

# Mutational analysis reveals a novel role for hepatitis C virus NS5A domain I in cyclophilin-dependent genome replication

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## Abstract

The hepatitis C virus (HCV) NS5A protein is comprised of three domains (D1–3). Previously, we observed that two alanine substitutions in D1 (V67A, P145A) abrogated replication of a genotype 2a isolate (JFH-1) sub-genomic replicon (SGR) in Huh7 cells, but this phenotype was partially restored in Huh7.5 cells. Here we demonstrate that five additional residues, surface-exposed and proximal to V67 or P145, exhibited the same phenotype. In contrast, the analogous mutants in a genotype 3a isolate (DBN3a) SGR exhibited different phenotypes in each cell line, consistent with fundamental differences in the functions of genotypes 2 and 3 NS5A. The difference between Huh7 and Huh7.5 cells was reminiscent of the observation that cyclophilin inhibitors are more potent against HCV replication in the former and suggested a role for D1 in cyclophilin dependence. Consistent with this, all JFH-1 and DBN3a mutants exhibited increased sensitivity to cyclosporin A treatment compared to wild-type. Silencing of cyclophilin A (CypA) in Huh7 cells inhibited replication of both JFH-1 and DBN3a. However, in Huh7.5 cells CypA silencing did not inhibit JFH-1 wild-type, but abrogated replication of all the JFH-1 mutants, and both DBN3a wild-type and all mutants. CypB silencing in Huh7 cells had no effect on DBN3a, but abrogated replication of JFH-1. CypB silencing in Huh7.5 cells had no effect on either SGR. Lastly, we confirmed that JFH-1 NS5A D1 interacted with CypA *in vitro*. These data demonstrate both a direct involvement of NS5A D1 in cyclophilin-dependent genome replication and functional differences between genotype 2 and 3 NS5A.

## INTRODUCTION

Hepatitis C virus (HCV) belongs to the *Hepacivirus* genus of the *Flaviviridae* family and is now formally named as the species *Hepacivirus hominis*. HCV infection is one of the leading causes of acute and chronic hepatitis, leading to chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC). According to the WHO 58 million people are infected with HCV worldwide and nearly 300000 deaths every year are attributed to this virus [1]. HCV is classified into eight genotypes with an uneven geographical distribution. Notably genotype 3 is the second most prevalent worldwide and is associated with more severe pathology than other genotypes [2]. Genotype 3 infected patients are also most likely to exhibit resistance to direct acting antivirals (DAA), which are currently in widespread clinical use and are highly efficacious (over 95% sustained virological response [SVR]) [3].

HCV is enveloped with a positive-sense, single-stranded RNA of approximately 9.6 kb [4]. The genome contains a single large open reading frame encoding a 3000-residue polyprotein flanked by 5' and 3' untranslated regions. The polyprotein is cleaved by viral and host proteases into three structural (Core, E1 and E2), and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. Of note, NS3 protease, NS5A and NS5B RNA-dependent RNA polymerase are the targets of DAAs.

NS5A is a phosphoprotein with multiple roles in the HCV life cycle. NS5A comprises an N-terminal amphipathic  $\alpha$ -helix membrane anchor and three domains (D1, D2 and D3) which are connected by two solvent-exposed, low-complexity sequences

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**Keywords:** hepatitis C virus; genotype; non-structural 5A protein (NS5A); genome replication; sub-genomic replicon; cyclophilin; cyclosporin.

**Abbreviations:** CsA, cyclosporin; Cyp, cyclophilin; EC50, effective concentration 50%; GST, glutathione-S-transferase; HCV, hepatitis C virus; NS5A, non-structural protein 5A; SGR, Sub-genomic replicon.

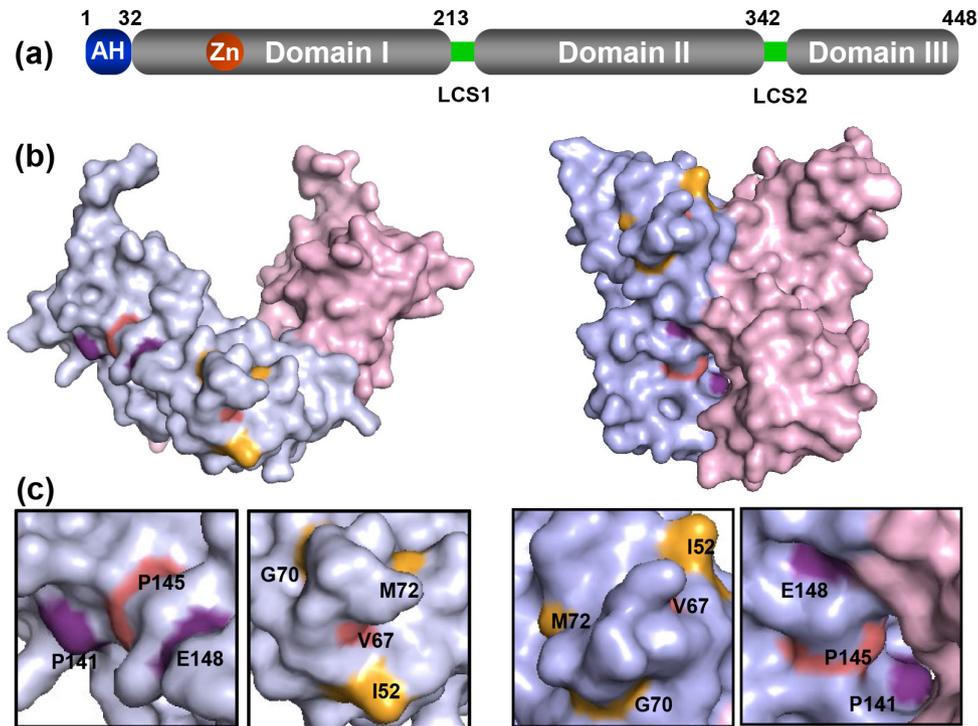
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Five supplementary figures and one supplementary table are available with the online version of this article.

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**Fig. 1.** Structure of NS5A D1 and location of mutated residues. (a) Structure of NS5A illustrating the three domains. AH: amphipathic helix (purple), LCS: low complexity sequence (yellow). (b, c) Conserved and surface exposed residues proximal to V67 and P145 (red) are displayed in the two NS5A D1 (genotype 1b) structures 1ZH1 (b) and 3FQM (c).

(LCS-I and LCS-II) (Fig. 1a). D1 is the only structured domain and is required for genome replication, in addition our recent data has shown that D1 is also involved in virus assembly [5], likely by blocking a novel function of PKR [6]. D2 is a disordered domain that has been reported to interact with cyclophilin A (CypA) [7, 8], a member of the Cyp family of peptidyl prolyl isomerases required for HCV genome replication [9]. D3 has also been reported to interact with CypA [10], and NS5B has been reported to be regulated by binding to the related CypB [11]. Cyps are one of the most abundant proteins in the cytoplasm (0.1–0.4% of total protein content) and are expressed in all tissues [12]. The mechanisms by which Cyps contribute to viral replication remain obscure, however they have been proposed to both induce conformational changes in target proteins (as a result of isomerase activity), and/or regulate protein complex formation [13]. An emerging theme is that Cyps protect either viral particles or replication complexes from cellular antiviral factors such as innate immune sensors [13]. Notably, both CypA and CypB are targets of the immunosuppressive drug cyclosporin A (CsA), and consequently CsA was demonstrated to inhibit HCV genome replication [14].

In this study, we use a combination of mutagenesis and silencing of Cyp expression to extend our understanding of the role of Cyps in HCV biology by showing that NS5A D1 also contributes to the dependence of HCV on Cyps for genome replication. We further show that two HCV genotypes (2a and 3a) exhibit differential requirements for CypA and CypB.

## METHODS

### Cell lines

Huh7 (human hepatocellular carcinoma) [15] and Huh7.5 cells (a derivative of Huh7 from which a stable subgenomic replicon was ‘cured’ by IFN treatment and which exhibit a defect in RIG-I) [16, 17] were used for electroporation. Cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS), 100 IU penicillin ml<sup>-1</sup>, 100 µg ml<sup>-1</sup> streptomycin and 1% non-essential amino acids (Lonza) in a humidified incubator at 37 °C, 5% CO<sub>2</sub>.

### Plasmid and virus constructs

The JFH-1 derived SGR with a luciferase reporter (mSGR-luc-JFH1) was described previously [18], and contains BamHI/AfeI restriction sites flanking the NS5A coding sequence. The DBN3a SGR was generated from the DBN3acc genotype 3a infectious clone [19], and was described recently [20]. NS5A mutations were constructed using Q5 Site-Directed Mutagenesis Kit (New England BioLabs; E0554S). For *E. coli* expression the NS5A D1 coding sequence (amino acids 35–215) was PCR amplified and

cloned into pET-28a-Sumo using BamHI/XhoI restriction sites. Lentivirus constructs for delivery of shRNA to silence CypA and CypB, or CRISPR-Cas9 mediated silencing of PKR or RIG-I were kindly provided by Professor Greg Towers (UCL) and used as described [21]. The EGFP-RIG-I plasmid was kindly provided by Professor Andrew Macdonald (University of Leeds). CypA wild-type and H126Q catalytically inactive mutant cloned in pGEX-6p-2 were described previously [7].

### Antibodies

The following primary antibodies were used: rabbit anti-CypA (Enzo; BML-SA296-0100), rabbit anti-CypB (Abcam; ab16045), rabbit anti-PKR (Abcam; ab32035), rabbit anti-RIG-I (Cell Signalling; 3743), mouse-anti- $\beta$ -Actin (Sigma Aldrich; A1978), sheep anti-GST (in-house) and sheep-anti-NS5A (in-house) [22]. Secondary IRDye 680 and 800 labelled antibodies were obtained from Li-COR.

### Electroporation and luciferase assay

Huh7 and Huh7.5 cells were washed in ice-cold PBS. Cells ( $5 \times 10^6$ ) were resuspended in ice-cold PBS and electroporated with 2  $\mu$ g of RNA at 950  $\mu$ F, 270 V. Cells were resuspended in complete media and then seeded separately into 96-well plates at  $3 \times 10^4$  cells/well. At the indicated times post-electroporation (hpe), cells were harvested into 30  $\mu$ l passive lysis buffer (PLB; Promega), incubated for 15 min at room temperature and stored at  $-80^\circ\text{C}$  until assayed. Luciferase activity was assessed (Promega) on a FluoStar Optima luminometer. Data were recorded as relative light units (RLU).

### CsA assay ( $EC_{50}$ )

Huh7.5 cells were electroporated and seeded as described above in 96-well plates. CsA (Sigma-Aldrich) was resuspended in dimethyl sulfoxide (DMSO) as 20 mM stock, diluted as required and added to cells at 4 hpe. Cells were harvested and luciferase activity determined as above. Data were modelled using the model of log (agonist) vs. response and  $EC_{50}$  values were calculated with GraphPad Prism.

### Lentivirus production and construction of stable knockout cell lines

HEK293T cells in 10 cm dishes were transfected with 1  $\mu$ g packaging plasmid p8.91, 1  $\mu$ g envelope plasmid pMDG encoding VSV-G protein and 1.5  $\mu$ g transfer plasmid pHIV-SIREN encoding shRNA to CypA or CypB, or pLentiCRISPR v2 encoding sgRNA to PKR or RIG-I as described [21]. Lentivirus supernatants were collected at 48 h and filtered through a 0.45  $\mu$ m syringe. Huh7 or Huh7.5 cells were seeded into six well plates at density of  $2.5 \times 10^5$  cells/well and transduced with 1 ml/well lentivirus and 8  $\mu$ g ml $^{-1}$  polybrene for 24 h. Transduced cells were selected using 2.5  $\mu$ g ml $^{-1}$  puromycin at 72 h post-transduction.

### Western blotting

Cells were washed twice in ice-cold PBS and lysed in GLB (1% Triton X-100, 120 mM KCl, 30 mM NaCl, 5 mM MgCl $_2$ , 10% glycerol (v/v), and 10 mM piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES)-NaOH, pH 7.2) with protease and phosphatase inhibitors (Roche; 5892791001). Then, 20  $\mu$ g of each sample were denatured at  $95^\circ\text{C}$  for 5 min and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 50% (v/v) Odyssey blocking buffer (Li-COR) diluted in 1 x Tris-buffered saline containing Tween-20 (TBS-T) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). Membranes were probed with primary antibodies (1:1000 dilution) at  $4^\circ\text{C}$  overnight, and stained with IRDye labelled anti-mouse (700 nm) and anti-rabbit (800 nm) secondary antibodies for 1 h at room temperature (RT). Membranes were imaged on a LI-COR Odyssey Sa Imager.

### His-SUMO-NS5A D1 and GST-CypA expression, purification and pulldown assay

Plasmids were freshly transformed into *Escherichia coli* BL21 (DE3) pLysS and grown at  $37^\circ\text{C}$  until  $OD_{600}$  values reached 0.6–0.8. Protein expression was induced by 100  $\mu$ M isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at  $18^\circ\text{C}$  for 6 h. Cells were recovered by centrifugation at 8000 g for 15 min and resuspended in 50 ml binding buffer (100 mM Tris pH 8.2, 200 mM NaCl, 20 mM imidazole), supplemented with 40  $\mu$ l DNase, 40  $\mu$ l RNaseA, 2 mg ml $^{-1}$  lysozyme and protease inhibitors (Roche) per 1 litre of culture. After incubation on ice for 30 min, samples were sonicated: 10 microns for 12 pulses of 20 s separated by 20 s at  $4^\circ\text{C}$ . After centrifugation at 4000 g for 1 h at  $4^\circ\text{C}$  twice, supernatants were filtered through a 0.45  $\mu$ m syringe filter. Samples were transferred to 1 ml HisTrap FF or GST-agarose columns (Cytiva) equilibrated with binding buffer and the columns were washed three times using five column volumes of binding buffer. The samples were eluted with binding buffer containing 250 mM imidazole or GST elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and dialysed against 20 mM Tris-HCl, pH 8.2, 150 mM NaCl and 10% (v/v) glycerol for His-SUMO-NS5A D1 or 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10% (v/v) glycerol for GST-CypA.

Then, 20  $\mu$ l of GST beads was transferred into 1.5 ml Eppendorf tubes. After washing the beads twice with 400  $\mu$ l binding buffer (100 mM Tris, 0.5 M NaCl, 1% Triton X100), 5  $\mu$ g GST (diluted with washing buffer) protein was added to beads and incubated at  $4^\circ\text{C}$  for 2 h. Beads were washed five times with binding buffer for 5 min and blocked overnight in 400  $\mu$ l washing buffer with 3% BSA. Subsequently, 1.5  $\mu$ g His-Sumo NS5A D1 protein diluted with washing buffer was added and incubated at  $4^\circ\text{C}$  for 2 h.

After five washes using binding buffer, the bound material was eluted with 20  $\mu$ l SDS sample buffer, heated for 10 min at 95 °C and analysed by Western blotting.

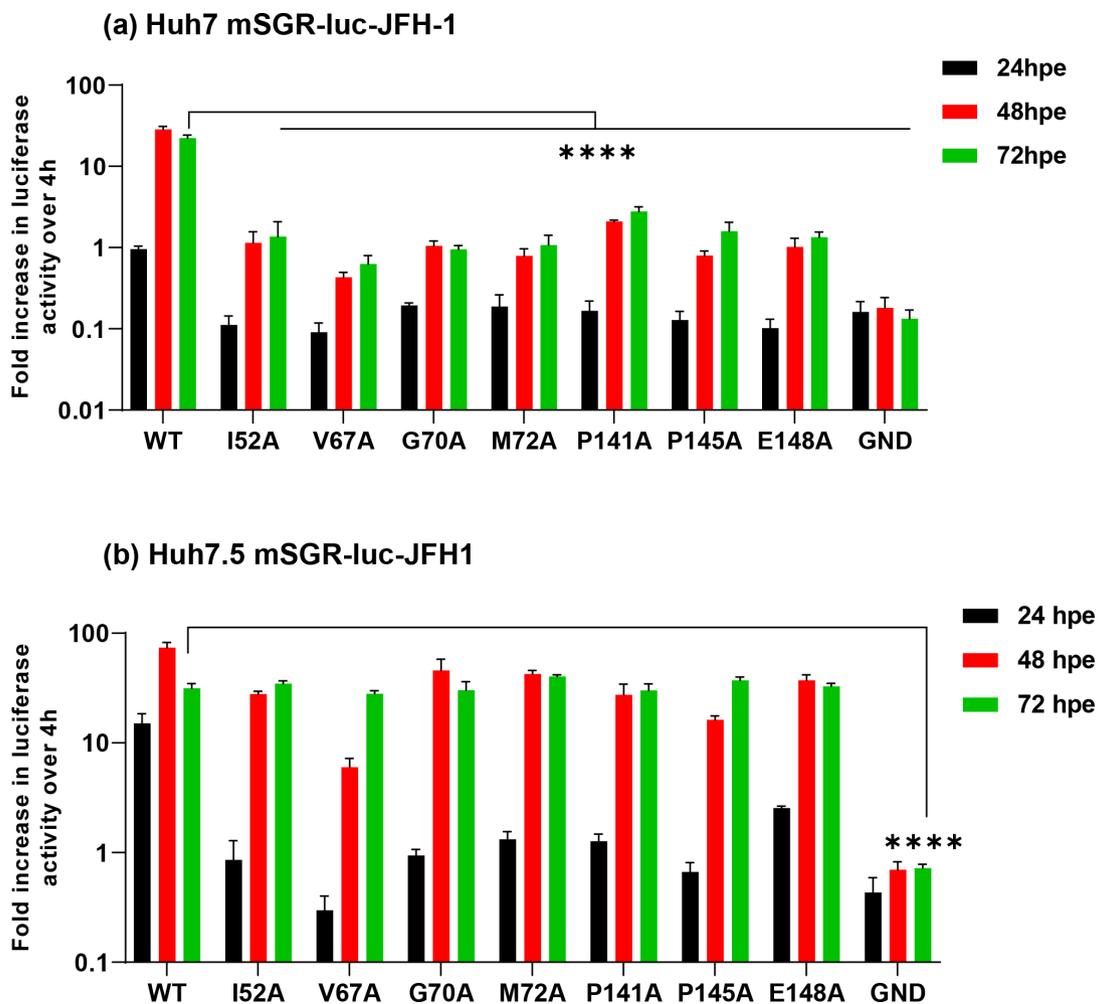
### Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t* tests on GraphPad Prism version 9.30. \*\*\*\* ( $P < 0.0001$ ) and \*\*\* ( $P < 0.001$ ) indicate significant difference from wild-type ( $n \geq 3$ ). Data in histograms are displayed as the means  $\pm$  S.E.

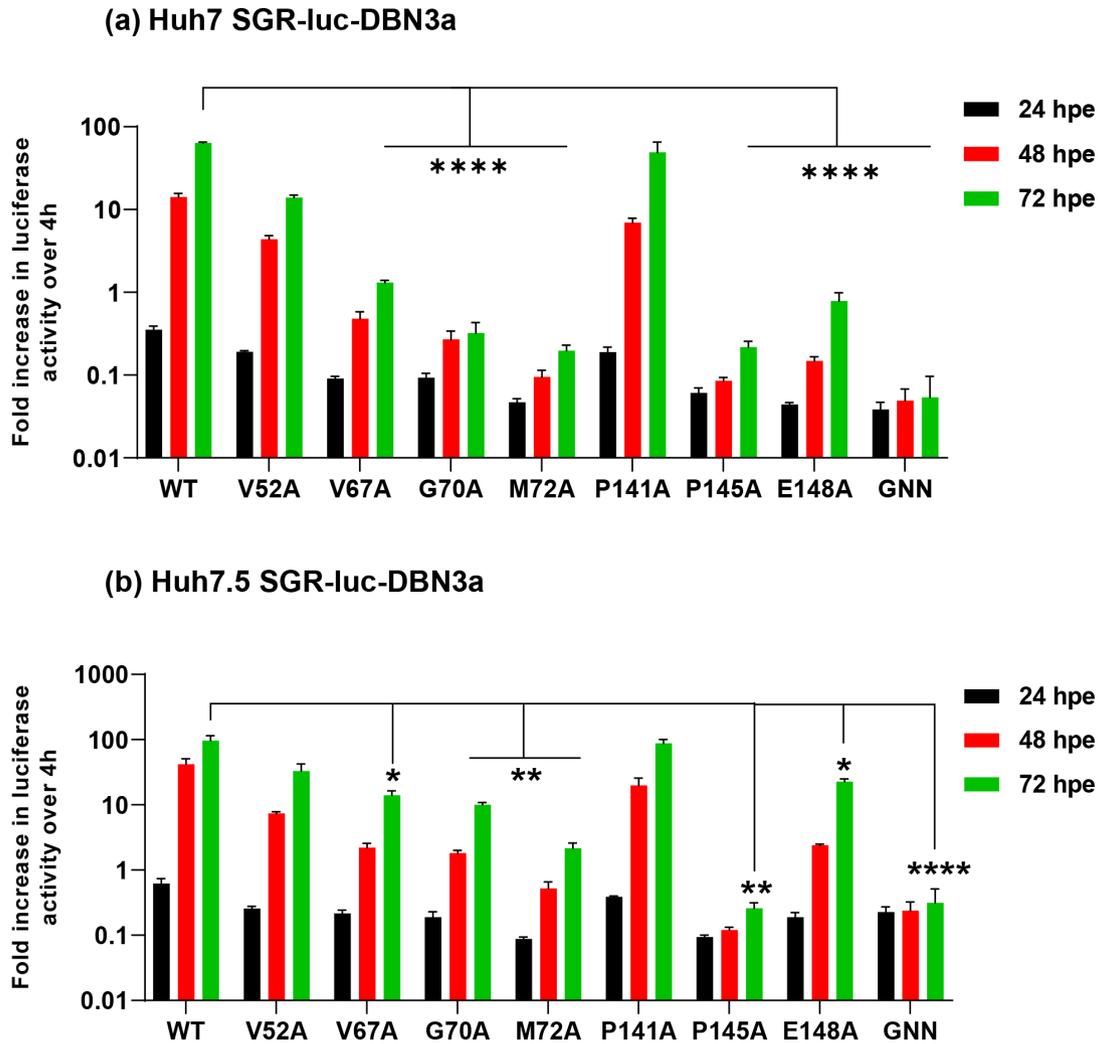
## RESULTS

### Two clusters of surface exposed residues in NS5A D1 exhibit partial defects in replication

We previously showed that alanine substitution of residues V67 and P145 within NS5A D1 resulted in a partial defect in genome replication in Huh7 cells but exhibited no replication phenotype in Huh7.5 cells [5]. To further understand this phenotype we sought to identify additional residues which also exhibited the cell line specific replication requirements of V67 and P145. To achieve this goal, we interrogated the two genotype 1b monomeric structures of NS5A domain I (PDB 1ZH1 and 3FQM) and identified 15 surface exposed and conserved residues proximal to V67 and P145 [6]. These residues were targeted for alanine scanning mutagenesis in the context of an SGR containing a firefly luciferase reporter and unique restriction sites engineered either side of the NS5A coding sequence to facilitate sub-cloning (mSGR-luc-JFH-1) [18, 23]. Analysis of the replication of this panel of NS5A D1 mutant SGRs in both Huh7 and Huh7.5 cell lines revealed several distinct phenotypes ranging from no effect



**Fig. 2.** Genome replication phenotypes of JFH-1 mutants in Huh7 and Huh7.5 cells. *In vitro* transcribed mSGR-luc-JFH-1 RNAs containing the indicated mutations were electroporated into (a) Huh7 and (b) Huh7.5 cells. Firefly luciferase activity was measured at 4, 24, 48 and 72 hpe and the data were normalized with respect to 4 hpe. A full dataset of all 15 mutants has recently been published [6].  $N=3$ , significant differences from WT denoted by \*\*\*\* ( $P < 0.0001$ ).

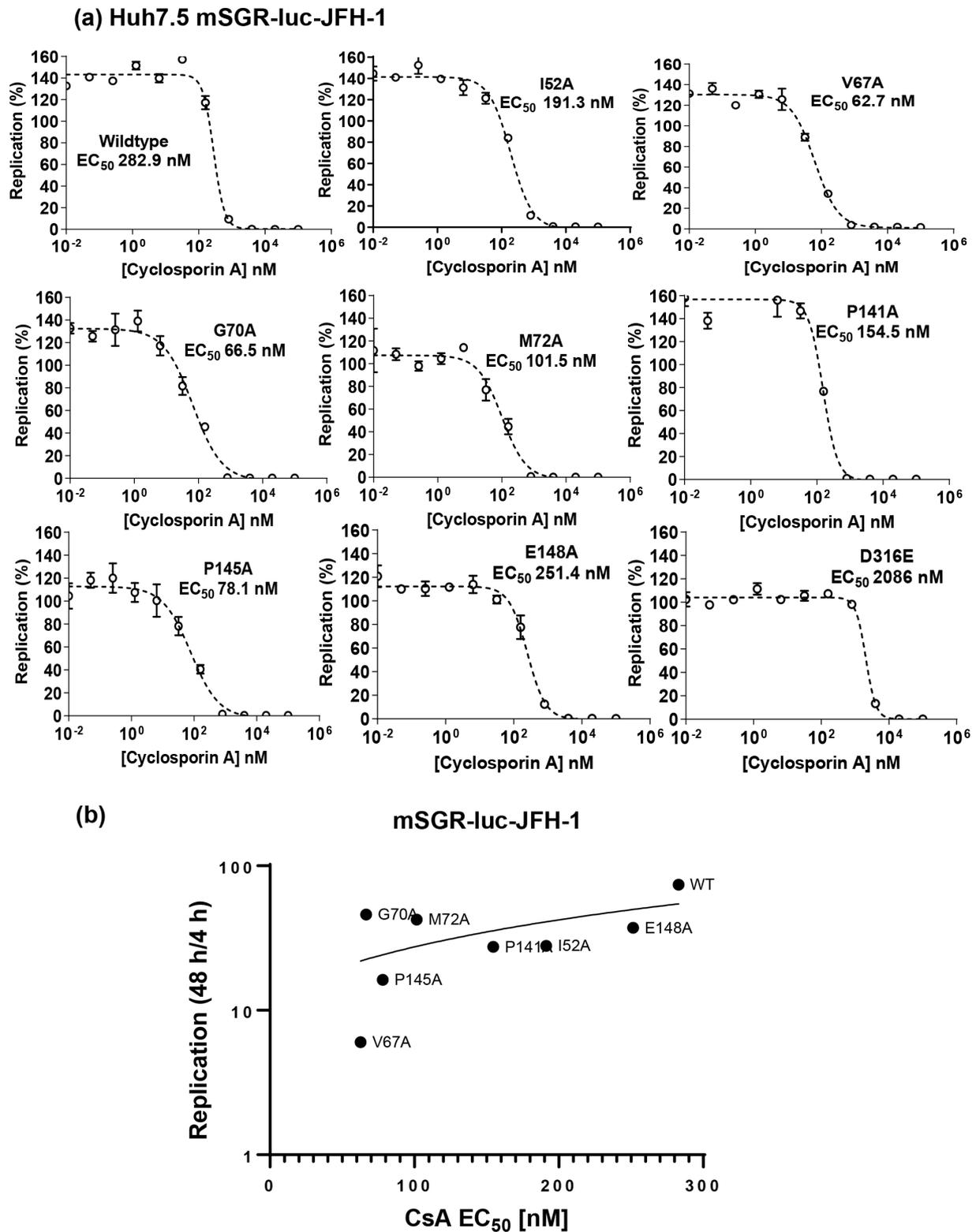


**Fig. 3.** Genome replication phenotypes of DBN3a mutants in Huh7 and Huh7.5 cells. *In vitro* transcribed SGR-luc-DBN3a RNAs containing the indicated mutations were electroporated into (a) Huh7 and (b) Huh7.5 cells. Firefly luciferase activity was measured at 4, 24, 48 and 72 hpe and the data were normalized with respect to 4 hpe.  $N=3$ , significant differences from WT denoted by \* ( $P<0.05$ ), \*\* ( $P<0.01$ ) and \*\*\*\* ( $P<0.0001$ ).

to complete abrogation of replication in both cell lines [6]. Here we focus our attention on five residues that exhibited the same genome replication phenotype as V67A and P145A, i.e. a significant defect in Huh7 cells which was partially or completely restored to wild-type levels in Huh7.5 cells (Fig. 2). Three of these residues were proximal to V67 (I52, G70 and M72), and two were proximal to P145 (P141 and E148) (Fig. 1b, c). To quantify the restoration of replicative capacity in Huh7.5 cells, the ratios of wild-type:mutant luciferase values were calculated for both Huh7 and Huh7.5 cells at 48 and 72 hpe (Table S1A). Although there were considerable differences in the replicative capacity, the wild-type:mutant ratios were dramatically lower in Huh7.5 cells, both at 48 hpe but particularly at 72 hpe where they all approximated to a value of 1, indicating no replication defect compared to wild-type. We conclude that for JFH-1, genome replication of a subset of mutations in NS5A D1 is impaired in Huh7 cells but not in Huh7.5 cells.

### D1 mutant replication phenotypes are not conserved in the context of a genotype 3a SGR

The majority of molecular studies of HCV have been performed using the genotype 2a JFH-1 isolate, however recently infectious clones have been developed for other genotypes [24, 25]. Of particular interest is genotype 3 as this is associated with more rapid fibrosis progression, a higher incidence of steatosis and hepatocellular carcinoma, and lower response rates to DAA treatment. As part of a broader programme of work to compare the functions of NS5A between different genotypes, we extended this current analysis to the genotype 3a isolate, DBN3a [19]. We recently developed an SGR derived from the DBN3a infectious clone [20] termed SGR-luc-DBN3a, and we generated the same seven alanine substitutions as described in Fig. 2 for JFH-1. Note that all of



**Fig. 4.** CsA sensitivity analysis of JFH-1 NS5A D1 mutants. (a) Huh7.5 cells were electroporated with mSGR-luc-JFH-1 RNAs containing the indicated mutations, seeded into 96-well plates and treated with five-fold serial dilutions of CsA. Cells were harvested at 48 hpe and firefly luciferase activity measured. Data were normalized to the DMSO control and processed using the EC<sub>50</sub> model of GraphPad 9.30. (b) CsA EC<sub>50</sub> values were plotted against replicative capacity of each mutant (as determined by the ratio of firefly luciferase at 48 hpe to 4 hpe).

these residues are conserved apart from I52 which is V52 in DBN3a (Fig. S1). Replication of the mutant panel was evaluated in both Huh7 and Huh7.5 cells (Fig. 3).

Again, to quantify the differences in replication between Huh7 and Huh7.5 cells, the ratios of wild-type:mutant luciferase values were calculated for both cell lines at both 48 and 72 hpe (Table S1B). This analysis revealed significant differences from JFH-1. Firstly, P145 was essential for replication in both cell lines, whereas both V52 and P141 appeared to be dispensable and not significantly different from wild-type in both cell lines (although V52A exhibited a modest defect). V67A, G70A, M72A and E148A all showed replication defects that were more pronounced in Huh7 than Huh7.5 cells. These data point to fundamental differences in the functions of JFH-1 and DBN3a NS5A.

### D1 mutants exhibit increased sensitivity to cyclosporin A (CsA) treatment

The differences that we observed between the two cell lines, in particular for JFH-1, were reminiscent of a study published recently [21] demonstrating that cyclophilin (Cyp) inhibitors were more potent against HCV replication in Huh7 compared to Huh7.5 cells. We reasoned that our data might point to a role for D1 in Cyp dependence. To address this we determined the CsA sensitivity of the JFH-1 mutant panel in Huh7.5 cells – we chose these cells as all the mutants were able to replicate efficiently in these cells, and in contrast the impaired replication in Huh7 cells might confound interpretation of the data. As a control we used the well characterized D2 mutant D316E which was previously shown to be highly resistant to CsA [8]. As expected, D316E showed a seven-fold increase in CsA  $EC_{50}$  over wild-type (Fig. 4), in contrast all of the D1 mutants were more sensitive to CsA with reduced  $EC_{50}$  values compared to wild-type. The same was true for DBN3a, with all mutants that could replicate in Huh7.5 cells showing a reduced CsA  $EC_{50}$  value compared to wild-type (Fig. 5). We considered that the increased sensitivity to CsA could be a consequence of reduced replicative capacity so to test this we plotted the  $EC_{50}$  value against the replication level (as measured by the ratio of firefly luciferase between 48/4h). For both JFH-1 (Fig. 4b) and DBN3a (Fig. 5b) there was a trend towards increased CsA sensitivity with lower replication, this was more pronounced for DBN3a whereas for JFH-1 there were significant outliers, for example G70A and M72A. We conclude that for both JFH-1 and DBN3a, CsA sensitivity is multifactorial, with contributions from overall replicative capacity and a direct role of D1, possibly by interactions with Cyps that contribute to genome replication.

### D1 mutants reveal differential cyclophilin dependence of JFH-1 and DBN3a genome replication

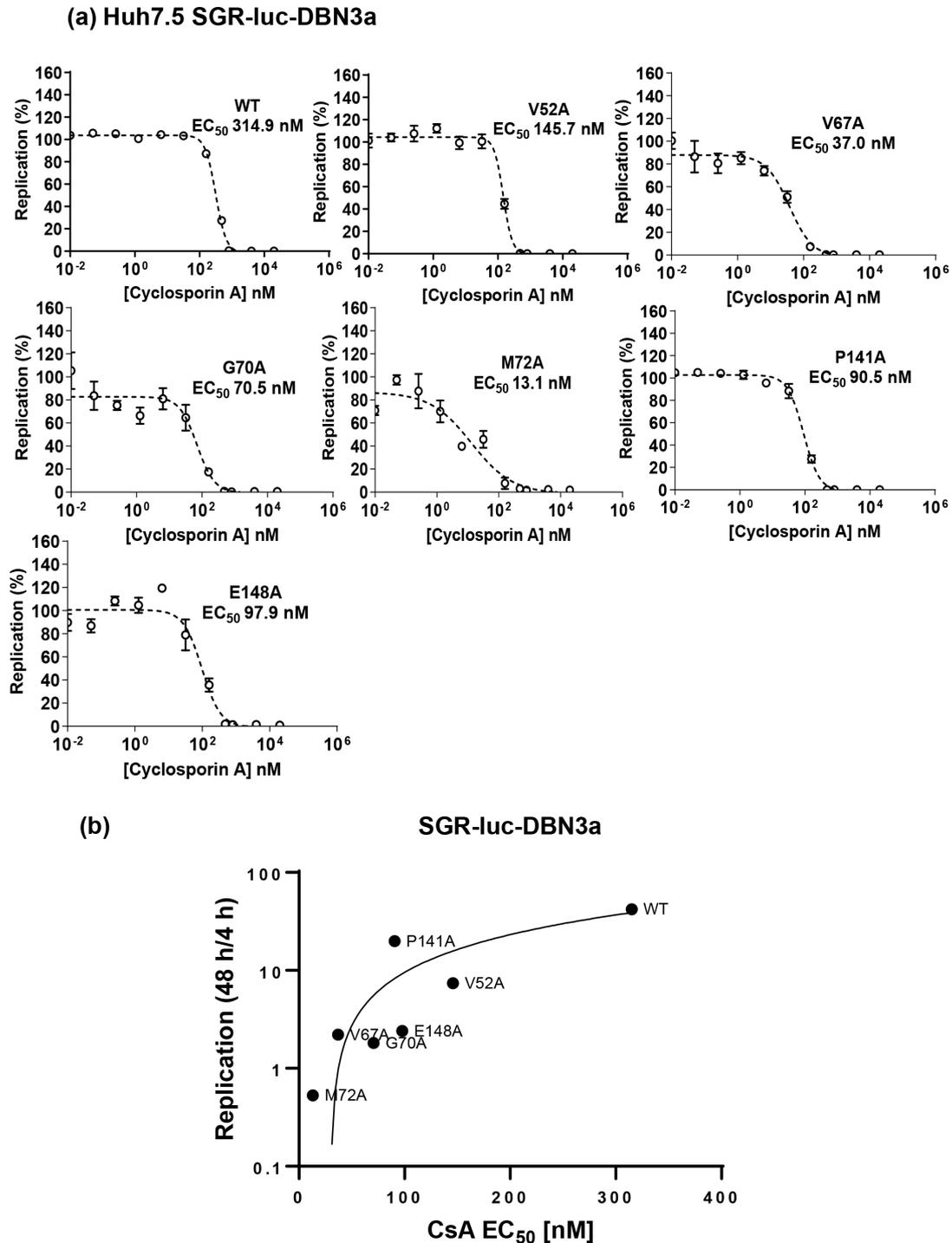
CsA binds to and inhibits Cyps, although 18 of these enzymes have been identified in humans only two (CypA and CypB) have been shown to be required for HCV genome replication [9, 11]. Given the changes in CsA sensitivity exhibited by the D1 mutants we therefore hypothesised that either CypA and/or CypB might regulate D1 function in genome replication. To test this we generated CypA and CypB silenced Huh7 and Huh7.5 cell lines using lentivirus delivery of shRNA, with a non-specific shRNA as control. Silencing was verified by Western blotting (Fig. 6a, b) and these cell lines were then electroporated with RNAs from the two panels of NS5A D1 mutant SGRs (JFH-1 and DBN3a).

Reassuringly, in the shCTRL cell lines the profile of replication for all SGRs mirrored that seen in parental Huh7 and Huh7.5 (compare Fig. 2a with 6c/d, and 3a with 7a/b), confirming that lentivirus transduction and/or puromycin selection did not affect HCV replication. For both JFH-1 and DBN3a replication of both wild-type and the mutants was completely abolished by silencing of CypA in Huh7 cells (Figs 6e and 7c). In Huh7.5 CypA silenced cells JFH-1 wild-type retained the ability to replicate, albeit reduced compared to parental and shCTRL cells (Fig. 6f). However, none of the JFH-1 mutants were able to replicate in CypA-silenced Huh7.5 cells, with the exception of E148A which exhibited a very low level of activity (Fig. 6f). In contrast, for DBN3a no replication was observed in CypA-silenced Huh7.5 cells for either wild-type or the mutants (Fig. 7d). Our data are thus consistent with the previous observation that CypA is critical for HCV replication in Huh7 cells, but not in Huh7.5 cells [21], however this conclusion is only true for JFH-1. In support of this hypothesis we also screened the complete set of JFH-1 mutants in both CypA silenced Huh7 and Huh7.5 cells (Fig. S1). None of the mutants could replicate in CypA-silenced Huh7 cells, however those mutants that replicated as wild-type in Huh7 cells (S71A, C142A, Q143A, P147A, C190A and E191A) [6] retained the ability to replicate in CypA silenced Huh7.5 cells. In comparison, DBN3a genome replication is absolutely dependent on CypA in both Huh7 and Huh7.5 cells.

The situation in CypB silenced cells was very different. For JFH-1 only the wild-type could replicate in CypB silenced Huh7 cells (Fig. 6g), whereas in Huh7.5 cells CypB silencing had no effect, being comparable to parental (Fig. 2b) or shCTRL cells (Fig. 6h). We conclude that JFH-1 is partially dependent on CypB in Huh7 cells but independent of CypB in Huh7.5 cells. In other words, mutants with reduced replicative capacity are rendered more dependent on CypB and are thus unable to replicate in CypB-silenced Huh7 cells. In contrast, CypB silencing had no effect at all on DBN3a replication in either cell line (Fig. 7e, f). Thus DBN3a exhibits no dependence on CypB compared to JFH-1.

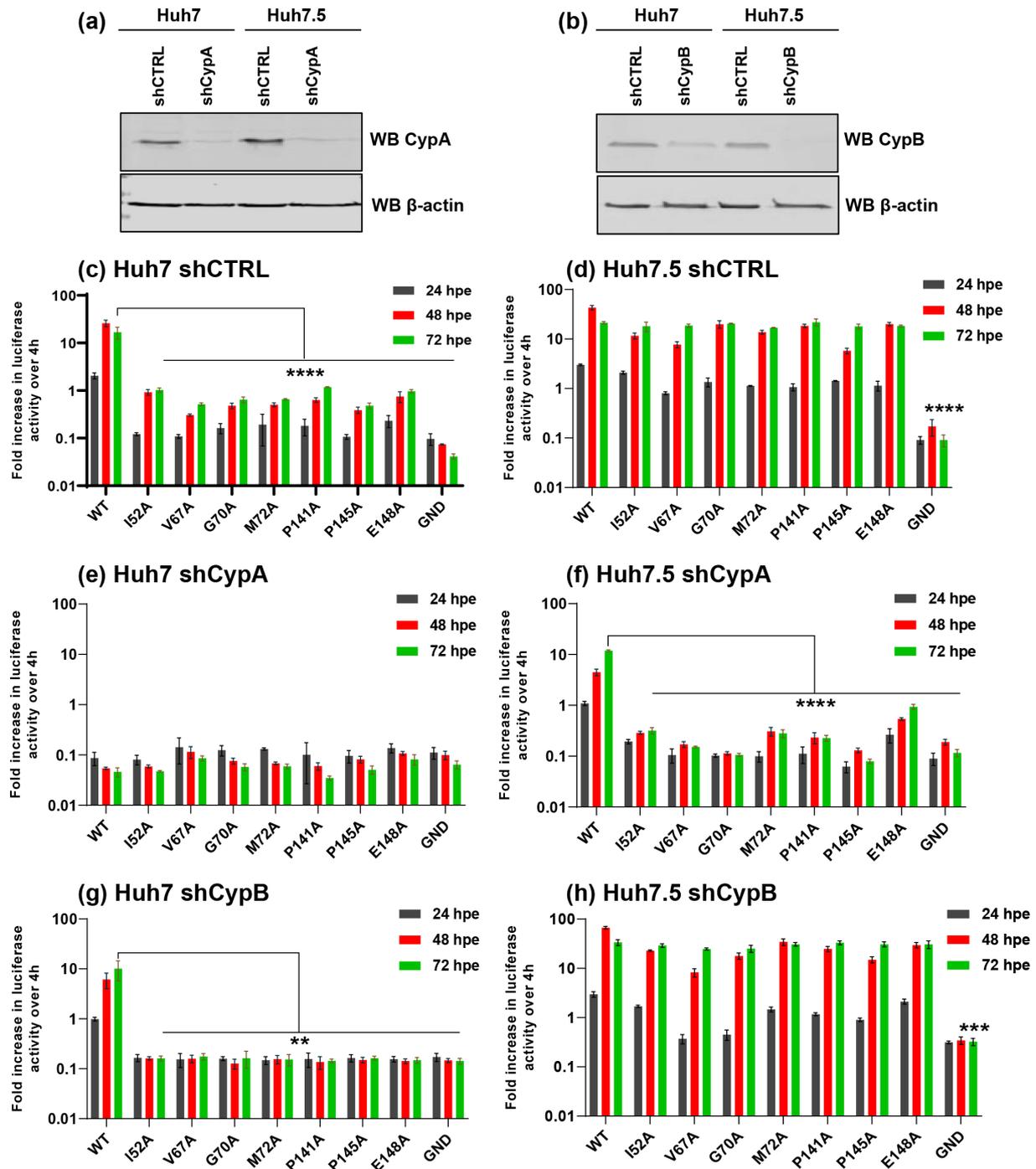
### NS5A D1 interacts with CypA

Previously it has been demonstrated that CypA binds to a proline-rich region within NS5A D2, with prolines in D2 also acting as substrates for the peptidyl-prolyl isomerase activity of CypA [26]. As we observed that the panel of D1 mutants resulted in



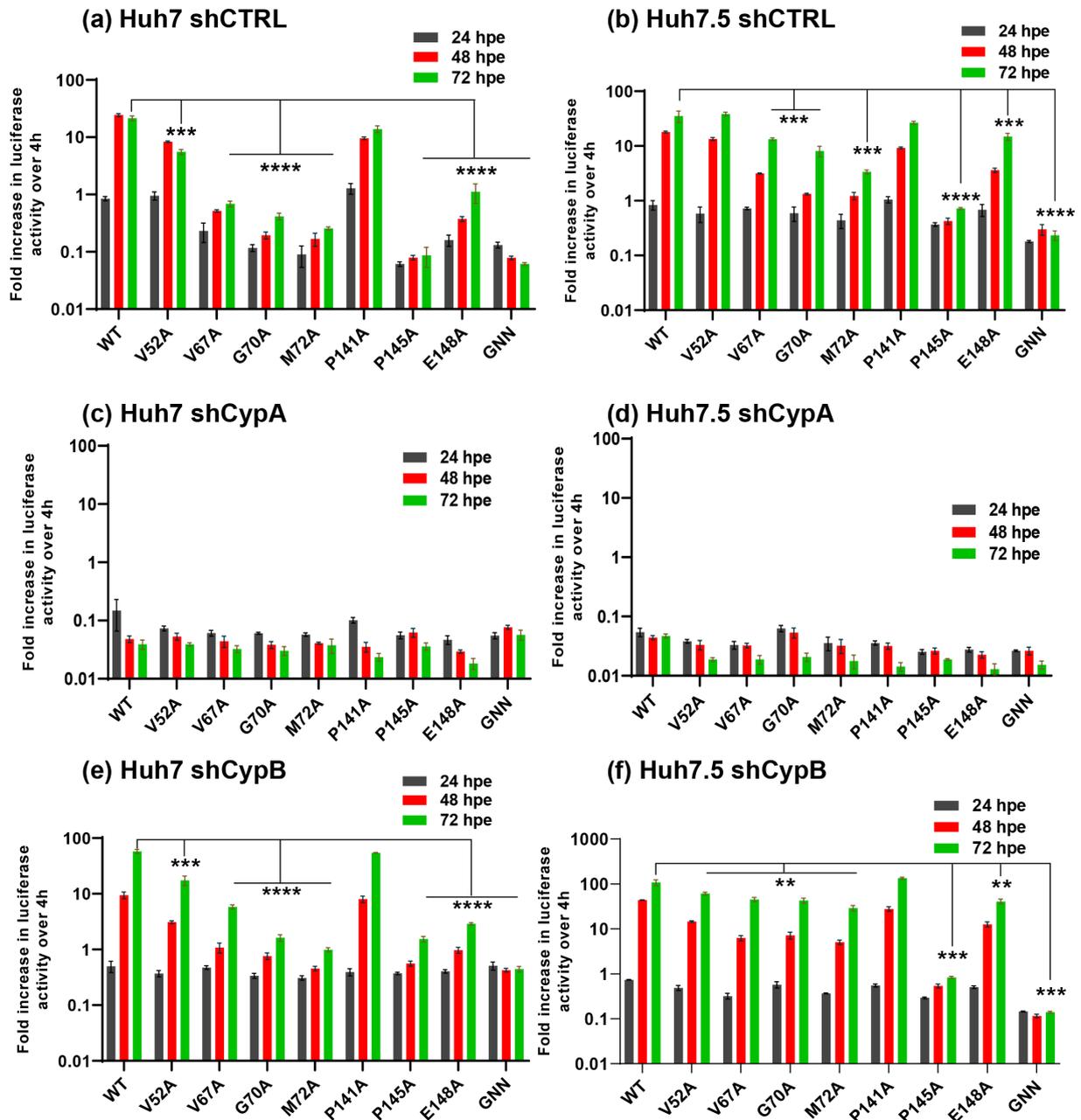
**Fig. 5.** CsA sensitivity analysis of DBN3a NS5A D1 mutants. (a) Huh7.5 cells were electroporated with SGR-luc-DBN3a RNAs containing the indicated mutations, seeded into 96-well plates and treated with five-fold serial dilutions of CsA. Cells were harvested at 48 hpe and firefly luciferase activity measured. Data were normalized to the DMSO control and processed using the  $EC_{50}$  model of GraphPad 9.30. (b) CsA  $EC_{50}$  values were plotted against replicative capacity of each mutant (as determined by the ratio of firefly luciferase at 48 hpe to 4 hpe).

increased sensitivity of genome replication to CsA, and abrogated the ability of the wild-type JFH-1 SGR to replicate in CypA-silenced Huh7.5 cells, we proposed that CypA might also interact with D1. To test this, we performed an *in vitro* GST-pulldown assay using GST-CypA (wild-type or H126Q catalytically inactive mutant) as baits to precipitate purified His-SUMO tagged NS5A D1 (residues 35–215). As shown in Fig. 8a, wild-type CypA did indeed precipitate NS5A D1, whereas the CypA-H126Q



**Fig. 6.** Genome replication phenotypes of JFH-1 mutants in Cyp silenced cells. (a, b) CypA (a) and Cyp B (b) expression was detected in silenced Huh7 and Huh7.5 cells by Western blot. (c–h) *In vitro* transcribed mSGR-luc-JFH-1 RNAs containing the indicated mutations were electroporated into the indicated cell lines. Luciferase activity was measured at 4, 24, 48 and 72 hpe and the data were normalized with respect to 4 hpe.  $N=3$ , significant differences from WT denoted by \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ) and \*\*\*\* ( $P<0.0001$ ).

mutant was unable to do so suggesting that the interaction of D1 with CypA is dependent on the active isomerase site. To confirm this hypothesis we demonstrated that the CypA-NS5A D1 interaction was inhibited by the presence of CsA (Fig. 8b). Lastly we expressed the JFH-1 D1 mutant I52A as an exemplar, and investigated the effect of this mutation on the interaction with CypA. As shown in Fig. 8c, I52A was not precipitated by GST-CypA (wild-type or H126Q). We verified input levels and integrity of the purified His-SUMO tagged NS5A D1 protein samples by Coomassie staining and Western blot (Fig. S2A). We also confirmed that two other D1 mutants (V67A and P145A) disrupted the D1 CypA interaction (Fig. S2B). We conclude from these data that

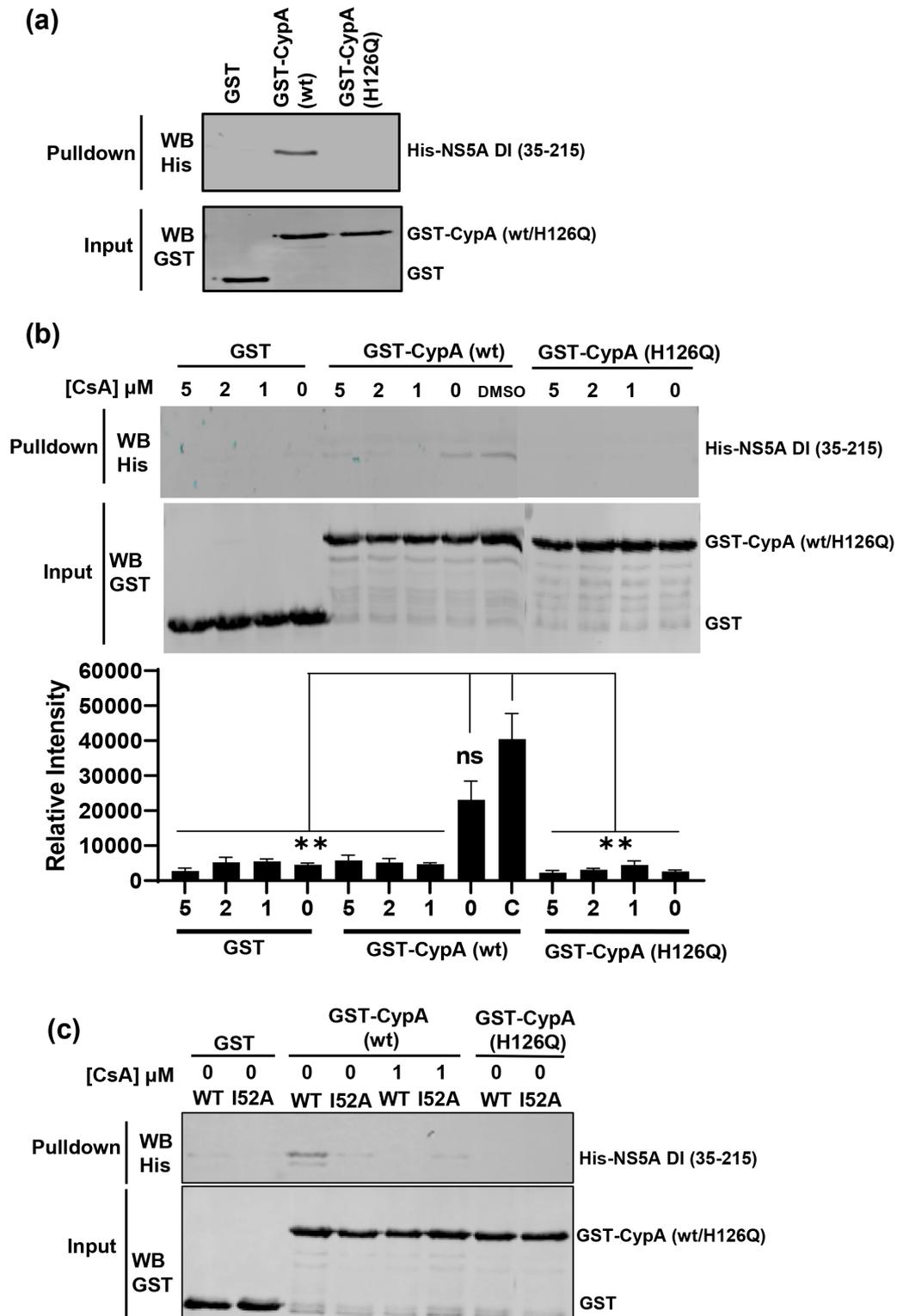


**Fig. 7.** Genome replication phenotypes of DBN3a mutants in Cyp silenced cells. (a–f) *In vitro* transcribed SGR-luc-DBN3a RNAs containing the indicated mutations were electroporated into the indicated cell lines. Luciferase activity was measured at 4, 24, 48 and 72 hpe and the data were normalized with respect to 4 hpe.  $N=3$ , significant differences from WT denoted by \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ) and \*\*\*\* ( $P<0.0001$ ).

NS5A D1 is able to physically interact with CypA and this interaction was dependent on the peptidyl-prolyl isomerase activity of CypA. This interaction may contribute to the CypA dependence of HCV genome replication.

## DISCUSSION

The data presented in this paper attest to significant differences in the functions of NS5A D1 from JFH-1 and DBN3a. This is perhaps surprising given that the D1 amino acid sequence is 72% identical between the two isolates (Fig S3). However, it is well documented that genotype 3 HCV is associated with more severe disease progression and higher resistance to DAAs, particularly those targeting NS5A D1 [3]. A plausible explanation is that these characteristics are driven by subtle differences in protein structure and/or interactions with cellular factors. In this regard, our data also show that JFH-1 and DBN3a exhibit a differential



**Fig. 8.** Interaction between JFH-1 NS5A D1 and CypA. (a) GST, GST-CypA (wild-type) and GST-CypA (H126Q) were purified and bound to glutathione-agarose as a bait to precipitate purified His-SUMO tagged JFH-1 NS5A D1 (amino acids 35–215). The precipitates were analysed by Western blotting for the His-tag (top), and inputs verified by GST Western blotting (bottom). (b) Indicated concentrations of CsA were added to GST or GST-CypA loaded glutathione-agarose for 10 min before the addition of His-SUMO tagged JFH-1 NS5A D1 and analysis as in (a). Representative Western blots shown. Graph below shows quantification.  $N=3$ , differences from WT denoted by ns (non-significant), \*\* ( $P<0.01$ ). (c) His-SUMO tagged JFH-1 NS5A D1 (wild-type or I52A mutant) were added to GST or GST-CypA loaded glutathione-agarose and analysed as in (a) and (b). Where indicated, CsA was added prior to addition of the NS5A D1 prey.

requirement for CypA and CypB in genome replication. Specifically, DBN3a is more dependent on CypA than JFH-1, as wild-type DBN3a failed to replicate in either Huh7 or Huh7.5 in which CypA was silenced, whereas wild-type JFH-1 was able to replicate in CypA silenced Huh7.5 (Figs 5f and 6d). Whilst this difference could be due to many factors such as genomic RNA binding ability [27], interactions with other viral proteins or protein stability, these data are most plausibly explained by the hypothesis that JFH-1 NS5A D1 binds a cellular antiviral factor that in the absence of CypA blocks HCV genome replication, whereas DBN3a D1 is unable to target this factor, rendering it more sensitive to CypA silencing. It is tempting to speculate that this might in part explain the different kinetics of DBN3a and JFH-1 genome replication, with DBN3a exhibiting a significant lag phase before replication proceeds (compare Figs 2 and 3). This hypothesis is supported by the observation that JFH-1 wild-type also exhibits a lag in CypA silenced Huh7.5 cells (Fig. 6f). The identity of the putative cellular factor remains elusive, however, one possibility is the antiviral effector PKR which is proposed to be regulated by CypA [21] and was reported to be expressed at lower levels in Huh7.5. However, in our hands we saw very little effect on genome replication when PKR is silenced in either Huh7 or Huh7.5 cells (Fig. S4), although in other work we have shown a novel role for D1 in blocking PKR-mediated inhibition of virus assembly [6]. It is also documented that Huh7.5 are heterozygous for a dominant negative mutant of RIG-I [16]. Again, we do not believe that RIG-I inactivation is the explanation as either RIG-I silencing in Huh7 cells, or exogenous RIG-I over-expression in Huh7.5 cells, had no effect on JFH-1 SGR replication (Fig. S5).

In contrast, DBN3a did not exhibit any requirement for CypB (Fig. 7e, f) compared to JFH-1 for which only wild-type was able to replicate in CypB silenced Huh7 cells. CypB was initially reported to interact with NS5B and stimulate RNA-dependent RNA polymerase activity [11], however CypB has also been reported to interact with NS5A D2 [26]. Our data are consistent with the suggestion that CypB also targets NS5A D1 of JFH-1 but not that of DBN3a. Clearly the differential requirement of the two isolates for the two Cyps demonstrates that these two cellular factors are not redundant with each other, and likely function via distinct mechanisms to facilitate viral genome replication.

All mutants in D1 tested in this study increased the sensitivity to CsA (Figs 4 and 5). Furthermore, in the case of JFH-1 they blocked replication in CypA silenced Huh7.5 cells and CypB silenced Huh7 cells suggesting that the residues mutated may be involved in direct interactions with the two Cyps, at least for JFH-1. The direct interaction of D1 with CypA *in vitro*, and the loss of the CypA interaction exhibited by I52A (Fig. 8), V67A and P145A (Fig. S3), provides some supporting evidence for this hypothesis. The disruption of this interaction by CsA or a mutation in the CypA active site suggests that CypA might function to isomerise D1, in this regard it is noteworthy that D1 is proline rich (20 prolines in JFH-1, 19 in DBN3a – Fig. S3). As the mutated residues that modify Cyp sensitivity are spatially distal (Fig. 1b, c), it is possible that CypA binds to and isomerises prolines at multiple sites on D1. The interaction of NS5A with CypA has also been shown to correlate with dimerization [28], as the E148A mutation resulted in a two-fold reduction of both dimerization and CypA interactions measured using a mammalian two-hybrid system. Additionally we have previously shown that V67A and P145A disrupt *in vitro* dimerization of NS5A [5]. Although there is no direct evidence that the NS5A-CypA interaction requires dimerization, it is formally possible that D1 dimerization might expose a CypA binding site in D2. It is also possible that CypA is recruited by D1 and then functions to isomerise D2, as we have no information about how the three domains of NS5A are orientated or interact with each other in the native protein.

Further studies in our laboratory are in progress to dissect out the molecular differences between JFH-1 and DBN3a NS5A, as well as the pathways associated with CypA and PKR that regulate the virus lifecycle.

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#### Conflicts of interest

The authors confirm there are no conflicts of interest.

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