4 Methods

4.1 Molecular biology

4.1.1 Digestion with restriction endonucleases

Restriction endonucleases are enzymes that catalyze the hydrolysis of the DNA phosphodiester bond. They are frequently used in molecular biology to prepare DNA fragments for ligation or to analyze DNA plasmids. Restriction endonucleases recognize specific DNA sequences (4-8bp) that are oftentimes palindromic. Digestion of double-stranded DNA with restriction endonucleases either produces blunt ends or sticky ends, depending on the nature of the enzyme.
1 unit (1U) of a restriction enzyme is defined as the amount of enzyme that catalyzes the cleavage of 1μg DNA in 1h.

4.1.2 Agarose gel electrophoresis

Linearized DNA fragments can be separated by agarose gel electrophoresis according to their size. The DNA is detected by the means of ethidium bromide, a dye that intercalates into the DNA double helix.
Agarose is resuspended in TAE buffer at the appropriate concentration (0.5-2% agarose). The suspension is heated until the agarose is completely dissolved and ethidium bromide is added (50ng/ml). The solution is cooled to 50-60°C and poured into a horizontal gel chamber that is stuffed with a comb. Once the gel has become solid, it can be loaded with DNA.
DNA samples are mixed with DNA sample buffer. Samples are electrophoresed at ~90V for 1h using TAE buffer as the running buffer. DNA is detected by the means of UV irradiation.

TAE buffer	40mM Tris/HCl, pH 8.0
	40mM sodium acetate
	1mM EDTA

DNA sample buffer (5X)	10mM Tris/HCl, pH 8.0
	100mM NaCl
	30% glycerol
	0.25% bromophenol blue

4.1.3 Determining the concentration of nucleic acids

The amount of DNA or RNA in aqueous solution can be quantified by measuring the absorbance at λ= 260nm. At this wavelength, an extinction of 1.0 corresponds to the concentration of 50µg/ml of double-stranded DNA, 40µg/ml of RNA or 30µg/ml of oligonucleotide.

The purity of a DNA/RNA preparation can be assessed by measuring the absorbance at λ= 280nm at the same time: For pure samples, the ratio OD_{260}/OD_{280} should amount to ~1.8. Lower values indicate a contamination with protein or phenol.

4.1.4 Isolation of DNA fragments from agarose gels

DNA fragments were isolated from agarose gels using the Qiaquick Gel Extraction Kit according to the manufacturer's instructions. In principle, the DNA is solubilized from the gel slize by the means of chaotropic salts. The solubilized DNA is bound to a silicagel matrix under high-salt conditions. After a washing step, the DNA is eluted under low-salt conditions.

4.1.5 Dephosphorylation of linearized DNA

Linearized vectors that are prepared for ligation are usually dephosphorylated at the 5' end in order to prevent religation of the vector. Dephosphorylation is catalyzed by the enzyme alkaline phosphatase. Alkaline phosphatase was used according to the manufacturer's instructions.

4.1.6 DNA ligation

DNA ligase is an enzyme that catalyzes the formation of a phosphodiester bond between the 5' phosphate group and the 3' hydroxyl group of a DNA molecule. This is frequently used during

cloning to insert a gene of interest into an expression vector. In this case, the DNA ligase catalyzes the formation of a phosphodiester bond between the vector and the insert.
In general, 150ng of linearized vector are incubated with a 3-5 fold excess of the insert in the presence of T4 DNA ligase. Ligation is performed at 16°C over night. The ligation product is immediately used for the transformation of *E.coli*.

4.1.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method that allows the amplification of specific DNA fragments. It capitalizes on the property of the DNA polymerase to extend a short piece of double-stranded DNA. Specificity is determined by two oligodeoxynucleotides that hybridize to the 5' and the 3' end of the fragment to be amplified.
For analytical PCRs, the DNA polymerase of *Thermus aquaticus* was used (BIOTAQ DNA polymerase). It displays a temperature optimum of 72°C. However, it does not show any proofreading activity, leading to an error rate of 1 in 10^5 bases.
For preparative PCRs, a DNA polymerase with proofreading activity was used (Herculase Enhanced DNA polymerase). It displays a temperature optimum of 68°C. As a consequence of the proofreading activity, the error rate is much lower (1 in 10^6 bases).
PCR was performed according to the following protocol:

10-100ng	DNA template (100ng)
2µM	Forward primer
2µM	Backward primer
1mM each	Deoxynucleotides (dATP/dCTP/dGTP/dTTP)
2mM	$MgCl_2$
1 U	BIOTAQ DNA polymerase
	in PCR reaction buffer

The PCR was performed under the following conditions:

Step 1	2min.	Initial denaturation at 95°C
Step 2	1min.	Denaturation at 95°C
Step 3	1min.	Annealing at 55-60°C
Step 4	1min./1kb	Primer extension at 72°C
Step 5	10min.	Final extension at 72°C

Steps 2-4 were repeated 24-29 times.

4.1.8 DNA sequencing

DNA sequencing is based on the method of chain termination that was originally developed by Sanger (Sanger et al., 1977). It relies on the polymerase chain reaction as described in chapter 4.1.7. However, in addition to the deoxynucleotides, 2',3' dideoxynucleotides are added. Whenever the dideoxynucleotides are incorporated into the growing DNA strand, chain termination occurs because the dideoxynucleotides do not possess a free 3' hydroxyl group. As a consequence, the PCR products constitute a heterogenous mixture of DNA fragments with each fragment having incorporated exactly one dideoxynucleotide at the 3' end. Dideoxynucleotides are coupled to different fluorescent dyes. Once the PCR is complete, the fragments can be separated by gel electrophoresis or capillary electrophoresis. The DNA sequence is determined by measuring the fluorescence signal that corresponds to each fragment.
Automated sequencing of DNA was performed using the ABI PRISM ready reaction dye deoxy terminator cycle sequencing kit. Sequencing was performed at a central facility in the Robert-Koch-Institut.

4.1.9 Generation of chemically competent *E.coli*

Bacteria are usually reluctant to take up foreign DNA. However, by treating the bacteria with divalent metal ions, they are rendered competent to do so.
In order to generate chemically competent bacteria, 800ml of LB medium are inoculated with 10ml of an overnight-culture of the respective *E.coli* strain. Bacteria are grown to an optical density of OD_{600}=0.5. Bacteria are pelleted by centrifugation using sterile centrifuge tubes and

subsequently resuspended in buffer TFB1. After incubating the bacteria on ice for 90min., the bacteria are again pelleted by centrifugation and subsequently resuspended in 15-20ml of buffer TFB2. The suspension is aliquoted (100-500µl) and immediately frozen in liquid nitrogen. Chemically competent bacteria are stored at -80°C.

Buffer TFB1	30mM potassium acetate, pH 5.8
	100mM $RbCl_2$
	50mM $MnCl_2$
	10mM $CaCl_2$
	15% glycerol
Buffer TFB2	10mM MOPS, pH 8.0
	10mM $RbCl_2$
	75mM $CaCl_2$
	15% glycerol

4.1.10 Transformation of *E.coli*

Chemically competent *E.coli* (see chapter 4.1.9) are transformed with the ligation product (see chapter 4.1.6) or with a plasmid using the heat-shock protocol. In brief, 100µl of chemically competent *E.coli* are added to ~100ng of DNA and incubated on ice for 30min. Next, they are incubated at 42°C for exactly 90s (heat shock). After cooling the bacteria on ice for 5min, 900µl of LB medium are added to the bacterial suspension. Bacteria are incubated at 37°C for 1h before being plated on agar plates containing the suitable antibiotic to select for the transformed bacteria. Agar plates are incubated at 37°C over night to allow for the growth of bacterial colonies.

LB medium	1% Bactotrypton
	0.5% Yeast extract
	0.5% NaCl
	in H_2O, pH 7.0

4.1.11 Plasmid isolation

Plasmids were isolated from recombinant *E.coli* using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. This kit is based on the method that was originally published by Binboim and Doly (Birnboim and Doly, 1979). It relies on the lysis of bacteria under alkaline conditions in the presence of SDS. Under these conditions, both chromosomal DNA and plasmid DNA are denatured. However, upon neutralization, plasmid DNA is selectively renatured whereas chromosomal DNA remains insoluble. Soluble (plasmid) DNA is subsequently purified by the means of an anion exchange chromatography and eluted under high salt conditions. The final purification of the DNA involves the precipitation by the means of isopropanol. The precipitate is washed and air-dryed before being dissolved in ddH_2O.

4.2 Cell biology

4.2.1 Cultivation and infection of Sf9 cells

Sf9 cells were grown in complete SFII900 medium. Cells were cultivated in 100ml suspension cultures (90rpm) at 28°C. As soon as the cells reached a density of $1x10^7$ cells/ml, they were diluted 1:10 with fresh medium (usually twice a week).

Sf9 cells were infected in 100ml suspension cultures at a density of $1x10^6$ cells/ ml. For infection, cells were cultivated in HyQ Sfx insect medium. 48h post infection, infected cells were harvested by centrifugation (150xg for 10min.).

Complete SFII900 medium	SFII900 medium supplemented with 10% FCS and 2mM glutamine

4.2.2 Cultivation and transfection of HuH-7 cells

HuH-7 cells were grown in complete DMEM at 37°C. Cells were cultivated in adherent cell culture flasks ($175cm^2$) at 37°C, 6% CO_2 and >90% humidity. As soon as the cells reached confluency, they were washed with PBS and detached by addition of trypsin/EDTA. Activity of trypsin was terminated by addition of complete DMEM. Cells were seeded at different dilutions (1:3 to 1:5) in order to ascertain optimal growth.

HuH-7 cells were transfected using ExGen500 according to the manufacturer's instructions. In brief, $1x10^5$ cells HuH-7 cells were seeded in a 35mm dish. On the next day, cells were transfected with 0.5-1.5 µg of plasmid DNA. Cells were harvested or analyzed by immunofluorescence 48h post transfection.

Complete DMEM	DMEM supplemented with	10% FCS 1U/ml penicillin 1U/ml streptomycin 2mM glutamine
PBS	40g NaCl 1g KCl 5.75g Na_2HPO_4 x $2H_2O$ 1g KH_2PO_4 ad 5l H_2O	

4.2.3 Generation of recombinant baculoviruses.

The gene of interest was inserted into the transfer vector pBacPak9 or pVL1393. Correct insertion was verified by DNA sequencing.

Recombinant baculoviruses were generated using the BaculoGOLD transfection kit according to the manufacturer's instructions. In brief, $4x10^6$ Sf9 cells were seeded in a $25cm^2$ flask. Cells were transfected with the transfer vector containing the gene of interest together with linearized baculovirus DNA. Recombinant baculoviruses were generated by homologous recombination of the transfer vector with the linearized baculovirus DNA. The supernatant (4ml) containing the recombinant virus was harvested 7 days post transfection (passage 1).

0.6ml of the supernatant were used to infect Sf9 cells in a 60ml suspension culture ($2x10^6$ cells/ml). The cells were grown for 7 days to allow for virus propagation to occur. The supernatant was harvested by centrifugation (150xg for 10min.) (passage 2).

5ml of the supernatant were used to infect Sf9 cells in a 100ml suspension culture ($3x10^6$ cells/ml). The cells were grown for 2 days to allow for virus propagation to occur. The supernatant was harvested by centrifugation (150xg for 10min.) (passage 3).

Virus titres were analyzed by a plaque assay (see chapter 4.2.4).

4.2.4 Plaque assay for recombinant baculoviruses.

Sf9 cells were seeded in 6-well plates at confluency (!). Cells were infected with serial dilutions of the recombinant baculovirus (5 dilutions per virus) for 2h on a shaker. Meanwhile, low-melting temperature agarose was diluted in HyQ Sfx insect medium (4% w/v). The solution was boiled to melt the agarose and kept at 41°C in the water bath. Once the infection was completed, the agarose solution (4%) was diluted with an equal volume of fresh pre-warmed (37°C) HyQ Sfx insect medium. The virus was aspired from the 6-well plates and the cells were overlaid with the agarose solution (2%). Once the agarose had become solid, it was overlaid with 1ml of HyQ Sfx insect medium to prevent the overlay from drying out. The infected cells were incubated at 28°C for 7 days.

To score for production of viral plaques, the cells were stained with MTT according to the manufacturer's instructions. The MTT assay is a viability assay that allows the discrimination between alive (uninfected) and dead (infected cells). Infected cell colonies were counted to calculate the viral titre.

4.2.5 Reporter gene assay

Reporter gene assays were performed in order to analyze the impact of NS5A on the basal activation level of the transcription factors SRF, AP-1, NF-κB and STAT-3. To that end, $1x10^5$ cells were seeded in a 35mm well. On the next day, cells were transiently transfected with 0.3µg of reporter plasmid (pSRE-Luc, pAP-1-Luc, pNF-κB-Luc or pSTAT3-TA-Luc) coupled to *Photinus pyralis* luciferase and 1.0µg of pcDNA-NS5A using Exgen500 according to the manufacturer's instructions. As a negative control, HuH-7 cells were transiently transfected with 1.0µg of pcDNA-NS5A *inverse*. This plasmid contains the NS5A coding sequence in 3'-5' orientation, leading to the expression of a nonsense mRNA. To monitor transfection efficiency, HuH-7 cells were cotransfected with 0.3µg of a reporter plasmid harbouring *Renilla reniformis* luciferase as a reporter gene (pRL-TK). *Renilla reniformis* luciferase expression is driven by the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter that is constitutively active. Cells were harvested 48h post transfection in reporter gene assay lysis buffer (Roche). Lysates were analyzed by measuring both *Photinus pyralis* and *Renilla reniformis* luciferase levels using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. *Photinus pyralis* luciferase levels were normalized with regard to *Renilla reniformis* luciferase levels.

In order to analyze differential activation of the transcription factors SRF, AP-1, NF-κB and STAT-3 in HCV replicon cells in comparison to naïve HuH-7 cells, cells were transfected with 0.3µg of the inducible reporter plasmid (pSRE-Luc, pAP-1-Luc, pNF-κB-Luc or pSTAT3-TA-Luc) and 0.3µg of the constitutive reporter plasmid (pRL-TK). Reporter gene expression was determined as described above.

4.2.6 HCV replication assays

To monitor HCV replication, bicistronic subgenomic reporter replicon cell lines HuH-7 I_{389}/NS3-3`/LucUbiNeo-ET were used (Frese et al., 2003). In these cells, the luciferase gene of the firefly *Photinus pyralis* is fused to the subgenomic HCV replicon RNA. Luciferase levels have been shown to be tightly correlated with HCV RNA replication in these replicons. This allows an easy quantification of HCV replication by luciferase reporter gene assay.
Cells were either incubated with small-molecule inhibitors of MEK (PD98059, U0126) or c-Raf1 (BAY43-9006) for 6h. Alternatively, cells were transfected with c-Raf1-specific siRNA or negative control siRNA (see chapter 4.2.7). Cells were lysed in reporter gene assay lysis buffer (Roche). Lysates were analyzed by automated addition of the luciferase substrate buffer and subsequent determination of chemoluminescence.

Luciferase substrate buffer	20mM tricine, pH 7.8
	470µM luciferin
	530µM ATP
	1mM basic $MgCO_3$
	2.7mM $MgSO_4$
	0.1mM EDTA
	33.3mM DTT

4.2.7 Silencing of c-Raf1 by siRNA

To silence c-Raf1 by siRNA, a silencer validated siRNA directed against c-Raf1 was used. HCV replicon cells were transfected with siRNAs using siPORT Lipid according to manufacturer's instructions. Briefly, $1x10^5$ cells were seeded in a 35mm well. On the next day, cells were transfected with 200nM silencer validated siRNA using siPORT Lipid. Cells were harvested 48h

post transfection. Silencing of c-Raf1 was monitored by Western blotting using a c-Raf1-specific antiserum (Santa Cruz).

4.3 Protein biochemistry

4.3.1 Purification of *E.coli*-derived NS5A by *Strep*Tactin Sepharose chromatography

A bacterial expression plasmid that codes for a fusion protein of NS5A with an N-terminal *Strep* tag and a C-terminal V5-epitope was generated (pASK-IBA7-NS5A-V5). DH5α were transformed with this plasmid and expression of NS5A was induced for 2h at 37°C by addition of 200ng/ml anhydrotetracyclin. Bacteria were harvested by centrifugation, resuspended in buffer W supplemented with 0.5% NP-40 and lysed by French Press (15000-25000psi). The lysate was cleared by ultracentrifugation (50000xg) and the supernatant was applied to *Strep*Tactin Sepharose column to allow Strep-NS5A-V5 to bind to the column. Next, the column was washed with 15ml of buffer W. Strep-NS5A-V5 was eluted from the column in 6 fractions (500µl each) by addition of buffer E.

Buffer W	100mM Tris/HCl, pH 8.0
	150mM NaCl
	1mM EDTA
Buffer E	100mM Tris/HCl, pH 8.0
	150mM NaCl
	1mM EDTA
	5mM desthiobiotin

4.3.2 Purification of Sf9 cell-derived NS5A(211-449) and NS5A by *Strep*Tactin Sepharose chromatography

Sf9 cells were seeded at a density of 10^6 cells/ml in a 100ml suspension culture. Cells were infected with 2ml of recombinant baculovirus (passage 3) coding for a truncated version of NS5A (NS5A (211-449)) fused to the *Strep* tag at the N-terminus and the V5 epitope at the C-terminus (AcNPV Strep-NS5A(211-449)-V5). 48h post infection, cells were harvested by centrifugation (150xg for 10min.) and resuspended in 15ml of buffer W. Cells were lysed by

French Press (15000-25000psi). The lysate was cleared by ultracentrifugation (50000xg) and the supernatant was applied to *Strep*Tactin Sepharose column to allow Strep-NS5A(211-449)-V5 to bind to the column. Next, the column was washed with 15ml of buffer W. Strep-NS5A(211-449)-V5 was eluted from the column in 6 fractions (500µl each) by addition of buffer E.
Strep-NS5A-V5 was purified according to the same protocol using AcNPV Strep-NS5A-V5. However, whereas Strep-NS5A(211-449)-V5 was purified in the absence of detergent, full-length NS5A was purified in the presence of 0.5% NP-40 (Huang et al., 2004).

4.3.3 Purification of GST-Raf by glutathione Sepharose chromatography

Sf9 cells were seeded at a density of 10^6 cells/ml in a 100ml suspension culture. Cells were infected with 2ml of recombinant baculovirus coding for c-Raf1 fused to glutathione S-transferase (GST) at the N-terminus (AcNPV GST-Raf). 48h post infection, cells were harvested by centrifugation (150xg for 10min.) and resuspended in 15ml of PBS supplemented with 0.5% NP-40. Cells were lysed by French Press (15000-25000psi). The lysate was cleared by ultracentrifugation (50000xg) and the supernatant was applied to the glutathione Sepharose column to allow GST-Raf to bind to the column. Next, the column was washed with 15ml of PBS. GST-Raf was eluted from the column in 6 fractions (500µl each) by addition of GST buffer E.
The inactive mutant GST-Raf K375W was purified accordingly using AcNPV GST-Raf K375W.
In order to get rid of kinases associated with GST-Raf, an additional washing step with RIPA buffer was performed before eluting the protein from the column (Janosch et al., 1996).

GST buffer E	20mM Tris/HCl, pH 8.0
	10mM glutathione
RIPA buffer	50mM Tris/HCl pH 7.2
	150mM NaCl
	0.1% SDS
	1% deoxycholate
	1% triton x-100

4.3.4 Kinase assay

Purified Strep-NS5A-V5 and highly purified GST-Raf were diluted in kinase buffer (5X). The *in vitro* phosphorylation was performed at 30°C for 15min. in the presence of 10µCi [γ-^{32}P]ATP. Samples were denatured by addition of SDS sample buffer and boiled for 5min. at 95°C. Samples were electrophoresed on a 10% SDS-PAGE and the dried gel was subjected to autoradiography.

Kinase buffer (5X)	125mM Tris/HCl, pH 7.2
	125mM 2-glycerophosphate
	50mM $MgCl_2$
	5mM DTT

4.3.5 Affinity chromatography approach to identify potential binding partners of NS5A.

Sf9 cells were seeded at a density of 10^6 cells/ml in a 100ml suspension culture. Cells were infected with 2ml of recombinant baculovirus (passage 3) coding for NS5A fused to the *Strep* tag at the N-terminus and the V5 epitope at the C-terminus (AcNPV Strep-NS5A-V5). 48h post infection, cells were harvested by centrifugation (150xg for 10min.) and resuspended in 15ml of buffer W. Cells were lysed by French Press (15000-25000psi). The lysate was cleared by ultracentrifugation (50000xg) and the supernatant was applied to *Strep*Tactin Sepharose column to allow Strep-NS5A-V5 to bind to the column. After washing the column with 15ml of buffer W, it was incubated with whole cell lysate derived from human hepatoma cells (HuH-7) to allow potential interaction partners to bind to the immobilized NS5A. After another washing step with buffer W, Strep-NS5A-V5 was eluted in 6 fractions (0.5ml) by addition of buffer E.

As a control, a fusion protein of Hepatitis-B virus Core (HBc) with an N-terminal *Strep* tag was produced by infection of Sf9 cells with AcNPV Strep-HBc and treated accordingly. As additional control, a *Strep*Tactin Sepharose column was incubated with hepatoma cell lysate only to analyze unspecific binding to the column material.

4.3.6 SDS-PAGE

Protein samples were prepared for electrophoresis by addition of SDS sample buffer (5X). Samples were heated to 95°C for 3-5min.

Samples were electrophoresed in a discontinuous SDS-polyacrylamide gel (Laemmli, 1970). Denatured protein samples migrate quickly though the large pores of the stacking gel (5% acrylamide) before entering the narrow pores of the running gel (10-15% acrylamide). An additional focusing effect is obtained by shifting the pH from 6.8 in the stacking gel to 8.8 in the running gel: Glycine, which is the main constituent of the running buffer, has an isoelectric point of ~6.0. This means that, once glycine enters the stacking gel, it assumes its zwitterionic form and is essentially uncharged. The resulting deficiency of charge carriers is synonymous with an increased electrical resistance R and – according to Ohm's law (E=RI) - and an increased electric field E in the stacking gel. In response to this increased field, proteins are accelerated in the stacking gel relative to the running gel.
SDS-polyacrylamide gels were poured as a block of 10 minigels (9x6cm). The composition of the gels is summarized in the following table.

Table 4.1 Composition of the stacking gel

	5% gel
Rotiphoresegel 40	5ml
Stacking gel buffer (4X)	10ml
H_2O	25ml
TEMED	40µl
Ammonium peroxo disulfate (10%)	200µl

Table 4.2 Composition of the running gel

	10% gel	12% gel	15% gel
Rotiphoresegel 40	20ml	24ml	30ml
Running gel buffer (4X)	20ml	20ml	20ml
H_2O	40ml	36ml	30ml
TEMED	60µl		
Ammonium peroxo disulfate (10%)	200µl		

SDS sample buffer (5X)	0.25M Tris/HCl, pH 6.8 50% Glycerin 8% SDS 0.5M DTT 0.5% bromophenol blue
Electrophoresis buffer (10X)	250mM Tris base 1.92M Glycine 1 % SDS
Stacking gel buffer (4X)	0.5 M Tris/HCl, pH 6.8 0.4 % SDS
Running gel buffer (4X)	1.5 M Tris/HCl, pH 8.8 0.4 % SDS

4.3.7 Silver staining of SDS-polyacrylamide gels

Silver staining of SDS-polyacrylamide gels is a very sensitive method that allows the detection of proteins in lower nanogram range (~10ng). Under the buffer conditions used, redox-active groups in the electrophoresed proteins catalyze the selective reduction of silver ions in the presence of formaldehyde. The resulting silver crystal seeds can be visualized by addition of the developer.

Once the electrophoresis is completed, the SDS-polyacrylamide gel is soaked in fixative for 30min. Next, the gel is transferred to the conditioner for 20min. Once conditioning is completed, the gel is rinsed at least three times (5 min. each) in ddH_2O. Then, the gel is soaked with silver nitrate solution for 20min. before it is developed by addition of the developer. Development is stopped by addition of acetic acid.

Fixative	25% Ethanol 10% Acetic acid

Conditioner	0.4M Sodium acetate, pH6.0 30% Ethanol 4.4mM Sodium thiosulfate 1% Glutaraldehyde
Silver nitrate solution	0.1% Silver nitrate 0.01% Formaldehyde
Developer	2.5% Sodium carbonate 0.015% Formaldehyde

4.3.8 Western blot

To detect specific proteins, proteins were electrophoresed on an SDS-polyacrylamide gel and transferred to a Hybond-P membrane by Western blotting. The transfer was performed under semi-dry conditions using a discontinuous buffer system (1-1.5mA/cm^2 for 50min.). The blot was set up as follows:

Cathode (-)
6 Whatman papers soaked with cathode buffer
SDS-polyacrylamide gel
Hybond P membrane
2 Whatman papers soaked with anode buffer II
4 Whatman papers soaked with anode buffer I
Anode (+)

Anode buffer I	300mM Tris base 20% Ethanol
Anode buffer II	25mM Tris base 20% Ethanol
Cathode buffer	40mM isocaproic acid 20% Ethanol

Once the transfer to the Hybond-P membrane was completed, unspecific binding sites were blocked by incubating the membrane with blocking solution. Next, the membrane was incubated with the primary antibody diluted in blocking solution (1-3h on the shaker). Then, the blot was rinsed three times (5min. each) with PBS-T. The primary antibody was visualized by incubating the blot with peroxidase-conjugated secondary antibody (HRP-linked ECL rabbit IgG or HRP-linked ECL mouse IgG). After rinsing the blot again with PBS-T, it was developed using the ECL Western blotting Reagent according to the manufacturer's instructions.

PBS-T	0.05% Tween 20 in PBS
Blocking solution	2% Skim milk powder in PBS-T

In some instances, the blocking solution was modified to contain 10% bovine serum albumin (BSA) instead of 2% milk powder. In case of the phosphorylation-state-specific antibodies (anti-phospho-MEK and anti-active MAPK), the Western blot was performed according to the manufacturer's instructions.

4.4 Immunological methods

4.4.1 Generation and purification of the NS5A-specific antiserum

A rabbit was immunized with pooled eluate fractions of purified NS5A(211-449) (see chapter 4.3.2). 1ml of pooled eluate contained ~300µg of protein. For the first immunization, 1ml of the pooled eluate was diluted with 1ml of Freund's complete adjuvant and injected subcutaneously into the rabbit. Two weeks later, 1ml of the pooled eluate was diluted with 1ml of Freund's incomplete adjuvant and applied by the same route in order to boost the immune response. A final injection with 1ml of pooled eluate diluted in 1ml of Freund's incomplete adjuvant was given another four weeks later. Two weeks after the last injection, the rabbit was sacrificed and the serum was harvested by centrifugation of the peripheral blood.

In order to purify the antiserum by affinity chromatography, purified Strep-NS5A(211-449)-V5 was coupled to an NHS Sepharose column according to manufacturer's instructions. In brief, Strep-NS5A(211-449)-V5 was dialyzed against coupling buffer over night at 4°C. NHS Sepharose was activated by pulsing it with 1mM HCl. Strep-NS5A(211-449)-V5 was then

coupled to NHS Sepharose for 4h at room temperature. The Strep-NS5A(211-449)-V5-coupled Sepharose was repeatedly washed with buffer WB and buffer WA in order to get rid of non-covalently associated proteins. The column was equilibrated with PBS.
The equilibrated column was loaded with 10ml of rabbit-derived antiserum. Then, the column was washed with PBS. The purified antiserum was eluted with buffer AE. In order to prevent denaturation of the purified antiserum, the pH was instantaneously shifted back to 7.2 by addition PBS (10X).

Coupling buffer	0.2M $NaHCO_3$, pH 8.0 0.5M NaCl
Buffer WB	100mM Tris/HCl, pH 8.0 500mM NaCl
Buffer WA	100mM Sodium acetate, pH 4.0 500mM NaCl
Buffer AE	100mM Glycine, pH 2.7

4.4.2 Immunoprecipitation

For immunoprecipitation from Sf9 cells, Sf9 cells were infected with recombinant baculoviruses (AcNPV Strep-NS5A-V5 and/or AcNPV GST-Raf) as described above. Cells were lysed in TX buffer and the lysate was cleared by centrifugation. For preclearance, the supernatant was incubated with protein A/G agarose for 30min. at 4°C. Next, antibodies were added for precipitation as indicated (anti-NS5A: rabbit-derived polyclonal antiserum; anti-Raf1: BD Transduction Laboratories) and the samples were incubated on a shaker for 2-4h at 4°C. Then, protein A/G agarose was added to pull down the precipitates and the samples were incubated for 1h at 4°C. Finally, precipitates were washed twice with buffer WI and once with buffer WII. Precipitates were solubilized by addition of SDS sample buffer (1X), incubated at 95°C for 3 min. and analyzed by SDS-PAGE and subsequent Western blotting using either anti-V5 (Invitrogen) or anti-Raf1 (Santa Cruz).

TX buffer	100mM Tris/HCl, pH 7.5 137mM NaCl 0.1% triton X-100 1mM DTT
Buffer WI	100mM Tris/HCl, pH 7.5 500mM LiCl
Buffer WII	40mM Tris/HCl, pH 7.5

4.4.3 Immunofluorescence analysis

HuH-7 cells were transfected as described in chapter 4.2.2 and fixed 48h post transfection. Fixation was performed as indicated using either cold ethanol or 4% paraformaldehyde. Cells were permeabilized using PBS supplemented with 1% triton X-100. Unspecific binding was blocked using PBS-T supplemented with 10% BSA for 1h. The primary antibody was diluted in blocking solution and applied for 1-3h. Cells were washed three times with PBS-T and subsequently, primary antibodies were visualized using secondary antibodies labeled with FITC and Cy3. Secondary antibodies were carefully chosen to exclude artifacts due to cross-reactivity. Immunofluorescence staining was analyzed by confocal laser scanning microscopy.

4.5 Generation and analysis of NS5A-transgenic mice

4.5.1 Generation of NS5A-transgenic mice

The NS5A expression cassette was cloned to express NS5A under the control of the mouse albumin promoter (~340bp) and the corresponding enhancer (~2kb) (Chisari et al., 1989; Pinkert et al., 1987). The β-globin intron was included to increase the level of transcription (Brinster et al., 1988). NS5A itself is expressed as a fusion protein with an N-terminal hexa-histidine tag and a C-terminal V5 epitope. A BGH poly-adenylation site was included downstream of the NS5A coding sequence.

The NS5A-transgenic mice were generated by Dr. Kurt Reifenberg (University of Mainz). In brief, the NS5A expression cassette was linearized by a combined restriction digest with NotI and XhoI. The larger (slower migrating) fragment was excised from the gel and purified. The

fragment was then microinjected into FVB embryos. The transgenic embryos were implanted into pseudopregnant FVB mice and the offspring was analyzed for presence of the transgene by PCR.

4.5.2 Detection of the NS5A-transgene by PCR analysis

To distinguish between NS5A-transgenic animals and non-transgenic littermates, mouse-tail biopsies were taken at the age of 3-4 weeks. DNA was extracted from mouse-tail biopsies according to a standard protocol: Mouse-tails biopsies were lysed in 500µl of lysis buffer at 55°C over night. Cellular protein was precipitated by addition of 250µl of saturated sodium chloride solution. Samples were centrifuged (4500g for 10min.). The supernatant (500µl) was precipitated with 1ml of cold ethanol (98%). DNA was pelleted by centrifugation (16000g for 10min.), washed with 500µl of ethanol (70%) and dried at room temperature. The dried DNA pellet was solubilized in 100µl of ddH_2O (37°C for at least 2h).

Lysis buffer	17mM Tris/HCl, pH 7.5
	17mM EDTA
	170mM NaCl
	0.85% SDS
	0.2mg/ml proteinase K

Chromosomal DNA preparations were analyzed by polymerase chain reaction (PCR). PCR was performed according to the following protocol:

2µl	chromosomal DNA
2.5µl	PCR reaction buffer (10X)
0.75µl	Magnesium chloride (50mM)
1µl	Deoxynucleotides (dATP/dCTP/dGTP/dTTP), 25mM each
0.1µl	Primer tb059 (100µM)
0.1µl	Primer tb060 (100µM)
0.1µl	Primer tb061 (100µM)
0.1µl	Primer tb062 (100µM)
0.25µl	BIOTAQ DNA polymerase (5U/µl)
ad 25µl	ddH_2O

Whereas the primer pair tb059/060 enabled the amplification of an NS5A-specific sequence, the primer pair tb061/062 targeted the tubulin gene. The latter was used as control to assay whether the DNA preparation was intact and suitable for PCR analysis.
The PCR was performed under the following conditions:

Step 1	2min.	Initial denaturation at 95°C
Step 2	1min.	Denaturation at 95°C
Step 3	1min.	Annealing at 57°C
Step 4	1min.	Primer extension at 72°C
Step 5	10min.	Final extension at 72°C

Steps 2-4 were repeated 34 times.

4.5.3 RNA isolation and RT-PCR analysis

RNA isolation was performed using TRIZOL according to the manufacturer's instructions. In brief, 100mg of frozen liver tissue (-80°C) were homogenized in 1ml of TRIZOL. Phase separation was obtained by addition of 0.2ml of chloroform and subsequent centrifugation (12000xg for 15min.). The aqueous phase was transferred to a fresh tube and the RNA was precipitated by addition of 0.5ml of isopropanol. The RNA was pelleted by centrifugation (12000xg for 10min.), washed with 500µl of ethanol (70%) and dried at room temperature. The dried RNA pellet was solubilized in 100µl of DEPC-treated ddH_2O (10min. at 55°C). Integrity of the liver RNA was analyzed by agarose gel electrophoresis: If the RNA is intact, the 18S rRNA and the 28S rRNA should be detected at ~2kb and ~5kb respectively.
Intact RNA was reverse transcribed using AMV reverse transcriptase (RT) according to the manufacturer's instructions. In brief, 4µg of RNA were subjected to DNAse digestion (30min. at 37°C). DNAse was heat-inactivated (10min. at 65°C). Half of the sample was stored to provide a DNAse-positive RT-negative control. The other half was reverse transcribed using an oligo-dT primer (1h at 45°C).
The resulting cDNA was analyzed by PCR using either albumin-specific primers (fw- and bw-albumin) or NS5A-specific primers (tb037 and tb039). The PCR was performed under the conditions described in chapter 4.5.2. However, the PCR was terminated after 30 cycles. As

mentioned above, both the RT-negative and the RT-positive samples were analyzed. A signal in the RT-negative sample indicates a potential contamination with chromosomal DNA.

4.5.4 Analysis of NS5A expression by Western blotting

100mg of frozen liver tissue (-80°C) was homogenized in 1ml of denaturing protein sample buffer. The lysate was subjected to ultrasound treatment (5 pulses, 5 cycles per pulse) to induce fragmentation of chromosomal DNA. Lysates were analyzed by SDS-PAGE and subsequent Western blotting using a V5 specific antibody to detect the H6-NS5A-V5 fusion protein.

Denaturing protein sample buffer	200mM Tris/HCl, pH 8.8
	5mM EDTA
	0,1% bromophenol blue
	10% sucrose
	3.3% SDS
	2% 2-mercaptoethanol

4.5.5 Immunohistochemical analysis of liver cryo-sections

Frozen livers (-80°C) were subjected to cryo-sectioning using a cryotome. Cryo-sections were prepared at a thickness of 5µm and immediately fixed in 4% paraformaldehyde. Sections were permeabilized using PBS supplemented with 1% triton X-100. Unspecific binding was blocked using PBS-T supplemented with 10% BSA for 1h. NS5A was detected using the rabbit-derived NS5A-specific antiserum. The NS5A-specific antiserum was diluted in blocking solution and applied for 1-3h. Cells were washed three times with PBS-T and subsequently, the primary antibody was visualized using a Cy3-coupled secondary antibody. As a control, the actin cytoskeleton was stained using FITC-coupled phalloidin.