RESEARCH ARTICLE

NS5A Domain 1 and Polyprotein Cleavage Kinetics Are Critical for Induction of Double-Membrane Vesicles Associated with Hepatitis C Virus Replication

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ABSTRACT **Induction of membrane rearrangements in the cytoplasm of infected cells is a hallmark of positive-strand RNA viruses. These altered membranes serve as scaffolds for the assembly of viral replication factories (RFs). We have recently shown that hepatitis C virus (HCV) infection induces endoplasmic reticulum-derived double-membrane vesicles (DMVs) representing the major constituent of the RF within the infected cell. RF formation requires the concerted action of nonstructural action of nonstructural protein (NS)3, -4A, protein (NS)3 -4A, -4B, -5A, and -5B. Although the sole expression of NS5A is sufficient to induce DMV formation, its efficiency is very low. In this study, we dissected the determinants within NS5A responsible for DMV formation and found that RNA-binding domain 1 (D1) and the amino-terminal membrane anchor are indispensable for this process. In contrast, deletion of NS5A D2 or D3 did not affect DMV formation but disrupted RNA replication and virus assembly, respectively. To identify *cis*- and *trans*-acting factors of DMV formation, we established a *trans* cleavage assay. We found that induction of DMVs requires full-length NS3, whereas a helicase-lacking mutant was unable to trigger DMV formation in spite of efficient polyprotein cleavage. Importantly, a mutation accelerating cleavage kinetics at the NS4B-5A site diminished DMV formation, while the insertion of an internal ribosome entry site mimicking constitutive cleavage at this boundary completely abolished this process. These results identify key determinants governing the biogenesis of the HCV RF with possible implications for our understanding of how RFs are formed in other positive-strand RNA viruses.**

IMPORTANCE **Like all positive-strand RNA viruses, hepatitis C virus (HCV) extensively reorganizes intracellular membranes to allow efficient RNA replication. Double-membrane vesicles (DMVs) that putatively represent sites of HCV RNA amplification are induced by the concerted action of viral and cellular factors. However, the contribution of individual proteins to this process remains poorly understood. Here we identify determinants in the HCV replicase that are required for DMV biogenesis. Major contributors to this process are domain 1 of nonstructural protein 5A and the helicase domain of nonstructural protein 3. In addition, efficient DMV induction depends on *cis* cleavage of the viral polyprotein, as well as tightly regulated cleavage kinetics. These results identify key determinants governing the biogenesis of the HCV replication factory with possible implications for our understanding of how this central compartment is formed in other positive-strand RNA viruses.**

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A feature common to infections by all positive-strand RNA viruses is the remodeling of intracellular membranes creating miniorganelles or "replication factories" where RNA amplification and eventually also virion assembly take place (reviewed in reference 1). These structures facilitate coordination of the different steps of the replication cycle, i.e., RNA translation, replication, and assembly, but might also shield viral RNA from degradation and recognition by the host's immune surveillance. Two classes of morphologically distinct replication factories have been proposed, the invaginated vesicle/spherule type and the double-membrane vesicle (DMV) type (reviewed in reference 2). We and others have recently shown that DMVs represent the main component of membrane rearrangements induced by hepatitis C virus (HCV) (3, 4). They are derived from the endoplasmic reticulum (ER) and accumulate in the cytoplasm of infected cells. Owing to their sponge-like appearance, HCV-remodeled membranes have been designated the "membranous web" (4–6). Importantly, purified DMVs contain active HCV replicase, arguing that these structures represent viral replication sites (7).

The HCV genome is an uncapped single-stranded RNA of ~9.6 kb that contains a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs) (reviewed in reference 8). After release of the viral RNA genome into the cytoplasm, it serves as mRNA and is used for cap-independent translation via the internal ribosome entry site (IRES) located within the 5′ UTR (reviewed in reference 8). The resulting polyprotein is co- and posttranslationally processed by cellular and viral proteases into 10 different proteins that are required for RNA replication and virion formation (reviewed in reference 9). The N-terminal region of the polyprotein comprises the structural proteins core and en-

velope protein 1 (E1) and E2 that build up the virus particle. The viroporin p7 and nonstructural protein 2 (NS2) are accessory factors required for the assembly of infectious HCV particles (reviewed in reference 10). The minimal HCV replicase comprises the remaining nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B (11). NS3 is composed of two domains comprising an N-terminal serine protease that is activated by interaction with NS4A and responsible for proteolytic maturation of the replicase proteins and an NTPase/RNA helicase domain formed by the C-terminal two-thirds of NS3 (reviewed in reference 12). Highly hydrophobic NS4B is believed to build the scaffold of the viral replication complex and forms oligomeric complexes that are important for the formation of DMVs (13–16). NS5A is an RNA-binding phosphoprotein containing an N-terminal amphipathic α-helix (AH) that stably tethers the protein to intracellular membranes (17, 18) (reviewed in reference 19). NS5A is composed of three domains (D1 to D3) that are separated by low-complexity sequence I (LCSI) and LCSII. D1 is the main determinant of RNA replication, whereas D3 plays a major role in the assembly of infectious virus particles, probably by interacting with the core protein (20–23). In fact, major parts of D3 can be deleted without affecting RNA replication and D3 tolerates in-frame insertion of heterologous proteins such as green fluorescent protein (GFP) (24). The exact role of D2 remains to be determined, since a large segment within this domain can be deleted with no significant effect on RNA replication and virus production in cell culture (20); however, a recent study has demonstrated that D2 is required to suppress the activation of the interferon response (25). NS5B is a tail-anchored RNA-dependent RNA polymerase (8).

HCV RNA replication is linked to membrane alterations that are induced by the concerted action of viral and cellular proteins (reviewed in reference 26). We have shown earlier that induction of the membranous web requires viral replicase polyprotein NS3-5B. The only protein able to induce DMVs and predominantly multimembrane vesicles on its own was NS5A, but with only very low efficiency compared to that obtained by the expression of an NS3-5B polyprotein (4). In the present study, we identified the determinants within NS5A required for DMV formation and analyzed the role of polyprotein cleavage in *cis* and *trans* for the establishment of the membranous HCV replication factory.

RESULTS

Domain 1 of NS5A is essential for the formation of DMVs. Given the prominent role of NS5A in HCV-induced membrane rearrangements (4), we characterized the involvement of the three NS5A domains in the formation of DMVs and correlated this property with viral RNA replication. To this end, we generated several deletion mutations by removing each NS5A domain individually in the context of genotype 2a isolate JFH-1 (27) (Fig. 1A). The mutations were introduced into a subgenomic luciferase reporter replicon, and *in vitro* transcripts were transfected into Huh7 cells. Consistent with our earlier report (20), we found that only the deletion of a segment of domain 2 (ΔD2short) or all of domain 3 (ΔD3) was tolerated (Fig. 1B), whereas removal of D1 or all of D2 blocked RNA replication (Fig. 1B).

To study the impact of these deletion mutations on DMV formation, we used a T7 RNA polymerase-based expression system that is sufficient to induce membranous web formation in the absence of HCV RNA replication (4). Mutations were introduced into an NS3-5B expression construct allowing cytoplasmic expression of the HCV polyprotein (Fig. 1C). Expression and cleavage of the polyprotein in transfected Huh7-Lunet/T7 cells that stably express the T7 RNA polymerase were determined by Western blotting with different NS5A-monospecific antibodies to account for the removal of epitopes residing in different NS5A domains. As shown in Fig. 1C, the polyproteins were properly processed, with the exception of ΔD1, where we observed two proteins that, on the basis of their apparent molecular weights and immunoreactivities with the monospecific antisera, were identified as NS5A and an uncleaved NS4B-5A precursor. Nevertheless, we note that fully processed NS4B was also generated by this mutant and the abundance of NS5A and NS4B was comparable to that in the other mutants.

We next determined the capacity of these mutants to induce DMV formation by using electron microscopy (EM) of cryofixed cells that had been transfected with the corresponding expression constructs (Fig. 2). Two parameters were used for EM analysis. First, the abundance of DMVs per cell profile, which was determined by counting the DMVs residing in the cytoplasmic area (measured in square micrometers and calculated as the cell surface of a given cell section minus the nuclear region, which is devoid of DMVs). To account for the possibility that DMVs reside in different planes, sections taken at different cell levels were included in our quantification. Second, the DMV diameter was measured as the distance between the two farthest opposed points on the membrane of a given DMV to account for the fact that DMVs most often are elliptical. We note that an elliptical rather than a circular appearance of DMVs might be caused by compression artifacts generated during sample fixation or sectioning. However, this possible artifact did not affect our analysis because we always used the wild type as a reference for comparison.

By using this approach, we found that removal of D1 (ΔD1) led to complete abrogation of DMV formation but induced fragmentation and swelling of the ER (Fig. 2B; Table 1). Interestingly, the deletion mutant that lacked all of D2 (ΔD2long) and was unable to replicate (20) (Fig. 1B) still induced the formation of DMVs (Fig. 2C) but with significantly reduced efficiency, as revealed by quantification of the number of DMVs per cell surface area (Fig. 2I; Table 1). Moreover, in contrast to wild-type NS3-5B, these DMVs exhibited an average diameter of 133 ± 20 nm (n = 90), which is ~30 nm smaller than the majority of the DMVs detected in cells expressing wild-type NS3-5B (Fig. 2H; Table 1). In addition, these DMVs were confined in smaller clusters, in contrast to the more interspersed distribution of wild-type-induced DMVs.

In the NS3-5B polyprotein containing a small deletion of D2 (ΔD2short), RNA replication was only moderately affected (Fig. 1B) and DMVs with wild-type morphology were induced (Fig. 2D and H; Table 1). Also, in this mutant, efficiency of DMV formation was reduced (Fig. 2I; Table 1). Removal of the complete domain 3 (ΔD3) had no effect on RNA replication but selectively abolished the assembly of infectious HCV particles (20). As expected, this mutation induced DMVs (Fig. 2E) but with a diameter of 220 ± 52 nm (n = 90), which is ~50 nm larger than that of wild-type DMVs (Fig. 2H; Table 1).

The results described so far suggested that neither D2 nor D3 are required for DMV formation. To corroborate this observation, we generated two additional deletion mutants: ΔD2D3, lacking both domains and LCSII, and mutant ΔLCSI-D3, lacking in addition LCSI (Fig. 1A). Both mutants still induced the formation

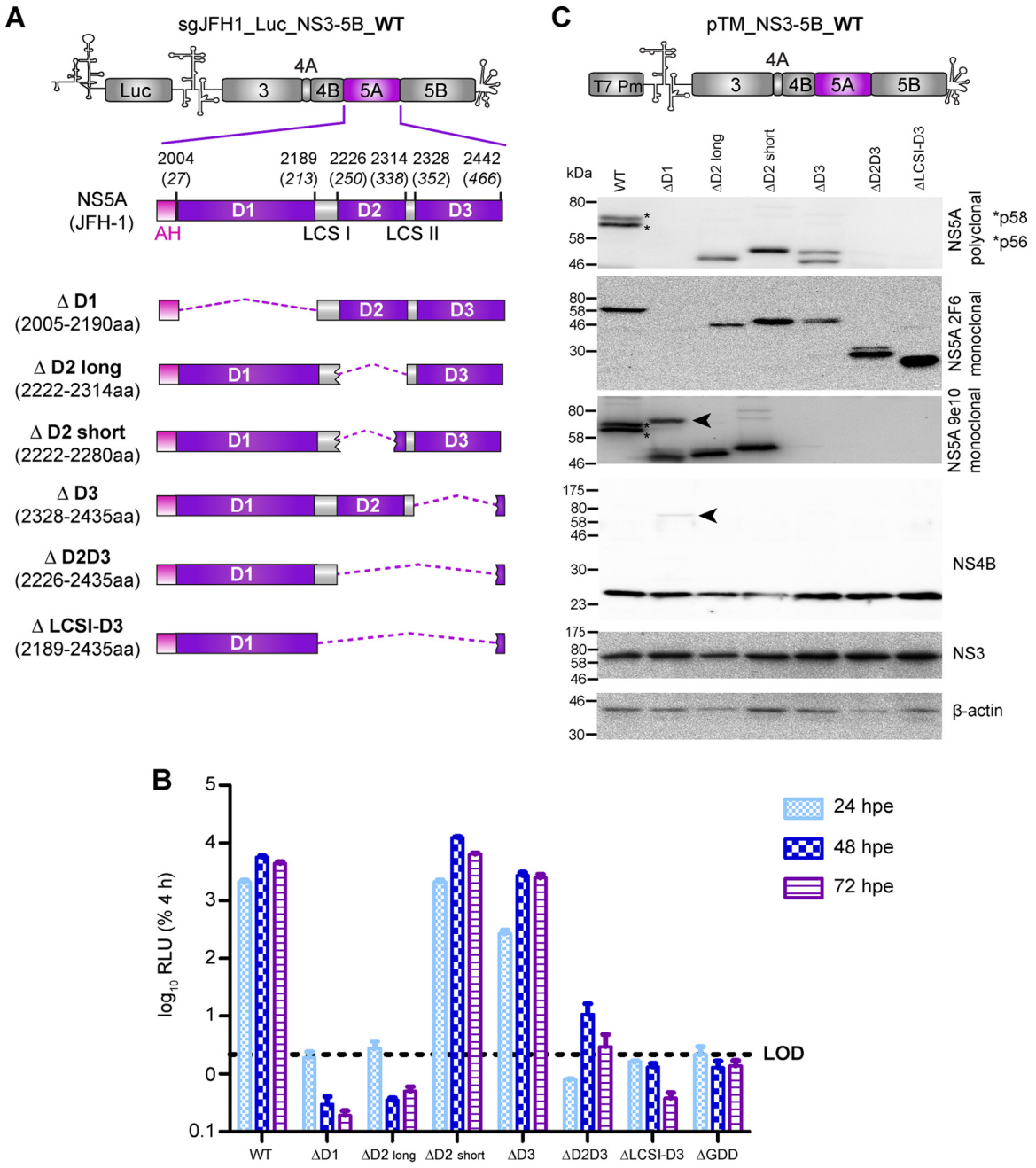

FIG 1 Deletions within NS5A and their impact on RNA replication and polyprotein cleavage. (A) Schematic representation of the subgenomic replicons used in this study. The 3′ UTR of HCV and the IRES of encephalomyocarditis virus downstream of the firefly luciferase gene (Luc; gray box) are shown as stem-loop structures. An enlarged view of the organization of the NS5A domain is shown below. The numbers at the top refer to amino acid residues of the JFH-1 polyprotein, and numbers in parentheses refer to residues of NS5A. Deletion mutants are shown below; the names of the mutants are shown to the left of the illustrations. The numbers in parentheses refer to the amino acid (aa) residues of the polyprotein that were removed. (B) Replication kinetics of NS5A deletion mutants. Huh7.5 cells were transfected with subgenomic Luc reporter replicons. Cells were harvested 4, 24, 48, and 72 h post electroporation (hpe), and the luciferase activity in the lysates was determined. Values were normalized to the 4-h value, reflecting transfection efficiency. A replication-deficient mutant encoding an inactive NS5B polymerase served as a negative control (ΔGDD). RLU, relative light units; WT, wild type; LOD, limit of detection. (C) Polyprotein processing of NS5A mutants. The basic construct used for expression of the NS3-5B polyprotein and NS5A mutant proteins derived therefrom is shown on the top. It contains the promoter (Pm) of the T7 RNA polymerase (gray box) and the IRES of encephalomyocarditis virus upstream of the polyprotein coding region. Huh7-Lunet/T7 cells were transfected with expression constructs specified above the lanes, and 24-h later, cells were lysed and HCV proteins were detected by Western blotting with various NS5A-, NS4B-, and NS3-specific antibodies as specified on the right of each panel. β-Actin served as a loading control. The values on the left are the molecular masses of the standards used. The uncleaved NS4B-ΔD15A precursor detected in the NS5A (9E10)- and NS4B-specific immunoblot analysis is indicated by arrowheads. The two phosphorylated variants of wild-type NS5A in the upper panel are labeled with asterisks, and they are referred to as p56 and p58.

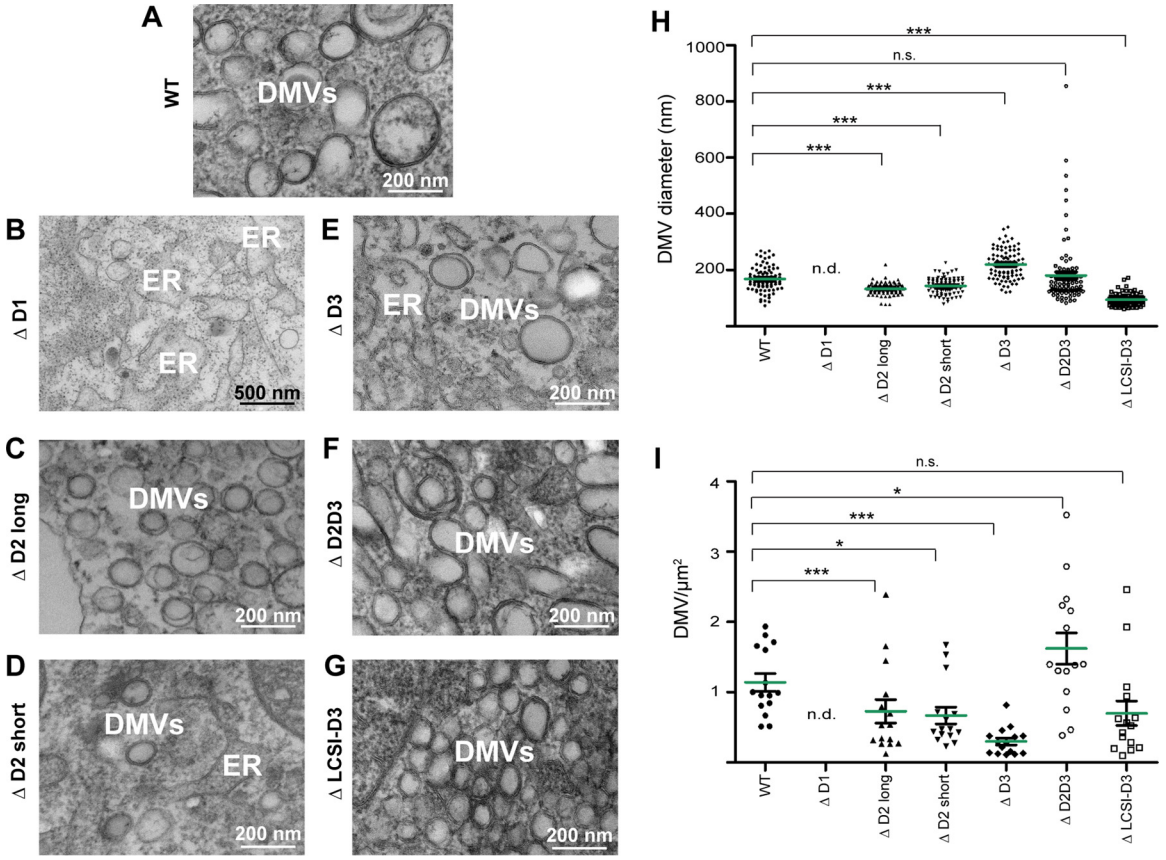

FIG 2 Deletions within NS5A and their impact on the formation of DMVs. (A to G) Cells were transfected with the expression constructs shown to the left of the panels, and 24 h later, cells were fixed and processed for EM by cryofixation and epon embedding. (H, I) Quantitative analyses of DMV diameters (H) and numbers of DMVs per square micrometer of cell surface area (I). Fifteen cell profiles from two independent experiments (10 and 5 cell profiles, respectively) were analyzed. The *P* values shown were calculated by using unpaired Student *t* tests; n.s., nonsignificant; *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; n.d., not determined because of absence of DMVs; WT, wild type. Green horizontal lines represent mean values.

of DMVs (Fig. 2F and G; Table 1), arguing that NS5A D1 is the critical determinant of membranous web formation. Consistent with this assumption, when correlative-light EM (CLEM)-based analysis of cells with proven expression of an NS3-5B polyprotein lacking NS5A D1 was used, no DMVs were detected (Fig. 3). Note that the field of view selected contains many mitochondria and especially lipid droplets (LDs) that were often found randomly clustered in certain regions of a cell. In cells containing large

TABLE 1 Impact of mutations in NS5A on the number and size of DMVs in NS3-5B polyprotein-expressing cells (pTM system) or in cells transfected with subgenomic replicons[a]

	Expression system (pTM)		Replicon system		
Name	DMV diam (nm)	No. of DMVs/μm^2	Replication competence (48/4 hpe)	DMV diam (nm)	No. of DMVs/μm^2
WT	168 ± 43	1.14 ± 0.48	5643	161 ± 32	0.11 ± 0.07
ΔD1	NA[b]	None	0.3	NA	None
ΔD2long	133 ± 20	0.73 ± 0.64	0.35	NA	None
ΔD2short	144 ± 28	0.67 ± 0.46	12,311	162 ± 34	0.11 ± 0.02
ΔD3	220 ± 52	0.3 ± 0.19	2751	167 ± 35	0.04 ± 0.02
ΔD2D3	181 ± 117	1.62 ± 0.87	10.7	NA	None
ΔLCSI-D3	95 ± 20	0.7 ± 0.67	1.32	NA	None
GBV-C 8-13	198 ± 101	0.21 ± 0.16	369	147 ± 38	0.08 ± 0.05
GBV-B 8-13	NA	None	0.31	NA	None
C59G	NA	None	0.31	NA	None
C142A	157 ± 48	0.47 ± 0.35	9049	123 ± 32	0.11 ± 0.05

[a] Note that for the pTM constructs, 15 cell profiles from two independent experiments (10 and 5, respectively) were counted to calculate the DMVs per square micrometer, while for the subgenomic replicons, only 5 cell profiles from one experiment were counted. For further details of quantification, see Text S1 in the supplemental material. Diameters and numbers of DMVs are averages ± standard deviations. Replication competence is given as the ratio of luciferase activity measured 48 h and 4 h after transfection to normalize for transfection efficiency.

[b] NA, not applicable.

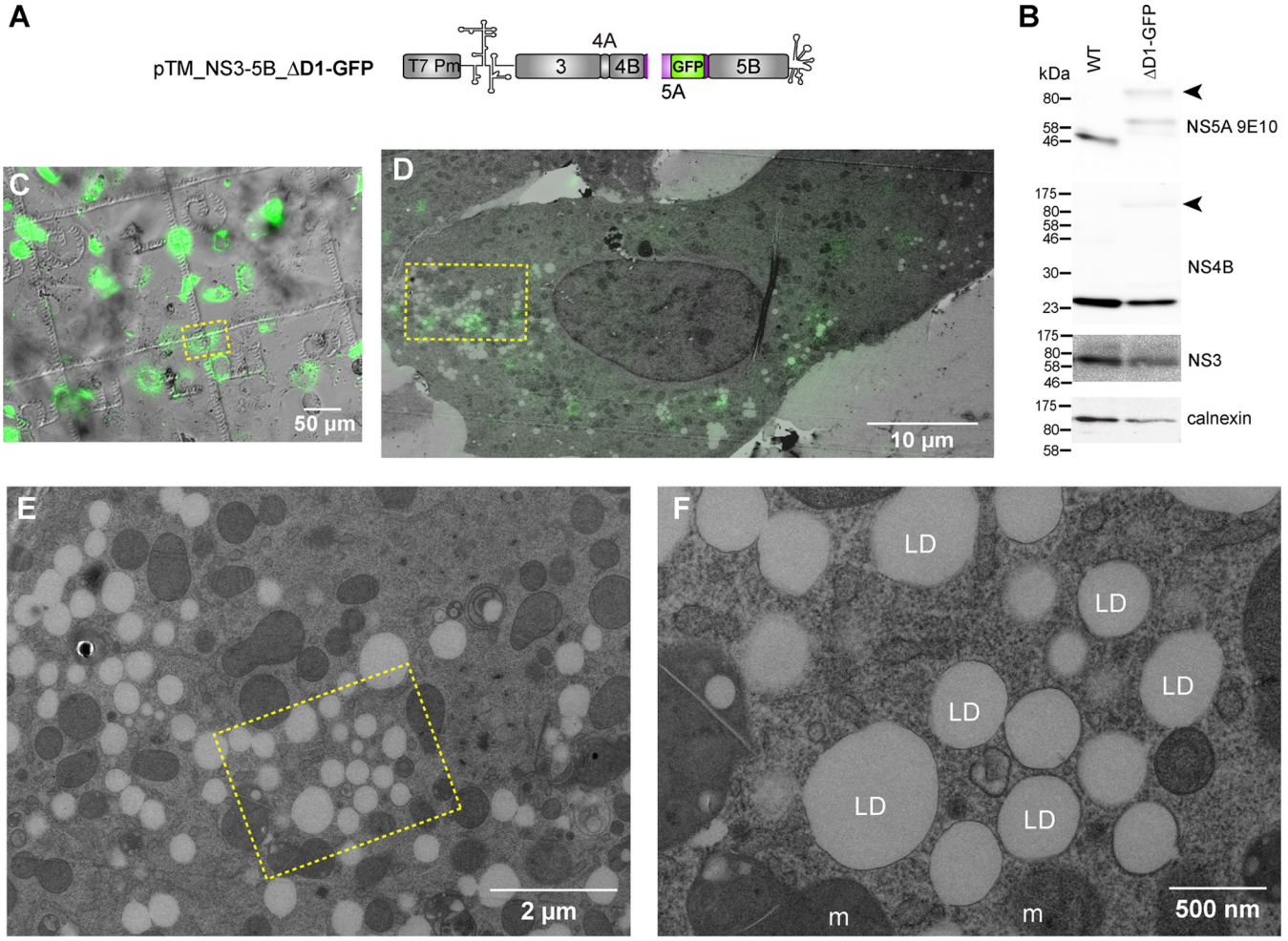

FIG 3 Essential role of NS5A D1 in DMV formation. (A) Schematic of the NS3-5B polyprotein expression construct lacking NS5A D1 and containing a GFP insertion in D3 (pTM_NS3-5B_ΔD1-GFP). Note that this GFP insertion does not affect the functionality of NS5A (24). Pm, promoter. (B) Abundance of variant NS5A, NS4B, and NS3 proteins in Huh7-Lunet/T7 cells transfected with pTM_NS3-5B_ΔD1-GFP in comparison with that in cells transfected with pTM_NS3-5B_WT. Cell lysates prepared 24 h after transfection were analyzed by Western blotting with monospecific antibodies. The uncleaved NS4B-5A precursor is indicated by an arrowhead. WT, wild type. (C) Twenty-four hours after transfection, Huh7-Lunet/T7 cells were analyzed by fluorescence microscopy to allocate GFP-positive cells grown on patterned sapphire discs. (D) Superposition of a low-magnification electron micrograph and the fluorescence image of the cell boxed in panel C. (E and F) Higher-magnification micrographs of the boxed area of this cell, revealing high numbers of LDs but an absence of DMVs.

amounts of DMVs, mitochondria were frequently found at the periphery of DMV-containing areas, whereas LDs were found either within these areas or surrounding them (not shown). Taken together, these results showed that the intrinsically disordered regions of NS5A (LCSI up to D3) are dispensable for DMV induction yet required for RNA replication (D2) or virus assembly (D3) (20), respectively. Thus, the defect of mutant ΔD2long in RNA replication is not linked to a lack of DMV formation.

Conserved residues in the amino-terminal AH and the zinc-binding domain of NS5A are essential for the formation of DMVs. To assess further the importance of NS5A D1 and the N-terminal AH for the induction of DMVs, we analyzed a set of previously described mutations within these regions that specifically disturb features of the AH, abrogate zinc binding of subdomain 1a, or disturb the disulfide bond of subdomain 1b (Fig. 4A). In the AH, which is required for HCV RNA replication (17), two mutations reported earlier (28) in the context of HCV isolate Con1 (genotype 1) were inserted into the JFH-1 isolate (genotype 2a). In these mutants, originally designated GBV-C 8-13 and GBV-B 8-13, the amphipathic character of the helix was maintained but highly conserved N-terminal amino acid residues 8 to 13 of NS5A were replaced with the homologous sequences of closely related GB virus C (GBV-C) and GBV-B, respectively (Fig. 4A). In agreement with previous results (28), the replication of mutant GBV-C 8-13 was clearly lower than that of the wild type (Fig. 4B), which we found to correlate with reduced efficiency of DMV formation in the expression system (Fig. 4G; Table 1). Mutant GBV-B 8-13 was replication incompetent (Fig. 4B) and unable to induce DMVs upon expression of the corresponding NS3-5B polyprotein in Huh7-Lunet/T7 cells, whereas single-membrane vesicles (SMVs) were still detectable (Fig. 4D; Table 1). Importantly, steady-state levels of these variant NS5A proteins were comparable to those of the wild type (Fig. 4C). Thus, specific residues within the NS5A N-terminal AH are required for the biogenesis of DMVs.

NS5A contains a zinc-binding motif in the N-terminal part of

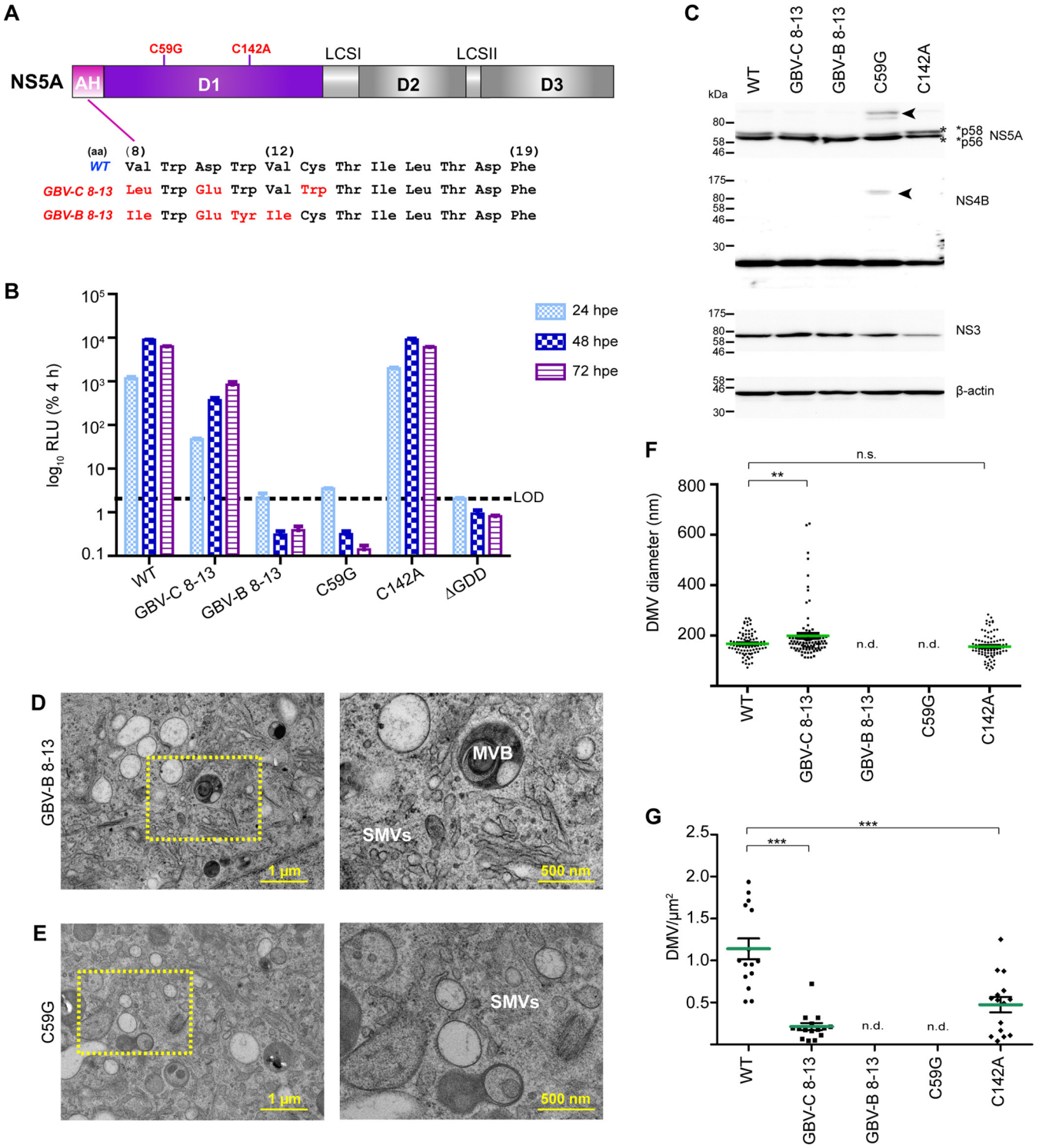

FIG 4 Determinants within the N-terminal AH and D1 of NS5A required for DMV formation. (A) Schematic representation of the domain organization of NS5A and the positions of the mutations introduced. The amino acid (aa) sequence of the N-terminal AH is shown at the bottom, with the amino acid residues that were replaced in red. (B) Replication kinetics of NS5A mutants as determined with subgenomic luciferase reporter replicons. For further details, see the legend to Fig. 1. RLU, relative light units; hpe, hours post electroporation; LOD, limit of detection. (C) Abundance of HCV proteins in Huh7-Lunet/T7 cells transfected with NS3-5B polyprotein expression constructs. Cell lysates prepared 24 h after transfection were analyzed by Western blotting with monospecific antibodies. The uncleaved NS4B-5A precursor is indicated by an arrowhead. WT, wild type. (D and E) Cells transfected in the same way were grown on sapphire discs and processed for EM as described in the legend to Fig. 2. Representative images of the mutants specified to the left of the panels are shown. Magnified views of the boxed areas are shown to the right. MVB, multivesicular body. (F and G) Quantitative analysis of EM images. For further details, see the legend to Fig. 2. The data presented for the wild type are the same as those shown in Fig. 2H and I because the mutant sets shown in Fig. 2 and 4 were analyzed in parallel, along with the wild type. n.d., not determined; n.s., nonsignificant; **, $P < 0.001$; ***, $P < 0.0001$.

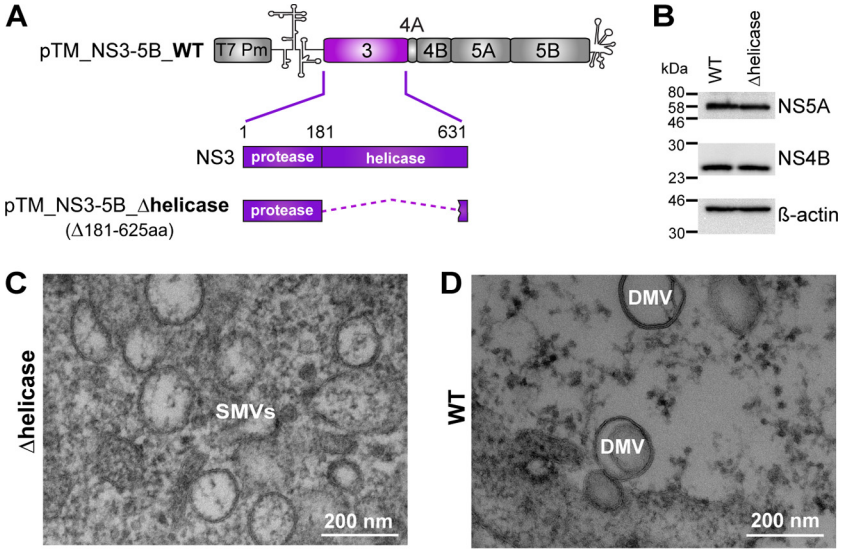

FIG 5 Formation of DMVs requires the NS3 helicase domain. (A) Schematic drawing of polyprotein expression constructs. Numbers refer to the JFH-1 polyprotein; For further details, see the legend to Fig. 1A. Pm, promoter. (B) Western blot analysis of lysates of cells harvested 24 h after transfection. β-Actin served as a loading control. WT, wild type. (C and D) Analyses of transfected cells by EM. Cells grown on sapphire discs were transfected with the constructs shown to the left of the panels, and 24 h later, cells were processed for EM as described in the legend to Fig. 2. Note that only SMVs were found in the helicase deletion mutant (30 cell profiles from two independent experiments analyzed).

D1 (designated subdomain D1a) that is absolutely required for NS5A structure integrity (29). A C59G mutation affecting the cysteine residue coordinating Zn^{2+} binding inhibited HCV RNA replication (18) (Fig. 4B) and abrogated the formation of DMVs, whereas SMVs were still detectable (Fig. 4E). Interestingly, this mutant also displayed a cleavage defect at the NS4B-5A site, but its steady-state level of processed NS5A was comparable to that of the other mutants (Fig. 4C). In contrast, an alanine substitution for cysteine 142 (C142A), reported to form a disulfide bond within subdomain 1b of NS5A (29), affected neither polyprotein cleavage (Fig. 4C) nor HCV RNA replication (Fig. 4B). However, this mutant induced DMVs with lower efficiency than the wild type in the pTM system (Fig. 4F and G) but not in cells transfected with the replicon (Table 1).

In conclusion, these results suggest that both the N-terminal membrane anchor and structural integrity of NS5A D1 are essential prerequisites for DMV formation.

DMV formation requires the NS3 helicase domain. Although we and others have shown earlier that efficient polyprotein cleavage in *trans* can be achieved by using only the N-terminal NS3 protease domain and the NS4A cofactor (30, 31), we did not know whether this minimal protease was sufficient to induce DMVs or whether full-length NS3 would be required. Therefore, we generated an NS3-5B polyprotein expression construct lacking the helicase domain (Fig. 5A). Upon the transfection of Huh7-Lunet/T7 cells with this construct, we observed efficient polyprotein cleavage (Fig. 5B). However, this mutant was unable to induce DMVs and only SMVs were observed (Fig. 5C). These results show that the helicase domain of NS3 is required for the induction of DMVs. Therefore, all subsequent *trans* cleavage assays were conducted with full-length NS3.

Expression of NS3-4A in *cis* is required for efficient formation of DMVs. In order to determine the *cis* and *trans* requirements for DMV formation, we established a polyprotein *trans* cleavage assay. This assay is based on cell lines stably expressing NS3-4A and transient coexpression of an NS4B-5B polyprotein substrate from a separate RNA (Fig. 6A). As a negative control, we used a cell line stably expressing an inactive full-length NS3-4A protease mutant in which the active-site serine residue had been replaced with an alanine residue (S139A) (32). Western blot analysis revealed proper polyprotein cleavage by wild-type NS3-4A, whereas only uncleaved precursor and presumably degradation products were found in cells containing the proteolytically inactive NS3-4A protein (Fig. 6B). While only large SMVs were found in the latter case (Fig. 6D), vesicles composed of two or more lipid bilayers were detected in cells containing the active NS3-4A protein (Fig. 6C). However, the number of DMVs induced in this *trans* cleavage setting was very low compared to that induced by a *cis*-cleaving NS3-5B polyprotein (0.0063/μm^2 versus 1.07/μm^2; Fig. 6F). Moreover, vesicles induced upon *trans* cleavage of the polyprotein had an electron-dense interior, indicative of a protein-rich content, which was not found in DMVs induced by the NS3-5B polyprotein. These results suggested that polyprotein cleavage in *cis* is required for efficient formation of DMVs and that DMVs detected in the *trans* cleavage system might represent different structures.

Regulated cleavage at the NS4B-5A junction determines DMV biogenesis. For several RNA viruses, it has been shown that processing intermediates serve distinct functions in the replication cycle. In HCV, polyprotein processing by NS3-4A is a tightly regulated process that occurs in a preferential order with rather protracted cleavage at the NS4B-5A site (33–37). Interestingly, mutations altering cleavage kinetics at this site are detrimental for HCV RNA replication, but the underlying mechanism has not been studied (38). Assuming that cleavage kinetics might play an important role in the biogenesis of the membranous HCV replication factory, we determined the impact of altered NS4B-5A cleavage kinetics on the formation of DMVs. On the basis of a

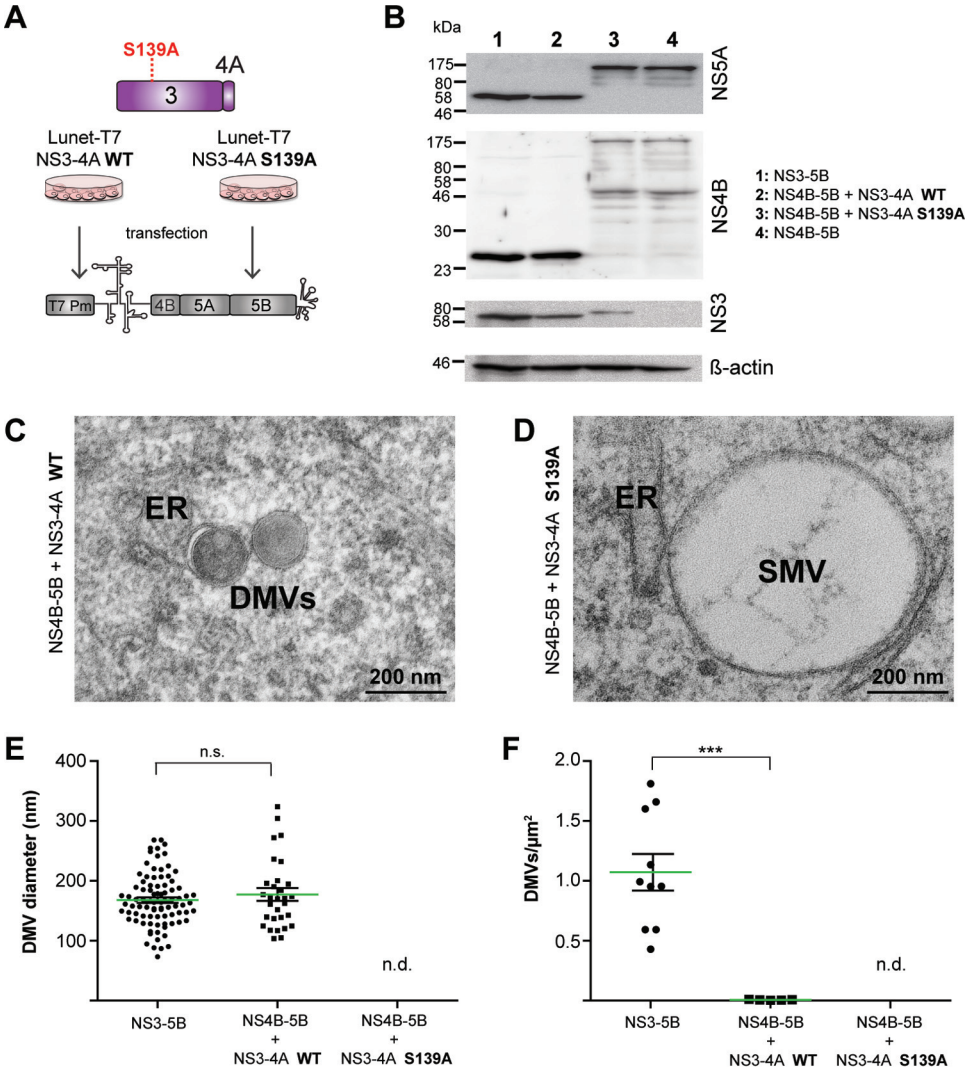

FIG 6 Efficiency of DMV formation is determined by polyprotein cleavage in *cis*. (A) Schematic of the experimental approach used for *trans* cleavage of the polyprotein. Huh7-Lunet/T7-derived cell lines stably expressing wild-type (WT) NS3-4A or an inactive protease (S139A) were transfected with the NS4B-5B polyprotein expression construct. (B) Western blot analysis of cell lysates prepared 24 h after transfection. The constructs and cell lines used are specified on the right. β-Actin served as a loading control. (C and D) Representative EM images of cells expressing the NS4B-5B polyprotein and wild-type NS3-4A or an inactive NS3 protease mutant. (E and F) Quantitative analyses of DMV diameters (E) and numbers of DMVs per square micrometer of cell surface area. (F) From two independent experiments, a total of 90 and 30 DMVs and 10 and 5 cell profiles were analyzed for NS3-5B- and NS4B-5B + NS3-4A WT-expressing cells, respectively. n.d., not determined; n.s., nonsignificant; ***, $P < 0.0001$.

recent report (38), we generated mutant P43VV, exhibiting accelerated cleavage kinetics due to two valine substitutions for proline and isoleucine residues at the P4 and P3 positions of the cleavage site (Fig. 7A, Fast mutant). Indeed, when it was expressed in the context of an NS3-5B polyprotein, much less uncleaved NS4B-5A was found with this mutant, consistent with accelerated cleavage at this site (Fig. 7A). Moreover, consistent with the earlier report (38), we found that the P43VV double mutation, inserted into a subgenomic replicon, reduced RNA replication up to 100-fold at early time points posttransfection (Fig. 7B). When it was tested in the context of the NS3-5B polyprotein expression construct, the formation of DMVs (Fig. 7C) with a regular morphology was observed (Fig. 7D). However, DMV abundance was significantly lower than that in the wild type (Fig. 7E), arguing that regulated cleavage at the NS4B-5A site is important for efficiency of HCV-induced membrane rearrangements and thus RNA replication.

To corroborate this hypothesis, we expressed an NS3-5B polyprotein lacking any NS4B-5A precursor because of the insertion of a heterologous encephalomyocarditis virus IRES between the NS4B and NS5A coding regions (Fig. 8A). This construct did not support RNA replication (Fig. 8A), suggesting that an unprocessed NS4B-5A precursor might play an important role in this process. Expression of the corresponding polyprotein in Huh7-Lunet/T7 cells revealed NS4B abundance to a level comparable to that of the wild type, whereas the abundance of NS5A was slightly reduced but it was still well detectable (Fig. 8B). Importantly, the IRES insertion mutant did not support DMV formation but instead induced SMVs (Fig. 8C). These results suggest that regulated cleavage at the NS4B-5A junction plays a pivotal role in the biogenesis of HCV replication factories and that an NS4B-5A precursor appears to be required for DMV formation.

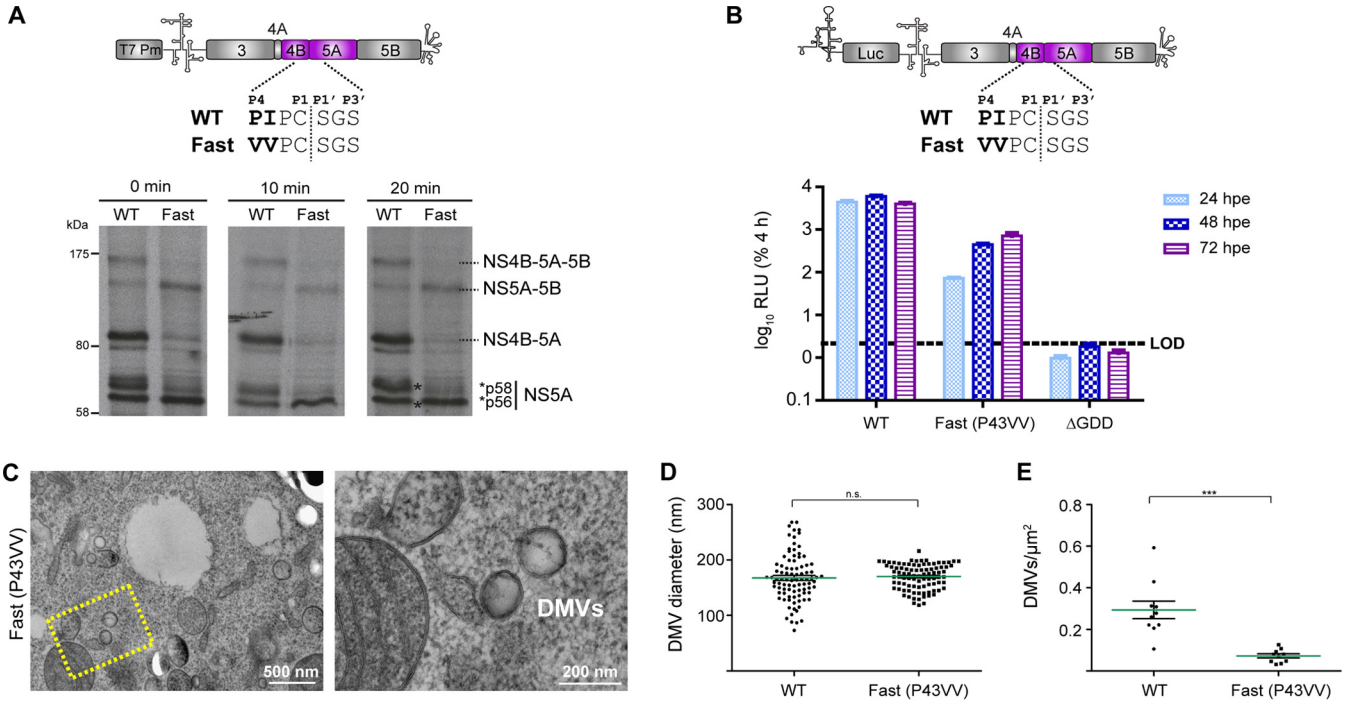

FIG 7 Enhancement of cleavage kinetics at the NS4B-5A site negatively affects RNA replication and DMV formation. (A) The basic polyprotein expression construct is depicted at the top (cf. Fig. 1A). The introduced double mutation reported to accelerate cleavage kinetics at the NS4B-5A site (20) is shown at the bottom (construct P43VV fast). Huh7-Lunet/T7 cells were transfected with each of these constructs and 1 day later incubated with [^{35}S]methionine/cysteine-containing medium for 1 h. The medium was removed, and cells were harvested (0 min) or incubated in nonradioactive medium for 10 or 20 min. NS5A-containing proteins were isolated by NS5A-specific immunoprecipitation and analyzed by SDS-PAGE and autoradiography. HCV proteins are specified on the right, and the positions of molecular weight marker proteins are shown on the left. Pm, promoter; WT, wild type. (B) Replication kinetics of subgenomic luciferase reporter replicons containing cleavage site mutations. The wild type and the NS5B polymerase-dead mutant (ΔGDD) served as positive and negative controls, respectively. LOD, limit of detection. For further details, see the legend to Fig. 1B. Luc, luciferase; RLU, relative light units; hpe, hours postel ectroporation. (C) Representative EM images of cells expressing the NS3-5B polyprotein containing the cleavage site mutation. The right panel is an enlarged view of the boxed region in the left panel. (D and E) Quantitative analyses of DMV diameters (D) and numbers of DMVs per cell surface area (E). Ten cell profiles from two independent experiments were analyzed.

DISCUSSION

Positive-strand RNA viruses, including HCV, replicate their genomes in association with intracellular membranes (1). However, it remains largely unknown how viral proteins can promote the formation of these remarkable membrane alterations. In the present study, we explored the role of viral NS proteins in the formation of HCV-induced DMVs. We reported earlier that induction of the membranous web requires the concerted action of all of the HCV replicase proteins (4). Although the sole expression of NS5A was sufficient to induce vesicles containing two or predominantly more lipid bilayers, DMVs were just sporadically observed. Only when we expressed the polyprotein comprising NS3 to NS5B was DMV formation abundant, arguing for a complex and coordinated interplay between the viral replicase factors to induce these membrane rearrangements. This assumption is supported by the observation that mutations residing, e.g., in NS4B can also drastically reduce DMV formation or alter their morphology even though NS5A is present (16). Thus, while NS5A can induce DMVs on its own, additional determinants are required for the proper formation of the membranous HCV replication factory. To gain further insights into these determinants, in the present study, we mapped the elements within NS5A that are required for DMV biogenesis. We found that the N-terminal AH and D1 are the primary determinants of this process. This is consistent with the observation that D1 is essential for RNA replication. Moreover, this domain binds RNA (29, 39), but it remains to be determined whether this property plays a role in DMV formation. The observation that the ΔD2short mutant does not produce hyperphosphorylated NS5A in spite of replication and DMV formation efficiency comparable to those of the wild type suggests that hyperphosphorylation of NS5A is not strictly required for DMV formation. Although we cannot rule out the possibility that, in the case of this NS5A variant, the basal and hyperphosphorylated forms have very similar electrophoretic mobility and thus are not separated by conventional gel electrophoresis, our conclusion is in keeping with the notion that hyperphosphorylation is critical for assembly, whereas basal phosphorylation appears to favor RNA replication (40–42).

We reported earlier that isolated DMVs contain HCV replicase proteins and enzymatically active replicase complexes, arguing that DMVs are the site of HCV replication (7). Consistent with this assumption, in this study, we observed a correlation between the amount of DMVs detected in cells containing replicating HCV RNA and replication fitness as determined with subgenomic replicons (Table 1). However, this correlation appeared less strict when ectopic expression of the polyprotein was used to induce DMVs. For instance, the ΔD2short and C142A mutants both replicated like the wild type and induced comparable numbers of

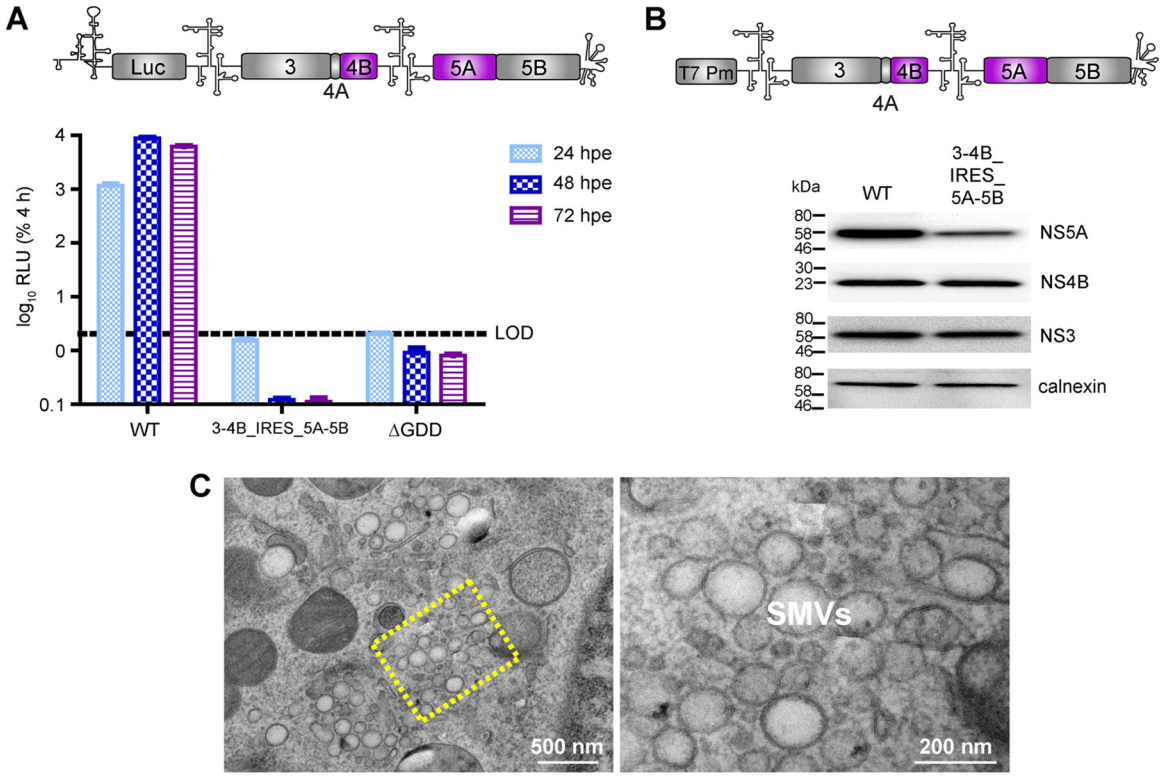

FIG 8 Uncleaved NS4B-5A is required for RNA replication and DMV formation. (A) The design of the subgenomic luciferase reporter replicon containing the IRES of encephalomyocarditis virus between NS4B and NS5A is shown on the top. Replication kinetics, as determined by transient transfection of Huh7.5 cells, are displayed below. Values were normalized to the 4-h values, reflecting transfection efficiency. For further details, see the legend to Fig. 1B. Luc, luciferase; hpe, hours post electroporation; RLU, relative light units; LOD, limit of detection; WT, wild type. (B) Schematic representation of the expression construct transfected into Huh7-Lunet/T7 cells. Twenty-four hours after transfection, cells were lysed and the abundance of HCV NS proteins was determined by Western blotting. Calnexin served as a loading control. (C) In parallel, a fraction of the cells was fixed and analyzed by EM as described in the legend to Fig. 2. Note that only SMVs were detected in cells transfected with the IRES insertion mutant. A total of 30 cell profiles obtained from two independent experiments were analyzed. The image on the right is a higher-magnification view of the area boxed on the left.

DMVs in replicon-containing cells, whereas significantly fewer DMVs were observed in the expression system. Although the DMV number in the expression-based system was, in general, ~10-fold higher than in replicon-containing cells, variability was also much higher and therefore quantitative information is difficult to gain from this system. Nevertheless, owing to high efficiency of DMV formation, the expression system is ideally suited to obtain qualitative information, especially about mutants that do not support HCV RNA replication.

By investigating the contribution of polyprotein cleavage by NS3-4A on HCV-induced membrane remodeling, we made two important observations. First, in spite of efficient polyprotein *trans* cleavage by the minimal NS3 protease domain, full-length NS3 was required for DMV formation. Thus, the helicase domain plays an important role in membrane remodeling by the HCV proteins. Whether this is due to structural constraints mediated, e.g., by helicase interaction with other viral or cellular proteins or viral RNA or whether helicase activity *per se* is needed for membrane remodeling remains to be determined. Second, we found that expression of the viral protease in *trans*, together with the NS4B-5B polyprotein substrate, induced only very low numbers of DMVs, in spite of highly efficient *trans* cleavage. Thus, high efficiency of DMV formation requires a contingent NS3-5B polyprotein acting in *cis*.

Along the same line, we found that regulated polyprotein cleavage kinetics play an important role in DMV formation, as well as RNA replication. HCV polyprotein cleavage occurs in a preferential order, with NS4B-5A representing a rather stable precursor (33–37). Interestingly, a mutation previously reported to accelerate cleavage at this site (38) diminished the formation of DMVs, correlating with a reduction of HCV RNA replication. Furthermore, elimination of the NS4B-5A precursor by the insertion of a heterologous IRES between NS4B and NS5A completely blocked DMV biogenesis, arguing that this precursor plays an important role in the formation of the membranous HCV replication factory. The reason why NS4B-5A intermediates are required for DMV formation and RNA replication might be related to a slow process of NS4B or NS5A maturation to allow proper folding, dimerization/oligomerization, or a posttranslational modification such as phosphorylation. In addition, proper folding might play an important role in the interaction of NS5A with host cell factors, e.g., those involved in cellular membrane homeostasis (19, 26). In this case, the NS4B-5A precursor would exert a spatiotemporal regulation of these interactions. Additionally, features of the NS4B-NS5A precursor itself might play a distinct role in initial steps of HCV membrane-remodeling events. For instance, the linkage of AHs in the NS4B C-terminal domain and at the very N

terminus of NS5A in the precursor might exert a membrane activity different from that of the fully processed proteins.

Distinct membrane-remodeling activities of protein precursors have been reported for poliovirus (43), which, similar to HCV, induces DMVs in infected cells (44). Moreover, in the severe acute respiratory syndrome coronavirus, three full-length transmembrane-containing nonstructural proteins (nsp3, -4, and -6) are needed to induce the formation of DMV-like structures that are morphologically similar to those found in infected cells (45). Single expression of each of these nsps leads to a distinct membrane rearrangement such as membrane proliferation or induction of vesicles, but DMV formation appears to require the concerted action of all three nsps (45). Along these lines, it is also known that the expression of replicase subunits nsp2 to nsp7 of arteriviruses induces membrane changes similar to those in infected cells (46), with nsp2 and nsp3 playing the main role in inducing a membrane-bound scaffold for the arterivirus replication complex (46). However, conversely to our findings, cleavage of the nsp2/3 junction by the nsp2 protease was not essential for the formation of DMVs (47).

In conclusion, we demonstrate that DMVs are induced by a tightly regulated and predominantly *cis*-acting NS3-5B polyprotein. Key factors revealed in the present study are the NS5A N-terminal AH and D1, as well as regulated polyprotein cleavage in *cis* by full-length NS3-4A. Deciphering how host cell proteins and lipids are subverted by these viral factors to induce membrane rearrangements, finally leading to the formation of membranous replication factories, will be the next important step.

MATERIALS AND METHODS

Plasmid constructs. For descriptions of the plasmids used in this study, see Text S1 in the supplemental material.

Generation and maintenance of cell lines. For information about the cell lines used in this study, see Text S1 in the supplemental material.

***In vitro* transcription and transfection of cell lines.** Synthesis of *in vitro* transcripts and transfection of replicon RNAs by electroporation have been described in detail elsewhere (48). pTM vectors allowing the expression of JFH-1-derived polyprotein fragments were transfected with the Mirus TransIT-LT1 Transfection Reagent (Mirus Bio LLC) according to the manufacturer's instructions.

Transient-replication assay. For quantification of the replication efficiency of subgenomic replicons containing a firefly luciferase gene, luciferase activity contained in cell lysates was determined at different time points after electroporation as described elsewhere (49).

Immunoblot analysis. Western blot analysis was performed as described previously (49), with antibodies specified in Text S1 in the supplemental material.

High-pressure freezing and freeze substitution. Cells grown on sapphire discs were subjected to high-pressure freezing and subsequent epon embedding as previously described (4). EM analyses of the embedded cells and quantifications were performed as described in Text S1 in the supplemental material.

CLEM. Huh7-Lunet/T7 cells grown on patterned sapphire discs and transfected with pTM_NS3-5B_ΔD1-GFP were subjected to CLEM as previously reported (4).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00759-15/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.

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