The HCV Replicase Complex and Viral RNA Synthesis

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Abstract Replication of hepatitis C virus (HCV) is tightly linked to membrane alterations designated the membranous web, harboring the viral replicase complex. In this chapter we describe the morphology and 3D architecture of the HCV-induced replication organelles, mainly consisting of double membrane vesicles, which are generated by a concerted action of the nonstructural proteins NS3 to NS5B. Recent studies have furthermore identified a number of host cell proteins and lipids contributing to the biogenesis of the membranous web, which are discussed in this chapter. Viral RNA synthesis is tightly associated with these membrane alterations and mainly driven by the viral RNA dependent RNA polymerase NS5B. We summarize our current knowledge of the structure and function of NS5B, the role of *cis*-acting replication elements at the termini of the genome in regulating RNA synthesis and the contribution of additional viral and host factors to viral RNA synthesis, which is still ill defined.

Keywords Replication organelles/factories/complexes • Membranous web • Membrane rearrangements • Double membrane vesicles • Replicase • Nonstructural proteins • Virus-cell proteins/lipids interactions • RdRp • Polymerase • Cis-acting replication elements • Host factor • RNA synthesis

Abbreviations

CRE	*cis*-acting replication element
DENV	Dengue virus
DMV	Double membrane vesicle
DRM	Detergent resistant membranes
EM	Electron microscopy
ER	Endoplasmic reticulum
ET	Electron tomography

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T. Miyamura et al. (eds.), *Hepatitis C Virus I*, DOI 10.1007/978-4-431-56098-2_8

FAPP2	Four-phosphate-adaptor protein 2
GFP	Green fluorescence protein
HCV	Hepatitis C virus
IRES	Internal ribosome entry site
JFH1	Japanese fulminant hepatitis 1
LD	Lipid droplet
MMV	Multimembrane vesicle
MW	Membranous web
NS	Non structural
OSBP	Oxysterol-binding protein
PI4KIIIα	Phosphatidylinositol 4-kinase IIIα
PI4P	Phosphatidylinositol 4-phosphate
RdRp	RNA dependent RNA polymerase
SMV	Single membrane vesicle

1 Introduction

The genome of HCV encompasses a single ~9,600 nts long RNA molecule containing one large open reading frame (ORF) that is flanked by non-translated regions (NTRs), important for viral translation and replication. The viral genome is not capped and the 5′NTR contains an internal ribosome entry site (IRES), enabling viral translation. Upon release into the cytoplasm of an infected cell, the genome is translated into a polyprotein, which is co- and posttranslationally cleaved into ten functional subunits by cellular and viral proteases: core, envelope glycoproteins E1 and E2, p7 and the nonstructural proteins (NS) NS2, NS3, NS4A, NS4B, NS5A and NS5B. Core to NS2 are primarily involved in the formation of infectious virus (reviewed in Lindenbach and Rice 2013), whereas the NS proteins NS3 to NS5B are necessary and sufficient for viral RNA replication, which will be the focus of this chapter. After translation and processing, the nonstructural proteins (NS3 to NS5B) induce intracellular membrane alterations designated the membranous web (MW) and harboring the viral replicase (Fig. 1). The formation of these replication factories is a common step during the replication of positive- strand RNA viruses (reviewed in Romero-Brey and Bartenschlager 2014). Although their function is not fully clear yet, it is generally assumed that they facilitate RNA synthesis by concentrating viral and host proteins involved in replication and that they might shield replication intermediates from detection by intracellular pattern recognition receptors to avoid activation of innate immune responses.

RNA synthesis is associated with these replication organelles, first generating a negative strand RNA genome, probably as part of a double stranded replication intermediate (dsRNA). Negative strand RNA is then the template for progeny positive strand RNA, which is produced in five- to tenfold excess. The newly synthesized positive strand RNA either re-enters a new translation/replication

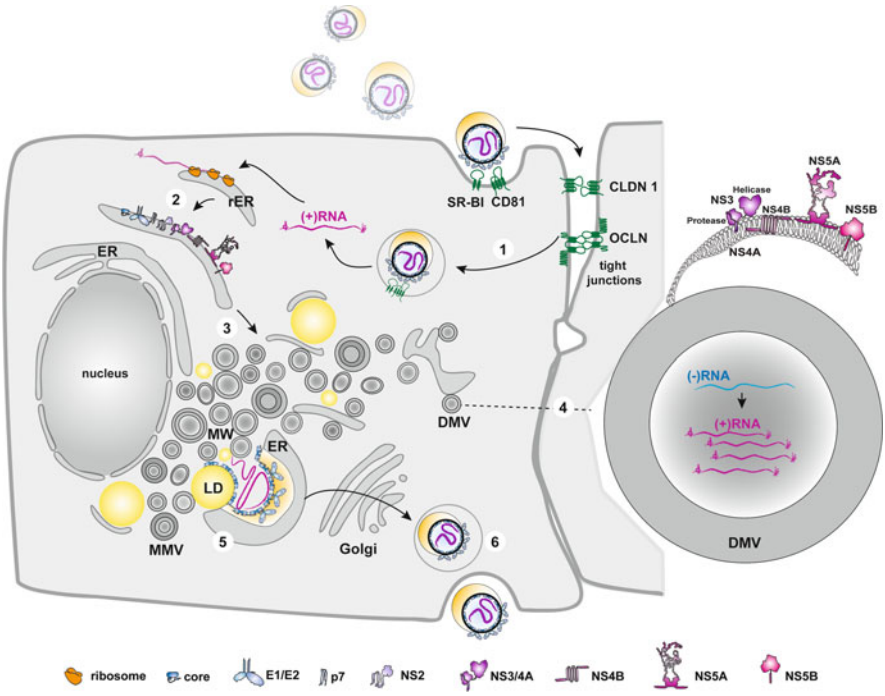

Fig. 1 Scheme of the HCV replication cycle (adapted from Bartenschlager et al. 2013). Following initial binding of HCV lipoviroparticle to scavenger receptor class B member 1 (SRB1) and CD81 and further interactions with the tight junction proteins claudin 1 (*CLDN1*) and occludin (*OCLN*), the virus enters the cell via receptor-mediated endocytosis (step 1, reviewed in Lindenbach and Rice 2013). The positive-sense single-stranded viral RNA genome is released into the cytoplasm and translated in an IRES-dependent manner at the rough ER. The resulting polyprotein is cleaved into ten mature proteins (step 2). Viral proteins, in conjunction with host cell factors, induce the formation of the membranous web (*MW*) mainly composed of double- and multi-membrane vesicles (DMVs and MMVs, respectively), usually in close proximity to lipid droplets (*LDs*) (step 3). RNA replication is thought to take place at DMVs and proceeds via a negative-sense copy ((−)RNA) that serves as a template for the production of excess amounts of positive-sense progeny RNAs ((+)RNA) (step 4, highlighted to the *right*). Assembly of HCV particles is supposed to take place in close proximity to the ER and LDs, where core protein and viral RNA accumulate (reviewed in Bartenschlager et al. 2011). The viral envelope is acquired by budding through the ER membrane in a process that is linked to lipoprotein synthesis (step 5). HCV particles are thought to be released via the constitutive secretory pathway (step 6)

cycle or is packaged into virions (Fig. 1). This chapter summarizes our current knowledge of HCV induced membrane alterations, providing a detailed view of viral and host cell proteins as well as lipids engaged in their biogenesis. We furthermore provide an overview of the role of the NS proteins and *cis*-acting elements in distinct steps of RNA synthesis.

2 Architecture of the HCV Replication Factories

The first visualization of HCV-induced intracellular changes in cell culture by transmission electron microscopy (TEM) was performed by Egger and coworkers (Egger et al. 2002) in U2-OS human osteosarcoma-derived cell lines inducibly expressing the entire HCV polyprotein, as well as individual HCV NS proteins. The main alterations found were vesicles of approximately 85 nm embedded in a membranous matrix of circular or very tightly undulating membranes that formed a rather compact structure. This alteration was designated the 'membranous web' (MW).

The MW structures were morphologically similar to web-like structures found in livers of HCV-infected chimpanzees (Pfeifer et al. 1980) and contained accumulations of HCV proteins, therefore it was proposed that the MW forms the viral replication complex in HCV-infected cells. This view was later supported by data showing that in cells harboring a bicistronic subgenomic HCV replicon (Lohmann et al. 1999a) the MW structures contained not only the HCV NS proteins, but also newly synthesized viral RNA (Gosert et al. 2003). These initial studies found that formation of the MW was triggered by the NS4B protein expressed in the absence of any other HCV protein, suggesting that this protein was playing a critical role in the formation of the MW. In addition, the sole expression of NS3/4A also induced vesicular membrane alterations, while this could not be observed for any of the other NS proteins. Additional HCV specific membrane alterations were observed, so called contiguous vesicles with an irregular shape that often surrounded the web, and vacuoles of dilated rER of various sizes, however, both structures were less frequent and contained less viral antigens (Table 1).

Ten years later, the use of cutting edge approaches like correlative light electron microscopy (CLEM) and electron tomography (ET), as well as the cryo-fixation of cells by means of high pressure freezing (HPF) have allowed us to gain further insights into the morphology and the 3D architecture of these HCV-induced membranous modifications with higher resolution (Romero-Brey et al. 2012). In this manner we found that HCV-infected cells show high abundance of double membrane vesicles (DMVs), having two lipid bilayers attached to each other (Fig. 2a). Furthermore, TEM analysis of infected Huh7.5 cells at different times post-infection revealed that the abundance of DMVs correlated with the amount of HCV RNA being synthesized, indicating that they might play a role in the replication of the HCV genome. Their diameter also increases over the course of infection from an average diameter of 125 nm 16 hpi to around 185 nm 48 hpi. Analysis by ET revealed also that approximately half of these double-layered vesicles remained connected through neck-like structures to their donor membranes at the ER. In addition, late in infection (from 36 hpi onwards), multi-membrane vesicles (MMVs) were observed, having an average diameter of 340 nm (Fig. 2b). These much more complicated structures might be generated from pre-existing DMVs that undergo secondary invaginations or from late synthesized large DMTs (double membrane tubules) that enwrap pre-existing DMVs. In our study, the abundance of DMVs correlated best with RNA synthesis, suggesting that DMVs might indeed

Table 1 Summary of HCV-induced structures described in ultrastructural analyses reported in the literature

Report	System/viral strain	Cell type	Fixation method	Structure	Size
Egger et al. 2002	Expression of the polyprotein/genotype 1a	U-2 OS human osteosarcoma-derived	2.5 % GA	Membranous web (MW)	85 nm
				Contiguous vesicles (CVs)	Not-provided
				Dilated ER vesicles	Various sizes (not provided)
Gosert et al. 2003	Subgenomic replicon/9–13 (genotype 1b)	Huh7	2.5 % GA	Membranous web (MW)	80–100 nm
Ferraris et al. 2010	Sugenomic replicon/JFH1 (genotype 2a)	Huh7.5	4 % PFA and 1 % GA	DMVs	200–500 nm
				MMVs	150–200 nm
Romero-Brey et al. 2012	Infection/Jc1 (genotype 2a)	Huh7.5	4 % PFA and 0.1 % GA + HPF	DMVs	~170 nm
	Subgenomic replicon/JFH1 (genotype 2a)	Huh7	HPF	MMVs	340 nm
	Expression of polyprotein/JFH1 (genotype 2a)	Huh7-Lunet T7	HPF		
Ferraris et al. 2013	Infection/JFH1 (genotype 2a)	Huh7.5	4 % PFA and 1 % GA	Vesicles in clusters (ViCs)	100–200 nm
				Contiguous vesicles (CVs)	~100 nm
				DMVs	150–1,000 nm

GA glutaraldehyde, *PFA* paraformaldehyde, *HPF* high-pressure freezing, *DMVs* Double Membrane Vesicles, *MMVs* Multi-membrane Vesicles

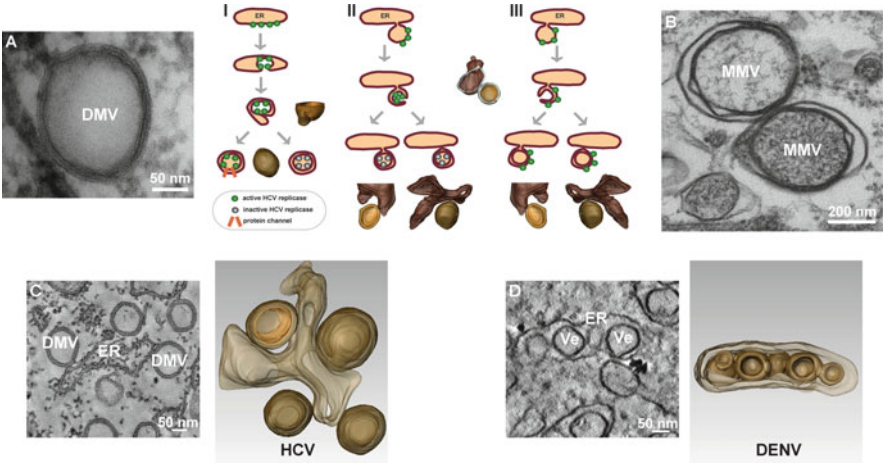

Fig. 2 3D architecture of HCV-induced structures. (**a**) DMVs, double membrane vesicles, are the main hallmark of HCV infection: they are composed of two lipid bilayers tightly opposed to each other. I–III represent different ways in which DMVs and active replicase might originate from the ER (Romero-Brey et al. 2012). (**b**) MMVs, multi-membrane vesicles, are found late in infection and consist of several circular concentric membranes. (**c**, **d**) Slices through electron tomograms (on the *left*) and 3D representation (on the *right*) of the HCV and DENV replication vesicles (reproduced with permission from Romero-Brey and Bartenschlager, 2014). (**c**) Some of the DMVs originated upon HCV infection remain connected to the ER via their outer membrane, indicating that they might be originated by evagination of ER membranes. (**d**) The DENV replication sites are formed as invaginations towards the ER lumen, generating several vesicles (*Ve*) inside the ER lumen

represent the sites of RNA replication. This view is supported by EM and biochemical data, showing that affinity-purified membranes from HCV replicon cells mainly consist of DMVs, that are able to support RNA synthesis in vitro (Paul et al. 2013).

DMVs and MMVs were also described previously in Huh 7.5 cells harboring a subgenomic replicon (JFH1) (Ferraris et al. 2010). Furthermore, both structures were also observed in purified membranes associated with HCV that were found to contain NS3, NS5A and HCV RNA. In a more recent study Ferraris et al. identified two additional types of membrane alterations using JFH1-infected cells: contiguous vesicles (CVs), small single-membrane vesicles, present in large numbers and widely distributed throughout the cytoplasm, with a more homogeneous size (around 100 nm). They were tightly associated with each other and tended to form a collar around lipid droplets (LDs). Vesicles in clusters (ViCs), small single-membrane vesicles of variable size (100–200 nm), were also found in infected cells, grouped together in well-delimited areas (Table 1, Ferraris et al. 2013). A 3D reconstruction of a complete HCV-infected cell revealed that all three membrane structures were tightly connected and closely associated with LD clusters (Ferraris et al. 2013). In this study, the number of CVs correlated with intracellular HCV RNA levels, arguing for a possible role of CVs in the early stages of viral replication. Alternatively, CVs might constitute the membranous platform for viral assembly. In fact, the core protein is present in these structures (16 %) as well as on the LD surface (81 %).

However, thus far visualization of virus assembly sites and virus particles in infected cells has not been possible, making this hypothesis difficult to prove. While most of the dsRNA signal was located within DMVs or at DMV membranes, ViCs were free of viral components and RNA and these structures as well as CVs were very rarely observed in cells with a subgenomic JFH1 replicon (Ferraris et al. 2010), or absent in cells infected with a JFH1 variant designated Jc1 (Romero-Brey et al. 2012), having the same replicase, but producing far higher amounts of virus (Pietschmann et al. 2006). Altogether, these data suggest that CVs and ViCs might arise from accumulations of structural proteins and are probably linked to assembly rather than being associated with RNA synthesis.

The differences observed for HCV-induced membrane alterations can most likely be attributed to variations in the cell culture models and fixation techniques. Still, recent studies agree that the predominant membranous structures detected in HCV-infected cells and in cells harboring subgenomic replicons are DMVs (Table 1). However it is not proven that these structures represent *bona fide* active replication factories. A functional link between DMVs and RNA replication is suggested by the fact that dsRNA, the presumptive RNA intermediate, localizes to the lumen of these vesicles (Ferraris et al. 2010), that purified DMVs contain HCV RNA (Ferraris et al. 2010), and also retain the ability to synthesize RNA in vitro (Paul et al. 2013). The most important argument against their role as active replication sites is the apparent absence of a pore or an opening connecting them with the surrounding cytosol, which is likely to be required to supply nucleotides and to deliver newly synthesized RNA to the cytoplasm. Only in ~10 % of DMVs has an opening towards the cytosol been observed, possibly indicating that only a minority of the DMVs are actively engaged in replication at a given time (Romero-Brey et al. 2012). Alternatively, many pores might be beyond the resolution limits of the methods used so far to visualize them. Thus it could be that proteinaceous (viral or cellular) or lipid channels might allow the transport of newly synthesized RNA towards the cytosol and the uptake of nucleotides from the cytosol (see below, Role of cell proteins and lipids in the biogenesis of the HCV replication sites). However, it still cannot be excluded that DMVs represent late stages of inactive replication organelles that originate from less prominent and less abundant active replication vesicles, e.g. from CVs (Ferraris et al. 2013), due to the lack of time-resolved data showing newly synthesized RNA in these structures.

3 Biogenesis of the Membranous Web and Comparison to Replication Organelles Induced by Different Members of the Family *Flaviviridae* and by Other Positive- Strand RNA Viruses

HCV, as the prototype virus of the genus *Hepacivirus*, seems to induce the formation of membrane-bound RNA factories that are distinct from those observed in cells infected with other members of the family *Flaviviridae*. Analysis by ET

revealed that DMVs are evaginations from the ER (Fig. 2c). Still it is not clear how they are generated and several alternative models might be envisaged (Fig. 2a, I–III) (Romero-Brey et al. 2012): (I) It could be that HCV proteins induce invaginations of the ER membrane, and the second membrane is acquired by a local contraction of the ER lumen. In this case enzymatically active HCV replicase (green dots) resides in the lumen of the invagination and remains active as long as the vesicle is linked to the cytosol. DMVs might be connected to the cytosol via protein channels formed by viral or host factors. Alternatively, such structures might be formed by autophagy, which has been proposed to play an essential role in MW formation (Sir et al. 2012). (II) HCV proteins or host cell machinery recruited by viral proteins might induce a single-membrane evagination from the ER membrane, which then undergoes a secondary invagination. These DMVs might initially contain an opening to the cytoplasm, which could later be closed to render the replicase inactive. The connection of the outer DMV membrane with the ER membrane could be pinched off by host factors. (III) A third possibility is that induction of DMVs follows the same pathway as described above, but the active replicase resides on the outside of the DMV. In this case, no connection of the DMV to the cytoplasm is required, explaining the low frequency of pores observed. However, this model seems unlikely, since viral replicase activity and RNA is highly resistant to proteases and nucleases, respectively, in replication complexes purified from replicon cells (Quinkert et al. 2005; Paul et al. 2013; Miyanari et al. 2003). This protection is detergent sensitive, arguing for the active replicase being shielded by membranes.

Interestingly, no prominent membrane alterations associated with RNA replication have been found in cells infected with the closely related pestiviruses, other than vesicles in multi-vesicular bodies (MVBs) containing dsRNA (Schmeiser et al. 2014).

Surprisingly, replication sites produced by viruses of the genus *Flavivirus* are entirely different from the HCV MW. Generally, flavivirus replication vesicles appear as single membrane invaginations in the lumen of the ER (Fig. 2d). These vesicles have been observed for instance in Dengue Virus (DENV)-infected cells (Welsch et al. 2009) having a diameter of 80–90 nm, as well as in Kunjin Virus -the Australian variant of West Nile Virus- (WNV_{KUN})- (Westaway et al. 1997b; Mackenzie et al. 2001; Gillespie et al. 2010) and Tick-Borne Encephalitis Virus (TBEV)-infected cells (Overby et al. 2010; Miorin et al. 2013). ET analysis of cells infected with either one of these three viruses revealed that such vesicles are frequently found in groups filling up the ER lumen and, therefore, they have been termed vesicle packets (VPs). Another aspect that distinguishes these vesicles from those seen in HCV-infected cells is that they remain connected to the cytosol via pores of ~10 nm diameter, which allows the export of the newly synthesized RNA to the flaviviral assembly sites. In fact, virions were found in close proximity to the pores of the replication vesicles, suggesting that replication factories could represent a continuous membrane network that provides a platform for the transport of viral proteins and genomes between sites of RNA replication, ribosome-containing compartments (RNA translation) and virus assembly sites (Welsch et al. 2009). In addition to these invaginations, convoluted membranes (CM) have been found in DENV (Welsch et al. 2009) and WNV_{KUN} (Westaway et al. 1997b; Mackenzie et al. 1996) infected

cells. Morphologically, CMs resemble smooth ER membranes, lack ribosomes and in the case of DENV are induced by the sole expression of NS4A (Roosendaal et al. 2006; Miller et al. 2007). Based on the localization of several NS proteins to these structures, it has been suggested that they may represent the site of RNA translation/polyprotein processing (Westaway et al. 1997a, b), or might represent a storage site for proteins and lipids involved in viral replication that can be recruited to vesicles upon demand (Welsch et al. 2009). However their exact role in the flaviviral life cycle needs still to be determined.

These findings indicate that all members of the genus *Flavivirus* studied to date, as well as HCV (genus *Hepacivirus*) utilize the ER as a source of membranes for the formation of their replication factories, although they seem to re-shape these membranes in a fundamentally different fashion: while members of the genus *Flavivirus* induce invaginations of the host membranes, HCV seems to form evaginations of these membranes, generating DMVs that look like protusions of the ER (Fig. 2c, d).

However, HCV is not the only positive- strand RNA virus inducing the formation of DMVs, which involves the formation of positive curvature membranes. Structures with two lipid bilayers have been also found in cells infected with positive-strand viruses classified within the families *Picornaviridae*, *Coronaviridae*, *Arteriviridae* and *Nodaviridae* (reviewed in Romero-Brey and Bartenschlager 2014). In contrast, alphaviruses and Rubella Virus are known to induce the formation of negatively curved membranes, initiated by invaginations of the pre-existing membrane bilayer and giving rise to vesicles (also called spherules or vacuoles) towards the lumen of the targeted cell organelle, as described above for members of the genus *Flavivirus* (reviewed in Romero-Brey and Bartenschlager 2014). The conservation of these two types of induced organelles in distantly related viruses supports the possibility of an evolutionary conserved mechanism, which might involve distinct cellular machineries.

4 Role of the Viral Proteins in the Formation of DMVs

As already discussed in this chapter, cells harboring subgenomic replicons contain DMVs as those found following HCV infection (Ferraris et al. 2010; Romero-Brey et al. 2012), indicating that the structural proteins are not only dispensable for replication (Lohmann et al. 1999a), but also for the formation of DMVs (Fig. 3a). This finding also provided an indirect indication that the function of these structures is related to the viral replication of HCV. Interestingly the replication efficiency of replicons correlates with the amount of DMVs that are found in the cytosol of cells transfected with replicons: replicon NS2-5B produces lower amount of DMVs than replicon NS3-5B (Romero-Brey et al. 2012). This is again an indirect evidence of the role of DMVs in replication.

The very same membrane alterations identified in replicon cells were also found upon expression of a NS3-5B polyprotein fragment demonstrating that formation of DMVs is solely induced by viral proteins independent from HCV RNA replication

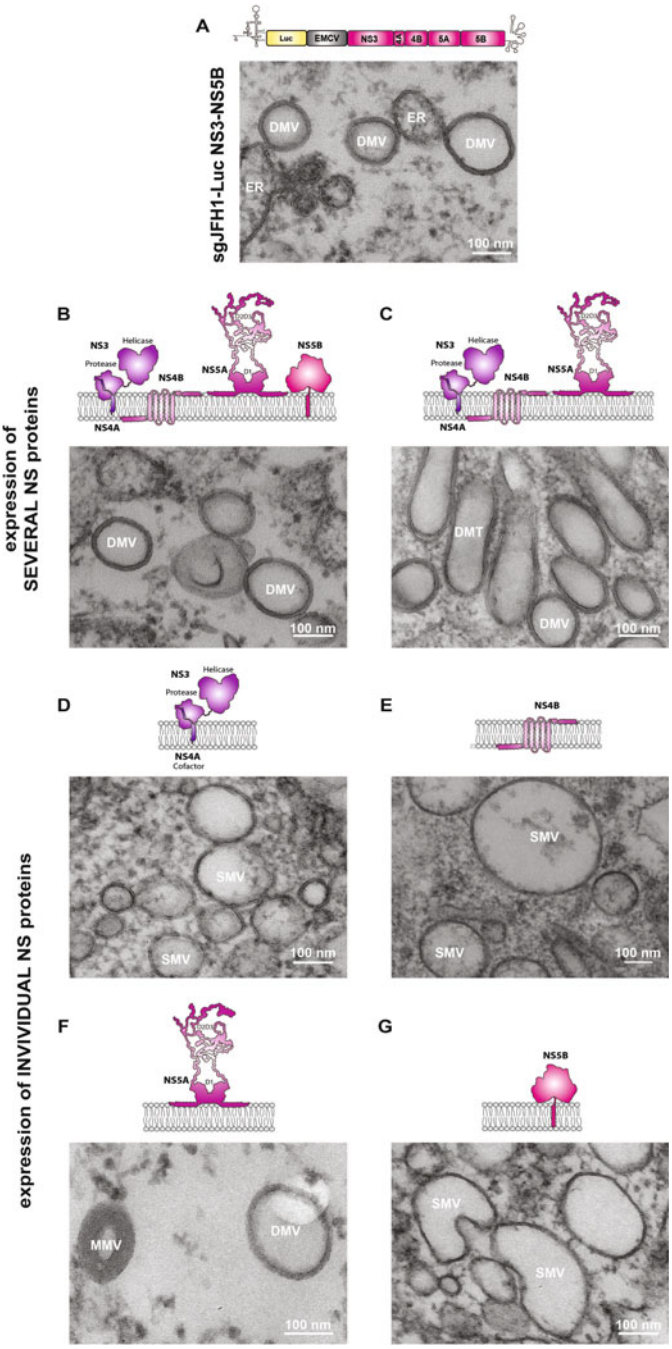

Fig. 3 Analysis of the formation of DMVs in cells harboring HCV subgenomic replicons (**a**) and upon expression of several NS proteins (**b**, **c**) and single proteins (**d**–**g**). The NS proteins were expressed in Huh7-Lunet T7 cells and membrane alterations were assessed by EM in cell sections after epon embedding. (**b**) Expression of all the NS replicase proteins (NS3-5B) induces the

(Romero-Brey et al. 2012) and also in the absence of the NTRs (Berger et al. 2014) (Fig. 3b). Therefore, expression systems can be used to assess the contribution of the replicase NS proteins to the formation of DMVs. In addition, the use of expression models allows studying the impact of inhibitors of viral replication and of mutations interfering with RNA synthesis on MW morphology (Romero-Brey et al. 2012).

Earlier studies already showed the potential of individual HCV proteins to induce membrane alterations: the NS3/4A complex induced the formation of large amounts of smooth SMVs and expression of NS4B was able to generate the MW (Egger et al. 2002), which was defined as compact clusters of ~85 nm vesicles. We obtained similar results following expression of NS3/4A and NS4B, but in addition also found specific membrane alterations upon expression of NS5A and NS5B (Fig. 3d–g, respectively). Importantly, expression of NS5A led to the formation of DMVs and MMVs, albeit with low abundance and different size as compared to NS3-NS5B expression, whereas NS5B induced enlargements of the ER occasionally containing invaginations. Therefore, all the replicase components were capable of inducing membrane vesiculation with NS5A having the highest potential to trigger membrane curvature. Some of these NS5A-induced structures were DMV-like vesicles, pointing to NS5A as an important contributor to DMV biogenesis. Still, the individual expression of the NS replicase components NS3/4A, NS4B, NS5A and NS5B did not lead to the formation of DMVs with the same abundance, morphology and heterogeneity as expression of NS3-5B (Romero-Brey et al. 2012).

Although our results showed that NS4B is clearly not able to induce the formation of DMVs and the complexity of the MW, it still plays an important role in triggering rearrangements of intracellular membranes. A recent study has demonstrated that NS4B oligomerizes through multiple conserved determinants and that oligomerization appears to be required for MW induction (Gouttenoire et al. 2010). Indeed, mutations affecting the highly conserved C-terminal domain

Fig. 3 (continued) formation of DMVs, morphologically identical to those found in infected cells. (**c**) Expression of an NS3 to 5A polyprotein fragment lacking NS5B (RdRp) induced the formation of small clusters of DMVs and elongated double membrane tubules (*DMTs*) having a highly variable diameter (166 ± 92 nm). (**d**–**g**) Analysis of the contribution of individual HCV proteins to the formation of the DMVs: NS3/4A, NS4B, NS5A or NS5B. (**c**) Note that NS3 and NS4A were not expressed individually, because both proteins form a stable membrane-associated complex and only this complex is physiologically relevant (Wolk et al. 2000). Expression of the NS3/4A complex led to the formation of swollen ER sheets and single membrane vesicles (*SMVs*) of variable diameter (203 ± 93 nm). (**d**) In cells expressing NS4B SMVs were also found having an average size of 325 nm (±168 nm). (**e**) Cells expressing NS5A alone showed vesicles containing several lipid bilayers in a concentric manner (MMVs, multimembrane vesicles) with an average diameter of 125 nm (±35 nm). Some of these vesicles displayed only two lipid bilayers and their morphology was indistinguishable from DMVs observed in HCV-infected cells or cells containing a subgenomic replicon. (**f**) Expression of NS5B induced enlarged ER sacs with an average diameter of 370 nm (±150 nm) and occasional curvature (The EM micrographs were taken from Romero-Brey et al. 2012, with permission)

impairing NS4B self-interaction resulted in the formation of aberrant DMVs, arguing for a central role of NS4B in the formation of functional replication compartments (Paul et al. 2011).

Although the molecular mechanisms orchestrating the formation of these complex structures are still poorly understood, it is now clear that all the replicase NS proteins (NS3/4A, NS4B, NS5A and NS5B) work in a concerted action to generate DMVs and the overall complexity of HCV replication sites. Even expression of NS3-5A, lacking the RNA-dependent RNA polymerase (RdRp), NS5B, generated different MW structures mainly consisting of elongated DMVs (so called DMTs, double membrane tubules, Fig. 3c), indicating that NS5B is not a mandatory building block for the formation of DMVs but still influences their morphology.

5 Inhibition of MW Formation by Direct Acting Antivirals

In agreement with the view that MW formation requires a complex interplay of all replicase proteins, recent studies brought up evidence that existing classes of replication inhibitors indeed act by interfering with MW biogenesis. NS5A inhibitors have been identified in high throughput screening with replicon cells and are characterized by a very high potency against HCV in vitro and in vivo (Gao et al. 2010). Among them the clinical lead compound Daclatasvir (DCV) has been approved for treatment of patients. This class of compounds indeed blocks the replication of HCV RNA already at the stage of the DMV formation (Berger et al. 2014). CLEM on cells expressing the NS3-5B polyprotein revealed unequivocally that co-treatment (addition of drug directly before DNA transfection) with NS5A inhibitors had no severe effect on the subcellular distribution of NS5A, but completely prevented biogenesis of DMVs. This complete abrogation of membranous vesicle formation was not found upon expression of an inhibitor-resistant NS5A mutant when the drug was applied after transfection. Therefore, highly potent DCV-like NS5A inhibitors disrupt DMV formation independent of RNA replication at a very early stage of the viral replication cycle.

Inhibition of NS4B is another way to interfere with MW formation. A recent study has shown that resistance mutations to intravenously administered Silibinin (Legalon-SIL [SIL]), which has been successfully used for HCV therapy, indeed map to NS4B (Esser-Nobis et al. 2013). Ultrastructural analyses revealed changes in the morphology of viral membrane alterations upon SIL treatment of a susceptible genotype 1b isolate, but not of a resistant NS4B mutant or genotype 2a. Most of the vesicles observed were MMVs and no DMVs, suggesting that SIL indeed modulated the morphology of viral replication sites. Other HCV inhibitors targeting NS4B might also act by interfering with MW morphogenesis, however, formal proof is lacking (Dvory-Sobol et al. 2010; Dufner-Beattie et al. 2014).

In addition to inhibitors directly targeting viral proteins and thereby interfering with MW formation, a number of drugs targeting host factors have been shown to change the morphology of the HCV replication sites. This includes inhibitors of

PI4KIIIα (Bianco et al. 2012), OSBP (Wang et al. 2014) and cyclophilins (Madan et al. 2014) and will be discussed in more detail in the next section. Generally, a surprisingly high number of drugs targeting various viral and cellular proteins seem to inhibit viral replication by disturbing the morphogenesis of the MW. Targeting viral replication sites might therefore be a promising strategy to develop inhibitors also to other positive- strand RNA viruses.

6 Role of Cellular Proteins and Lipids in the Biogenesis of the HCV Replication Sites

Literally hundreds of cellular proteins and lipids linked to viral RNA synthesis have been identified in numerous high content screenings (e.g. Li et al. 2009; Tai et al. 2009) and a comprehensive overview goes beyond the scope of this review, particularly since still little is known about their mechanisms of action. However, since several of those factors have recently been shown to be involved in the formation of the HCV replication sites, we will focus on the following here: PI4KIIIα, OSBP, VAP-A and VAP-B, cyclophilins, GBF1-Arf1-COPI, Nups, the autophagy machinery, Rabs, PSTPIP2 and PLA2G4C as summarized in Fig. 4.

6.1 Role of Cellular Lipids and Proteins Involved in Lipid Synthesis and Transport in the Biogenesis of the MW

The ability to shape a target membrane into a RNA replicating vesicle is not exclusively dependent on the ability of viral and cellular proteins to bend membranes. Indeed growing evidence shows that lipids are also key players in membrane-bound viral RNA synthesis (reviewed in Alvisi et al. 2011). Multiple reports have indicated that HCV modulates lipid metabolism (e.g. cholesterol and fatty acid biosynthesis) to promote viral replication (Kapadia and Chisari 2005; Su et al. 2002; Takano et al. 2011; Waris et al. 2007; Diamond et al. 2010). This modulation results in *de novo* lipid formation in order to increase membrane surface area, which is required for the formation of viral replication factories. A cellular cofactor involved in lipogenesis during HCV infection is the sterol regulatory element-binding protein (SREBP) (Waris et al. 2007). SREBPs are major regulators of lipid metabolism and major transcription factors for expression of genes required for lipid biosynthesis. Over-expression of NS4B has been shown to activate SREBP, leading to elevated levels of transcripts involved in lipogenesis such as fatty acid synthase (FASN) (Waris et al. 2007; Park et al. 2009). However, by using S1P-specific inhibitors SREBP-mediated lipogenesis was found to be dispensable for HCV RNA replication but required for assembly and release of progeny virus (Olmstead et al. 2012). Similarly modulation of triglyceride and cholesterol ester

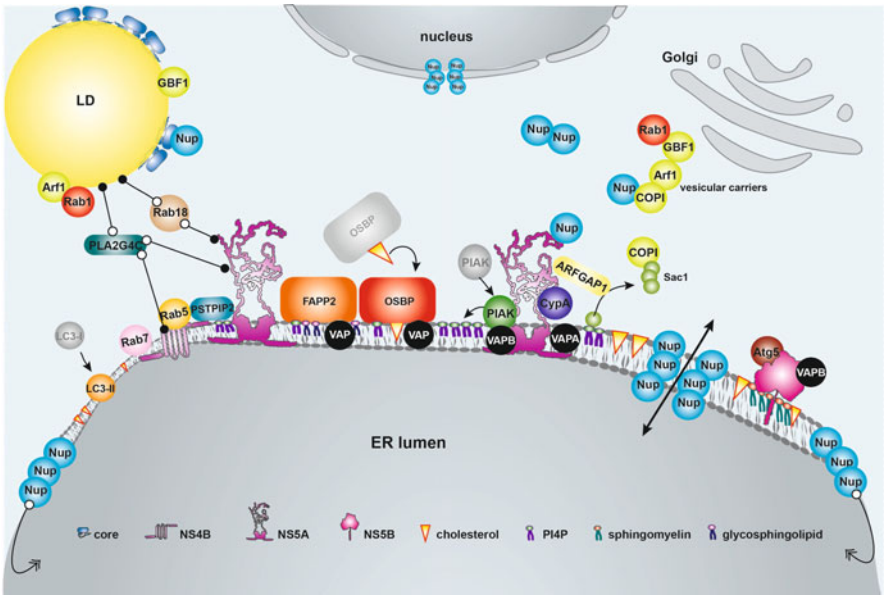

Fig. 4 Schematic representation of an ER membrane, depicting a map of interactions between the HCV NS proteins and several cellular cofactors that might be directly or indirectly involved in the formation and/or functionality of DMVs. For an understating of the different interactions please refer to the text

synthesis –lipids stored in LDs- seems to be also related to HCV assembly (Liefhebber et al. 2014).

Sphingolipid synthesis is also stimulated by HCV, resulting in globally and locally increased sphingomyelin levels that are required for replication and might contribute to detergent resistance of HCV replication sites (Hirata et al. 2012). In addition, sphingomyelin interacts with the NS5B polymerase in a genotype-specific manner, enhancing HCV RNA synthesis (Weng et al. 2010) (Fig. 4).

A very recent report stressed not only the importance of *de novo* synthesis of lipids, but also the need to limit the oxidative degradation of lipids, designated lipid peroxidation, to achieve robust replication of HCV in cell culture (Yamane et al. 2014). Thus lipid peroxidation, regulated in part through sphingosine kinase-2, severely restricts HCV replication of all genotypes in Huh-7 cells and primary human hepatoblasts and reduces the HCV-induced MW abundance. The genotype 2a JFH1 strain is an exception and is resistant to lipid peroxidation (Yamane et al. 2014). For further details, readers are referred to the chapter by Yamane and Lemon in this volume.

Besides generally enhancing cellular lipid content, HCV also exerts a more direct influence on the lipid composition of its replication sites. The HCV replication complex has been reported to be associated with lipid rafts (Aizaki et al. 2004; Shi et al. 2003). These membrane lipid microdomains are enriched for cholesterol,

sphingolipids and certain proteins and form nanoscale-ordered protein-lipid assemblies (reviewed in Simons and Sampaio 2011): they generally contain three- to fivefold the amount of cholesterol found in the surrounding bilayer (Anchisi et al. 2012). Consistent with this, analysis of the molecular composition of the membranous replication compartment revealed that HCV-remodeled membranes are highly enriched in cholesterol (Paul et al. 2013). Higher concentrations of this cone-shaped lipid on membranes enhances rigidity and stability, likely serving as an important structural component of HCV-remodeled membranes (Fig. 4). The accumulation of these lipids within HCV-induced membranes might help the bending of these membranes into DMVs, via the formation of lipid-enriched microdomains that favour the positive contouring of these membranes. Several mechanisms have been unraveled concerning how HCV modulates the lipid environment of its replication sites, including the activation of the lipid kinase PI4KIIIα to generate enhanced levels of PI4P at the replication sites, which in turn attracts lipid transport proteins delivering cholesterol and glycosphingolipids.

6.1.1 PI4KIIIα

One essential host factor implicated in HCV RNA replication is the phosphatidylinositol 4-kinase IIIα (PI4KIIIα), an ER-resident enzyme that catalyzes the synthesis of phosphatidylinositol 4-phosphate (PI4P). In mammalian cells, the family of PI4-kinases comprises two types with two isoforms each (PI4KIIα, PI4KIIβ, PI4KIIIα and PI4KIIIβ) differing in subcellular localization and being responsible for the synthesis of distinct PI4P pools (reviewed in Balla 2006). PI4KIIIα is present in the ER, the plasma membrane (Balla et al. 2005) and parts of the Golgi (Bianco et al. 2012). PI4P accumulates in HCV replicating cells due to recruitment and activation of PI4KIIIα (Reiss et al. 2011; Berger et al. 2011; Tai and Salloum 2011; Bianco et al. 2012; Hsu et al. 2010) by NS5A and NS5B (Reiss et al. 2013) (Fig. 4). The interaction site of PI4KIIIα with NS5A has been mapped to the carboxyterminal end of domain I, encompassing seven amino acids, which are essential for viral RNA replication and PI4KIIIα activation (Reiss et al. 2013). However, only the C-terminal half of PI4KIIIα is required for HCV replication (Harak et al. 2014). In addition to PI4KIIIα, several other studies also implicated a role of the Golgi resident PI4KIIIβ isoform, particularly in the replication of genotype 1 (Borawski et al. 2009; Hsu et al. 2010). This indeed suggests that PI4P metabolism has an essential role in HCV replication.

PI4P localizes mainly to the Golgi and the inner leaflet of the plasma membrane, where it fulfills important functions by providing "signatures" to distinct membrane compartments and by recruiting multiple factors involved in vesicle budding and lipid biosynthesis (Hammond et al. 2012). Activation of PI4KIIIα by HCV results in strongly elevated intracellular PI4P levels with an altered intracellular distribution, partially co-localizing with HCV replication sites (Reiss et al. 2011). PI4KIIIα thereby influences the morphogenesis of the HCV replication compartments, most likely involving altered levels of PI4P. Depletion of PI4KIIIα by RNA interference

or inhibitors, or by abrogating NS5A interaction, results in more homogenous MW structures consisting solely of DMVs with reduced diameter and organized in huge clusters (Reiss et al. 2011, 2013).

In addition to its role in MW morphogenesis, PI4KIIIα might also impact HCV replication in a PI4P-independent fashion, influencing regulation of NS5A phosphorylation, thereby facilitating synthesis of the p56 form of this NS protein (Reiss et al. 2013). Alternatively, PI4P may recruit cellular and/or viral proteins required for replication (Berger et al. 2009; Bishe et al. 2012).

6.1.2 Lipid Transporters: OSBP, FAPP2

In addition to a potential direct role in the morphogenesis of the MW, PI4P indirectly affects the lipid composition of membranes by attracting lipid transfer proteins, thereby orchestrating changes in lipid composition at HCV replication sites. Lipid transfer proteins (LTPs) are implicated in the nonvesicular transport of lipids within the cell. Several of these proteins contain an N-terminal pleckstrin homology (PH) domain that mediates PI4P binding and a C-terminal lipid transfer domain that interacts with a second lipid that needs to be transferred between membranes. The direction of transport is typically driven by PI4P concentration gradients. The ER membrane is depleted of PI4P by the phosphoinositide phosphatase Sac1 (the enzyme that catalyzing PI4P dephosphorylation), whereas oxysterol-binding protein (OSBP) regularly transfers PI4P from Golgi to ER and transports back cholesterol from ER to Golgi in a process assisted by VAP-A and -B (Mesmin et al. 2013) (described below).

OSBP was first shown to be important for HCV virion secretion (Amako et al. 2009, 2011). However, it was recently shown that OSBP is also essential for HCV RNA replication (Wang et al. 2014). Colocalization of OSBP and NS5A is lost when PI4KIIIα is silenced, suggesting that OSBP is recruited to the replication compartment via PI4P (Fig. 4). Using fluorescent cholesterol analogs, the authors confirmed that cholesterol is enriched in NS5A-positive structures (Wang et al. 2014), corroborating previous findings with purified DMVs (Paul et al. 2013). Furthermore, inhibition of OSBP caused clustering and reduction in the diameter of the membranous vesicles, mimicking the phenotype observed upon silencing of PI4KIIIα and suggesting that this phenotype in fact is mediated by cholesterol depletion. In summary, these results suggest that OSBP is a PI4P effector required for HCV replication membrane integrity and cholesterol trafficking. Activation of PI4KIIIα and enrichment of PI4P thereby generates a concentration gradient to deliver cholesterol to replication sites. Interestingly, OSBP is also required for poliovirus infection, suggesting that DMV-inducing viruses might generally require PI4P- and cholesterol- enriched membranes in order to form their replication compartments.

Recently, an additional PI4P-specific lipid transfer protein, the four-phosphate adaptor protein 2 (FAPP2), was shown to be important for HCV replication (Khan et al. 2014). FAPP2 exchanges PI4P with glycosphingolipids, which are thereby enriched in the MW, again due to PI4P concentration gradient generated by

activation of PI4KIIIα. Depletion of FAPP2 could be partially rescued by providing exogenous glycosphingolipids, arguing for a direct role of the lipid rather than the protein (Khan et al. 2014). Overall, HCV-mediated induction of PI4KIIIα has a fundamental impact on the lipid composition of the viral replication compartment beyond PI4P and might also substantially contribute to the lipid-raft properties of these membranes (Aizaki et al. 2004) and provide lipids required for virion formation (Aizaki et al. 2008).

6.2 *Role of Vesicular Trafficking in the Biogenesis of the MW*

6.2.1 VAPs

Two additional well-described host factors are the vesicle-associated membrane protein–associated proteins A and B (VAP-A and VAP-B) (Evans et al. 2004; Gao et al. 2004; Hamamoto et al. 2005) that are involved in ER homeostasis and vesicular trafficking (Wyles et al. 2002; Amarilio et al. 2005). In a recent study, Paul and colleagues found that these HCV–co-opted host proteins VAP-A and VAP-B are enriched in purified DMVs (Paul et al. 2013). These findings together with the fact that VAP-A interacts with OSBP to modify export from the ER (Amarilio et al. 2005), where the DMVs are being formed, suggests that an interplay of both proteins might help to re-shape ER membranes (Fig. 4). In addition, VAP-A and -B might be involved in the recruitment of lipid transport proteins like OSBP and FAPP2 and thereby help to shape the lipid composition of the HCV replication sites (Mesmin et al. 2013). Furthermore, VAP-A and VAP-B proteins have been shown to interact with NS5A in a phosphorylation-dependent manner (Evans et al. 2004; Gao et al. 2004; Hamamoto et al. 2005) and with NS5B (Hamamoto et al. 2005), highlighting again the central role of NS5A in the formation of the membranous replication factories (Fig. 4).

6.2.2 Rabs

The Ras superfamily of small GTPases is involved in the regulation of vesicle budding, transport and fusion with target membranes. They cycle between active (GTP-bound) and inactive (GDP-bound) forms, requiring cognate GTPase-activating proteins (GAPs) for catalysis and guanine nucleotide exchange factors (GEFs) for GTP binding. Rabs are associated with specific host membranes, for instance, Rab5 is bound to the early endosome (EE) where it regulates endocytosis and EE fusion, Rab7 is found in the late endosome (LE) and facilitates transport from LE to lysosomes, Rab6 is mostly associated with EE recycling to the Golgi as well as intra-Golgi traffic and Rab1 and 2 are mostly found in the Golgi and regulate

traffic between ER and the Golgi apparatus (reviewed in Mizuno-Yamasaki et al. 2012).

Rab5 has been shown to be involved in HCV genome replication (Stone et al. 2007; Berger et al. 2009) and the NS4B C-terminal domain might play a role in Rab5 recruitment to NS4B foci (Aligo et al. 2009) (Fig. 4), suggesting that the early endosome contributes to the formation of HCV replication sites. Another study demonstrated that Rab5 and 7 strongly co-localize with NS4B positive foci and Rab2, 5 and 7 are required for HCV RNA replication (Manna et al. 2010) (Fig. 4).

Rab1b, a regulator of membrane dynamics in the early secretory pathway (Sklan et al. 2007) and its negative regulator TBC1D20 (Sklan et al. 2007; Nevo-Yassaf et al. 2012) have been reported to be involved in the replication of HCV as well.

Rab18 is believed to promote physical interaction between LDs and ER membranes (Ozeki et al. 2005). Salloum and colleagues (2013) have shown that Rab18 promotes the physical association of the other replicase components with LDs through direct association between NS5A and the active, GTP-bound form of Rab18 (Fig. 4). Rab18 might, therefore, help to establish a physical coupling between the HCV replication and virion production. Consistent with this hypothesis, a recent report has shown that Rab18 is required for viral assembly (Dansako et al. 2014).

Altogether these findings suggest an involvement of several Rabs in the formation of HCV replication sites, which are likely dependent on a mechanism more complex than a simple remodeling of ER membranes.

6.2.3 GBF1-Arf1-COPI

Several independent studies point to a role for GBF1-ArfI-COPI, a protein complex involved in retrograde transport from *cis*-Golgi to the ER (reviewed in Bonifacino and Glick 2004; Donaldson and Jackson 2011), in the biogenesis of the MW. GBF1 (Golgi complex-specific BFA resistance factor 1) is a guanine nucleotide exchange factor (GEF), which is recruited to the membrane of the *cis*-Golgi and activates the small GTPase-protein Arf1 (ADP-ribosylation factor 1). Once activated by the binding of GTP, Arf1 in turn recruits different effectors, including the coat protein complex COPI, which then forms vesicular carriers. Recent reports indicate that the replication of HCV depends on the GBF1-Arf1-COPI complex (Tai et al. 2009; Goueslain et al. 2010; Matto et al. 2011; Zhang et al. 2012; Farhat et al. 2013), however the precise role of this pathway in HCV replication is still elusive.

Pharmacological inhibition of Arf1 by the fungal metabolite brefeldin A (BFA), has been shown to decrease HCV RNA replication (Tai et al. 2009; Goueslain et al. 2010; Matto et al. 2011). The inhibition of HCV replication by BFA is much stronger at the beginning of the infection than when the infection is already established (Goueslain et al. 2010), suggesting a crucial role at the onset of replication. EM analyses indicated that BFA does not block the formation of MW-like structures induced by expression of HCV proteins in a non-replicative

context, suggesting that GBF1 is probably not involved in the formation of HCV replication complexes but, rather, in their activity (Goueslain et al. 2010). HCV replication is almost as sensitive to BFA in BFA-resistant hepatoma-derived cells as in Huh-7 cells, suggesting that GBF1 might fulfill another function, in addition to regulation of the secretory pathway (Farhat et al. 2013). Goueslain and coworkers hypothesized that GBF1-associated mechanisms function to deliver proteins or lipids to the HCV replication sites (Goueslain et al. 2010). Indeed Arf1 and GBF1 seem to be involved in generating a PI4P-enriched environment supportive of HCV replication (Zhang et al. 2012). Along the same lines, it has been recently shown that ARFGAP1 is hijacked by NS5A to remove COPI cargo Sac1 from the site of HCV replication (Li et al. 2014). Since Sac1 catalyzes the PI4P dephosphorylation, its removal by ARFGAP1 might thereby help to maintain high levels of PI4P at HCV replication sites (Fig. 4).

6.3 Cyclophilins

Cyclophilins (Cyps) are ubiquitous molecular chaperones catalyzing the *cis-trans* isomerization of proline residues and hence are called peptidyl-prolyl *cis-trans*-isomerases (PPIases). Their isomerase activity is thought to be important for proper folding of certain proteins. At present, 16 Cyp members have been identified with seven major members found in humans (CypA-E, Cyp40, CypNK; (Wang and Heitman 2005)).

The role of Cyps in HCV replication was identified by the inhibition of HCV replication by Cyclosporin A (Watashi et al. 2003). Initially, CypB was supposed to be the key player in HCV replication (Watashi et al. 2005; Nakagawa et al. 2004), however, more recent evidence rather points to CypA (Yang et al. 2008; Kaul et al. 2009; Chatterji et al. 2009). For both CypA (Yang et al. 2008) and CypB (Watashi et al. 2005; Nakagawa et al. 2005; Heck et al. 2009), direct interactions with NS5B were reported, but more recently, binding sites in NS5A domain II and III have been identified and characterized by NMR as well (Hanoulle et al. 2009; Verdegem et al. 2011). Indeed, mutations conferring resistance to CypA inhibitors have been found in NS5A (Yang et al. 2010; Grise et al. 2012) as well as NS5B (Liu et al. 2009a). Initially, CypA was regarded as a host factor directly regulating RNA synthesis by *cis-trans* isomerization of particular proline residues in NS5A and NS5B, thereby modulating polyprotein processing kinetics (Kaul et al. 2009), RNA binding by NS5B (Watashi et al. 2005; Liu et al. 2009a), or recruitment of NS5B to replication sites (Liu et al. 2009b). A more recent study additionally suggests that CypA and NS5B share a binding site in NS5A, which might regulate replication (Rosnoblet et al. 2012). However, Madan and colleagues have reported that cyclosporine also blocks the *de novo* formation of DMVs, while having little effect on established membranous replication factories (Madan et al. 2014). Furthermore, this block was prevented by cyclosporine resistance mutations in NS5A. These data

suggest that CypA-dependent modification of NS5A is required for the biogenesis of the HCV replication compartment (Fig. 4).

6.4 Nups

Several components of the nuclear transport machinery have been shown to be involved in the life cycle of HCV (Kim et al. 1999; Suzuki et al. 2005; Yamanaka et al. 2002; Ide et al. 1996; Isoyama et al. 2002; Chung et al. 2000; de Chassey et al. 2008), including soluble nuclear transport factors (NTFs), many of which are members of a family of proteins termed karyopherins (Kaps). Kaps bind nuclear localization/import signal (NLS) or nuclear export signal (NES) containing molecules in the cytoplasm or nucleus and escort these cargos across the nuclear envelope (NE) through passageways formed by large macromolecular structures termed nuclear pore complexes (NPCs) (reviewed in (Wente and Rout 2010). Each NPC is comprised of ~30 distinct proteins, called nucleoporins (Nups), which form a cylindrical channel lined by Nups that facilitate movement of the NTF across the nuclear envelope.

Following HCV infection Neufeldt and colleagues (Neufeldt et al. 2013), observed an increase in cytoplasmic levels of various Nups and their recruitment to regions of cytoplasm containing HCV replication or assembly complexes. Consistent with these observations, they also demonstrated an association between various HCV proteins and specific Nups, as well as the NTFs Kap β3/IPO5 and Kap α. These interactions appear to play a role in the viral life cycle, as depletion of specific Nups or Kap β3/IPO5 inhibits HCV replication. Interaction of HCV proteins with Nups and Kaps could therefore potentially alter host cell nucleocytoplasmic transport to facilitate HCV replication. Alternatively, Nups may be recruited to the MW, in part, to usurp their functions in contouring of membranes (Neufeldt et al. 2013) (Fig. 4). The curvature of membrane domains at sites of viral particle budding into the ER lumen is indeed topologically similar to the nuclear pore membrane (Bartenschlager et al. 2010; Shimizu et al. 2011). Finally, Nups could act as a permeability barrier allowing NLS-containing molecules to access regions within the MW (Fig. 4). NLS-like sequences or NTF binding domains have been identified in core (Ide et al. 1996), NS5A (Suzuki et al. 2005), and NS3 (Kim et al. 1999). Moreover, previous studies have found that core and NS5A proteins interact with Kap β3/IPO5 and Kap α (Isoyama et al. 2002; Chung et al. 2000; de Chassey et al. 2008). NLS sequences have also been detected in several host-cell factors associated with the MW (Lee et al. 2011; Isken et al. 2007).

6.5 *Autophagy Machinery*

The morphological similarity of DMVs and autophagosomes argues in favour of the autophagy machinery as a player during their biogenesis. The autophagy machinery is implicated in the degradation and recycling of cellular material, being essential to maintain the cell homeostasis. It has been shown to play a major role for the replication of several other positive-strand RNA viruses, including poliovirus, coronaviruses and dengue virus (reviewed in Paul and Bartenschlager 2013).

In the case of HCV, the role of autophagy is still a matter of controversy. Ferraris et al. found that the fractions enriched in HCV-induced DMVs contain microtubule-associated protein 1 light chain 3-II (LC3-II), the lipidated species of LC3-I, a hallmark of autophagy induction (Fig. 4) (Ferraris et al. 2010). However, there are conflicting results concerning the stage of the HCV life cycle requiring the autophagy machinery. Hence, it has been proposed that autophagy is involved in HCV RNA translation (Dreux et al. 2009), initiation of RNA replication (Sir et al. 2008a, b; Guevin et al. 2010), production of infectious virus particles (Tanida et al. 2009) or suppression of the innate antiviral defense leading to viral persistence and chronic hepatitis (Ke and Chen 2011; Shrivastava et al. 2011; Granato et al. 2014). A recent study even suggests that autophagosomes indeed represent the actual HCV replication sites (Sir et al. 2012). However, other studies observed only limited co-localization of autophagy markers with HCV proteins and failed to detect autophagosome precursors early after infection which might develop into DMVs (Romero-Brey et al. 2012).

It might well be possible that individual factors of the autophagy pathway, rather than the complete machinery, are involved in the formation of the MW. Indeed it has been recently reported that NS4B forms a complex with Rab5 and Vps34 and induces autophagy (Su et al. 2011). Moreover, NS5B appears to interact via its thumb domain with the autophagy protein 5 (Atg5), which regularly initiates the formation of autophagic DMVs (Guevin et al. 2010).

Autophagy might also play a role in the formation of MMVs late in HCV infection (Fig. 2). MMVs could originate from autophagosomes engulfing DMVs as part of a cellular stress response induced by massive virus-induced membrane alterations, to degrade or recycle these structures after RNA synthesis has finished (Romero-Brey et al. 2012).

6.6 *PSTPIP2*

Proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2) is a protein with membrane-deforming activity critical for MW formation in HCV replication (Chao et al. 2012). Immunoprecipitation results indicated that PSTPIP2 has the potential to interact with NS4B and NS5A directly. PSTPIP2 was predominantly localized in detergent-resistant membranes (DRMs) where HCV replication occurs

(Shi et al. 2003). Importantly, PSTPIP2 knockdown caused a significant reduction in the formation of HCV- and NS4B-induced MWs, whereas its over-expression induced cytoplasmic tubular membranes, highlighting its ability to induce positive membrane curvature. Furthermore, a PSTPIP2 mutant defective in inducing membrane curvature failed to support HCV replication, confirming that the membrane-deforming ability of PSTPIP2 is essential for HCV replication.

Consistent with these results, the F-BAR domain of PSTPIP2 can bind to phosphatidylinositide (PI) lipids (Hu et al. 2009; Tsujita et al. 2006) and may thereby target them to intracellular membranes to induce the formation of membrane curvature, thus initiating and/or stabilizing the MW (Fig. 4).

6.7 PLA2G4C

The phospholipase A2 gamma group IVC (PLA2G4C) gene was identified as a host gene that is upregulated in expression upon HCV infection (Xu et al. 2012). While PLA2G4C was barely detectable in the hepatoma cell lines Huh7.5.1 and Lunet, its expression was enhanced after HCV infection, contributing to HCV replication and assembly and colocalizing with NS4B and NS5A. PLA2G4C is a membrane-bound, calcium-independent, cytosolic phospholipase (Stewart et al. 2002; Underwood et al. 1998) that hydrolyzes fatty acid from the sn-2 and sn-1 positions of phosphatidylcholine generating lipid signaling molecules such as arachidonic acid. EM analysis demonstrated that the MW formation was defective after PLA2G4C knockdown in HCV replicon-containing cells, suggesting that this protein is required for the biogenesis of the MW. In addition, PLA2G4C overexpression relocates the NS4B protein to LDs, where virion assembly occurs. Thus, PLA2G4C may bridge the steps of RNA replication and HCV assembly by translocation of replication complexes to LDs.

As mentioned above, NS5A is a key player in the formation of DMVs (Romero-Brey et al. 2012). Indeed, most of the cellular proteins that are known to have an impact in the morphology of DMVs are interaction partners of this multifunctional NS protein, including PI4KIIIα, VAP-A, VAP-B, cyclophilins, GBF1-Arf1-COPI, Nups, some members of the autophagy machinery, Rab18, PSTPIP2 and PLA2G4C (Fig. 4). However, the precise functions of host cell factors involved in the biogenesis of DMVs and the MW are still ill-defined and future analyses are needed to get further insight into their mechanisms of action. HCV RNA synthesis takes place in tight association with the MW. It is mainly driven by the viral polymerase NS5B and regulated by *cis*-acting replication elements with the help of other viral NS proteins and numerous host factors. The mechanistic details of viral RNA replication are still enigmatic due to the close connection between translation, polyprotein processing, morphogenesis of membrane alterations and RNA synthesis. However, the process of RNA replication must be tightly regulated, since it generates a five to tenfold excess of positive-strand RNA molecules compared to negative-strand RNA (Lohmann et al. 1999a; Quinkert et al. 2005; Keum

et al. 2012). In addition, HCV RNA levels in vivo (Lanford et al. 2011) and in cell culture (Keum et al. 2012; Quinkert et al. 2005) are quite limited compared to other positive- strand RNA viruses, which might be an important determinant to maintain persistence.

We have learned much about the functions of individual components of the replicase by reverse genetics, crystal structures of viral proteins, and biochemical analyses on purified proteins in vitro. This knowledge will be summarized in the next sections.

7 Linkage Between HCV RNA Synthesis and Membrane Alterations

For positive-strand RNA viruses the process of viral RNA synthesis is tightly linked to the biogenesis of virus-induced membrane alterations. However, surprisingly little is known about how these structures are functionally connected to RNA synthesis itself, because it has yet not been possible to generate replication competent mutants in absence of membrane alterations, whereas the morphology of the membrane alterations is not dependent on RNA replication in the case of HCV (Romero-Brey et al. 2012). Still, as detailed above, most current evidence suggests that RNA synthesis occurs within vesicular structures, most likely DMVs: (i) Most of the viral RNA, in particular almost all of the negative strand RNA, is nuclease-protected but detergent sensitive in cell extracts containing biochemically active viral replication complexes, so called CRCs (Quinkert et al. 2005; Miyanari et al. 2003), suggesting that viral replication intermediates are indeed shielded by membranes. (ii) The numbers and kinetics of appearance of DMVs coincides with RNA synthesis (Romero-Brey et al. 2012). (iii) Affinity purified membrane fractions from HCV replicon cells mainly consist of DMVs and retain the ability to synthesize viral RNA in vitro (Paul et al. 2013; Ferraris et al. 2010). (iv) Nascent RNA has been found associated to vesicular structures in EM studies (Gosert et al. 2003; Sir et al. 2012). However, at the moment it cannot be ruled out that RNA replication may be associated with alternative membrane structures, which could be less prominent in EM studies and, hence, DMVs might represent late stages of viral replication factories (Romero-Brey et al. 2012).

The reasons for the enclosure of RNA synthesis into vesicular structures is not fully clear yet. Viral induced membrane alterations might be a way to concentrate the active viral replicase proteins, RNA and host factors at specific sites. The replication vesicles might thereby also contribute to the template specificity of the replicase, by excluding cellular RNAs. This might represent an evolutionarily conserved mechanism, paralleling the encapsidation of the reverse transcription machinery in retroviruses (Schwartz et al. 2002). The membrane alterations might furthermore facilitate the separation of different steps of the viral replication cycle (translation, replication, assembly) by compartmentalization. Thus, after a certain

amount of proteins has been synthesized from a viral genome, a membrane invagination process driven by the NS proteins might stop translation by preventing access of new ribosomes (Quinkert et al. 2005). Another example is the separation of the viral RNA from the capsid protein, which is localized on LDs (Barba et al. 1997; Moradpour et al. 1996), to avoid packaging of genomes prior RNA replication. In addition, viral replication intermediates like dsRNA are potent molecular patterns recognized by cytosolic pattern recognition receptors (RIG-I, MDA5; reviewed in Reikine et al. 2014) and replication vesicles might therefore have a role in shielding them from detection by innate immune responses.

8 *Cis*-Acting Replication Elements

A number of *cis*-acting elements essential for RNA replication have been identified within or close to the 5′ and 3′ NTR by secondary structure predictions and characterized in detail by reverse genetic studies and biochemical/structural analyses (Fig. 5). However, their function in RNA replication likely involves regulating the initiation of negative and positive strand synthesis, which is mainly accomplished by the 3′ends of the positive and negative strand genome, respectively (Fig. 5b, c).

8.1 The 3′ End of the Positive Strand

Several important regulatory sequences are localized close to the 3′ end of the HCV genome: the 3′NTR, a *cis*-acting replication element (CRE) within the C-terminal NS5B coding region (designated SL3.2) and a stem-loop structure further upstream, termed 9005 (Fig. 5b). The 3′NTR is comprised of a so-called variable region, a polyU/UC tract of variable length, and a highly conserved 98-nt element designated X-tail or 3′X (Kolykhalov et al. 1996; Tanaka et al. 1995). The variable region encompasses predicted stem-loop structures surrounding the stop codon of the polyprotein. The variable region is not essential for viral replication, since deletion mutants in the replicon model and in vivo are still replication competent, albeit with strongly reduced efficiency (Yanagi et al. 1999; Friebe and Bartenschlager 2002; Yi and Lemon 2003a). The polyU/UC tract is composed of homopolymeric uridine stretches interspersed by individual cytosines and varies between 30 and 90 nucleotides in length between viral isolates (Kolykhalov et al. 1996). A minimal of 26–33 homopolymeric uridine residues, which must not be interrupted by cytosines, is essential and sufficient for efficient RNA replication in cell culture (Friebe and Bartenschlager 2002; You and Rice 2008). The position of this polyU stretch within the polyU/UC region is flexible (You and Rice 2008; Yi and Lemon 2003a). The functional significance of the polyU/UC tract is not clear yet, but it might provide an assembly platform for the viral replicase, since the helicase domain of

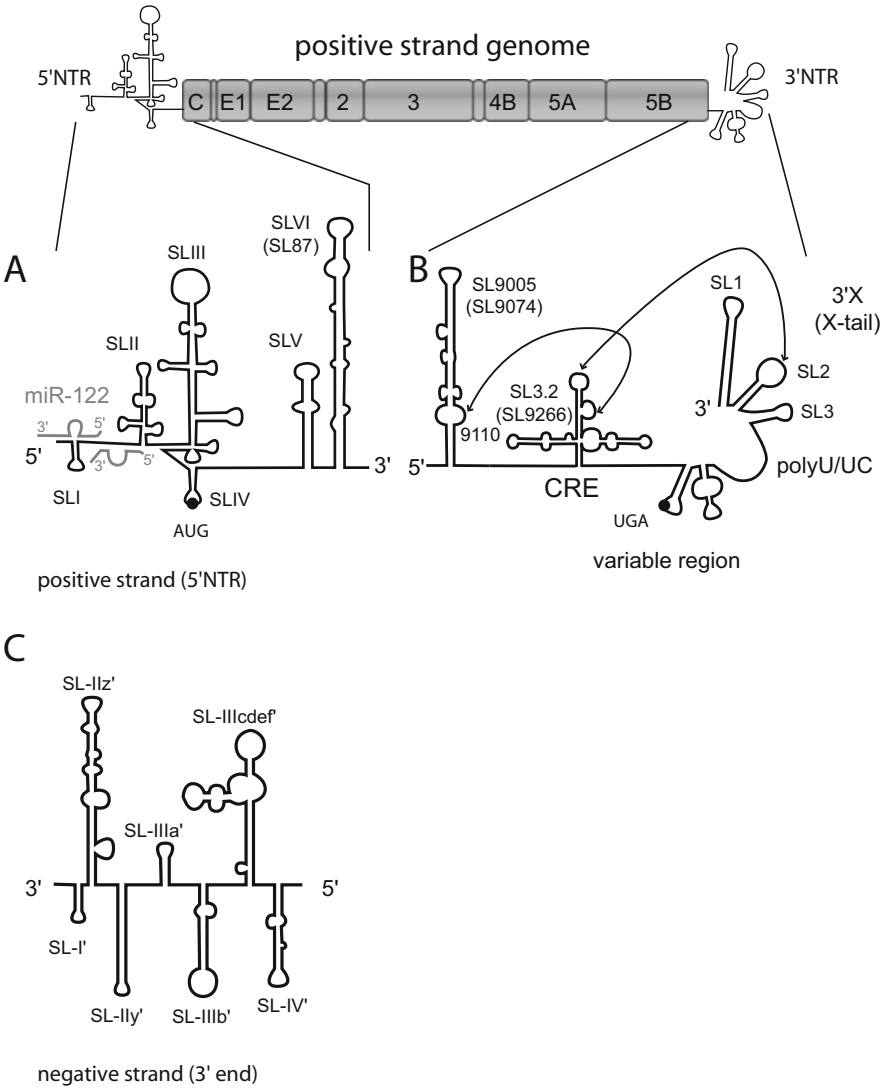

Fig. 5 Schematic representation of *cis*-acting replication elements. (**a**) 5′ end of the viral positive-strand including the IRES (Honda et al. 1996) and stem-loop structures in the core coding region (McMullan et al. 2007). Two copies of miR-122 binding to the 5′NTR are shown in grey (Machlin et al. 2011). (**b**) 3′ end of the viral positive strand (Blight and Rice 1997). Long range interactions of SL3.2 with sequences around 9110 (Tuplin et al. 2012), which is part of SL9005 (Chu et al. 2013), and with the loop region of SL2 (Friebe et al. 2005) are indicated by *arrows*. (**c**) 3′ end of the viral negative strand (Smith et al. 2002; Schuster et al. 2002). Alternative nomenclatures are given in brackets (Modified from Lohmann 2013)

NS3, NS5A and NS5B have been shown to efficiently bind to polyU (Gwack et al. 1996; Huang et al. 2005; Lohmann et al. 1997). The X-tail (Fig. 5b) was identified years after the cloning of the first HCV genome (Kolykhalov et al. 1996; Tanaka et al. 1995) and comprises three experimentally validated stem-loop structures (Blight and Rice 1997), which are all essential for RNA replication (Friebe and Bartenschlager 2002) and barely tolerate any mutations (Yi and Lemon 2003a, b). This region therefore is likely to be the main regulatory element for the initiation of negative strand synthesis; however, the precise mechanisms remain to be determined.

SL3.2 (Fig. 5b) is part of a cruciform-like RNA secondary structure located in the C-terminal coding sequence of NS5B and has been shown to be essential for viral replication by mutational analysis (You et al. 2004). The terminal loop of SL3.2 is engaged in a kissing-loop interaction with SL2 in the 3′NTR, which is mandatory for RNA replication (Friebe et al. 2005). The complementary of the loop-loop interaction is more important than the actual sequence, arguing for a functional role of the pseudoknot structure (Friebe et al. 2005). The bulge region of SL3.2 undergoes another long-range interaction with a *cis*-acting element around nt 9110 of the HCV genome (Tuplin et al. 2012). Mutual interaction of SL3.2 with this region and the X-tail is likely to play a regulatory role, e.g. possibly regulating a shift from translation to replication (Tuplin et al. 2012). A recent study found that region 9110 is part of an extended stem-loop-structure termed SL9074 (or SL9005 according to the reference isolate H77) and also identified additional stem-loop structures in the NS5B coding region that are critical for replication and assembly (Chu et al. 2013).

8.2 *The 3′ End of the Negative Strand and 5′NTR of the Positive Strand*

The 3′ end of the negative strand is complementary to the 5′NTR of the positive strand, and therefore the sequence of this part of the genome must fulfill two functions. In the positive strand, nt 44-350 form the IRES, which is essential for cap-independent translation of the polyprotein (Honda et al. 1996) (Fig. 5a). The complementary negative-strand sequence contains essential replication signals for positive-strand synthesis (Fig. 5c), with the 125 terminal nucleotides, encompassing SL-I′ and SL-IIz′, being indispensable for this function (Friebe et al. 2001; Friebe and Bartenschlager 2009). Interestingly, the secondary structures in the positive and negative strand, respectively, are very different, despite their complementarity (compare Fig. 5a–c Honda et al. 1996; Schuster et al. 2002; Smith et al. 2002), reflecting their different functions. This is particularly evident in the sequence following SL-I and SL-I′ respectively, which is single stranded in the positive strand, containing two miR-122 binding sites important for replication and translation (Jopling et al. 2005; Machlin et al. 2011), whereas the complementary

sequence is embedded in a strong stem-loop-structure (SL-IIz′; Fig. 5c Schuster et al. 2002; Smith et al. 2002). miR-122 binding therefore might protect the 5′NTR sequence from the formation of alternative secondary structures, which could interfere with IRES function, particularly with the folding of SL-II.

Additional conserved stem-loop structures have been predicted within the core coding region (Smith and Simmonds 1997). Indeed, disruption of SL-VI impaired viral RNA replication in cell culture and in vivo (McMullan et al. 2007; Vassilaki et al. 2008). This is surprising, since these sequences are included in replication-competent subgenomic replicons (Lohmann et al. 1999a). This stem-loop thus seems to have a regulatory role in the context of a full-length genome only.

9 The Viral Polymerase NS5B

9.1 Structure of NS5B

The RdRp NS5B is the central catalytic component of the viral replication complex and has been studied extensively in vitro and structurally, since the active enzyme can be expressed heterologously in insect cells and in *E. coli* (Al et al. 1998; Behrens et al. 1996). NS5B encompasses a catalytic domain, followed by a linker sequence and a C-terminal membrane insertion sequence, which tethers the polymerase to membranes (Fig. 6a). The membrane insertion sequence is essential for RNA replication in cell culture (Ivashkina et al. 2002; Moradpour et al. 2004), but not required for RNA synthesis in vitro using heterologously expressed, purified NS5B (Lohmann et al. 1997; Yamashita et al. 1998). The structure of the catalytic domain comprises a so-called right hand shape, common to many single-subunit polymerases, with fingers, thumb and palm subdomains (Fig. 6b, Ago et al. 1999; Lesburg et al. 1999; Bressanelli et al. 1999). Most published structures reveal a closed conformation, encircled on one side by the fingertips and on the other side by a linker and a so-called beta-flap (or β-hairpin). The linker or a variation thereof is common to *de novo* initiating RdRps (Butcher et al. 2001) whereas the beta-flap is restricted to polymerases from the *Flaviviridae*. Such closed NS5B structures imply two functional consequences. (i) The closed structure has only space for a single-stranded template and priming nucleotides. It is therefore supposed to represent the initiation state of the polymerase (Simister et al. 2009). However, after RNA synthesis switches from initiation to elongation, the linker has to be removed to allow egress of the primer-template duplex, causing a major structural rearrangement towards an open conformation (Fig. 6c). Such an "open" conformation with a large cavity capable of binding dsRNA was indeed found with a genotype 2a NS5B (Biswal et al. 2005), in a structure of NS5B complexed with a primer-template following deletion of the beta-flap (Mosley et al. 2012), and upon SAXS analysis in solution (Harrus et al. 2010). A recent structural analysis of NS5B further supports this concept (Appleby et al. 2015, comment by Bressanelli 2015).

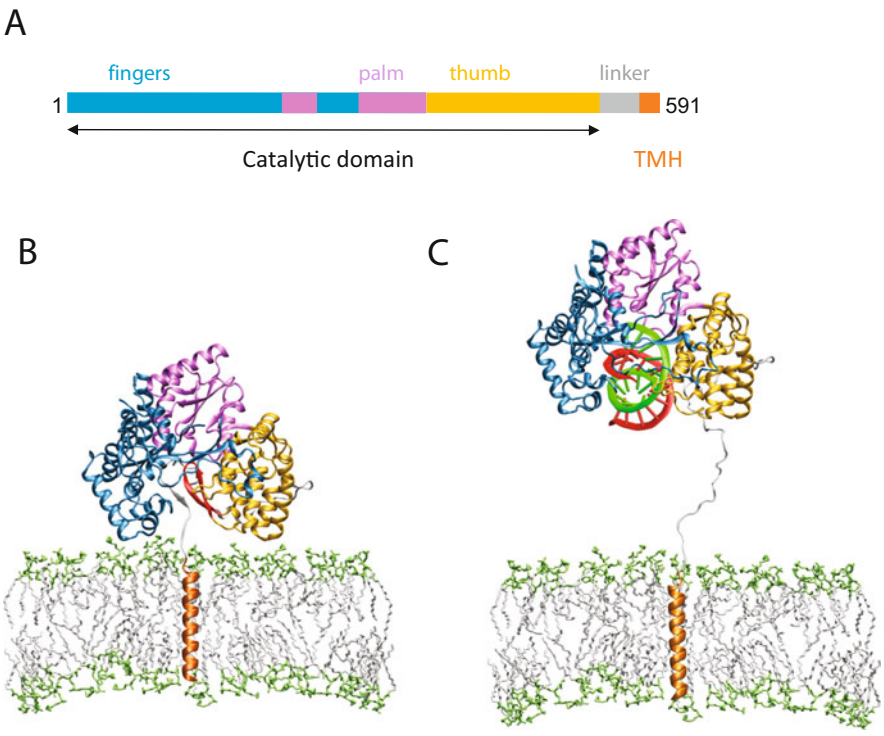

Fig. 6 Schematic subdomain composition of NS5B (**a**) and structure of full length NS5B (PDB entry 1GX6) and its association with the membrane via its C-terminal transmembrane helix (*TMH*) in its closed (panel **b**) and open conformation (panel **c**). The finger, thumb and palm subdomains are given in *blue*, *magenta* and *gold*, respectively. The C-terminal linker (*grey*) connects the core of the enzyme with the membrane insertion sequence (*orange*). (**b**) Structure of NS5B and proposed membrane topology in the so-called closed conformation, which is believed to represent the initiation state of the polymerase. Note that in this model the RNA-binding groove is hidden by the membrane, the so-called beta-flap is shown in red. (**c**) Model of NS5B in a hypothetical elongation mode, which releases the RNA binding groove by moving the linker sequence out of the active center upon switching from initiation to elongation (F. Penin, unpublished data). A double stranded RNA replication intermediate is modeled into the active site according to Mosley et al. (2012). Panels **b** and **c** are a generous gift from F. Penin

Several different stalled NS5B ternary complexes with polymerase, template and incoming nucleotides were generated using elegant strategies to the arrest the enzyme in distinct intermediate states during initiation and elongation. The resulting structures revealed that the beta-flap (here "beta-loop") indeed provides the major priming platform for *de novo* initiation and, together with the C-terminal linker, moves out of the active center, guiding the dsRNA upon opening of the structure. (ii) The enzymatic core of NS5B in its closed conformation appears to be tightly tethered to the membrane, when modeled to the C-terminal linker (Fig. 6b). It is therefore supposed that the switch from initiation to elongation also changes the position of the elongating polymerase relative to the membrane due to distortion

of the linker from the catalytic cleft (Fig. 6c, reviewed in Lohmann 2013). However, the functional significance of this altered topology of NS5B remains to be determined and the entire concept requires further experimental validation, since all structures published thus far lack the membrane insertion sequence.

Overall the role of the linker is not fully clarified yet in HCV NS5B. On the one hand, the entire linker can be deleted (so called ΔC47 or ΔC60 constructs), giving rise to strongly enhanced polymerase activity in vitro suggesting a negative regulatory function (Leveque et al. 2003). Such a mechanism might make sense since only a minority of NS5B molecules is supposed to be actively engaged in RNA synthesis (Quinkert et al. 2005). Self-inactivation of NS5B molecules not involved in RNA synthesis might therefore represent an immune evasion mechanism, since NS5B expressed in cells is capable of generating dsRNA from cellular templates (Ranjith-Kumar et al. 2011; Yu et al. 2012), inducing an intracellular immune response.

9.2 The Polymerase in Motion

Purified NS5B can initiate RNA synthesis either by a primer-dependent mechanism or *de novo* (Behrens et al. 1996; Lohmann et al. 1997; Luo et al. 2000; Zhong et al. 2000; Sun et al. 2000). Since the HCV genome has no terminal protein, *de novo* initiation is supposed to be the initiation mode of genome replication in vivo. The first step of *de novo* initiation after binding of the polymerase to a template is the synthesis of a di- or trinucleotide primer, which has to start with a purine base and requires high concentrations of the priming nucleotides (Ferrari et al. 2008; Luo et al. 2000). Primer synthesis requires a "platform" to support the first nucleotide, which has to move out of the active center upon addition of the third base. This platform has recently been shown to be provided by the beta-flap (Appleby et al. 2015), in line with the mechanism suggested for pestiviruses (Lescar and Canard 2009; Choi et al. 2004; D'Abramo et al. 2006). In addition, *de novo* initiation of RNA synthesis is stimulated by high GTP concentrations upon binding to allosteric sites (Lohmann et al. 1999b; Harrus et al. 2010). GTP stabilizes the nucleotides involved in primer synthesis (D'Abramo et al. 2006) and facilitates the step from initiation to elongation (Harrus et al. 2010). The closed conformation seen in almost all crystal structures of NS5B represents most likely the initiation state of the enzyme (Simister et al. 2009), a view supported by a recent study of multiple stalled ternary complexes (Appleby et al. 2015). The switch from initiation to elongation seems to be one of the rate limiting steps at least in vitro and a number of excess primers are synthesized, before the enzyme continues to processive elongation (Harrus et al. 2010). This step furthermore requires a major conformational change towards an open conformation, probable driven by the removal of the linker sequence to allow exit of the dsRNA, thereby likely relocating the entire enzymatic core (Fig. 6c). Position 405 in the thumb seems to be critical for efficient primer synthesis as well as for switching from initiation to elongation in vitro and

this residue is also an important determinant for replication efficiency in cell culture (Scrima et al. 2012; Schmitt et al. 2011).

Once RNA synthesis is initiated, NS5B elongates nascent RNA by about 100–400 nts per minute and is capable of processively copying an entire RNA genome in vitro (Oh et al. 1999; Lohmann et al. 1998; Simister et al. 2009) at nucleotide concentrations far lower than required for initiation (Lohmann et al. 1998; Jin et al. 2012). Elongation complexes are quite stable in vitro and can even be stalled and purified (Jin et al. 2012). Nothing is known so far about termination of RNA synthesis, and the enzyme might finally just fall off the template. However, it must be considered that NS5B as well as all other NS proteins involved in RNA synthesis are tightly linked to the membrane and buried in membrane alterations (Figs. 1 and 4). In vivo the template might move along the polymerase rather than the polymerase along the template; therefore, termination and re-initiation might be regulated processes as well.

RNA synthesis by NS5B is error prone and provides the molecular basis of the high genetic variability of HCV isolates, although a nucleotide excision mechanism has recently been reported for NS5B, which might allow limited error correction (Jin et al. 2013). An error rate of ca. 10^{-3} per site was determined in vitro with a strong bias towards G:U/U:G mismatches, which was mirrored by a 75-fold difference in transitions over transversions in vivo (Powdrill et al. 2011).

The natural initiation sites for RNA synthesis in vivo are supposed to be the 3′ ends of the viral positive and negative strand RNAs. In vitro, *de novo* initiation of RNA synthesis has no distinct sequence specificity and can even take place on circular templates (Ranjith-Kumar and Kao 2006) or homopolymers (poly C and polyU, Luo et al. 2000). However, the initiating nucleotide must be a purine (Zhong et al. 2000) and initiation seems most efficient on a single-stranded region of at least 3 nts adjacent to a stem-loop structure (Kao et al. 2000). This is exactly the configuration found at the 3′ end of the negative strand (Fig. 5c), whereas the 3′ end of the positive strand is buried in a strong stem-structure, therefore hardly giving rise to terminal *de novo* initiation in vitro (Fig. 5b Reigadas et al. 2001; Binder et al. 2007). This might suggest that additional viral or host factors are required for initiation of negative-strand synthesis, thereby allowing a tight regulation of RNA synthesis. In principle, primer-synthesis by the polymerase could also take place at a different site (e.g. at an internal CRE as in case of poliovirus Paul et al. 1998, 2000) and the polymerase/primer complex might then be moved to the end of the genome. However, there is as yet no experimental evidence for such a scenario.

9.3 Contribution of Other Viral Proteins to RNA Synthesis

The polymerase is capable of *de novo* initiation and can copy an entire genome without the help of other factors in vitro (Lohmann et al. 1998; Oh et al. 1999). Still, it is obvious that additional viral and cellular proteins substantially contribute to

RNA synthesis. However, we do not know much about the molecular details of regulation of RNA synthesis due to the limited availability of appropriate model systems (reviewed in Lohmann 2013). There is still a huge gap between reverse genetic studies, revealing the importance of *cis*-acting elements and viral proteins NS3 to NS5B for RNA replication in cells, and in vitro models that build on purified proteins, and that currently provide only limited mechanistic insight due to a lack of specificity (e.g. of the polymerase for viral templates).

A study using inter-genotypic chimeras revealed that the helicase-domain of NS3 (NS3 helicase), NS5A, NS5B and the NTRs must be derived from the same genotype for efficient RNA replication in cell culture, suggesting that these proteins may form the core of the replicase (Binder et al. 2007). This view is supported by a recently developed in vitro model (Mani et al. 2014) using a circular RNA complexed with human replication protein A as a template, and demonstrating that NS5A might help binding NS5B to the template. The helicase is subsequently recruited by NS5B and facilitates processive elongation (Mani et al. 2014). Stimulation of polymerase activity by the helicase has been found by others (Piccininni et al. 2002), as well as a regulation of helicase activity by the polymerase and the protease (Jennings et al. 2008; Zhang et al. 2005). Regarding NS5A, conflicting results have been published suggesting that low doses of NS5A stimulate NS5B activity, whereas high doses are inhibitory to the polymerase (Shirota et al. 2002; Quezada and Kane 2009).

The distinct role of the NS3 helicase activity has not been clarified yet, but the enzyme (i) might resolve strong stem-loop structures at the 3′ end of the genome to facilitate initiation of RNA synthesis by the polymerase, (ii) unwind double-stranded replication intermediates during RNA synthesis to support NS5B in the elongation phase, and/or (iii) help to strip proteins off the RNA or deliver RNA for packaging into virions by a recently demonstrated ssRNA translocase activity (Gu and Rice 2010). In addition, a recent reverse genetic study suggested that the linker sequence between the protease and helicase domains of NS3 has a regulatory role in replication and assembly (Kohlway et al. 2014).

NS5A is believed to be a central regulator of the viral replication cycle, and it is particularly difficult to separate its contribution to the biogenesis of the MW described earlier in this chapter (Romero-Brey et al. 2012) from essential functions in RNA synthesis and assembly. The RNA binding of NS5A, which is mediated mainly by domain I (Huang et al. 2005) and modulated by domains II and III (Foster et al. 2010) might clearly contribute to RNA synthesis as shown in vitro (Mani et al. 2014). The phosphorylation status of NS5A is also believed to have a major regulatory role in RNA synthesis. However, although a number of phosphorylation sites have recently been identified and analyzed by reverse genetics (Masaki et al. 2014; Ross-Thriepland and Harris 2014) it has not been possible yet to assign distinctly phosphorylated subspecies of NS5A to specific functions. Several replication enhancing mutations identified in replicon cells reduced the relative amount of hyperphosphorylated NS5A (p58 Appel et al. 2005; Blight et al. 2000), which brought up the concept that basally phosphorylated NS5A (p56) is required for RNA replication, whereas p58 favors assembly. Indeed, a recent study showed

positive evidence that CKIα mediated phosphorylation events leading to p58 synthesis are required for production of infectious virus (Masaki et al. 2014). In addition, hVAP-A, a cellular protein critical for RNA replication, preferentially binds to p56 and not to p58 (Evans et al. 2004), suggesting that differential phosphorylation of NS5A might regulate the viral life cycle by changing interactions with viral and host factors. Still, a comprehensive concept is missing concerning which distinct NS5A phosphorylation events govern different steps of the replication cycle and by which kinds of mechanisms.

NS4B may have a role in the regulation of replicase activity beyond MW formation. NS4B can bind RNA (Einav et al. 2004, 2008) and also has an NTPase activity. There is also genetic evidence for an interaction of NS4B with NS3 (Paredes and Blight 2008) and NS4B has been shown to inhibit NS5B in vitro (Piccininni et al. 2002).

9.4 Host Factors Involved in RNA Synthesis

Several of the host factors involved in MW formation that have been discussed previously in this chapter may have in addition a more direct role in the regulation of RNA synthesis (e.g. PI4KIIIα, CypA, sphingomyelin).

RNA-binding proteins are of course prime candidates for distinct functions in regulation of RNA synthesis. Twenty-six cellular proteins specifically binding to the IRES in the 5′NTR (Lu et al. 2004) and more than 70 interacting with the 3′NTR (Harris et al. 2006) have been identified in proteomic studies, but their distinct roles remain elusive. Several mechanisms have been brought up mediating the circularization of the viral genome, which might be important to stimulate translation, as in case of cellular messenger RNAs, or to switch between different steps of the viral life cycle, as shown for flaviviruses (reviewed in Villordo and Gamarnik 2009). For HCV, potential circularization sequences have been identified close to the termini of the genome (Romero-Lopez et al. 2014). Genome circulization might further be facilitated by RNA binding proteins, like the NF/NFAR (Isken et al. 2007) or the La protein (Kumar et al. 2013).

The liver specific microRNA miR-122 is a critical host factor in HCV replication in cell culture (Jopling et al. 2005) and in vivo (Lanford et al. 2010) and might substantially contribute to the liver tropism of HCV. miR-122 binds to two sites in the 5′NTR with extensive base pairing outside the seed sequence (Fig. 5a; Machlin et al. 2011), forming an unconventional ternary complex, encompassing the 5′ end of the viral genome (Mortimer and Doudna 2013). The mode of action of miR-122 in the viral replication cycle is not fully clarified yet, but it has been shown to stimulate translation (Henke et al. 2008) and to protect the genome from degradation by the exonucleases Xrn1 (Li et al. 2013) and Xrn2 (Sedano and Sarnow 2014).

10 Dynamics of RNA Synthesis and Open Questions

Mathematic modeling of the decline in circulating virus after the initiation of therapy revealed that about 10^{12} virions are produced per day in infected individuals (Neumann et al. 1998), suggesting a highly dynamic process for RNA replication in the infected liver. Recent studies using two photon microscopy or highly sensitive *in situ* hybridization revealed that 7–20 % (Liang et al. 2009) or 1–54 % (Wieland et al. 2014) of hepatocytes are infected and that the number of infected cells indeed correlates with viral load (Wieland et al. 2014; Mensa et al. 2013).

Detailed intracellular replication kinetics are available from subgenomic replicons (Binder et al. 2007) or after virus infection (Keum et al. 2012), as well as from a quantitative analysis of replication dynamics in persistent replicon cells (Quinkert et al. 2005). These data have been recently used to model replication dynamics *in silico* (Dahari et al. 2007; Binder et al. 2013). Altogether these studies suggest that each of the incoming positive strand RNA molecules is first translated to give rise to ca. 1,000 protein copies (Quinkert et al. 2005), which will then likely induce the formation of the MW to allow initiation of RNA synthesis. However, only a subfraction of less than 5 % of these protein copies seem to be engaged in the formation of viral replication sites and even less in enzymatic replicase activity (Miyanari et al. 2003; Quinkert et al. 2005). It remains elusive which mechanisms render a few replicase copies active and the majority inactive. However, recent *trans*-complementation analyses reveal that some deleterious mutations in NS5A can complement each other, suggesting the existence of several nonstructural protein complexes serving different functions (Fridell et al. 2011).

First viral negative-strand RNAs are detectable 4 h or 6 h after transfection or infection, respectively. At this time point, positive to negative strand ratios are ~1:1 (Binder et al. 2007; Keum et al. 2012), probably reflecting double-stranded replication intermediates (Targett-Adams et al. 2008). The initial log-phase of 4–6 h likely represents the time required for polyprotein translation, generation of the membranous replication compartment and RNA synthesis (100–400 nts/min in vitro) (Lohmann et al. 1998; Simister et al. 2009). After this time point, negative- and positive-strand RNA levels increase exponentially and asymmetrically, reaching a plateau at 24–48 h, with a +/− strand ratio of ~10:1. Roughly 1,000–5,000 positive and 100–500 negative strand RNA molecules per cell were reported for transient and steady state cell cultures and this number might represent a limit of Huh-7-based cell cultures (Quinkert et al. 2005; Keum et al. 2012; Blight et al. 2002), reflecting limiting host factors involved in RNA synthesis as suggested by recent mathematic modelling (Binder et al. 2013). Less efficient genotype 1 replicons exhibit much slower replication kinetics with no clear exponential phase and reach steady state replication levels at later time points (Binder et al. 2007; Krieger et al. 2001). The half-lives of viral NS proteins and viral positive-strand RNA in replicon cells have been shown to be 11–16 h (Pietschmann et al. 2001; Pause et al. 2003). It can therefore be estimated that only about 1,000

positive-strand RNA molecules are synthesized per day per cell by ca. 100 replicase complexes during persistent replication in cell culture (Quinkert et al. 2005).

It is generally not clear, how the progeny positive-strand RNA is released into the cytoplasm. This could involve NS5A and/or the translocase function of the NS3 helicase (Gu and Rice 2010), delivering the RNA through a direct, probably transient connection to the cytoplasm (Romero-Brey et al. 2012) or through a protein pore e.g. nuclear pore like complexes Neufeldt et al. 2013. Due to the low and limiting number of negative-strand RNA copies it seems plausible that negative-strand synthesis can be initiated only once from a positive-strand genome by a *cis*-acting protein complex translated on the same RNA. Initiation of negative-strand RNA would then require a preceding translation of the positive strand RNA, resulting in the formation of a new replication vesicle and each replication site would indeed contain only one negative strand RNA/replication intermediate. Such a model would also explain in part the asymmetry of RNA synthesis, resulting in a strong surplus of positive strands.

Many questions regarding the regulation and dynamics of RNA synthesis are still unresolved and some assumptions are highly speculative. We still have no clear concept how the shift from translation to RNA synthesis is accomplished, how the membrane alterations are functionally linked to RNA synthesis, how negative and positive strand RNA synthesis are initiated to achieve the asymmetric +/− strand ratio and which viral and cellular proteins are involved, just to name a few. Further mechanistic insights will require better defined in vitro models assembled from individual replicase components that allow dissection of the complexities governing HCV RNA synthesis.

11 Conclusions/Perspectives

Twenty five years after its discovery, a new era of HCV research is emerging due to the advent of efficient specific antiviral therapies. This huge success would not have been possible without enormous efforts in basic research, which provided important model systems for drug development as well as an understanding of the viral replication cycle that was essential for definition and evaluation of efficient targets for therapy. However, beyond antiviral drug development, HCV has become an outstandingly important role model for various aspects of virology and virus host interactions in general, including the biogenesis of viral replication sites, interactions with lipids and lipid metabolism, the establishment of persistence, etc. Although our knowledge has increased tremendously over the past years, which is partly reflected by this review, many issues are far from being clarified. This accounts particularly for mechanisms governing viral RNA replication, which remain difficult to address due to a plethora of *cis*-functions regulating translation, induction of membrane alterations, viral RNA synthesis and packaging of newly synthesized genomes into virions. The chances are high that several important questions surrounding the biology of positive-strand RNA viruses as well as of

underlying cellular mechanisms can be solved using HCV as a paradigm, now that an overwhelming toolbox has been generated, driven by the importance of HCV as a pathogen. Therefore, we have to keep research efforts on HCV engaged in high gear even in the era of effective antiviral therapy, to reach the next level of mechanistic understanding.

Acknowledgements The authors are grateful to Francois Penin for providing Fig. 6b, c. VL is supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (LO1556/1–2 and LO1556/4–1; FOR1202, TP 3 and TRR77, TP A1)

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