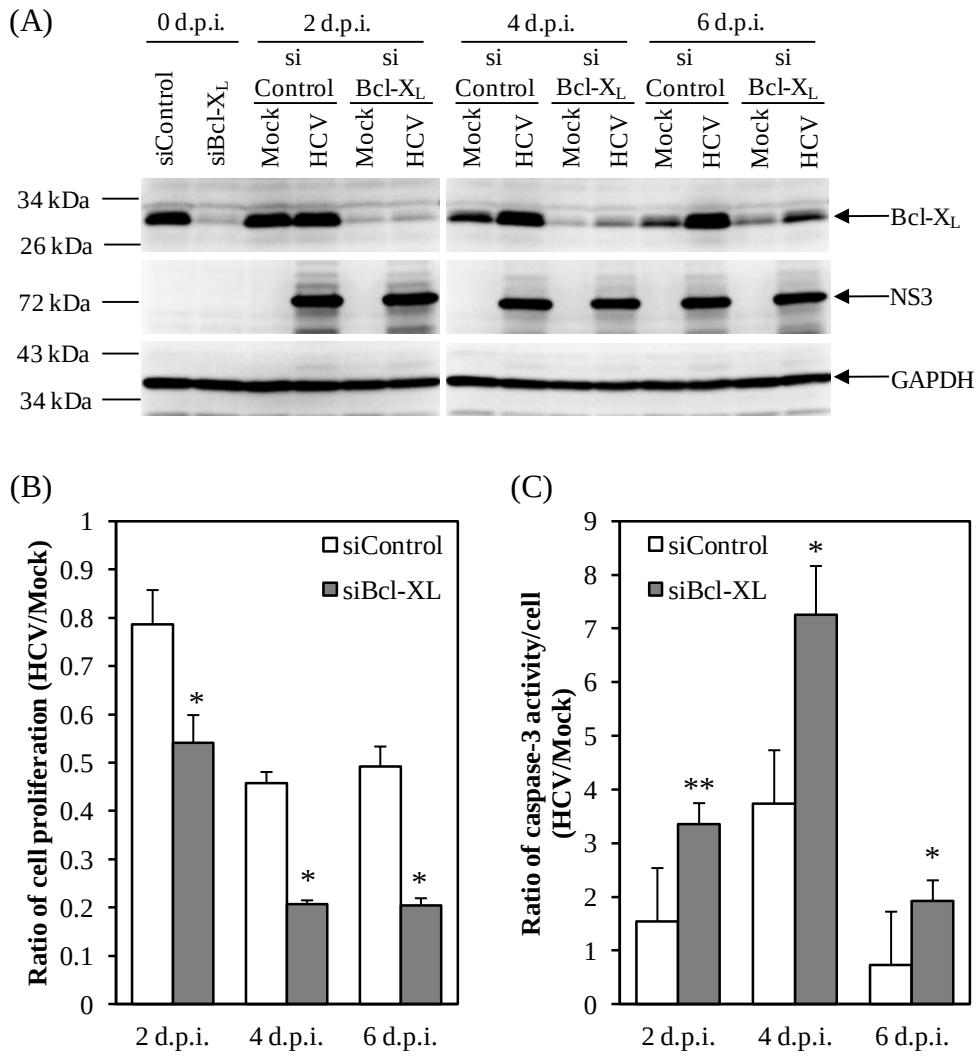

Fig. 5.6. Protein expression, cell proliferation and caspase-3 activation in Bcl-X_L knockdown cells infected with HCV. (A) Whole cell lysates were prepared at 0, 2, 4 and 6 days post-infection (d.p.i.) and subjected to Western blot analysis using antibodies against Bcl-X_L and NS3. The amounts of total cell lysates loaded were verified by measuring the level of endogenous GAPDH. Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. (B) Cell viability/proliferation of siBcl-X_L-treated (filled columns) and siControl-treated (empty columns) cells was measured using WST-1 assay at 0, 2, 4 and 6 d.p.i. (C) A Caspase-Glo 3/7 luminogenic assay system was used to measure the activation of caspase-3 in HCV-infected and mock-treated cells. Luminescence readings were normalised to cell numbers, as determined by WST-1 assay. Proliferation/caspase-3 activity of HCV-infected cells is expressed relative to the proliferation/caspase-3 activity of mock-treated cells (HCV/Mock) at each time point. The proliferation/caspase-3 activity of siControl-treated cells at 0 d.p.i. was arbitrarily expressed as 1.0 (data not shown). Data shown are representative of three independent experiments and statistically significant differences between siBcl-X_L-treated and siControl-treated cells at each time point are marked by asterisks (*P <0.05 and **P <0.01).

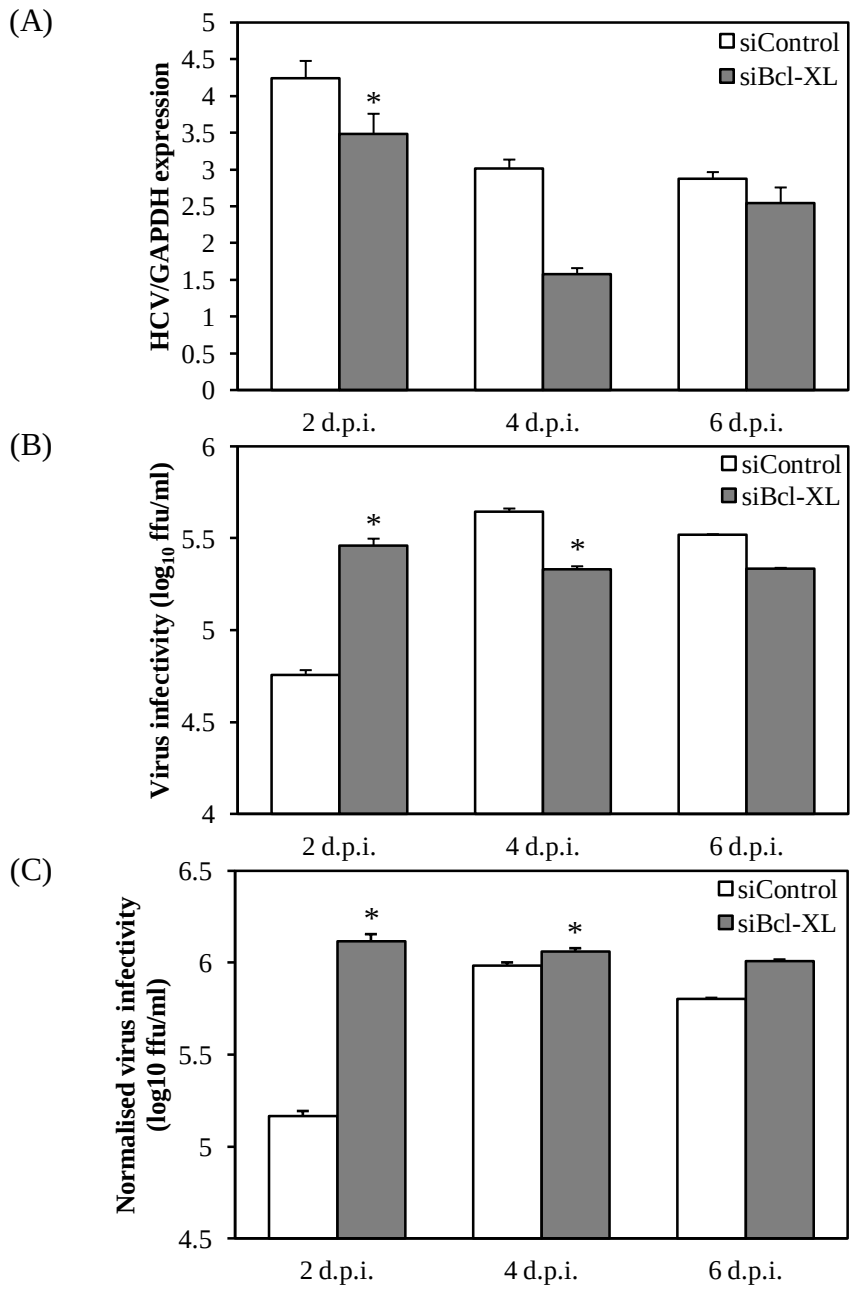

Fig. 5.7. Viral RNA replication and virus production in HCV-infected Bcl-X_L knockdown cells. (A) Total RNA was isolated from HCV-infected siBcl-X_L-treated (filled columns) and siControl-treated (empty columns) cells at 2, 4 and 6 days post-infection (d.p.i.) and reverse transcribed. HCV RNA replication levels were determined by quantitative real-time PCR analysis and normalised to endogenous GAPDH levels. (B) Culture supernatants of HCV-infected cells were collected at 2, 4 and 6 d.p.i. and used to inoculate naive Huh7.5 cells. Virus infectivity was determined at 24 h post-infection by indirect immunofluorescence analysis to detect HCV antigens using HCV-infected patient's serum and FITC-conjugated goat anti-human IgG. (C) The amount of virus produced per cell was determined by normalising the virus infectivity titres of the culture supernatants to cell proliferation levels as determined by WST-1 assay. Data shown are representative of three independent experiments and statistically significant differences between siBcl-X_L-treated and siControl-treated cells are marked by asterisks (*P <0.05).

5.4 Role of Mcl-1 in HCV infection of Huh7.5 cells

5.4.1 Effects of Mcl-1 overexpression on HCV-induced apoptosis, HCV RNA replication and virus production

Cells overexpressing Mcl-1 (M34) exhibited more proliferation than control cells (V4) up to 6 days after HCV infection (Figure 5.8A). This observation correlated with a decrease in caspase-3 activation in M34 cells compared to V4 cells at 2 and 4 d.p.i. (Figure 5.8B). These data show that Mcl-1, like Bcl-X_L, can confer protection against HCV-induced apoptosis during the early stages of infection. The HCV RNA content was slightly lower in M34 cells than in V4 cells, suggesting that high Mcl-1 levels may to some extent inhibit viral RNA replication (Figure 5.8C). A small decrease in the production of progeny virus was also observed in the M34 cells compared to the V4 cells, probably as a result of decreased viral RNA replication in the Mcl-1 stable cells (Figure 5.8D). Unlike the Bcl-X_L stable cells, only one suitable Mcl-1 stable clone was obtained in this study. Thus, it is not possible to ascertain if the effects observed here are clone-specific.

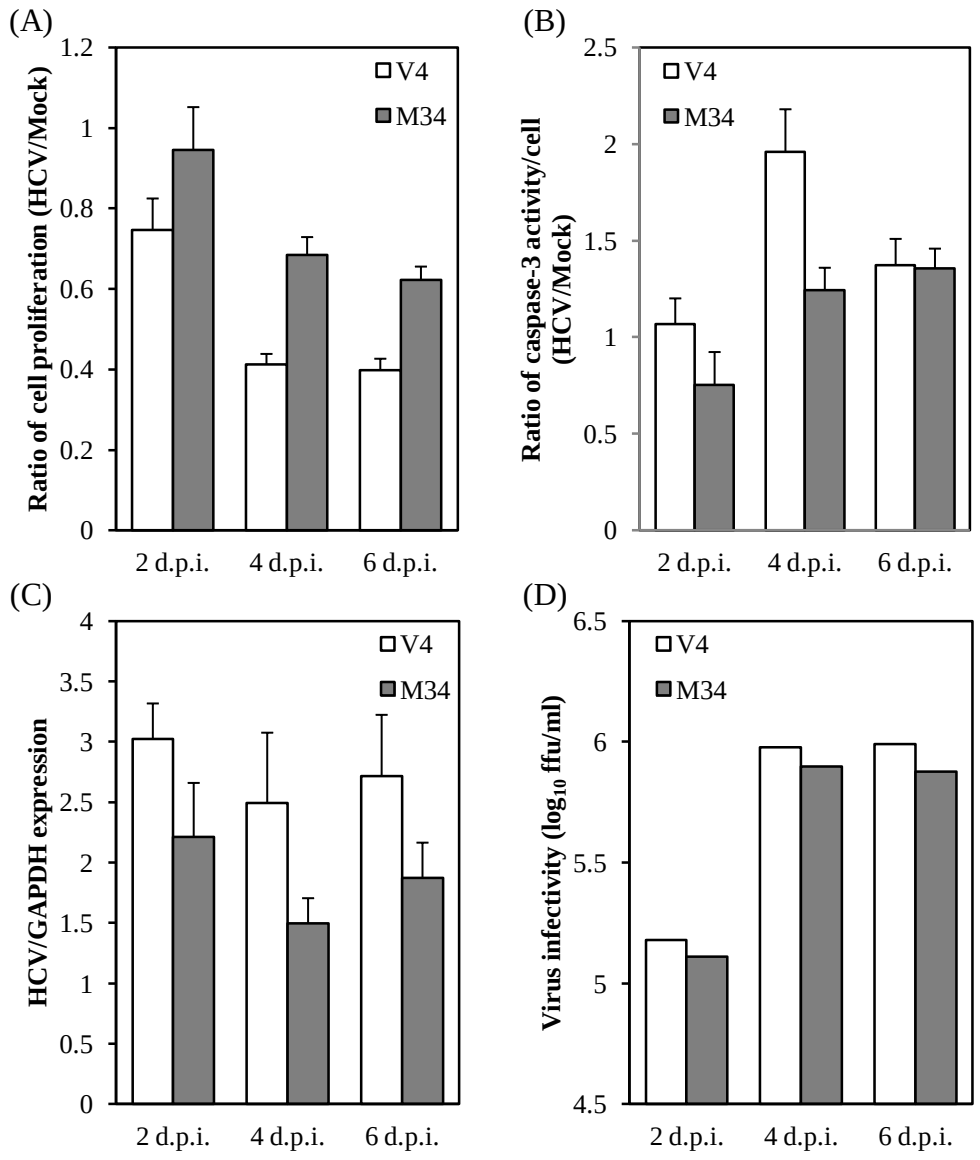

Fig. 5.8. Cell proliferation, caspase-3 activation, viral RNA replication and virus production in Mcl-1 stable cells infected with HCV. (A) Cell viability/proliferation of Mcl-1 stable (M34; filled columns) and vector control (V4; empty columns) cells was measured using WST-1 assay at 0, 2, 4 and 6 days post-infection (d.p.i.). (B) A Caspase-Glo 3/7 luminogenic assay system was used to measure the activation of caspase-3 in HCV-infected and mock-treated M34 and V4 cells. Luminescence readings were normalised to cell numbers, as determined by WST-1 assay. Proliferation/caspase-3 activity of HCV-infected cells is expressed relative to the proliferation/caspase-3 activity of mock-treated cells (HCV/Mock) at each time point. The proliferation/caspase-3 activity of V4 cells at 0 d.p.i. was arbitrarily expressed as 1.0 (data not shown). (C) Total RNA was isolated from HCV-infected M34 and V4 cells at 2, 4 and 6 d.p.i. and reverse transcribed. HCV RNA replication levels were determined by quantitative real-time PCR analysis and normalised to endogenous GAPDH levels. (D) Culture supernatants of HCV-infected cells were collected at 2, 4 and 6 d.p.i. and used to inoculate naive Huh7.5 cells. Virus infectivity was determined at 24 h post-infection by indirect immunofluorescence analysis to detect HCV antigens using HCV-infected patient's serum and FITC-conjugated goat anti-human IgG.

5.4.2 Depletion of Mcl-1 has no significant impact on HCV-induced apoptosis, HCV RNA replication and virus production

Similar to the observation previously made in wild-type Huh7.5 cells, HCV infection did not alter the expression of Mcl-1 in siControl-treated cells (Figure 5.9A). The knockdown of Mcl-1 expression had no effect on cell proliferation as well as apoptosis induction in HCV-infected samples (Figures 5.9B and 5.9C). This is rather surprising as we previously found that Mcl-1 overexpression protected HCV-infected cells against apoptosis, suggesting a role for it in HCV-induced apoptosis. A possible explanation for this disparity is that while Mcl-1 can contribute to the inhibition of HCV-induced apoptosis, other host factors with similar functions may also be involved and they may be able to compensate for the loss of Mcl-1 prosurvival activity in the siMcl-1-treated cells. As shown in Figure 5.10A, depletion of Mcl-1 did not affect viral RNA replication in the infected cells. Similarly, the production of progeny virus in Mcl-1 knockdown and control cells did not vary significantly (Figure 5.10B). Taken together, the findings of Mcl-1 overexpression and depletion on HCV infection have shown that aside from its ability to confer protection against HCV-induced apoptosis, Mcl-1 does not appear to play a major role in the early acute phase of HCV infection.

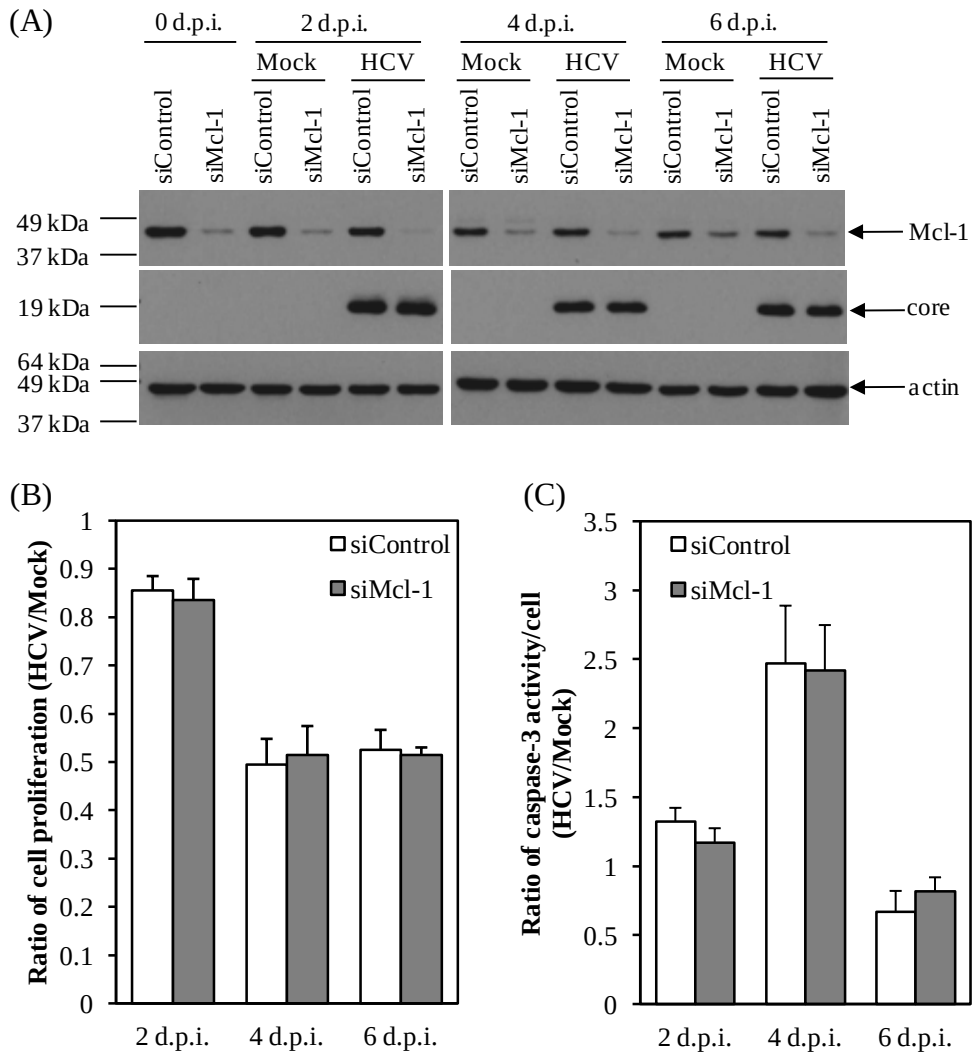

Fig. 5.9. Protein expression, cell proliferation and caspase-3 activity of Mcl-1 knockdown cells infected with HCV. (A) Whole cell lysates were prepared at 0, 2, 4 and 6 days post-infection (d.p.i.) and subjected to Western blot analysis using antibodies against Mcl-1 and core. The amounts of total cell lysates loaded were verified by measuring the level of endogenous actin. Specific bands are indicated by arrows on the right and molecular masses of markers are indicated on the left. (B) Cell viability/proliferation of siMcl-1-treated (filled columns) and siControl-treated (empty columns) cells was measured using WST-1 assay at 0, 2, 4 and 6 d.p.i. (C) A Caspase-Glo 3/7 luminogenic assay system was used to measure the activation of caspase-3 in HCV-infected and mock-treated cells. Luminescence readings were normalised to cell numbers, as determined by WST-1 assay. Proliferation/caspase-3 activity of HCV-infected cells is expressed relative to the proliferation/caspase-3 activity of mock-treated cells (HCV/Mock) at each time point. The proliferation/caspase-3 activity of siControl-treated cells at 0 d.p.i. was arbitrarily expressed as 1.0 (data not shown).

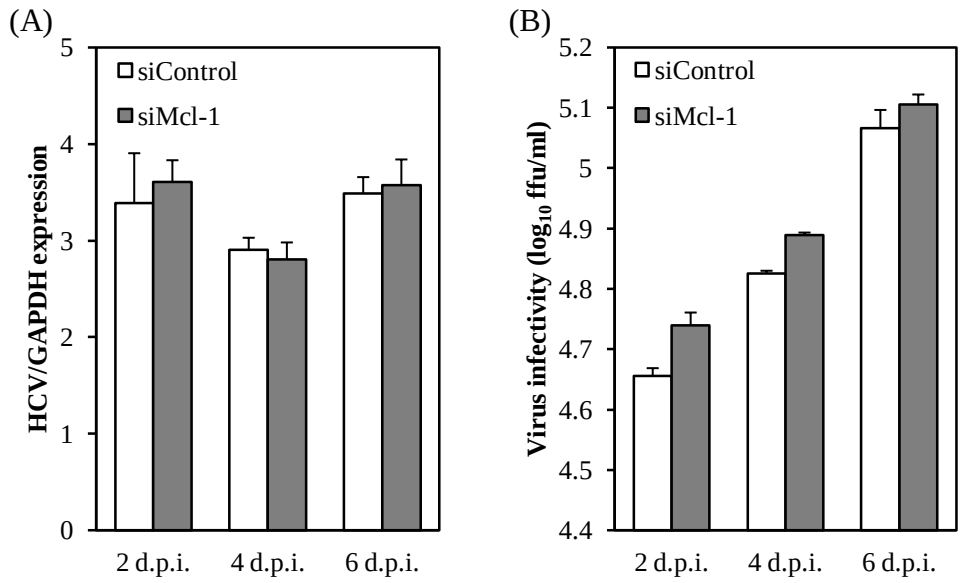

Fig. 5.10. Viral RNA replication and virus production in HCV-infected Mcl-1 knockdown cells. (A) Total RNA was isolated from HCV-infected siMcl-1-treated (filled columns) and siControl-treated (empty columns) cells at 2, 4 and 6 days post-infection (d.p.i.) and reverse transcribed. HCV RNA replication levels were determined by quantitative real-time PCR analysis and normalised to endogenous GAPDH levels. (B) Culture supernatants of HCV-infected cells were collected at 2, 4 and 6 d.p.i. and used to inoculate naive Huh7.5 cells. Virus infectivity was determined at 24 h post-infection by indirect immunofluorescence analysis to detect HCV antigens using HCV-infected patient's serum and FITC-conjugated goat anti-human IgG.

5.5 Discussion

In Chapter 4, we demonstrated that HCV induces the upregulation of Bcl-X_L in infected Huh7.5 cells. Prosurvival factor Bcl-X_L is constitutively expressed in the liver and acts as a critical apoptosis antagonist in hepatocytes (Takehara et al., 2004). It has been postulated that apoptosis inhibition may be beneficial for viruses as premature host cell death prevents completion of the viral replication cycle. Hence, many viruses have evolved anti-apoptotic strategies such as the expression of viral homologues of Bcl-2 in order to counteract altruistic host cell suicide, prolong the survival of infected cells and maximise progeny virus production [see reviews by (Cuconati and White, 2002; Hardwick and Bellows, 2003; Polster et al., 2004; White, 2006)]. On the other hand, other studies have shown that suppression of virus-induced apoptosis through the overexpression of host prosurvival proteins such as Bcl-2 and Bcl-X_L may inhibit the propagation of several other viruses via the modulation of NF-κB activity, viral glycosylation or viral release (Carthy et al., 2003; Marshall et al., 1999; Olsen et al., 1996; Scallan et al., 1997).

In the present study, we investigated the role of HCV-induced Bcl-X_L upregulation in infected cells. Using stable overexpression and gene silencing systems, we demonstrated that Bcl-X_L can modulate HCV-induced apoptosis, viral RNA replication and progeny virus production. Consistent with its protective role against apoptosis, high levels of Bcl-X_L expression inhibited HCV-induced apoptosis in infected stable cells (Figure 5.4B). Interestingly, a decrease in viral RNA replication was also observed in the infected Bcl-X_L stable cells (Figure 5.5A). In addition to apoptosis inhibition, Bcl-X_L has been

reported to inhibit autophagy through direct interaction with Beclin-1, a BH3-only protein and essential mediator of autophagy (Maiuri et al., 2007a; Maiuri et al., 2007b). Autophagy has been observed in HCV-infected hepatocytes and components of the autophagy machinery have been shown to be essential for initiating HCV replication (Dreux et al., 2009; Ke and Chen, 2011; Sir et al., 2008). Therefore, enhanced Bcl-X_L expression in the stable cells possibly impaired viral replication through the inhibition of Beclin-1-mediated autophagy. In addition, the Bcl-X_L stable cells also produced less infectious progeny virus compared to the control (Figures 5.5B). Reduced apoptosis-dependent virus release and impaired viral replication in the Bcl-X_L stable cells probably contributed to this decrease in virus production. The two independent Bcl-X_L stable clones (B60 and B69) that were examined in this study gave similar results, implying that the observations were directly due to the higher Bcl-X_L levels in these cells.

In agreement with the inhibition of apoptosis by Bcl-X_L overexpression, depletion of Bcl-X_L enhanced apoptosis induction in HCV-infected cells (Figure 5.6C). Collectively, these data provide further evidence that HCV-induced apoptosis *in vitro* is largely mediated via the mitochondrial apoptosis pathway, which can be antagonized or potentiated by the overexpression or depletion of certain prosurvival Bcl-2 members respectively (Wong and Puthalakath, 2008). Enhanced apoptosis in the HCV-infected Bcl-X_L knockdown cells affected both viral RNA replication and subsequent progeny virus production. Extensive cellular changes during apoptosis probably affected the HCV replication sites, thus resulting in decreased viral

RNA content in the Bcl-X_L knockdown cells (Figure 5.7A). In addition, high apoptosis levels promoted progeny virus release initially but also reduced the number of viable cells substantially (Figures 5.6B, 5.6C and 5.7B). The latter probably accounts for the decrease in total virus production observed at 4 and 6 d.p.i., even as the apoptosis level remained elevated (Figure 5.6C and 5.7B). In agreement with this, when cell numbers in the culture were taken into account, each Bcl-X_L knockdown cell was found to produce more virus than the control at all time points post-infection, although virus production at the later time points appeared to be limited by other factors such as viral RNA replication (Figure 5.7C).

Taken together, our findings demonstrate that high levels of Bcl-X_L expression in HCV-infected cells are unfavourable for virus propagation and thus, the upregulation of endogenous Bcl-X_L in infected cells may constitute an "antiviral" response. In this connection, Bcl-X_L upregulation may also serve to protect host cells against extensive HCV-induced apoptosis. On the other hand, HCV-induced upregulation of Bcl-X_L in infected cells may be beneficial for the virus. We postulate that Bcl-X_L upregulation may function to moderate the direct cytopathic effects of HCV in infected cells by blocking the intrinsic apoptosis pathway with the aim of maximising progeny virus production. Our data suggest that Bcl-X_L may also be involved in regulating HCV RNA replication (Figure 5.5A). This presents a possible additional pathway for modulating HCV-induced apoptosis as viral replication is closely associated with apoptosis induction (Zhu et al., 2007). Furthermore, prosurvival Bcl-2 members, namely Bcl-2 and Bcl-X_L, have been shown to

confer protection against Fas-mediated hepatic apoptosis (de la Coste et al., 1999; Lacronique et al., 1996). Thus, during an *in vivo* infection, the elevated Bcl-X_L levels may serve to protect HCV-infected cells against death receptor-mediated killing by cytotoxic lymphocytes, leading to evasion of host immune responses and viral persistence.

Interestingly, Bcl-X_L is frequently overexpressed and confers resistance to apoptosis in hepatocellular carcinoma (Takehara et al., 2001; Watanabe et al., 2002; Zhao et al., 2011). Although the link between HCV-induced Bcl-X_L upregulation and HCV-related hepatocellular carcinoma (HCC) is still unclear, Bcl-X_L expression appears to correlate with the presence of HCV in tumour cells (Zekri et al., 2011). Transcriptional regulation of Bcl-X_L expression has been reported to be mediated by members of the STAT, Rel/NF-κB, Ets and AP-1 transcription factor families [reviewed by (Grad et al., 2000; Sevilla et al., 2001)]. Modulation of these pathways by HCV might contribute to Bcl-X_L upregulation since we previously showed that Bcl-X_L was regulated at the transcriptional level during HCV infection (Figure 4.5). In relation to this, HCV has previously been shown to interfere with the Rel/NF-κB and Jak-STAT pathways (Blindenbacher et al., 2003; Heim et al., 1999; Tai et al., 2000). Of particular interest is the modulation of the STAT3 signalling pathway by HCV as it has been implicated in both the core protein-mediated and NS5A-mediated upregulation of Bcl-X_L expression (Sarcar et al., 2004; Yoshida et al., 2002).

In addition to Bcl-X_L, prosurvival Mcl-1 is also expressed in the liver and protects differentiated hepatocytes against apoptosis (Hikita et al., 2009).

As shown in Chapter 4, unlike Bcl-X_L, the expression of Mcl-1 remained unchanged in HCV-infected cells, indicating a lack of role for Mcl-1 in HCV infection. However, the overexpression of Mcl-1 conferred protection against HCV-induced apoptosis and slightly reduced viral RNA replication and progeny virus production, which is similar to the observations made with Bcl-X_L stable cells although to a smaller extent (Figure 5.8). Unfortunately, the effects of Mcl-1 overexpression were examined using only one Mcl-1 stable clone, thus the possibility of clone-specific effects cannot be ruled out. The lack of any observable effects of Mcl-1 silencing on host cell apoptosis, viral RNA replication and progeny virus production further suggests that the Mcl-1 overexpression results may be unreliable (Figures 5.9 and 5.10). Overall, the data suggest that, compared to Bcl-X_L, Mcl-1 does not play a major role in our HCV genotype 2a infection system. This correlates with the findings discussed in Chapter 3 which show that endogenous Mcl-1 does not interact with the BH3 domain of the HCV genotype 2a core protein and thus, does not contribute to core protein-induced apoptosis.

This study highlights the importance of viral-induced apoptosis in acute HCV infection. When host cell apoptosis is suppressed, viral propagation is impaired and thus, HCV circumvents this problem by directly inducing apoptosis in the infected cells (Deng et al., 2008; Kannan et al., 2011; Mateu et al., 2008; Walters et al., 2009). However, rapid and excessive host cell death is also unfavourable for the virus as it results in insufficient time and opportunities for the virus to complete its replication cycle. In accordance with this, a recent study on the hepatitis B virus (HBV)

demonstrated an inverse relationship between the degree of host cell apoptosis and the release of infectious virus particles (Arzberger et al., 2010). Therefore, a fine balance between cell survival and apoptosis and/or the timing of apoptosis induction is critical for HCV infection.

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS

Apoptosis of hepatocytes is a prominent feature of viral hepatitis C and is thought to be a major contributing factor towards HCV-induced liver injury. Induction of apoptosis in virus-infected cells may be a host response to eliminate the virus or a viral strategy to promote virus dissemination. Evaluation of liver tissue samples from patients chronically infected with HCV has thus far provided most of the *in vivo* evidence for apoptosis in HCV infection (Fischer et al., 2007). However, due to the lack of adequate animal models and the limited availability of biopsy material, the functional role of apoptosis during HCV infection and the molecular mechanisms underlying apoptosis induction remain unclear.

Using overexpression systems, there have been numerous attempts to define the potential roles of individual HCV proteins in regulating host cell apoptosis. However, conflicting apoptotic properties have been described for most of the viral proteins, probably due to differences in the experimental conditions, HCV genotypes, cell types and apoptotic stimuli used [reviewed by (Fischer et al., 2007)]. Moreover, the overexpression of proteins in these studies is incongruent with the very low levels of viral proteins usually expressed in HCV-infected patients. Taking these into consideration, interpretation of the currently available data in relation to possible events occurring in a natural infection is restricted.

Recently, *in vitro* infection studies using the JFH-1-based cell culture system have demonstrated HCV-induced apoptosis of infected hepatoma cells (Deng et al., 2008; Mateu et al., 2008). Subsequently, this infection system

has been used to perform genome-wide analysis of changes in host gene expression following HCV infection (Blackham et al., 2010; Liu et al., 2010; Walters et al., 2009). These studies provide a general overview as to which cellular pathways are likely to be modulated during acute HCV infection and among others, differential expression of apoptosis-related mRNAs and miRNAs was observed in HCV-infected cells. However, specific viral-host interactions that are involved in regulating apoptosis have not yet been clearly defined.

There is increasing evidence to suggest that hepatocyte apoptosis is an important mechanism in HCV-induced liver disease [reviewed by (Mengshol et al., 2007)]. Therefore, apoptotic signalling pathways may provide new targets for the treatment of hepatitis C. In relation to this, a clinical study demonstrated that the administration of ursodeoxycholic acid, an antiapoptotic agent, improved the response rate to IFN-α therapy in patients chronically infected with HCV who were initially unresponsive to IFN-α treatment alone (Fabbri et al., 2000). Further characterization of the molecular mechanisms governing HCV-associated apoptosis may lead to the identification of specific targets for potential antiviral therapy and open up challenging possibilities for the development of novel pharmacological strategies to eradicate HCV infection.

The research efforts described in this thesis attempt to gain a better understanding of the viral-host interactions that contribute to HCV-associated apoptosis. Our study focused on the identification and characterization of interactions between apoptosis-related host proteins and viral factors using

both cell culture overexpression and HCVcc systems. The findings of our study are summarized below:

a) It was demonstrated that the HCV genotype 1b core protein contains a functional BH3 domain that is essential for the induction of apoptosis in core protein-expressing Huh7 cells.

b) Coimmunoprecipitation experiments showed that the BH3 domain of the genotype 1b core protein mediates specific interaction with human Mcl-1.

c) Genotype 1b core protein-induced apoptosis was demonstrated to be dependent on the expression of proapoptotic Bax and inhibited by the overexpression of prosurvival Mcl-1 or Bcl-X_L. This provides further evidence that the genotype 1b core protein acts via the intrinsic apoptosis pathway.

d) Similar to the complementation between BH3-only proteins Noxa and Bad, the genotype 1b core protein was shown to functionally complement Bad, resulting in enhanced mitochondrial cytochrome c release and apoptosis.

e) Site-directed mutagenesis analysis showed that the three hydrophobic residues in the BH3 domain of the genotype 1b core protein are essential for apoptosis induction.

f) The ability of the genotype 1b core protein to induce more apoptosis than the genotype 2a core protein was shown to be due to an amino acid difference in one of these hydrophobic residues (residue 119).

g) It was demonstrated that a single amino acid substitution from V to L at residue 119 in the core protein of the HCV J6/JFH-1 strain (genotype 2a) correlates with its ability to interact with endogenous Mcl-1 and to induce higher levels of apoptosis in infected cells. This was also accompanied by enhanced progeny virus release.

h) Using the HCVcc system, HCV J6/JFH-1-P47 strain was shown to efficiently induce apoptosis in Huh7.5 cells during acute infection.

i) Gene expression analysis of apoptosis-related genes revealed that HCV J6/JFH-1-P47 infection of Huh7.5 cells induces upregulation of BIK, FAS, GADD45A and HRK and downregulation of BNIP3.

j) The expression levels of Bcl-X_L mRNA and protein were shown to be elevated in HCV J6/JFH-1-P47-infected Huh7.5 cells.

k) When the level of Bcl-X_L in infected cells was further increased by stable transfection, there was a reduction in HCV J6/JFH-1-P47-induced apoptosis, viral RNA replication and progeny virus release.

l) When the upregulation of Bcl-X_L in infected cells was prevented by RNA interference, HCV-J6/JFH-1-P47-infected cells displayed higher levels of apoptosis and an initial increase in progeny virus release.

m) However, enhanced apoptosis also resulted in a reduction in the number of viable cells and lower viral RNA replication was observed.

n) In contrast to Bcl-X_L, the expression of Mcl-1 was not affected by HCV J6/JFH-1-P47 infection and the knockdown of Mcl-1 had minimal effects on virus-induced apoptosis and viral replication.

In this study, we have demonstrated that the genotype 1b core protein is a novel BH3-only viral homologue that contributes to the induction of apoptosis during HCV infection by mimicking Noxa and interfering with the prosurvival function of Mcl-1 (Mohd-Ismail et al., 2009). These findings present one of the mechanisms possibly employed by the virus to regulate apoptosis in infected cells. Comparison of the BH3 domains of the genotype 1b and 2a core proteins revealed genotypic differences in core protein-induced apoptosis. Consequently, modification of the J6/JFH-1 genotype 2a virus through a substitution at residue 119 of the core protein produced a mutant virus [J6/JFH-1(V119L)] that induced higher levels of apoptosis and showed enhanced release of progeny virus from infected cells, indicating that apoptosis can be advantageous for viral spreading at the late stages of infection.

Despite lacking a functional BH3 domain in the core protein, the J6/JFH-1 genotype 2a virus still induced considerable apoptosis, thus indicating the presence of alternative pathways of HCV-induced apoptosis. Using gene expression analysis, we have identified five host apoptosis-related genes (BIK, BNIP3, FAS, GADD45A and HRK) that may be involved in these pathways. Although HRK mRNA level was found to be highly upregulated by HCV, this upregulation was not observed at the protein level, suggesting that HRK is unlikely to be important in HCV infection. While the

mRNA levels of the other four genes, which are involved in both the intrinsic and extrinsic apoptosis pathways, are strongly up- or down-regulated by HCV, the corresponding changes at protein level have to be confirmed in further studies. Then, further investigations are needed to determine their role in HCV-induced apoptosis and the mechanism of their regulation by viral proteins, as well as the specific apoptotic pathways possibly modulated by HCV.

In addition, we observed that HCV infection also significantly upregulates Bcl-X_L expression on the mRNA and protein levels in a time-dependent manner. The change in Bcl-X_L expression during HCV infection seems to keep the cells in a finely balanced state as HCV-induced apoptosis is consistently antagonized or potentiated by the overexpression or depletion of Bcl-X_L. Consistent with the J6/JFH-1(V119L) virus described above, there is a direct correlation between the level of apoptosis and release of progeny virus from infected cells. However, the reduction in viable cell number and cellular changes that are caused by premature apoptosis negatively impacted viral replication and total virus production, which suggests that HCV-induced upregulation of Bcl-X_L in infected cells may be beneficial for the virus.

Future studies are required to identify the viral factor(s) that contribute to HCV-induced Bcl-X_L upregulation, the molecular mechanisms of Bcl-X_L-mediated regulation of HCV infection and the interplay with other host factors such as the apoptosis-related genes that we have identified to be transcriptionally regulated by HCV. It will also be interesting to determine if HCV-induced Bcl-X_L upregulation is conserved across different HCV

genotypes and if it possibly contributes to hepatocarcinogenesis since Bcl-X_L has been found to be frequently overexpressed in HCC. Lastly, our findings provide interesting insights into HCV subversion of cellular apoptotic pathways and suggest that a fine balance between cell survival and apoptosis has to be achieved during acute infection. Further understanding of this phenomenon, particularly in relation to chronic infection and clinical observations, may potentially lead to the discovery of novel antiviral targets.

REFERENCES

Acosta-Rivero, N., Rodriguez, A., Mussachio, A., Poutou, J., Falcon, V., Torres, D., Aguilar, J.C., Linares, M., Alonso, M., Perez, A., *et al.* (2005). A C-terminal truncated hepatitis C virus core protein variant assembles in vitro into virus-like particles in the absence of structured nucleic acids. Biochem Biophys Res Commun *334*, 901-906.

Acton, S.L., Scherer, P.E., Lodish, H.F., and Krieger, M. (1994). Expression cloning of SR-BI, a CD36-related class B scavenger receptor. J Biol Chem *269*, 21003-21009.

Adams, J.M., and Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol *19*, 488-496.

Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., and Miyano, M. (1999). Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure *7*, 1417-1426.

Akazawa, Y., and Gores, G.J. (2007). Death receptor-mediated liver injury. Semin Liver Dis *27*, 327-338.

Aligo, J., Jia, S., Manna, D., and Konan, K.V. (2009). Formation and function of hepatitis C virus replication complexes require residues in the carboxy-terminal domain of NS4B protein. Virology *393*, 68-83.

Alisi, A., Giambartolomei, S., Cupelli, F., Merlo, P., Fontemaggi, G., Spaziani, A., and Balsano, C. (2003). Physical and functional interaction between HCV core protein and the different p73 isoforms. Oncogene *22*, 2573-2580.

Alsaleh, K., Delavalle, P.Y., Pillez, A., Duverlie, G., Descamps, V., Rouille, Y., Dubuisson, J., and Wychowski, C. (2010). Identification of basic amino acids at the N-terminal end of the core protein that are crucial for hepatitis C virus infectivity. J Virol *84*, 12515-12528.

Alter, H.J., Holland, P.V., Morrow, A.G., Purcell, R.H., Feinstone, S.M., and Moritsugu, Y. (1975). Clinical and serological analysis of transfusion-associated hepatitis. Lancet *2*, 838-841.

Alter, H.J., and Seeff, L.B. (2000). Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin Liver Dis *20*, 17-35.

Anderson, L., and Seilhamer, J. (1997). A comparison of selected mRNA and protein abundances in human liver. Electrophoresis *18*, 533-537.

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J.L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G., and Lotteau, V.

(2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol *76*, 6919-6928.

Angus, A.G., Dalrymple, D., Boulant, S., McGivern, D.R., Clayton, R.F., Scott, M.J., Adair, R., Graham, S., Owsianka, A.M., Targett-Adams, P., *et al.* (2010). Requirement of cellular DDX3 for hepatitis C virus replication is unrelated to its interaction with the viral core protein. J Gen Virol *91*, 122-132.

Appel, N., Pietschmann, T., and Bartenschlager, R. (2005). Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. J Virol *79*, 3187-3194.

Arikawa, E., Sun, Y., Wang, J., Zhou, Q., Ning, B., Dial, S.L., Guo, L., and Yang, J. (2008). Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC Genomics *9*, 328.

Ariumi, Y., Kuroki, M., Abe, K., Dansako, H., Ikeda, M., Wakita, T., and Kato, N. (2007). DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol *81*, 13922-13926.

Arzberger, S., Hosel, M., and Protzer, U. (2010). Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. J Virol *84*, 11994-12001.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science *281*, 1305-1308.

Aweya, J.J., and Tan, Y.J. (2011). Modulation of programmed cell death pathways by the hepatitis C virus. Front Biosci *16*, 608-618.

Azad, M.B., Chen, Y., Henson, E.S., Cizeau, J., McMillan-Ward, E., Israels, S.J., and Gibson, S.B. (2008). Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. Autophagy *4*, 195-204.

Bantel, H., Lugering, A., Heidemann, J., Volkmann, X., Poremba, C., Strassburg, C.P., Manns, M.P., and Schulze-Osthoff, K. (2004). Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. Hepatology *40*, 1078-1087.

Bantel, H., Lugering, A., Poremba, C., Lugering, N., Held, J., Domschke, W., and Schulze-Osthoff, K. (2001). Caspase activation correlates with the degree of inflammatory liver injury in chronic hepatitis C virus infection. Hepatology *34*, 758-767.

Bantel, H., and Schulze-Osthoff, K. (2003). Apoptosis in hepatitis C virus infection. Cell Death Differ *10 Suppl 1*, S48-58.

Bao, Q., and Shi, Y. (2007). Apoptosome: a platform for the activation of initiator caspases. Cell Death Differ *14*, 56-65.

Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M.J., Miyamura, T., *et al.* (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A *94*, 1200-1205.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1993). Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J Virol *67*, 3835-3844.

Bartenschlager, R., Lohmann, V., Wilkinson, T., and Koch, J.O. (1995). Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J Virol *69*, 7519-7528.

Barth, H., Schafer, C., Adah, M.I., Zhang, F., Linhardt, R.J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T.H., Depla, E., *et al.* (2003). Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem *278*, 41003-41012.

Bartosch, B., and Cosset, F.L. (2006). Cell entry of hepatitis C virus. Virology *348*, 1-12.

Bartosch, B., Dubuisson, J., and Cosset, F.L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med *197*, 633-642.

Battaglia, S., Benzoubir, N., Nobilet, S., Charneau, P., Samuel, D., Zignego, A.L., Atfi, A., Brechot, C., and Bourgeade, M.F. (2009). Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. PLoS One *4*, e4355.

Behrens, S.E., Tomei, L., and De Francesco, R. (1996). Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. Embo J *15*, 12-22.

Bellecave, P., Sarasin-Filipowicz, M., Donze, O., Kennel, A., Gouttenoire, J., Meylan, E., Terracciano, L., Tschopp, J., Sarrazin, C., Berg, T., *et al.* (2010). Cleavage of mitochondrial antiviral signaling protein in the liver of patients with chronic hepatitis C correlates with a reduced activation of the endogenous interferon system. Hepatology *51*, 1127-1136.

Benali-Furet, N.L., Chami, M., Houel, L., De Giorgi, F., Vernejoul, F., Lagorce, D., Buscail, L., Bartenschlager, R., Ichas, F., Rizzuto, R., *et al.* (2005). Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. Oncogene *24*, 4921-4933.

Berger, K.L., Cooper, J.D., Heaton, N.S., Yoon, R., Oakland, T.E., Jordan, T.X., Mateu, G., Grakoui, A., and Randall, G. (2009). Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. Proc Natl Acad Sci U S A *106*, 7577-7582.

Best, S.M., Wolfinbarger, J.B., and Bloom, M.E. (2002). Caspase activation is required for permissive replication of Aleutian mink disease parvovirus in vitro. Virology *292*, 224-234.

Bissig, K.D., Wieland, S.F., Tran, P., Isogawa, M., Le, T.T., Chisari, F.V., and Verma, I.M. (2010). Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. J Clin Invest *120*, 924-930.

Blackham, S., Baillie, A., Al-Hababi, F., Remlinger, K., You, S., Hamatake, R., and McGarvey, M.J. (2010). Gene expression profiling indicates the roles of host oxidative stress, apoptosis, lipid metabolism, and intracellular transport genes in the replication of hepatitis C virus. J Virol *84*, 5404-5414.

Blaho, J.A. (2004). Virus infection and apoptosis (issue II) an introduction: cheating death or death as a fact of life? Int Rev Immunol *23*, 1-6.

Blanchard, E., Belouzard, S., Goueslain, L., Wakita, T., Dubuisson, J., Wychowski, C., and Rouille, Y. (2006). Hepatitis C virus entry depends on clathrin-mediated endocytosis. J Virol *80*, 6964-6972.

Blight, K.J. (2007). Allelic variation in the hepatitis C virus NS4B protein dramatically influences RNA replication. J Virol *81*, 5724-5736.

Blight, K.J., McKeating, J.A., Marcotrigiano, J., and Rice, C.M. (2003). Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol *77*, 3181-3190.

Blight, K.J., McKeating, J.A., and Rice, C.M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol *76*, 13001-13014.

Blight, K.J., and Rice, C.M. (1997). Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. J Virol *71*, 7345-7352.

Blindenbacher, A., Duong, F.H., Hunziker, L., Stutvoet, S.T., Wang, X., Terracciano, L., Moradpour, D., Blum, H.E., Alonzi, T., Tripodi, M., *et al.* (2003). Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology *124*, 1465-1475.

Bomsztyk, K., Denisenko, O., and Ostrowski, J. (2004). hnRNP K: one protein multiple processes. Bioessays *26*, 629-638.

Bortolami, M., Kotsafti, A., Cardin, R., and Farinati, F. (2008). Fas / FasL system, IL-1beta expression and apoptosis in chronic HBV and HCV liver disease. J Viral Hepat *15*, 515-522.

Boson, B., Granio, O., Bartenschlager, R., and Cosset, F.L. (2011). A concerted action of hepatitis C virus p7 and nonstructural protein 2 regulates core localization at the endoplasmic reticulum and virus assembly. PLoS Pathog *7*, e1002144.

Boulant, S., Montserret, R., Hope, R.G., Ratinier, M., Targett-Adams, P., Lavergne, J.P., Penin, F., and McLauchlan, J. (2006). Structural determinants that target the hepatitis C virus core protein to lipid droplets. J Biol Chem *281*, 22236-22247.

Boulant, S., Targett-Adams, P., and McLauchlan, J. (2007). Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. J Gen Virol *88*, 2204-2213.

Boulant, S., Vanbelle, C., Ebel, C., Penin, F., and Lavergne, J.P. (2005). Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. J Virol *79*, 11353-11365.

Bowen, D.G., and Walker, C.M. (2005). Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature *436*, 946-952.

Boya, P., Pauleau, A.L., Poncet, D., Gonzalez-Polo, R.A., Zamzami, N., and Kroemer, G. (2004). Viral proteins targeting mitochondria: controlling cell death. Biochim Biophys Acta *1659*, 178-189.

Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J., *et al.* (1995). Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene *11*, 1921-1928.

Bradley, D., McCaustland, K., Krawczynski, K., Spelbring, J., Humphrey, C., and Cook, E.H. (1991). Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. J Med Virol *34*, 206-208.

Bradley, D.W., McCaustland, K.A., Cook, E.H., Schable, C.A., Ebert, J.W., and Maynard, J.E. (1985). Posttransfusion non-A, non-B hepatitis in chimpanzees. Physicochemical evidence that the tubule-forming agent is a small, enveloped virus. Gastroenterology *88*, 773-779.

Bradrick, S.S., Walters, R.W., and Gromeier, M. (2006). The hepatitis C virus 3'-untranslated region or a poly(A) tract promote efficient translation subsequent to the initiation phase. Nucleic Acids Res *34*, 1293-1303.

Brass, V., Bieck, E., Montserret, R., Wolk, B., Hellings, J.A., Blum, H.E., Penin, F., and Moradpour, D. (2002). An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. J Biol Chem *277*, 8130-8139.

Brenndorfer, E.D., Karthe, J., Frelin, L., Cebula, P., Erhardt, A., Schulte am Esch, J., Hengel, H., Bartenschlager, R., Sallberg, M., Haussinger, D., *et al.*

(2009). Nonstructural 3/4A protease of hepatitis C virus activates epithelial growth factor-induced signal transduction by cleavage of the T-cell protein tyrosine phosphatase. Hepatology *49*, 1810-1820.

Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R.L., Mathieu, M., De Francesco, R., and Rey, F.A. (1999). Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc Natl Acad Sci U S A *96*, 13034-13039.

Bukh, J., Miller, R.H., and Purcell, R.H. (1995). Biology and genetic heterogeneity of hepatitis C virus. Clin Exp Rheumatol *13 Suppl 13*, S3-7.

Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R.E., Govindarajan, S., Shapiro, M., St Claire, M., and Bartenschlager, R. (2002). Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc Natl Acad Sci U S A *99*, 14416-14421.

Bukh, J., Purcell, R.H., and Miller, R.H. (1994). Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci U S A *91*, 8239-8243.

Bungyoku, Y., Shoji, I., Makine, T., Adachi, T., Hayashida, K., Nagano-Fujii, M., Ide, Y.H., Deng, L., and Hotta, H. (2009). Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J Gen Virol *90*, 1681-1691.

Cai, Y., Liu, Y., Yu, D., and Zhang, X. (2003). Down-regulation of transcription of the proapoptotic gene BNip3 in cultured astrocytes by murine coronavirus infection. Virology *316*, 104-115.

Cai, Z., Zhang, C., Chang, K.S., Jiang, J., Ahn, B.C., Wakita, T., Liang, T.J., and Luo, G. (2005). Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J Virol *79*, 13963-13973.

Calabrese, F., Pontisso, P., Pettenazzo, E., Benvegnu, L., Vario, A., Chemello, L., Alberti, A., and Valente, M. (2000). Liver cell apoptosis in chronic hepatitis C correlates with histological but not biochemical activity or serum HCV-RNA levels. Hepatology *31*, 1153-1159.

Callens, N., Ciczora, Y., Bartosch, B., Vu-Dac, N., Cosset, F.L., Pawlotsky, J.M., Penin, F., and Dubuisson, J. (2005). Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. J Virol *79*, 15331-15341.

Canbay, A., Friedman, S., and Gores, G.J. (2004). Apoptosis: the nexus of liver injury and fibrosis. Hepatology *39*, 273-278.

Canbay, A., Taimr, P., Torok, N., Higuchi, H., Friedman, S., and Gores, G.J. (2003). Apoptotic body engulfment by a human stellate cell line is profibrogenic. Lab Invest *83*, 655-663.

Cao, Y., Hamada, T., Matsui, T., Date, T., and Iwabuchi, K. (2004). Hepatitis C virus core protein interacts with p53-binding protein, 53BP2/Bbp/ASPP2, and inhibits p53-mediated apoptosis. Biochem Biophys Res Commun *315*, 788-795.

Carrere-Kremer, S., Montpellier-Pala, C., Cocquerel, L., Wychowski, C., Penin, F., and Dubuisson, J. (2002). Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. J Virol *76*, 3720-3730.

Carthy, C.M., Yanagawa, B., Luo, H., Granville, D.J., Yang, D., Cheung, P., Cheung, C., Esfandiarei, M., Rudin, C.M., Thompson, C.B., *et al.* (2003). Bcl-2 and Bcl-xL overexpression inhibits cytochrome c release, activation of multiple caspases, and virus release following coxsackievirus B3 infection. Virology *313*, 147-157.

Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S.A., and Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell *9*, 351-365.

Chan, S.W., and Egan, P.A. (2005). Hepatitis C virus envelope proteins regulate CHOP via induction of the unfolded protein response. Faseb J *19*, 1510-1512.

Chang, J., Yang, S.H., Cho, Y.G., Hwang, S.B., Hahn, Y.S., and Sung, Y.C. (1998). Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. J Virol *72*, 3060-3065.

Chang, M.L., Chen, J.C., Chang, M.Y., Yeh, C.T., Lin, W.P., Liang, C.K., Huang, S.F., Dang, K.N., Chiu, C.T., and Lin, D.Y. (2008). Acute expression of hepatitis C core protein in adult mouse liver: Mitochondrial stress and apoptosis. Scand J Gastroenterol *43*, 747-755.

Chang, Y.H., Lin, H.H., Wang, Y.K., Chiu, W.T., Su, H.W., and Tang, M.J. (2007). Activation of caspase-8 and Erk-1/2 in domes regulates cell death induced by confluence in MDCK cells. J Cell Physiol *211*, 174-182.

Chao, D.T., and Korsmeyer, S.J. (1998). BCL-2 family: regulators of cell death. Annu Rev Immunol *16*, 395-419.

Charlton, M. (2001). Hepatitis C infection in liver transplantation. Am J Transplant *1*, 197-203.

Chen, C.M., You, L.R., Hwang, L.H., and Lee, Y.H. (1997). Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-beta receptor

modulates the signal pathway of the lymphotoxin-beta receptor. J Virol *71*, 9417-9426.

Chen, L., Willis, S.N., Wei, A., Smith, B.J., Fletcher, J.I., Hinds, M.G., Colman, P.M., Day, C.L., Adams, J.M., and Huang, D.C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell *17*, 393-403.

Chen, S.L., and Morgan, T.R. (2006). The natural history of hepatitis C virus (HCV) infection. Int J Med Sci *3*, 47-52.

Chen, Z., Zhu, Y., Ren, Y., Tong, Y., Hua, X., Zhu, F., Huang, L., Liu, Y., Luo, Y., Lu, W., *et al.* (2011). Hepatitis C virus protects human B lymphocytes from Fas-mediated apoptosis via E2-CD81 engagement. PLoS One *6*, e18933.

Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K., and Hardwick, J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. Science *278*, 1966-1968.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chiou, H.L., Hsieh, Y.S., Hsieh, M.R., and Chen, T.Y. (2006). HCV E2 may induce apoptosis of Huh-7 cells via a mitochondrial-related caspase pathway. Biochem Biophys Res Commun *345*, 453-458.

Chipuk, J.E., and Green, D.R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol *18*, 157-164.

Chipuk, J.E., Moldoveanu, T., Llambi, F., Parsons, M.J., and Green, D.R. (2010). The BCL-2 family reunion. Mol Cell *37*, 299-310.

Chiyo, T., Sekiguchi, S., Hayashi, M., Tobita, Y., Kanegae, Y., Saito, I., and Kohara, M. (2011). Conditional gene expression in hepatitis C virus transgenic mice without induction of severe liver injury using a non-inflammatory Cre-expressing adenovirus. Virus Res *160*, 89-97.

Choi, S.H., Park, K.J., Ahn, B.Y., Jung, G., Lai, M.M., and Hwang, S.B. (2006). Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase. Mol Cell Biol *26*, 3048-3059.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science *244*, 359-362.

Choo, Q.L., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., *et al.* (1991). Genetic

organization and diversity of the hepatitis C virus. Proc Natl Acad Sci U S A *88*, 2451-2455.

Chou, A.H., Tsai, H.F., Wu, Y.Y., Hu, C.Y., Hwang, L.H., Hsu, P.I., and Hsu, P.N. (2005). Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway. J Immunol *174*, 2160-2166.

Chung, Y.L., Sheu, M.L., and Yen, S.H. (2003). Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. Int J Cancer *107*, 65-73.

Ciccaglione, A.R., Marcantonio, C., Tritarelli, E., Equestre, M., Magurano, F., Costantino, A., Nicoletti, L., and Rapicetta, M. (2004). The transmembrane domain of hepatitis C virus E1 glycoprotein induces cell death. Virus Res *104*, 1-9.

Ciesek, S., and Manns, M.P. (2011). Hepatitis in 2010: the dawn of a new era in HCV therapy. Nat Rev Gastroenterol Hepatol *8*, 69-71.

Cocquerel, L., Duvet, S., Meunier, J.C., Pillez, A., Cacan, R., Wychowski, C., and Dubuisson, J. (1999). The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. J Virol *73*, 2641-2649.

Cocquerel, L., Meunier, J.C., Pillez, A., Wychowski, C., and Dubuisson, J. (1998). A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. J Virol *72*, 2183-2191.

Colin, J., Gaumer, S., Guenal, I., and Mignotte, B. (2009). Mitochondria, Bcl-2 family proteins and apoptosomes: of worms, flies and men. Front Biosci *14*, 4127-4137.

Colombo, M. (1999). Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. J Hepatol *31 Suppl 1*, 25-30.

Cormier, E.G., Tsamis, F., Kajumo, F., Durso, R.J., Gardner, J.P., and Dragic, T. (2004). CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci U S A *101*, 7270-7274.

Cuconati, A., and White, E. (2002). Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev *16*, 2465-2478.

Czabotar, P.E., Lee, E.F., van Delft, M.F., Day, C.L., Smith, B.J., Huang, D.C., Fairlie, W.D., Hinds, M.G., and Colman, P.M. (2007). Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc Natl Acad Sci U S A *104*, 6217-6222.

D'Agostino, D.M., Ranzato, L., Arrigoni, G., Cavallari, I., Belleudi, F., Torrisi, M.R., Silic-Benussi, M., Ferro, T., Petronilli, V., Marin, O., *et al.*

(2002). Mitochondrial alterations induced by the p13II protein of human T-cell leukemia virus type 1. Critical role of arginine residues. J Biol Chem *277*, 34424-34433.

Danial, N.N. (2007). BCL-2 family proteins: critical checkpoints of apoptotic cell death. Clin Cancer Res *13*, 7254-7263.

de la Coste, A., Fabre, M., McDonell, N., Porteu, A., Gilgenkrantz, H., Perret, C., Kahn, A., and Mignon, A. (1999). Differential protective effects of Bcl-xL and Bcl-2 on apoptotic liver injury in transgenic mice. Am J Physiol *277*, G702-708.

Degos, F., Christidis, C., Ganne-Carrie, N., Farmachidi, J.P., Degott, C., Guettier, C., Trinchet, J.C., Beaugrand, M., and Chevret, S. (2000). Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. Gut *47*, 131-136.

Delgrange, D., Pillez, A., Castelain, S., Cocquerel, L., Rouille, Y., Dubuisson, J., Wakita, T., Duverlie, G., and Wychowski, C. (2007). Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol *88*, 2495-2503.

Deng, L., Adachi, T., Kitayama, K., Bungyoku, Y., Kitazawa, S., Ishido, S., Shoji, I., and Hotta, H. (2008). Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol *82*, 10375-10385.

Deng, L., Nagano-Fujii, M., Tanaka, M., Nomura-Takigawa, Y., Ikeda, M., Kato, N., Sada, K., and Hotta, H. (2006). NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. J Gen Virol *87*, 1703-1713.

Dowling, A.J., Waterfield, N.R., Hares, M.C., Le Goff, G., Streuli, C.H., and ffrench-Constant, R.H. (2007). The Mcf1 toxin induces apoptosis via the mitochondrial pathway and apoptosis is attenuated by mutation of the BH3-like domain. Cell Microbiol *9*, 2470-2484.

Dreux, M., Gastaminza, P., Wieland, S.F., and Chisari, F.V. (2009). The autophagy machinery is required to initiate hepatitis C virus replication. Proc Natl Acad Sci U S A *106*, 14046-14051.

Dumoulin, F.L., von dem Bussche, A., Li, J., Khamzina, L., Wands, J.R., Sauerbruch, T., and Spengler, U. (2003). Hepatitis C virus NS2 protein inhibits gene expression from different cellular and viral promoters in hepatic and nonhepatic cell lines. Virology *305*, 260-266.

Duvet, S., Op De Beeck, A., Cocquerel, L., Wychowski, C., Cacan, R., and Dubuisson, J. (2002). Glycosylation of the hepatitis C virus envelope protein E1 occurs posttranslationally in a mannosylphosphoryldolichol-deficient CHO mutant cell line. Glycobiology *12*, 95-101.

Eckart, M.R., Selby, M., Masiarz, F., Lee, C., Berger, K., Crawford, K., Kuo, C., Kuo, G., Houghton, M., and Choo, Q.L. (1993). The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. Biochem Biophys Res Commun *192*, 399-406.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., and Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol *76*, 5974-5984.

Einav, S., Elazar, M., Danieli, T., and Glenn, J.S. (2004). A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. J Virol *78*, 11288-11295.

Einav, S., Sklan, E.H., Moon, H.M., Gehrig, E., Liu, P., Hao, Y., Lowe, A.W., and Glenn, J.S. (2008). The nucleotide binding motif of hepatitis C virus NS4B can mediate cellular transformation and tumor formation without Ha-ras co-transfection. Hepatology *47*, 827-835.

Elangovan, B., and Chinnadurai, G. (1997). Functional dissection of the pro-apoptotic protein Bik. Heterodimerization with anti-apoptosis proteins is insufficient for induction of cell death. J Biol Chem *272*, 24494-24498.

Elazar, M., Liu, P., Rice, C.M., and Glenn, J.S. (2004). An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. J Virol *78*, 11393-11400.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol *35*, 495-516.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F., and Sato, C. (1995). Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest *96*, 224-230.

Erdtmann, L., Franck, N., Lerat, H., Le Seyec, J., Gilot, D., Cannie, I., Gripon, P., Hibner, U., and Guguen-Guillouzo, C. (2003). The hepatitis C virus NS2 protein is an inhibitor of CIDE-B-induced apoptosis. J Biol Chem *278*, 18256-18264.

Ernst, E., Schonig, K., Bugert, J.J., Blaker, H., Pfaff, E., Stremmel, W., and Encke, J. (2007). Generation of inducible hepatitis C virus transgenic mouse lines. J Med Virol *79*, 1103-1112.

Evans, M.J., Rice, C.M., and Goff, S.P. (2004). Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc Natl Acad Sci U S A *101*, 13038-13043.

Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., and Rice, C.M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature *446*, 801-805.

Fabbri, C., Marchetto, S., Pezzoli, A., Accogli, E., Fusaroli, P., Azzaroli, F., Jaboli, M.F., Mazzeo, C., Montagnani, M., Festi, D., *et al.* (2000). Efficacy of ursodeoxycholic acid in association with alpha-interferon for chronic hepatitis C in alpha-interferon non-responder patients. Eur J Gastroenterol Hepatol *12*, 511-515.

Fadeel, B., and Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med *258*, 479-517.

Fadok, V.A., de Cathelineau, A., Daleke, D.L., Henson, P.M., and Bratton, D.L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J Biol Chem *276*, 1071-1077.

Failla, C., Tomei, L., and De Francesco, R. (1994). Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J Virol *68*, 3753-3760.

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., Shimizu, Y., Shapiro, M., Alter, H.J., and Purcell, R.H. (1996). Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc Natl Acad Sci U S A *93*, 15394-15399.

Faubion, W.A., and Gores, G.J. (1999). Death receptors in liver biology and pathobiology. Hepatology *29*, 1-4.

Feinstone, S.M., Kapikian, A.Z., Purcell, R.H., Alter, H.J., and Holland, P.V. (1975). Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med *292*, 767-770.

Feinstone, S.M., Mihalik, K.B., Kamimura, T., Alter, H.J., London, W.T., and Purcell, R.H. (1983). Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. Infect Immun *41*, 816-821.

Feld, J.J., and Hoofnagle, J.H. (2005). Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature *436*, 967-972.

Feldstein, A.E., Canbay, A., Angulo, P., Taniai, M., Burgart, L.J., Lindor, K.D., and Gores, G.J. (2003). Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology *125*, 437-443.

Ferraro-Peyret, C., Quemeneur, L., Flacher, M., Revillard, J.P., and Genestier, L. (2002). Caspase-independent phosphatidylserine exposure during apoptosis of primary T lymphocytes. J Immunol *169*, 4805-4810.

Fimia, G.M., Tripodi, M., and Alonzi, T. (2003). Transgenic models for Hepatitis C virus pathogenesis. Cell Death Differ *10 Suppl 1*, S16-18.

Fischer, R., Baumert, T., and Blum, H.E. (2007). Hepatitis C virus infection and apoptosis. World J Gastroenterol *13*, 4865-4872.

Fletcher, J.I., and Huang, D.C. (2008). Controlling the cell death mediators Bax and Bak: puzzles and conundrums. Cell Cycle *7*, 39-44.

Frick, D.N., Rypma, R.S., Lam, A.M., and Gu, B. (2004). The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. J Biol Chem *279*, 1269-1280.

Friebe, P., and Bartenschlager, R. (2002). Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. J Virol *76*, 5326-5338.

Friebe, P., Boudet, J., Simorre, J.P., and Bartenschlager, R. (2005). Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. J Virol *79*, 380-392.

Friebe, P., Lohmann, V., Krieger, N., and Bartenschlager, R. (2001). Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. J Virol *75*, 12047-12057.

Fu, N.Y., Sukumaran, S.K., and Yu, V.C. (2007). Inhibition of ubiquitin-mediated degradation of MOAP-1 by apoptotic stimuli promotes Bax function in mitochondria. Proc Natl Acad Sci U S A *104*, 10051-10056.

Gale, M., Jr., Kwieciszewski, B., Dossett, M., Nakao, H., and Katze, M.G. (1999). Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. J Virol *73*, 6506-6516.

Gale, M.J., Jr., Korth, M.J., Tang, N.M., Tan, S.L., Hopkins, D.A., Dever, T.E., Polyak, S.J., Gretch, D.R., and Katze, M.G. (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology *230*, 217-227.

Gallinari, P., Brennan, D., Nardi, C., Brunetti, M., Tomei, L., Steinkuhler, C., and De Francesco, R. (1998). Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. J Virol *72*, 6758-6769.

Galluzzi, L., Brenner, C., Morselli, E., Touat, Z., and Kroemer, G. (2008). Viral control of mitochondrial apoptosis. PLoS Pathog *4*, e1000018.

Garry, R.F., and Dash, S. (2003). Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. Virology *307*, 255-265.

Gastaminza, P., Cheng, G., Wieland, S., Zhong, J., Liao, W., and Chisari, F.V. (2008). Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J Virol *82*, 2120-2129.

Georgel, P., Schuster, C., Zeisel, M.B., Stoll-Keller, F., Berg, T., Bahram, S., and Baumert, T.F. (2010). Virus-host interactions in hepatitis C virus infection: implications for molecular pathogenesis and antiviral strategies. Trends Mol Med *16*, 277-286.

Germi, R., Crance, J.M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R.W., Zarski, J.P., and Drouet, E. (2002). Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. J Med Virol *68*, 206-215.

Gillissen, B., Essmann, F., Graupner, V., Starck, L., Radetzki, S., Dorken, B., Schulze-Osthoff, K., and Daniel, P.T. (2003). Induction of cell death by the BH3-only Bcl-2 homolog Nbk/Bik is mediated by an entirely Bax-dependent mitochondrial pathway. Embo J *22*, 3580-3590.

Gillissen, B., Essmann, F., Hemmati, P.G., Richter, A., Oztop, I., Chinnadurai, G., Dorken, B., and Daniel, P.T. (2007). Mcl-1 determines the Bax dependency of Nbk/Bik-induced apoptosis. J Cell Biol *179*, 701-715.

Goffard, A., Callens, N., Bartosch, B., Wychowski, C., Cosset, F.L., Montpellier, C., and Dubuisson, J. (2005). Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J Virol *79*, 8400-8409.

Goffard, A., and Dubuisson, J. (2003). Glycosylation of hepatitis C virus envelope proteins. Biochimie *85*, 295-301.

Gottwein, J.M., Scheel, T.K., Jensen, T.B., Lademann, J.B., Prentoe, J.C., Knudsen, M.L., Hoegh, A.M., and Bukh, J. (2009). Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. Hepatology *49*, 364-377.

Gouttenoire, J., Montserret, R., Kennel, A., Penin, F., and Moradpour, D. (2009). An amphipathic alpha-helix at the C terminus of hepatitis C virus nonstructural protein 4B mediates membrane association. J Virol *83*, 11378-11384.

Grad, J.M., Zeng, X.R., and Boise, L.H. (2000). Regulation of Bcl-xL: a little bit of this and a little bit of STAT. Curr Opin Oncol *12*, 543-549.

Grakoui, A., McCourt, D.W., Wychowski, C., Feinstone, S.M., and Rice, C.M. (1993). A second hepatitis C virus-encoded proteinase. Proc Natl Acad Sci U S A *90*, 10583-10587.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., and Schulte-Hermann, R. (1995). In situ detection of fragmented DNA

(TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology *21*, 1465-1468.

Hacker, G. (2000). The morphology of apoptosis. Cell Tissue Res *301*, 5-17.

Hacker, G., and Weber, A. (2007). BH3-only proteins trigger cytochrome c release, but how? Arch Biochem Biophys *462*, 150-155.

Hahn, C.S., Cho, Y.G., Kang, B.S., Lester, I.M., and Hahn, Y.S. (2000). The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. Virology *276*, 127-137.

Haid, S., Pietschmann, T., and Pecheur, E.I. (2009). Low pH-dependent hepatitis C virus membrane fusion depends on E2 integrity, target lipid composition, and density of virus particles. J Biol Chem *284*, 17657-17667.

Hamacher-Brady, A., Brady, N.R., Logue, S.E., Sayen, M.R., Jinno, M., Kirshenbaum, L.A., Gottlieb, R.A., and Gustafsson, A.B. (2007). Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ *14*, 146-157.

Han, J., Sabbatini, P., and White, E. (1996). Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. Mol Cell Biol *16*, 5857-5864.

Haqshenas, G., Mackenzie, J.M., Dong, X., and Gowans, E.J. (2007). Hepatitis C virus p7 protein is localized in the endoplasmic reticulum when it is encoded by a replication-competent genome. J Gen Virol *88*, 134-142.

Hara, Y., Hino, K., Okuda, M., Furutani, T., Hidaka, I., Yamaguchi, Y., Korenaga, M., Li, K., Weinman, S.A., Lemon, S.M., *et al.* (2006). Hepatitis C virus core protein inhibits deoxycholic acid-mediated apoptosis despite generating mitochondrial reactive oxygen species. J Gastroenterol *41*, 257-268.

Hardwick, J.M., and Bellows, D.S. (2003). Viral versus cellular BCL-2 proteins. Cell Death Differ *10 Suppl 1*, S68-76.

Harper, N., Hughes, M., MacFarlane, M., and Cohen, G.M. (2003). Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. J Biol Chem *278*, 25534-25541.

Harris, C.A., and Johnson, E.M., Jr. (2001). BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J Biol Chem *276*, 37754-37760.

Hay, S., and Kannourakis, G. (2002). A time to kill: viral manipulation of the cell death program. J Gen Virol *83*, 1547-1564.

He, L., Perkins, G.A., Poblenz, A.T., Harris, J.B., Hung, M., Ellisman, M.H., and Fox, D.A. (2003). Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. Proc Natl Acad Sci U S A *100*, 1022-1027.

He, L.F., Alling, D., Popkin, T., Shapiro, M., Alter, H.J., and Purcell, R.H. (1987). Determining the size of non-A, non-B hepatitis virus by filtration. J Infect Dis *156*, 636-640.

He, Y., Staschke, K.A., and Tan, S.L. (2006). HCV NS5A: A Multifunctional Regulator of Cellular Pathways and Virus Replication. In Hepatitis C Viruses: Genomes and Molecular Biology, S.L. Tan, ed. (Norfolk (UK)).

Heck, J.A., Meng, X., and Frick, D.N. (2009). Cyclophilin B stimulates RNA synthesis by the HCV RNA dependent RNA polymerase. Biochem Pharmacol *77*, 1173-1180.

Heim, M.H., Moradpour, D., and Blum, H.E. (1999). Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J Virol *73*, 8469-8475.

Henke, J.I., Goergen, D., Zheng, J., Song, Y., Schuttler, C.G., Fehr, C., Junemann, C., and Niepmann, M. (2008). microRNA-122 stimulates translation of hepatitis C virus RNA. Embo J *27*, 3300-3310.

Herzer, K., Sprinzl, M.F., and Galle, P.R. (2007). Hepatitis viruses: live and let die. Liver Int *27*, 293-301.

Herzer, K., Weyer, S., Krammer, P.H., Galle, P.R., and Hofmann, T.G. (2005). Hepatitis C virus core protein inhibits tumor suppressor protein promyelocytic leukemia function in human hepatoma cells. Cancer Res *65*, 10830-10837.

Higuchi, H., Bronk, S.F., Taniai, M., Canbay, A., and Gores, G.J. (2002). Cholestasis increases tumor necrosis factor-related apoptotis-inducing ligand (TRAIL)-R2/DR5 expression and sensitizes the liver to TRAIL-mediated cytotoxicity. J Pharmacol Exp Ther *303*, 461-467.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci U S A *88*, 5547-5551.

Hijikata, M., Shimizu, Y.K., Kato, H., Iwamoto, A., Shih, J.W., Alter, H.J., Purcell, R.H., and Yoshikura, H. (1993). Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J Virol *67*, 1953-1958.

Hikita, H., Takehara, T., Shimizu, S., Kodama, T., Li, W., Miyagi, T., Hosui, A., Ishida, H., Ohkawa, K., Kanto, T., *et al.* (2009). Mcl-1 and Bcl-xL

cooperatively maintain integrity of hepatocytes in developing and adult murine liver. Hepatology *50*, 1217-1226.

Hinds, M.G., and Day, C.L. (2005). Regulation of apoptosis: uncovering the binding determinants. Curr Opin Struct Biol *15*, 690-699.

Hiramatsu, N., Hayashi, N., Katayama, K., Mochizuki, K., Kawanishi, Y., Kasahara, A., Fusamoto, H., and Kamada, T. (1994). Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology *19*, 1354-1359.

Hollander, M.C., and Fornace, A.J., Jr. (2002). Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. Oncogene *21*, 6228-6233.

Honda, M., Beard, M.R., Ping, L.H., and Lemon, S.M. (1999). A phylogenetically conserved stem-loop structure at the 5' border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. J Virol *73*, 1165-1174.

Hope, R.G., and McLauchlan, J. (2000). Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J Gen Virol *81*, 1913-1925.

Houghton, M., and Abrignani, S. (2005). Prospects for a vaccine against the hepatitis C virus. Nature *436*, 961-966.

Hsieh, T.Y., Matsumoto, M., Chou, H.C., Schneider, R., Hwang, S.B., Lee, A.S., and Lai, M.M. (1998). Hepatitis C virus core protein interacts with heterogeneous nuclear ribonucleoprotein K. J Biol Chem *273*, 17651-17659.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C.M., and McKeating, J.A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A *100*, 7271-7276.

Huang, L., Hwang, J., Sharma, S.D., Hargittai, M.R., Chen, Y., Arnold, J.J., Raney, K.D., and Cameron, C.E. (2005a). Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. J Biol Chem *280*, 36417-36428.

Huang, Y.P., Zhang, S.L., Cheng, J., Wang, L., Guo, J., Liu, Y., Yang, Y., Zhang, L.Y., Bai, G.Q., Gao, X.S., *et al.* (2005b). Screening of genes of proteins interacting with p7 protein of hepatitis C virus from human liver cDNA library by yeast two-hybrid system. World J Gastroenterol *11*, 4709-4714.

Hussy, P., Langen, H., Mous, J., and Jacobsen, H. (1996). Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology *224*, 93-104.

Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997). harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). Embo J *16*, 1686-1694.

Inohara, N., Gourley, T.S., Carrio, R., Muniz, M., Merino, J., Garcia, I., Koseki, T., Hu, Y., Chen, S., and Nunez, G. (1998). Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J Biol Chem *273*, 32479-32486.

Ivashkina, N., Wolk, B., Lohmann, V., Bartenschlager, R., Blum, H.E., Penin, F., and Moradpour, D. (2002). The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. J Virol *76*, 13088-13093.

Jacotot, E., Ferri, K.F., El Hamel, C., Brenner, C., Druillennec, S., Hoebeke, J., Rustin, P., Metivier, D., Lenoir, C., Geuskens, M., *et al.* (2001). Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2. J Exp Med *193*, 509-519.

Jennings, T.A., Chen, Y., Sikora, D., Harrison, M.K., Sikora, B., Huang, L., Jankowsky, E., Fairman, M.E., Cameron, C.E., and Raney, K.D. (2008). RNA unwinding activity of the hepatitis C virus NS3 helicase is modulated by the NS5B polymerase. Biochemistry *47*, 1126-1135.

Jennings, T.A., Mackintosh, S.G., Harrison, M.K., Sikora, D., Sikora, B., Dave, B., Tackett, A.J., Cameron, C.E., and Raney, K.D. (2009). NS3 helicase from the hepatitis C virus can function as a monomer or oligomer depending on enzyme and substrate concentrations. J Biol Chem *284*, 4806-4814.

Jin, D.Y., Wang, H.L., Zhou, Y., Chun, A.C., Kibler, K.V., Hou, Y.D., Kung, H., and Jeang, K.T. (2000). Hepatitis C virus core protein-induced loss of LZIP function correlates with cellular transformation. Embo J *19*, 729-740.

Jones, C.T., Murray, C.L., Eastman, D.K., Tassello, J., and Rice, C.M. (2007). Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol *81*, 8374-8383.

Jones, D.M., Patel, A.H., Targett-Adams, P., and McLauchlan, J. (2009). The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus. J Virol *83*, 2163-2177.

Jopling, C.L., Norman, K.L., and Sarnow, P. (2006). Positive and negative modulation of viral and cellular mRNAs by liver-specific microRNA miR-122. Cold Spring Harb Symp Quant Biol *71*, 369-376.

Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science *309*, 1577-1581.

Joyce, M.A., Walters, K.A., Lamb, S.E., Yeh, M.M., Zhu, L.F., Kneteman, N., Doyle, J.S., Katze, M.G., and Tyrrell, D.L. (2009). HCV induces oxidative and ER stress, and sensitizes infected cells to apoptosis in SCID/Alb-uPA mice. PLoS Pathog *5*, e1000291.

Kafrouni, M.I., Brown, G.R., and Thiele, D.L. (2001). Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. J Immunol *167*, 1566-1574.

Kamegaya, Y., Hiasa, Y., Zukerberg, L., Fowler, N., Blackard, J.T., Lin, W., Choe, W.H., Schmidt, E.V., and Chung, R.T. (2005). Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. Hepatology *41*, 660-667.

Kanai, K., Kako, M., and Okamoto, H. (1992). HCV genotypes in chronic hepatitis C and response to interferon. Lancet *339*, 1543.

Kaneko, T., Tanji, Y., Satoh, S., Hijikata, M., Asabe, S., Kimura, K., and Shimotohno, K. (1994). Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. Biochem Biophys Res Commun *205*, 320-326.

Kang, S.M., Kim, S.J., Kim, J.H., Lee, W., Kim, G.W., Lee, K.H., Choi, K.Y., and Oh, J.W. (2009). Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-alpha-mediated apoptosis. Cancer Lett *279*, 230-237.

Kannan, R.P., Hensley, L.L., Evers, L.E., Lemon, S.M., and McGivern, D.R. (2011). Hepatitis C virus infection causes cell cycle arrest at the level of initiation of mitosis. J Virol *85*, 7989-8001.

Kanzawa, T., Zhang, L., Xiao, L., Germano, I.M., Kondo, Y., and Kondo, S. (2005). Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene *24*, 980-991.

Kao, C.F., Chen, S.Y., Chen, J.Y., and Wu Lee, Y.H. (2004). Modulation of p53 transcription regulatory activity and post-translational modification by hepatitis C virus core protein. Oncogene *23*, 2472-2483.

Kapadia, S.B., Barth, H., Baumert, T., McKeating, J.A., and Chisari, F.V. (2007). Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J Virol *81*, 374-383.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., and Shimotohno, K. (1990). Sequence diversity of hepatitis C viral genomes. Mol Biol Med *7*, 495-501.

Kato, N., Ootsuyama, Y., Ohkoshi, S., Nakazawa, T., Sekiya, H., Hijikata, M., and Shimotohno, K. (1992). Characterization of hypervariable regions in the

putative envelope protein of hepatitis C virus. Biochem Biophys Res Commun *189*, 119-127.

Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., Nagayama, K., Tanaka, T., and Wakita, T. (2001). Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol *64*, 334-339.

Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R.K., Murthy, K., Wakita, T., and Liang, T.J. (2007). Production of infectious hepatitis C virus of various genotypes in cell cultures. J Virol *81*, 4405-4411.

Kaukinen, P., Sillanpaa, M., Kotenko, S., Lin, R., Hiscott, J., Melen, K., and Julkunen, I. (2006). Hepatitis C virus NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression. Virol J *3*, 66.

Kaul, A., Stauffer, S., Berger, C., Pertel, T., Schmitt, J., Kallis, S., Zayas, M., Lohmann, V., Luban, J., and Bartenschlager, R. (2009). Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics. PLoS Pathog *5*, e1000546.

Kaul, A., Woerz, I., Meuleman, P., Leroux-Roels, G., and Bartenschlager, R. (2007). Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J Virol *81*, 13168-13179.

Kawamura, H., Govindarajan, S., Aswad, F., Machida, K., Lai, M.M., Sung, V.M., and Dennert, G. (2006). HCV core expression in hepatocytes protects against autoimmune liver injury and promotes liver regeneration in mice. Hepatology *44*, 936-944.

Ke, P.Y., and Chen, S.S. (2011). Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. J Clin Invest *121*, 37-56.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kim, D.W., Gwack, Y., Han, J.H., and Choe, J. (1995). C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. Biochem Biophys Res Commun *215*, 160-166.

Kim, H., Tu, H.C., Ren, D., Takeuchi, O., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., and Cheng, E.H. (2009). Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. Mol Cell *36*, 487-499.

Kim, J.L., Morgenstern, K.A., Griffith, J.P., Dwyer, M.D., Thomson, J.A., Murcko, M.A., Lin, C., and Caron, P.R. (1998). Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. Structure *6*, 89-100.

Kim, J.L., Morgenstern, K.A., Lin, C., Fox, T., Dwyer, M.D., Landro, J.A., Chambers, S.P., Markland, W., Lepre, C.A., O'Malley, E.T., *et al.* (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell *87*, 343-355.

Kim, Y.K., Kim, C.S., Lee, S.H., and Jang, S.K. (2002). Domains I and II in the 5' nontranslated region of the HCV genome are required for RNA replication. Biochem Biophys Res Commun *290*, 105-112.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo J *14*, 5579-5588.

Kittlesen, D.J., Chianese-Bullock, K.A., Yao, Z.Q., Braciale, T.J., and Hahn, Y.S. (2000). Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. J Clin Invest *106*, 1239-1249.

Koch, J.O., and Bartenschlager, R. (1999). Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. J Virol *73*, 7138-7146.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J., *et al.* (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science *278*, 294-298.

Kou, Y.H., Chou, S.M., Wang, Y.M., Chang, Y.T., Huang, S.Y., Jung, M.Y., Huang, Y.H., Chen, M.R., Chang, M.F., and Chang, S.C. (2006). Hepatitis C virus NS4A inhibits cap-dependent and the viral IRES-mediated translation through interacting with eukaryotic elongation factor 1A. J Biomed Sci *13*, 861-874.

Krieger, N., Lohmann, V., and Bartenschlager, R. (2001). Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol *75*, 4614-4624.

Kubli, D.A., Ycaza, J.E., and Gustafsson, A.B. (2007). Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. Biochem J *405*, 407-415.

Kurosaka, K., Takahashi, M., Watanabe, N., and Kobayashi, Y. (2003). Silent cleanup of very early apoptotic cells by macrophages. J Immunol *171*, 4672-4679.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J.E., Bonzon, C., Sullivan, B.A., Green, D.R., and Newmeyer, D.D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell *17*, 525-535.

Kwo, P.Y., Lawitz, E.J., McCone, J., Schiff, E.R., Vierling, J.M., Pound, D., Davis, M.N., Galati, J.S., Gordon, S.C., Ravendhran, N., *et al.* (2010). Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. Lancet *376*, 705-716.

Kwun, H.J., and Jang, K.L. (2003). Dual effects of hepatitis C virus Core protein on the transcription of cyclin-dependent kinase inhibitor p21 gene. J Viral Hepat *10*, 249-255.

Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., *et al.* (1996). Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. Nat Med *2*, 80-86.

Lai, M.M., and Ware, C.F. (2000). Hepatitis C virus core protein: possible roles in viral pathogenesis. Curr Top Microbiol Immunol *242*, 117-134.

Lan, K.H., Sheu, M.L., Hwang, S.J., Yen, S.H., Chen, S.Y., Wu, J.C., Wang, Y.J., Kato, N., Omata, M., Chang, F.Y., *et al.* (2002). HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. Oncogene *21*, 4801-4811.

Lane, J.D., Vergnolle, M.A., Woodman, P.G., and Allan, V.J. (2001). Apoptotic cleavage of cytoplasmic dynein intermediate chain and p150(Glued) stops dynein-dependent membrane motility. J Cell Biol *153*, 1415-1426.

Lau, J.Y., Xie, X., Lai, M.M., and Wu, P.C. (1998). Apoptosis and viral hepatitis. Semin Liver Dis *18*, 169-176.

Lavanchy, D. (2011). Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect *17*, 107-115.

Lavillette, D., Pecheur, E.I., Donot, P., Fresquet, J., Molle, J., Corbau, R., Dreux, M., Penin, F., and Cosset, F.L. (2007). Characterization of fusion determinants points to the involvement of three discrete regions of both E1 and E2 glycoproteins in the membrane fusion process of hepatitis C virus. J Virol *81*, 8752-8765.

Lechner, F., Gruener, N.H., Urbani, S., Uggeri, J., Santantonio, T., Kammer, A.R., Cerny, A., Phillips, R., Ferrari, C., Pape, G.R., *et al.* (2000). CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. Eur J Immunol *30*, 2479-2487.

Lee, E.F., Czabotar, P.E., van Delft, M.F., Michalak, E.M., Boyle, M.J., Willis, S.N., Puthalakath, H., Bouillet, P., Colman, P.M., Huang, D.C., *et al.* (2008). A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. J Cell Biol *180*, 341-355.

Lee, J.W., Soung, Y.H., Kim, S.Y., Nam, S.W., Kim, C.J., Cho, Y.G., Lee, J.H., Kim, H.S., Park, W.S., Kim, S.H., *et al.* (2004). Inactivating mutations of

proapoptotic Bad gene in human colon cancers. Carcinogenesis *25*, 1371-1376.

Lee, M.N., Jung, E.Y., Kwun, H.J., Jun, H.K., Yu, D.Y., Choi, Y.H., and Jang, K.L. (2002). Hepatitis C virus core protein represses the p21 promoter through inhibition of a TGF-beta pathway. J Gen Virol *83*, 2145-2151.

Lee, S.H., Kim, Y.K., Kim, C.S., Seol, S.K., Kim, J., Cho, S., Song, Y.L., Bartenschlager, R., and Jang, S.K. (2005). E2 of hepatitis C virus inhibits apoptosis. J Immunol *175*, 8226-8235.

Lee, S.K., Park, S.O., Joe, C.O., and Kim, Y.S. (2007). Interaction of HCV core protein with 14-3-3epsilon protein releases Bax to activate apoptosis. Biochem Biophys Res Commun *352*, 756-762.

Lei, Y., Moore, C.B., Liesman, R.M., O'Connor, B.P., Bergstralh, D.T., Chen, Z.J., Pickles, R.J., and Ting, J.P. (2009). MAVS-mediated apoptosis and its inhibition by viral proteins. PLoS One *4*, e5466.

Lerat, H., Honda, M., Beard, M.R., Loesch, K., Sun, J., Yang, Y., Okuda, M., Gosert, R., Xiao, S.Y., Weinman, S.A., *et al.* (2002). Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology *122*, 352-365.

Leroux-Roels, G. (2005). Development of prophylactic and therapeutic vaccines against hepatitis C virus. Expert Rev Vaccines *4*, 351-371.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., and Weber, P.C. (1999). Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat Struct Biol *6*, 937-943.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S., and Korsmeyer, S.J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183-192.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale, M., Jr., and Lemon, S.M. (2005a). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A *102*, 2992-2997.

Li, S., Ye, L., Yu, X., Xu, B., Li, K., Zhu, X., Liu, H., Wu, X., and Kong, L. (2009). Hepatitis C virus NS4B induces unfolded protein response and endoplasmic reticulum overload response-dependent NF-kappaB activation. Virology *391*, 257-264.

Li, X.D., Sun, L., Seth, R.B., Pineda, G., and Chen, Z.J. (2005b). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A *102*, 17717-17722.

Liberman, E., Fong, Y.L., Selby, M.J., Choo, Q.L., Cousens, L., Houghton, M., and Yen, T.S. (1999). Activation of the grp78 and grp94 promoters by hepatitis C virus E2 envelope protein. J Virol *73*, 3718-3722.

Liefhebber, J.M., Brandt, B.W., Broer, R., Spaan, W.J., and van Leeuwen, H.C. (2009). Hepatitis C virus NS4B carboxy terminal domain is a membrane binding domain. Virol J *6*, 62.

Lin, C., Thomson, J.A., and Rice, C.M. (1995). A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J Virol *69*, 4373-4380.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., *et al.* (2005). Complete replication of hepatitis C virus in cell culture. Science *309*, 623-626.

Lindenbach, B.D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A.J., McKeating, J.A., Lanford, R.E., Feinstone, S.M., Major, M.E., Leroux-Roels, G., *et al.* (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc Natl Acad Sci U S A *103*, 3805-3809.

Lindenbach, B.D., Pragai, B.M., Montserret, R., Beran, R.K., Pyle, A.M., Penin, F., and Rice, C.M. (2007). The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J Virol *81*, 8905-8918.

Liu, Q., Bhat, R.A., Prince, A.M., and Zhang, P. (1999). The hepatitis C virus NS2 protein generated by NS2-3 autocleavage is required for NS5A phosphorylation. Biochem Biophys Res Commun *254*, 572-577.

Liu, Q., Tackney, C., Bhat, R.A., Prince, A.M., and Zhang, P. (1997). Regulated processing of hepatitis C virus core protein is linked to subcellular localization. J Virol *71*, 657-662.

Liu, X., Wang, T., Wakita, T., and Yang, W. (2010). Systematic identification of microRNA and messenger RNA profiles in hepatitis C virus-infected human hepatoma cells. Virology *398*, 57-67.

Lo, S.Y., Selby, M.J., and Ou, J.H. (1996). Interaction between hepatitis C virus core protein and E1 envelope protein. J Virol *70*, 5177-5182.

Lohmann, V., Korner, F., Dobierzewska, A., and Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol *75*, 1437-1449.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science *285*, 110-113.

Lorenz, I.C., Marcotrigiano, J., Dentzer, T.G., and Rice, C.M. (2006). Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. Nature *442*, 831-835.

Love, R.A., Parge, H.E., Wickersham, J.A., Hostomsky, Z., Habuka, N., Moomaw, E.W., Adachi, T., and Hostomska, Z. (1996). The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell *87*, 331-342.

Lovell, J.F., Billen, L.P., Bindner, S., Shamas-Din, A., Fradin, C., Leber, B., and Andrews, D.W. (2008). Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell *135*, 1074-1084.

Lu, Y.W., Tan, T.L., Chan, V., and Chen, W.N. (2006). The HBSP gene is expressed during HBV replication, and its coded BH3-containing spliced viral protein induces apoptosis in HepG2 cells. Biochem Biophys Res Commun *351*, 64-70.

Lundin, M., Monne, M., Widell, A., Von Heijne, G., and Persson, M.A. (2003). Topology of the membrane-associated hepatitis C virus protein NS4B. J Virol *77*, 5428-5438.

Machida, K., Tsukiyama-Kohara, K., Seike, E., Tone, S., Shibasaki, F., Shimizu, M., Takahashi, H., Hayashi, Y., Funata, N., Taya, C., *et al.* (2001). Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. J Biol Chem *276*, 12140-12146.

Madan, V., Castello, A., and Carrasco, L. (2008). Viroporins from RNA viruses induce caspase-dependent apoptosis. Cell Microbiol *10*, 437-451.

Maillard, P., Krawczynski, K., Nitkiewicz, J., Bronnert, C., Sidorkiewicz, M., Gounon, P., Dubuisson, J., Faure, G., Crainic, R., and Budkowska, A. (2001). Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. J Virol *75*, 8240-8250.

Maiuri, M.C., Criollo, A., Tasdemir, E., Vicencio, J.M., Tajeddine, N., Hickman, J.A., Geneste, O., and Kroemer, G. (2007a). BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L). Autophagy *3*, 374-376.

Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., *et al.* (2007b). Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. Embo J *26*, 2527-2539.

Major, M.E., Dahari, H., Mihalik, K., Puig, M., Rice, C.M., Neumann, A.U., and Feinstone, S.M. (2004). Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. Hepatology *39*, 1709-1720.

Majumder, M., Ghosh, A.K., Steele, R., Zhou, X.Y., Phillips, N.J., Ray, R., and Ray, R.B. (2002). Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology *294*, 94-105.

Malhi, H., and Gores, G.J. (2008). Cellular and molecular mechanisms of liver injury. Gastroenterology *134*, 1641-1654.

Mamiya, N., and Worman, H.J. (1999). Hepatitis C virus core protein binds to a DEAD box RNA helicase. J Biol Chem *274*, 15751-15756.

Mandal, D., Mazumder, A., Das, P., Kundu, M., and Basu, J. (2005). Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. J Biol Chem *280*, 39460-39467.

Marshall, A., Rushbrook, S., Davies, S.E., Morris, L.S., Scott, I.S., Vowler, S.L., Coleman, N., and Alexander, G. (2005). Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection. Gastroenterology *128*, 33-42.

Marshall, W.L., Datta, R., Hanify, K., Teng, E., and Finberg, R.W. (1999). U937 cells overexpressing bcl-xl are resistant to human immunodeficiency virus-1-induced apoptosis and human immunodeficiency virus-1 replication. Virology *256*, 1-7.

Marusawa, H., Hijikata, M., Chiba, T., and Shimotohno, K. (1999). Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J Virol *73*, 4713-4720.

Mateu, G., Donis, R.O., Wakita, T., Bukh, J., and Grakoui, A. (2008). Intragenotypic JFH1 based recombinant hepatitis C virus produces high levels of infectious particles but causes increased cell death. Virology *376*, 397-407.

Matsumoto, M., Hwang, S.B., Jeng, K.S., Zhu, N., and Lai, M.M. (1996). Homotypic interaction and multimerization of hepatitis C virus core protein. Virology *218*, 43-51.

McCaffrey, K., Boo, I., Poumbourios, P., and Drummer, H.E. (2007). Expression and characterization of a minimal hepatitis C virus glycoprotein E2 core domain that retains CD81 binding. J Virol *81*, 9584-9590.

McCaughan, G.W., McGuinness, P.H., Bishop, G.A., Painter, D.M., Lien, A.S., Tulloch, R., Wylie, B.R., and Archer, G.T. (1992). Clinical assessment and incidence of hepatitis C RNA in 50 consecutive RIBA-positive volunteer blood donors. Med J Aust *157*, 231-233.

McHutchison, J.G., Manns, M.P., Muir, A.J., Terrault, N.A., Jacobson, I.M., Afdhal, N.H., Heathcote, E.J., Zeuzem, S., Reesink, H.W., Garg, J., *et al.* (2010). Telaprevir for previously treated chronic HCV infection. N Engl J Med *362*, 1292-1303.

McLauchlan, J. (2000). Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. J Viral Hepat *7*, 2-14.

McLauchlan, J., Lemberg, M.K., Hope, G., and Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. Embo J *21*, 3980-3988.

McLean, J.E., Ruck, A., Shirazian, A., Pooyaei-Mehr, F., and Zakeri, Z.F. (2008). Viral manipulation of cell death. Curr Pharm Des *14*, 198-220.

Memon, S.A., Moreno, M.B., Petrak, D., and Zacharchuk, C.M. (1995). Bcl-2 blocks glucocorticoid- but not Fas- or activation-induced apoptosis in a T cell hybridoma. J Immunol *155*, 4644-4652.

Mengshol, J.A., Golden-Mason, L., and Rosen, H.R. (2007). Mechanisms of Disease: HCV-induced liver injury. Nat Clin Pract Gastroenterol Hepatol *4*, 622-634.

Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., *et al.* (2001). Hepatitis C virus replication in mice with chimeric human livers. Nat Med *7*, 927-933.

Merino, D., Giam, M., Hughes, P.D., Siggs, O.M., Heger, K., O'Reilly, L.A., Adams, J.M., Strasser, A., Lee, E.F., Fairlie, W.D., *et al.* (2009). The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. J Cell Biol *186*, 355-362.

Meyer, K., Basu, A., Saito, K., Ray, R.B., and Ray, R. (2005). Inhibition of hepatitis C virus core protein expression in immortalized human hepatocytes induces cytochrome c-independent increase in Apaf-1 and caspase-9 activation for cell death. Virology *336*, 198-207.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature *437*, 1167-1172.

Mi, J., Li, Z.Y., Ni, S., Steinwaerder, D., and Lieber, A. (2001). Induced apoptosis supports spread of adenovirus vectors in tumors. Hum Gene Ther *12*, 1343-1352.

Mihm, S., Fayyazi, A., Hartmann, H., and Ramadori, G. (1997). Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology *25*, 735-739.

Mita, E., Hayashi, N., Iio, S., Takehara, T., Hijioka, T., Kasahara, A., Fusamoto, H., and Kamada, T. (1994). Role of Fas ligand in apoptosis induced by hepatitis C virus infection. Biochem Biophys Res Commun *204*, 468-474.

Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., and Shimotohno, K. (2007). The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol *9*, 1089-1097.

Mohd-Ismail, N.K., Deng, L., Sukumaran, S.K., Yu, V.C., Hotta, H., and Tan, Y.J. (2009). The hepatitis C virus core protein contains a BH3 domain that regulates apoptosis through specific interaction with human Mcl-1. J Virol *83*, 9993-10006.

Molina, S., Castet, V., Fournier-Wirth, C., Pichard-Garcia, L., Avner, R., Harats, D., Roitelman, J., Barbaras, R., Graber, P., Ghersa, P., *et al.* (2007). The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J Hepatol *46*, 411-419.

Montserret, R., Saint, N., Vanbelle, C., Salvay, A.G., Simorre, J.P., Ebel, C., Sapay, N., Renisio, J.G., Bockmann, A., Steinmann, E., *et al.* (2010). NMR structure and ion channel activity of the p7 protein from hepatitis C virus. J Biol Chem *285*, 31446-31461.

Moorman, J.P., Prayther, D., McVay, D., Hahn, Y.S., and Hahn, C.S. (2003). The C-terminal region of hepatitis C core protein is required for Fas-ligand independent apoptosis in Jurkat cells by facilitating Fas oligomerization. Virology *312*, 320-329.

Moradpour, D., Brass, V., Bieck, E., Friebe, P., Gosert, R., Blum, H.E., Bartenschlager, R., Penin, F., and Lohmann, V. (2004). Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. J Virol *78*, 13278-13284.

Moradpour, D., Englert, C., Wakita, T., and Wands, J.R. (1996). Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology *222*, 51-63.

Morchang, A., Yasamut, U., Netsawang, J., Noisakran, S., Wongwiwat, W., Songprakhon, P., Srisawat, C., Puttikhunt, C., Kasinrerk, W., Malasit, P., *et al.* (2011). Cell death gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis. Virus Res *156*, 25-34.

Moriishi, K., Mochizuki, R., Moriya, K., Miyamoto, H., Mori, Y., Abe, T., Murata, S., Tanaka, K., Miyamura, T., Suzuki, T., *et al.* (2007). Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A *104*, 1661-1666.

Morishima, N. (1999). Changes in nuclear morphology during apoptosis correlate with vimentin cleavage by different caspases located either upstream or downstream of Bcl-2 action. Genes Cells *4*, 401-414.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med *4*, 1065-1067.

Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. (1997). Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol *78 (Pt 7)*, 1527-1531.

Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M.R., Pedrazzini, E., Bonatti, S., and Migliaccio, G. (2002). Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology *293*, 31-43.

Munakata, T., Nakamura, M., Liang, Y., Li, K., and Lemon, S.M. (2005). Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C virus NS5B RNA-dependent RNA polymerase. Proc Natl Acad Sci U S A *102*, 18159-18164.

Murray, C.L., Jones, C.T., Tassello, J., and Rice, C.M. (2007). Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J Virol *81*, 10220-10231.

Nakai, K., Okamoto, T., Kimura-Someya, T., Ishii, K., Lim, C.K., Tani, H., Matsuo, E., Abe, T., Mori, Y., Suzuki, T., *et al.* (2006). Oligomerization of hepatitis C virus core protein is crucial for interaction with the cytoplasmic domain of E1 envelope protein. J Virol *80*, 11265-11273.

Nechushtan, A., Smith, C.L., Lamensdorf, I., Yoon, S.H., and Youle, R.J. (2001). Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. J Cell Biol *153*, 1265-1276.

Neddermann, P., Clementi, A., and De Francesco, R. (1999). Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. J Virol *73*, 9984-9991.

Neddermann, P., Quintavalle, M., Di Pietro, C., Clementi, A., Cerretani, M., Altamura, S., Bartholomew, L., and De Francesco, R. (2004). Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. J Virol *78*, 13306-13314.

Neumann, A.U., Lam, N.P., Dahari, H., Gretch, D.R., Wiley, T.E., Layden, T.J., and Perelson, A.S. (1998). Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science *282*, 103-107.

NIH (2002a). National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C 2002 (June 10-12, 2002). Gastroenterology *123*, 2082-2099.

NIH (2002b). NIH Consensus Statement on Management of Hepatitis C: 2002. NIH Consens State Sci Statements *19*, 1-46.

Nomura-Takigawa, Y., Nagano-Fujii, M., Deng, L., Kitazawa, S., Ishido, S., Sada, K., and Hotta, H. (2006). Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J Gen Virol *87*, 1935-1945.

Oberst, A., Pop, C., Tremblay, A.G., Blais, V., Denault, J.B., Salvesen, G.S., and Green, D.R. (2010). Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. J Biol Chem *285*, 16632-16642.

Ogino, T., Fukuda, H., Imajoh-Ohmi, S., Kohara, M., and Nomoto, A. (2004). Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. J Virol *78*, 11766-11777.

Ohata, K., Hamasaki, K., Toriyama, K., Matsumoto, K., Saeki, A., Yanagi, K., Abiru, S., Nakagawa, Y., Shigeno, M., Miyazoe, S., *et al.* (2003). Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. Cancer *97*, 3036-3043.

Okamoto, K., Mori, Y., Komoda, Y., Okamoto, T., Okochi, M., Takeda, M., Suzuki, T., Moriishi, K., and Matsuura, Y. (2008). Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. J Virol *82*, 8349-8361.

Okazaki, M., Hino, K., Fujii, K., Kobayashi, N., and Okita, K. (1996). Hepatic Fas antigen expression before and after interferon therapy in patients with chronic hepatitis C. Dig Dis Sci *41*, 2453-2458.

Olsen, C.W., Kehren, J.C., Dybdahl-Sissoko, N.R., and Hinshaw, V.S. (1996). bcl-2 alters influenza virus yield, spread, and hemagglutinin glycosylation. J Virol *70*, 663-666.

Op De Beeck, A., Voisset, C., Bartosch, B., Ciczora, Y., Cocquerel, L., Keck, Z., Foung, S., Cosset, F.L., and Dubuisson, J. (2004). Characterization of functional hepatitis C virus envelope glycoproteins. J Virol *78*, 2994-3002.

Otsuka, M., Kato, N., Lan, K., Yoshida, H., Kato, J., Goto, T., Shiratori, Y., and Omata, M. (2000). Hepatitis C virus core protein enhances p53 function through augmentation of DNA binding affinity and transcriptional ability. J Biol Chem *275*, 34122-34130.

Otsuka, M., Kato, N., Taniguchi, H., Yoshida, H., Goto, T., Shiratori, Y., and Omata, M. (2002). Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. Virology *296*, 84-93.

Owen, D.M., Huang, H., Ye, J., and Gale, M., Jr. (2009). Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology *394*, 99-108.

Owsianka, A.M., and Patel, A.H. (1999). Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology *257*, 330-340.

Pallaoro, M., Lahm, A., Biasiol, G., Brunetti, M., Nardella, C., Orsatti, L., Bonelli, F., Orru, S., Narjes, F., and Steinkuhler, C. (2001). Characterization of the hepatitis C virus NS2/3 processing reaction by using a purified precursor protein. J Virol *75*, 9939-9946.

Pang, P.S., Jankowsky, E., Planet, P.J., and Pyle, A.M. (2002). The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. Embo J *21*, 1168-1176.

Panickar, K.S., Nonner, D., and Barrett, J.N. (2005). Overexpression of Bcl-xl protects septal neurons from prolonged hypoglycemia and from acute ischemia-like stress. Neuroscience *135*, 73-80.

Park, C.Y., Jun, H.J., Wakita, T., Cheong, J.H., and Hwang, S.B. (2009). Hepatitis C virus nonstructural 4B protein modulates sterol regulatory element-binding protein signaling via the AKT pathway. J Biol Chem *284*, 9237-9246.

Park, K.J., Choi, S.H., Choi, D.H., Park, J.M., Yie, S.W., Lee, S.Y., and Hwang, S.B. (2003). Hepatitis C virus NS5A protein modulates c-Jun N-terminal kinase through interaction with tumor necrosis factor receptor-associated factor 2. J Biol Chem *278*, 30711-30718.

Pavio, N., Battaglia, S., Boucreux, D., Arnulf, B., Sobesky, R., Hermine, O., and Brechot, C. (2005). Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-beta pathway. Oncogene *24*, 6119-6132.

Pavio, N., Taylor, D.R., and Lai, M.M. (2002). Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J Virol *76*, 1265-1272.

Penin, F., Brass, V., Appel, N., Ramboarina, S., Montserret, R., Ficheux, D., Blum, H.E., Bartenschlager, R., and Moradpour, D. (2004). Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J Biol Chem *279*, 40835-40843.

Pestka, J.M., Zeisel, M.B., Blaser, E., Schurmann, P., Bartosch, B., Cosset, F.L., Patel, A.H., Meisel, H., Baumert, J., Viazov, S., *et al.* (2007). Rapid

induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc Natl Acad Sci U S A *104*, 6025-6030.

Petros, A.M., Olejniczak, E.T., and Fesik, S.W. (2004). Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta *1644*, 83-94.

Phan, T., Kohlway, A., Dimberu, P., Pyle, A.M., and Lindenbach, B.D. (2011). The acidic domain of hepatitis C virus NS4A contributes to RNA replication and virus particle assembly. J Virol *85*, 1193-1204.

Pianko, S., Patella, S., Ostapowicz, G., Desmond, P., and Sievert, W. (2001). Fas-mediated hepatocyte apoptosis is increased by hepatitis C virus infection and alcohol consumption, and may be associated with hepatic fibrosis: mechanisms of liver cell injury in chronic hepatitis C virus infection. J Viral Hepat *8*, 406-413.

Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K.D., and McCarthy, J.E. (2002). Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J Biol Chem *277*, 45670-45679.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F.L., *et al.* (2006). Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A *103*, 7408-7413.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D., and Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol *76*, 4008-4021.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A.J., Houghton, M., Rosa, D., Grandi, G., *et al.* (1998). Binding of hepatitis C virus to CD81. Science *282*, 938-941.

Piodi, A., Chouteau, P., Lerat, H., Hezode, C., and Pawlotsky, J.M. (2008). Morphological changes in intracellular lipid droplets induced by different hepatitis C virus genotype core sequences and relationship with steatosis. Hepatology *48*, 16-27.

Ploss, A., Evans, M.J., Gaysinskaya, V.A., Panis, M., You, H., de Jong, Y.P., and Rice, C.M. (2009). Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature *457*, 882-886.

Pokrovskii, M.V., Bush, C.O., Beran, R.K., Robinson, M.F., Cheng, G., Tirunagari, N., Fenaux, M., Greenstein, A.E., Zhong, W., Delaney, W.E.t., *et al.* (2011). Novel mutations in a tissue culture-adapted hepatitis C virus strain improve infectious-virus stability and markedly enhance infection kinetics. J Virol *85*, 3978-3985.

Polster, B.M., Pevsner, J., and Hardwick, J.M. (2004). Viral Bcl-2 homologs and their role in virus replication and associated diseases. Biochim Biophys Acta *1644*, 211-227.

Pop, C., and Salvesen, G.S. (2009). Human caspases: activation, specificity, and regulation. J Biol Chem *284*, 21777-21781.

Prikhod'ko, E.A., Prikhod'ko, G.G., Siegel, R.M., Thompson, P., Major, M.E., and Cohen, J.I. (2004). The NS3 protein of hepatitis C virus induces caspase-8-mediated apoptosis independent of its protease or helicase activities. Virology *329*, 53-67.

Prince, A.M., Brotman, B., Grady, G.F., Kuhns, W.J., Hazzi, C., Levine, R.W., and Millian, S.J. (1974). Long-incubation post-transfusion hepatitis without serological evidence of exposure to hepatitis-B virus. Lancet *2*, 241-246.

Prokop, A., Wrasidlo, W., Lode, H., Herold, R., Lang, F., Henze, G., Dorken, B., Wieder, T., and Daniel, P.T. (2003). Induction of apoptosis by enediyne antibiotic calicheamicin thetaII proceeds through a caspase-mediated mitochondrial amplification loop in an entirely Bax-dependent manner. Oncogene *22*, 9107-9120.

Qin, W., Luo, H., Nomura, T., Hayashi, N., Yamashita, T., and Murakami, S. (2002). Oligomeric interaction of hepatitis C virus NS5B is critical for catalytic activity of RNA-dependent RNA polymerase. J Biol Chem *277*, 2132-2137.

Quinsay, M.N., Lee, Y., Rikka, S., Sayen, M.R., Molkentin, J.D., Gottlieb, R.A., and Gustafsson, A.B. (2010). Bnip3 mediates permeabilization of mitochondria and release of cytochrome c via a novel mechanism. J Mol Cell Cardiol *48*, 1146-1156.

Rahmani, Z., Huh, K.W., Lasher, R., and Siddiqui, A. (2000). Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. J Virol *74*, 2840-2846.

Ray, R.B., Lagging, L.M., Meyer, K., and Ray, R. (1996). Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol *70*, 4438-4443.

Ray, R.B., Meyer, K., and Ray, R. (2000). Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. Virology *271*, 197-204.

Ray, R.B., and Ray, R. (2001). Hepatitis C virus core protein: intriguing properties and functional relevance. FEMS Microbiol Lett *202*, 149-156.

Realdon, S., Gerotto, M., Dal Pero, F., Marin, O., Granato, A., Basso, G., Muraca, M., and Alberti, A. (2004). Proapoptotic effect of hepatitis C virus

CORE protein in transiently transfected cells is enhanced by nuclear localization and is dependent on PKR activation. J Hepatol *40*, 77-85.

Riedl, S.J., and Salvesen, G.S. (2007). The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol *8*, 405-413.

Rouille, Y., Helle, F., Delgrange, D., Roingeard, P., Voisset, C., Blanchard, E., Belouzard, S., McKeating, J., Patel, A.H., Maertens, G., *et al.* (2006). Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. J Virol *80*, 2832-2841.

Rubbia-Brandt, L., Quadri, R., Abid, K., Giostra, E., Male, P.J., Mentha, G., Spahr, L., Zarski, J.P., Borisch, B., Hadengue, A., *et al.* (2000). Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol *33*, 106-115.

Ruggieri, A., Harada, T., Matsuura, Y., and Miyamura, T. (1997). Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology *229*, 68-76.

Sabile, A., Perlemuter, G., Bono, F., Kohara, K., Demaugre, F., Kohara, M., Matsuura, Y., Miyamura, T., Brechot, C., and Barba, G. (1999). Hepatitis C virus core protein binds to apolipoprotein AII and its secretion is modulated by fibrates. Hepatology *30*, 1064-1076.

Sacco, R., Tsutsumi, T., Suzuki, R., Otsuka, M., Aizaki, H., Sakamoto, S., Matsuda, M., Seki, N., Matsuura, Y., Miyamura, T., *et al.* (2003). Antiapoptotic regulation by hepatitis C virus core protein through up-regulation of inhibitor of caspase-activated DNase. Virology *317*, 24-35.

Saito, K., Meyer, K., Warner, R., Basu, A., Ray, R.B., and Ray, R. (2006). Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. J Virol *80*, 4372-4379.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.

Sakai, A., Claire, M.S., Faulk, K., Govindarajan, S., Emerson, S.U., Purcell, R.H., and Bukh, J. (2003). The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc Natl Acad Sci U S A *100*, 11646-11651.

Santolini, E., Migliaccio, G., and La Monica, N. (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. J Virol *68*, 3631-3641.

Santolini, E., Pacini, L., Fipaldini, C., Migliaccio, G., and Monica, N. (1995). The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J Virol *69*, 7461-7471.

Sarcar, B., Ghosh, A.K., Steele, R., Ray, R., and Ray, R.B. (2004). Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. Virology *322*, 51-60.

Sarfraz, S., Hamid, S., Ali, S., Jafri, W., and Siddiqui, A.A. (2009). Modulations of cell cycle checkpoints during HCV associated disease. BMC Infect Dis *9*, 125.

Sarfraz, S., Hamid, S., Siddiqui, A., Hussain, S., Pervez, S., and Alexander, G. (2008). Altered expression of cell cycle and apoptotic proteins in chronic hepatitis C virus infection. BMC Microbiol *8*, 133.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. Embo J *17*, 1675-1687.

Scallan, M.F., Allsopp, T.E., and Fazakerley, J.K. (1997). bcl-2 acts early to restrict Semliki Forest virus replication and delays virus-induced programmed cell death. J Virol *71*, 1583-1590.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R.M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R., and Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. Embo J *21*, 5017-5025.

Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C.M., Blum, H.E., and Moradpour, D. (2001). Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J Biol Chem *276*, 44052-44063.

Schofield, D.J., Bartosch, B., Shimizu, Y.K., Allander, T., Alter, H.J., Emerson, S.U., Cosset, F.L., and Purcell, R.H. (2005). Human monoclonal antibodies that react with the E2 glycoprotein of hepatitis C virus and possess neutralizing activity. Hepatology *42*, 1055-1062.

Schulze-Osthoff, K., Walczak, H., Droge, W., and Krammer, P.H. (1994). Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol *127*, 15-20.

Schwarz, A.K., Grove, J., Hu, K., Mee, C.J., Balfe, P., and McKeating, J.A. (2009). Hepatoma cell density promotes claudin-1 and scavenger receptor BI expression and hepatitis C virus internalization. J Virol *83*, 12407-12414.

Sevilla, L., Zaldumbide, A., Pognonec, P., and Boulukos, K.E. (2001). Transcriptional regulation of the bcl-x gene encoding the anti-apoptotic Bcl-xL protein by Ets, Rel/NFkappaB, STAT and AP1 transcription factor families. Histol Histopathol *16*, 595-601.

Shan, Y., Chen, X.G., Huang, B., Hu, A.B., Xiao, D., and Guo, Z.M. (2005). Malignant transformation of the cultured human hepatocytes induced by hepatitis C virus core protein. Liver Int *25*, 141-147.

Shi, S.T., Polyak, S.J., Tu, H., Taylor, D.R., Gretch, D.R., and Lai, M.M. (2002). Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology *292*, 198-210.

Shibue, T., and Taniguchi, T. (2006). BH3-only proteins: integrated control point of apoptosis. Int J Cancer *119*, 2036-2043.

Shimakami, T., Hijikata, M., Luo, H., Ma, Y.Y., Kaneko, S., Shimotohno, K., and Murakami, S. (2004). Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. J Virol *78*, 2738-2748.

Shimazu, T., Degenhardt, K., Nur, E.K.A., Zhang, J., Yoshida, T., Zhang, Y., Mathew, R., White, E., and Inouye, M. (2007). NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition. Genes Dev *21*, 929-941.

Shirota, Y., Luo, H., Qin, W., Kaneko, S., Yamashita, T., Kobayashi, K., and Murakami, S. (2002). Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J Biol Chem *277*, 11149-11155.

Siavoshian, S., Abraham, J.D., Kieny, M.P., and Schuster, C. (2004). HCV core, NS3, NS5A and NS5B proteins modulate cell proliferation independently from p53 expression in hepatocarcinoma cell lines. Arch Virol *149*, 323-336.

Simmonds, P. (2004). Genetic diversity and evolution of hepatitis C virus--15 years on. J Gen Virol *85*, 3173-3188.

Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., *et al.* (2005). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology *42*, 962-973.

Simmonds, P., Holmes, E.C., Cha, T.A., Chan, S.W., McOmish, F., Irvine, B., Beall, E., Yap, P.L., Kolberg, J., and Urdea, M.S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol *74 (Pt 11)*, 2391-2399.

Simonin, Y., Disson, O., Lerat, H., Antoine, E., Biname, F., Rosenberg, A.R., Desagher, S., Lassus, P., Bioulac-Sage, P., and Hibner, U. (2009). Calpain activation by hepatitis C virus proteins inhibits the extrinsic apoptotic signaling pathway. Hepatology *50*, 1370-1379.

Sir, D., Chen, W.L., Choi, J., Wakita, T., Yen, T.S., and Ou, J.H. (2008). Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. Hepatology *48*, 1054-1061.

Soguero, C., Joo, M., Chianese-Bullock, K.A., Nguyen, D.T., Tung, K., and Hahn, Y.S. (2002). Hepatitis C virus core protein leads to immune suppression and liver damage in a transgenic murine model. J Virol *76*, 9345-9354.

Song, Y., Friebe, P., Tzima, E., Junemann, C., Bartenschlager, R., and Niepmann, M. (2006). The hepatitis C virus RNA 3'-untranslated region strongly enhances translation directed by the internal ribosome entry site. J Virol *80*, 11579-11588.

Soo, H.M., Garzino-Demo, A., Hong, W., Tan, Y.H., Tan, Y.J., Goh, P.Y., Lim, S.G., and Lim, S.P. (2002). Expression of a full-length hepatitis C virus cDNA up-regulates the expression of CC chemokines MCP-1 and RANTES. Virology *303*, 253-277.

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H., and Walczak, H. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity *12*, 599-609.

Steinmann, E., Penin, F., Kallis, S., Patel, A.H., Bartenschlager, R., and Pietschmann, T. (2007). Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. PLoS Pathog *3*, e103.

Strosberg, A.D., Kota, S., Takahashi, V., Snyder, J.K., and Mousseau, G. (2010). Core as a novel viral target for hepatitis C drugs. Viruses *2*, 1734-1751.

Subramanian, T., Vijayalingam, S., Lomonosova, E., Zhao, L.J., and Chinnadurai, G. (2007). Evidence for involvement of BH3-only proapoptotic members in adenovirus-induced apoptosis. J Virol *81*, 10486-10495.

Tabor, E., Gerety, R.J., Drucker, J.A., Seeff, L.B., Hoofnagle, J.H., Jackson, D.R., April, M., Barker, L.F., and Pineda-Tamondong, G. (1978). Transmission of non-A, non-B hepatitis from man to chimpanzee. Lancet *1*, 463-466.

Tai, C.L., Chi, W.K., Chen, D.S., and Hwang, L.H. (1996). The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). J Virol *70*, 8477-8484.

Tai, D.I., Tsai, S.L., Chen, Y.M., Chuang, Y.L., Peng, C.Y., Sheen, I.S., Yeh, C.T., Chang, K.S., Huang, S.N., Kuo, G.C., *et al.* (2000). Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. Hepatology *31*, 656-664.

Tait, S.W., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol *11*, 621-632.

Takamatsu, M., Fujita, T., and Hotta, H. (2001). Suppression of serum starvation-induced apoptosis by hepatitis C virus core protein. Kobe J Med Sci *47*, 97-112.

Takehara, T., Liu, X., Fujimoto, J., Friedman, S.L., and Takahashi, H. (2001). Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology *34*, 55-61.

Takehara, T., Tatsumi, T., Suzuki, T., Rucker, E.B., 3rd, Hennighausen, L., Jinushi, M., Miyagi, T., Kanazawa, Y., and Hayashi, N. (2004). Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology *127*, 1189-1197.

Tan, K.O., Tan, K.M., Chan, S.L., Yee, K.S., Bevort, M., Ang, K.C., and Yu, V.C. (2001). MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. J Biol Chem *276*, 2802-2807.

Tanaka, M., Nagano-Fujii, M., Deng, L., Ishido, S., Sada, K., and Hotta, H. (2006). Single-point mutations of hepatitis C virus NS3 that impair p53 interaction and anti-apoptotic activity of NS3. Biochem Biophys Res Commun *340*, 792-799.

Tanaka, T., Kato, N., Cho, M.J., Sugiyama, K., and Shimotohno, K. (1996). Structure of the 3' terminus of the hepatitis C virus genome. J Virol *70*, 3307-3312.

Tanji, Y., Hijikata, M., Satoh, S., Kaneko, T., and Shimotohno, K. (1995). Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J Virol *69*, 1575-1581.

Tansey, M.G., and Szymkowski, D.E. (2009). The TNF superfamily in 2009: new pathways, new indications, and new drugs. Drug Discov Today *14*, 1082-1088.

Targett-Adams, P., Hope, G., Boulant, S., and McLauchlan, J. (2008). Maturation of hepatitis C virus core protein by signal peptide peptidase is required for virus production. J Biol Chem *283*, 16850-16859.

Tasaka, M., Sakamoto, N., Itakura, Y., Nakagawa, M., Itsui, Y., Sekine-Osajima, Y., Nishimura-Sakurai, Y., Chen, C.H., Yoneyama, M., Fujita, T., *et al.* (2007). Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response. J Gen Virol *88*, 3323-3333.

Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N., and Lai, M.M. (1999). Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science *285*, 107-110.

Tedbury, P., Welbourn, S., Pause, A., King, B., Griffin, S., and Harris, M. (2011). The subcellular localization of the hepatitis C virus non-structural protein NS2 is regulated by an ion channel-independent function of the p7 protein. J Gen Virol *92*, 819-830.

Tellinghuisen, T.L., Marcotrigiano, J., Gorbalenya, A.E., and Rice, C.M. (2004). The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem *279*, 48576-48587.

Tellinghuisen, T.L., Marcotrigiano, J., and Rice, C.M. (2005). Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature *435*, 374-379.

Thiel, H.J., Collett, M. S., Gould, E. A., Heinz, F. X., Houghton, M., Meyers, G., Purcell, R. H., and Rice, C. M. (2005). Family Flaviviridae. In Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses, C.M. Fauquet, Mayo, M. A., Maniloff, J., Desselberger, U., Ball, L. A., ed. (San Diego, Academic Press), pp. 979-996.

Thimme, R., Oldach, D., Chang, K.M., Steiger, C., Ray, S.C., and Chisari, F.V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med *194*, 1395-1406.

Thompson, A.A., Zou, A., Yan, J., Duggal, R., Hao, W., Molina, D., Cronin, C.N., and Wells, P.A. (2009). Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. Biochemistry *48*, 906-916.

Thomssen, R., Bonk, S., Propfe, C., Heermann, K.H., Kochel, H.G., and Uy, A. (1992). Association of hepatitis C virus in human sera with beta-lipoprotein. Med Microbiol Immunol *181*, 293-300.

Thomssen, R., Bonk, S., and Thiele, A. (1993). Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. Med Microbiol Immunol *182*, 329-334.

Thoren, F., Romero, A., Lindh, M., Dahlgren, C., and Hellstrand, K. (2004). A hepatitis C virus-encoded, nonstructural protein (NS3) triggers dysfunction and apoptosis in lymphocytes: role of NADPH oxidase-derived oxygen radicals. J Leukoc Biol *76*, 1180-1186.

Timmer, J.C., and Salvesen, G.S. (2007). Caspase substrates. Cell Death Differ *14*, 66-72.

Timpe, J.M., Stamataki, Z., Jennings, A., Hu, K., Farquhar, M.J., Harris, H.J., Schwarz, A., Desombere, I., Roels, G.L., Balfe, P., *et al.* (2008). Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology *47*, 17-24.

Tong, T., Ji, J., Jin, S., Li, X., Fan, W., Song, Y., Wang, M., Liu, Z., Wu, M., and Zhan, Q. (2005). Gadd45a expression induces Bim dissociation from the cytoskeleton and translocation to mitochondria. Mol Cell Biol *25*, 4488-4500.

Tong, W.Y., Nagano-Fujii, M., Hidajat, R., Deng, L., Takigawa, Y., and Hotta, H. (2002). Physical interaction between hepatitis C virus NS4B protein and CREB-RP/ATF6beta. Biochem Biophys Res Commun *299*, 366-372.

Tsutsumi, T., Suzuki, T., Shimoike, T., Suzuki, R., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K., and Miyamura, T. (2002). Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. Hepatology *35*, 937-946.

Tumurbaatar, B., Sun, Y., Chan, T., and Sun, J. (2007). Cre-estrogen receptor-mediated hepatitis C virus structural protein expression in mice. J Virol Methods *146*, 5-13.

Uhlmann, E.J., Subramanian, T., Vater, C.A., Lutz, R., and Chinnadurai, G. (1998). A potent cell death activity associated with transient high level expression of BCL-2. J Biol Chem *273*, 17926-17932.

Uren, R.T., Dewson, G., Chen, L., Coyne, S.C., Huang, D.C., Adams, J.M., and Kluck, R.M. (2007). Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. J Cell Biol *177*, 277-287.

Valli, M.B., Crema, A., Lanzilli, G., Serafino, A., Bertolini, L., Ravagnan, G., Ponzetto, A., Menzo, S., Clementi, M., and Carloni, G. (2007). Molecular and cellular determinants of cell-to-cell transmission of HCV in vitro. J Med Virol *79*, 1491-1499.

van Delft, M.F., and Huang, D.C. (2006). How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res *16*, 203-213.

Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R., and Greenberg, A.H. (2000). BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol *20*, 5454-5468.

Vassilaki, N., and Mavromara, P. (2009). The HCV ARFP/F/core+1 protein: production and functional analysis of an unconventional viral product. IUBMB Life *61*, 739-752.

Vieyres, G., Dubuisson, J., and Patel, A.H. (2011). Characterization of antibody-mediated neutralization directed against the hypervariable region 1 of hepatitis C virus E2 glycoprotein. J Gen Virol *92*, 494-506.

Vieyres, G., Thomas, X., Descamps, V., Duverlie, G., Patel, A.H., and Dubuisson, J. (2010). Characterization of the envelope glycoproteins associated with infectious hepatitis C virus. J Virol *84*, 10159-10168.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., *et al.* (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med *11*, 791-796.

Walensky, L.D. (2006). BCL-2 in the crosshairs: tipping the balance of life and death. Cell Death Differ *13*, 1339-1350.

Walewski, J.L., Keller, T.R., Stump, D.D., and Branch, A.D. (2001). Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. Rna *7*, 710-721.

Walters, K.A., Syder, A.J., Lederer, S.L., Diamond, D.L., Paeper, B., Rice, C.M., and Katze, M.G. (2009). Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. PLoS Pathog *5*, e1000269.

Wang, Q.M., Hockman, M.A., Staschke, K., Johnson, R.B., Case, K.A., Lu, J., Parsons, S., Zhang, F., Rathnachalam, R., Kirkegaard, K., *et al.* (2002). Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. J Virol *76*, 3865-3872.

Watanabe, J., Kushihata, F., Honda, K., Mominoki, K., Matsuda, S., and Kobayashi, N. (2002). Bcl-xL overexpression in human hepatocellular carcinoma. Int J Oncol *21*, 515-519.

Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y., and Shimotohno, K. (2005). Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell *19*, 111-122.

Waterhouse, N.J., Clarke, C.J., Sedelies, K.A., Teng, M.W., and Trapani, J.A. (2004). Cytotoxic lymphocytes; instigators of dramatic target cell death. Biochem Pharmacol *68*, 1033-1040.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B., and Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev *14*, 2060-2071.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Weiner, A.J., Brauer, M.J., Rosenblatt, J., Richman, K.H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q.L., Houghton, M., *et al.* (1991). Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology *180*, 842-848.

Westphal, D., Dewson, G., Czabotar, P.E., and Kluck, R.M. (2011). Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta *1813*, 521-531.

White, E. (2006). Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis. Cell Death Differ *13*, 1371-1377.

WHO (1997). Hepatitis C: global prevalence. Wkly Epidemiol Rec *72*, 341-344.

WHO (2000). Hepatitis C--global prevalence (update). Wkly Epidemiol Rec *75*, 18-19.

WHO (2009). Viral Cancers: Hepatitis C Virus. World Health Organization, Geneva, Switzerland. http://www.who.int/vaccine_research/diseases/viral_cancers/en/index2.html.

WHO (2011). Hepatitis C. World Health Organization, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs164/en/.

Willis, S.N., and Adams, J.M. (2005). Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol *17*, 617-625.

Willis, S.N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J.I., Adams, J.M., and Huang, D.C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev *19*, 1294-1305.

Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P., *et al.* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.

Wolk, B., Sansonno, D., Krausslich, H.G., Dammacco, F., Rice, C.M., Blum, H.E., and Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. J Virol *74*, 2293-2304.

Wong, W.W., and Puthalakath, H. (2008). Bcl-2 family proteins: the sentinels of the mitochondrial apoptosis pathway. IUBMB Life *60*, 390-397.

Xu, J., Liu, S., Xu, Y., Tien, P., and Gao, G. (2009). Identification of the nonstructural protein 4B of hepatitis C virus as a factor that inhibits the antiviral activity of interferon-alpha. Virus Res *141*, 55-62.

Yamaga, A.K., and Ou, J.H. (2002). Membrane topology of the hepatitis C virus NS2 protein. J Biol Chem *277*, 33228-33234.

Yamaguchi, A., Tazuma, S., Nishioka, T., Ohishi, W., Hyogo, H., Nomura, S., and Chayama, K. (2005). Hepatitis C virus core protein modulates fatty acid metabolism and thereby causes lipid accumulation in the liver. Dig Dis Sci *50*, 1361-1371.

Yamanaka, T., Uchida, M., and Doi, T. (2002). Innate form of HCV core protein plays an important role in the localization and the function of HCV core protein. Biochem Biophys Res Commun *294*, 521-527.

Yamashita, T., Kaneko, S., Shirota, Y., Qin, W., Nomura, T., Kobayashi, K., and Murakami, S. (1998). RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. J Biol Chem *273*, 15479-15486.

Yang, F., Robotham, J.M., Nelson, H.B., Irsigler, A., Kenworthy, R., and Tang, H. (2008). Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. J Virol *82*, 5269-5278.

Yang, X.J., Liu, J., Ye, L., Liao, Q.J., Wu, J.G., Gao, J.R., She, Y.L., Wu, Z.H., and Ye, L.B. (2006). HCV NS2 protein inhibits cell proliferation and induces cell cycle arrest in the S-phase in mammalian cells through down-regulation of cyclin A expression. Virus Res *121*, 134-143.

Yao, K., and Vakharia, V.N. (2001). Induction of apoptosis in vitro by the 17-kDa nonstructural protein of infectious bursal disease virus: possible role in viral pathogenesis. Virology *285*, 50-58.

Yao, N., Hesson, T., Cable, M., Hong, Z., Kwong, A.D., Le, H.V., and Weber, P.C. (1997). Structure of the hepatitis C virus RNA helicase domain. Nat Struct Biol *4*, 463-467.

Yao, Z.Q., Nguyen, D.T., Hiotellis, A.I., and Hahn, Y.S. (2001). Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. J Immunol *167*, 5264-5272.

Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S.I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J.R., and Kohara, M. (1998). The native form and maturation process of hepatitis C virus core protein. J Virol *72*, 6048-6055.

Yi, M., Ma, Y., Yates, J., and Lemon, S.M. (2007). Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J Virol *81*, 629-638.

Yoshida, T., Hanada, T., Tokuhisa, T., Kosai, K., Sata, M., Kohara, M., and Yoshimura, A. (2002). Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. J Exp Med *196*, 641-653.

You, L.R., Chen, C.M., Yeh, T.S., Tsai, T.Y., Mai, R.T., Lin, C.H., and Lee, Y.H. (1999). Hepatitis C virus core protein interacts with cellular putative RNA helicase. J Virol *73*, 2841-2853.

You, S., Stump, D.D., Branch, A.D., and Rice, C.M. (2004). A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. J Virol *78*, 1352-1366.

Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*, 47-59.

Yu, G.Y., Lee, K.J., Gao, L., and Lai, M.M. (2006). Palmitoylation and polymerization of hepatitis C virus NS4B protein. J Virol *80*, 6013-6023.

Yu, K.L., Jang, S.I., and You, J.C. (2009). Identification of in vivo interaction between Hepatitis C Virus core protein and 5' and 3' UTR RNA. Virus Res *145*, 285-292.

Zampino, R., Ingrosso, D., Durante-Mangoni, E., Capasso, R., Tripodi, M.F., Restivo, L., Zappia, V., Ruggiero, G., and Adinolfi, L.E. (2008). Microsomal triglyceride transfer protein (MTP) -493G/T gene polymorphism contributes to fat liver accumulation in HCV genotype 3 infected patients. J Viral Hepat *15*, 740-746.

Zeisel, M.B., Koutsoudakis, G., Schnober, E.K., Haberstroh, A., Blum, H.E., Cosset, F.L., Wakita, T., Jaeck, D., Doffoel, M., Royer, C., *et al.* (2007). Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology *46*, 1722-1731.

Zekri, A.R., Bahnassy, A.A., Hafez, M.M., Hassan, Z.K., Kamel, M., Loutfy, S.A., Sherif, G.M., El-Zayadi, A.R., and Daoud, S.S. (2011). Characterization of chronic HCV infection-induced apoptosis. Comp Hepatol *10*, 4.

Zender, L., Hutker, S., Mundt, B., Waltemathe, M., Klein, C., Trautwein, C., Malek, N.P., Manns, M.P., Kuhnel, F., and Kubicka, S. (2005). NFkappaB-mediated upregulation of bcl-xl restrains TRAIL-mediated apoptosis in murine viral hepatitis. Hepatology *41*, 280-288.

Zeuzem, S. (2004). Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C: who responds less well? Ann Intern Med *140*, 370-381.

Zhan, S.S., Jiang, J.X., Wu, J., Halsted, C., Friedman, S.L., Zern, M.A., and Torok, N.J. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology *43*, 435-443.

Zhang, J., and Ney, P.A. (2009). Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ *16*, 939-946.

Zhang, L., Yu, J., Park, B.H., Kinzler, K.W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. Science *290*, 989-992.

Zhang, N., Hartig, H., Dzhagalov, I., Draper, D., and He, Y.W. (2005). The role of apoptosis in the development and function of T lymphocytes. Cell Res *15*, 749-769.

Zhao, X., Ogunwobi, O.O., and Liu, C. (2011). Survivin inhibition is critical for bcl-2 inhibitor-induced apoptosis in hepatocellular carcinoma cells. PLoS One *6*, e21980.

Zheng, X.M., Wang, Y., and Pallen, C.J. (1992). Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. Nature *359*, 336-339.

Zheng, Y., Gao, B., Ye, L., Kong, L., Jing, W., Yang, X., and Wu, Z. (2005). Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. J Microbiol *43*, 529-536.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., and Chisari, F.V. (2005). Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A *102*, 9294-9299.

Zhu, H., Dong, H., Eksioglu, E., Hemming, A., Cao, M., Crawford, J.M., Nelson, D.R., and Liu, C. (2007). Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. Gastroenterology *133*, 1649-1659.

Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M.M. (1998). Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. J Virol *72*, 3691-3697.

Zhu, N., Ware, C.F., and Lai, M.M. (2001). Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. Virology *283*, 178-187.

APPENDICES

Appendix 1: Gene table of the Human Apoptosis RT^2 Profiler PCR Array

Position	Unigene	GeneBank	Symbol	Description
A01	Hs.431048	NM_005157	ABL1	C-abl oncogene 1, receptor tyrosine kinase
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1
A03	Hs.552567	NM_001160	APAF1	Apoptotic peptidase activating factor 1
A04	Hs.370254	NM_004322	BAD	BCL2-associated agonist of cell death
A05	Hs.377484	NM_004323	BAG1	BCL2-associated athanogene
A06	Hs.523309	NM_004281	BAG3	BCL2-associated athanogene 3
A07	Hs.194726	NM_004874	BAG4	BCL2-associated athanogene 4
A08	Hs.485139	NM_001188	BAK1	BCL2-antagonist/killer 1
A09	Hs.624291	NM_004324	BAX	BCL2-associated X protein
A10	Hs.193516	NM_003921	BCL10	B-cell CLL/lymphoma 10
A11	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2
A12	Hs.227817	NM_004049	BCL2A1	BCL2-related protein A1
B01	Hs.516966	NM_138578	BCL2L1	BCL2-like 1
B02	Hs.283672	NM_020396	BCL2L10	BCL2-like 10 (apoptosis facilitator)
B03	Hs.469658	NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)
B04	Hs.410026	NM_004050	BCL2L2	BCL2-like 2
B05	Hs.486542	NM_014739	BCLAF1	BCL2-associated transcription factor 1
B06	Hs.435556	NM_016561	BFAR	Bifunctional apoptosis regulator
B07	Hs.591054	NM_001196	BID	BH3 interacting domain death agonist
B08	Hs.475055	NM_001197	BIK	BCL2-interacting killer (apoptosis-inducing)

Position	Unigene	GeneBank	Symbol	Description
B09	Hs.710305	NM_004536	NAIP	NLR family, apoptosis inhibitory protein
B10	Hs.696238	NM_001166	BIRC2	Baculoviral IAP repeat-containing 2
B11	Hs.127799	NM_001165	BIRC3	Baculoviral IAP repeat-containing 3
B12	Hs.356076	NM_001167	XIAP	X-linked inhibitor of apoptosis
C01	Hs.150107	NM_016252	BIRC6	Baculoviral IAP repeat-containing 6
C02	Hs.348263	NM_033341	BIRC8	Baculoviral IAP repeat-containing 8
C03	Hs.145726	NM_001205	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1
C04	Hs.646490	NM_004330	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2
C05	Hs.144873	NM_004052	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
C06	Hs.131226	NM_004331	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
C07	Hs.550061	NM_004333	BRAF	V-raf murine sarcoma viral oncogene homolog B1
C08	Hs.405153	NM_006092	NOD1	Nucleotide-binding oligomerization domain containing 1
C09	Hs.200242	NM_032587	CARD6	Caspase recruitment domain family, member 6
C10	Hs.446146	NM_014959	CARD8	Caspase recruitment domain family, member 8
C11	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
C12	Hs.5353	NM_001230	CASP10	Caspase 10, apoptosis-related cysteine peptidase
D01	Hs.466057	NM_012114	CASP14	Caspase 14, apoptosis-related cysteine peptidase
D02	Hs.368982	NM_032982	CASP2	Caspase 2, apoptosis-related cysteine peptidase
D03	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase
D04	Hs.138378	NM_001225	CASP4	Caspase 4, apoptosis-related cysteine peptidase
D05	Hs.213327	NM_004347	CASP5	Caspase 5, apoptosis-related cysteine peptidase
D06	Hs.654616	NM_032992	CASP6	Caspase 6, apoptosis-related cysteine peptidase
D07	Hs.9216	NM_001227	CASP7	Caspase 7, apoptosis-related cysteine peptidase
D08	Hs.599762	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase
D09	Hs.329502	NM_001229	CASP9	Caspase 9, apoptosis-related cysteine peptidase

Position	Unigene	GeneBank	Symbol	Description
D10	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5
D11	Hs.592244	NM_000074	CD40LG	CD40 ligand
D12	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator
E01	Hs.249129	NM_001279	CIDEA	Cell death-inducing DFFA-like effector a
E02	Hs.642693	NM_014430	CIDEB	Cell death-inducing DFFA-like effector b
E03	Hs.38533	NM_003805	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
E04	Hs.380277	NM_004938	DAPK1	Death-associated protein kinase 1
E05	Hs.484782	NM_004401	DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide
E06	Hs.86131	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain
E07	Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)
E08	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
E09	Hs.80409	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha
E10	Hs.87247	NM_003806	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)
E11	Hs.643120	NM_000875	IGF1R	Insulin-like growth factor 1 receptor
E12	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
F01	Hs.1116	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)
F02	Hs.632486	NM_021960	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)
F03	Hs.513667	NM_003946	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)
F04	Hs.499094	NM_013258	PYCARD	PYD and CARD domain containing
F05	Hs.103755	NM_003821	RIPK2	Receptor-interacting serine-threonine kinase 2
F06	Hs.241570	NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)
F07	Hs.591834	NM_003844	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a
F08	Hs.521456	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
F09	Hs.81791	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b
F10	Hs.279594	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A

Position	Unigene	GeneBank	Symbol	Description
F11	Hs.443577	NM_014452	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21
F12	Hs.462529	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25
G01	Hs.355307	NM_001242	CD27	CD27 molecule
G02	Hs.654459	NM_001561	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9
G03	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
G04	Hs.501497	NM_001252	CD70	CD70 molecule
G05	Hs.654445	NM_001244	TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8
G06	Hs.654481	NM_000546	TP53	Tumor protein p53
G07	Hs.523968	NM_005426	TP53BP2	Tumor protein p53 binding protein, 2
G08	Hs.697294	NM_005427	TP73	Tumor protein p73
G09	Hs.460996	NM_003789	TRADD	TNFRSF1A-associated via death domain
G10	Hs.522506	NM_021138	TRAF2	TNF receptor-associated factor 2
G11	Hs.510528	NM_003300	TRAF3	TNF receptor-associated factor 3
G12	Hs.8375	NM_004295	TRAF4	TNF receptor-associated factor 4
H01	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H03	Hs.523185	NM_012423	RPL13A	Ribosomal protein L13a
H04	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H05	Hs.520640	NM_001101	ACTB	Actin, beta
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07-H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10-H12	N/A	SA_00103	PPC	Positive PCR Control

Appendix 2: Apoptosis-related genes excluded from PCR array analysis

Position	Gene symbol	Description
A11	BCL2	B-cell CLL/lymphoma 2
B11	BIRC3	Baculoviral IAP repeat-containing 3
C02	BIRC8	Baculoviral IAP repeat-containing 8
C11	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
D01	CASP14	Caspase 14, apoptosis-related cysteine peptidase
D08	CASP8	Caspase 8, apoptosis-related cysteine peptidase
D10	CD40	CD40 molecule, TNF receptor superfamily member 5
D11	CD40LG	CD40 ligand
E01	CIDEA	Cell death-inducing DFFA-like effector a
E08	FASLG	Fas ligand (TNF superfamily, member 6)
E12	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
F04	PYCARD	PYD and CARD domain containing
F06	TNF	Tumor necrosis factor (TNF superfamily, member 2)
G01	CD27	CD27 molecule
G02	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9
G04	CD70	CD70 molecule
G05	TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8
G08	TP73	Tumor protein p73
G11	TRAF3	TNF receptor-associated factor 3

Appendix 3: Additional differentially expressed apoptosis-related genes in HCV-infected Huh7.5 cells

Position	Gene symbol	Fold Up- or Down-Regulation[a]	*P* value[b]
C06	BNIP3L	-2.45	0.04
D05	CASP5	2.26	0.009
E02	CIDEB	-7.32	0.006
F11	TNFRSF21	2.17	0.02
F12	TNFRSF25	-2.26	0.03
G03	TNFSF10	-3.34	0.004

[a] Average fold changes of at least 2-fold observed at 6 days post-infection are listed.
[b] *P* values of less than 0.05 are considered to be statistically significant. (n = 3)