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Cyclophilin A allows the allosteric regulation of a structural motif in the disordered domain 2 of NS5A and thereby fine-tunes HCV RNA replication

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Implicated in numerous human diseases, intrinsically disordered proteins (IDPs) are dynamic ensembles of interconverting conformers that often contain many proline residues. Whether and how proline conformation regulates the functional aspects of IDPs remains an open question, however. Here, we studied the disordered domain 2 of nonstructural protein 5A (NS5A-D2) of hepatitis C virus (HCV). NS5A-D2 comprises a short structural motif (PW-turn) embedded in a proline-rich sequence, whose interaction with the human prolyl isomerase cyclophilin A (CypA) is essential for viral RNA replication. Using NMR, we show here that the PW-turn motif exists in a conformational equilibrium between folded and disordered states. We found that the fraction of conformers in the NS5A-D2 ensemble that adopt the structured motif is allosterically modulated both by the *cis/trans* isomerization of the surrounding prolines that are CypA substrates and by substitutions conferring resistance to cyclophilin inhibitor. Moreover, we noted that this fraction is directly correlated with HCV RNA replication efficiency. We conclude that CypA can fine-tune the

dynamic ensemble of the disordered NS5A-D2, thereby regulating viral RNA replication efficiency.

Hepatitis C virus (HCV)⁷ is a positive sense single-stranded RNA (~9.6 kb) virus, belonging to the *Flaviviridae* family. It is estimated that worldwide ~71 million people have a chronic HCV infection (www.who.int/mediacentre/factsheets/fs164/en/). This can lead to progressive hepatic injuries, such as cirrhosis or even hepatocellular carcinoma (2). Each year, chronic hepatitis C is responsible for the death of roughly 400,000 people with ~25% of all liver cancer cases being linked to HCV infection.

The HCV genome has one large ORF, encoding a polyprotein precursor that is proteolytically processed into 10 mature proteins by both host and viral proteases. The structural proteins (core, E1 and E2) constitute the viral particle, and the nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are involved in virion formation and RNA replication (3, 4). NS3 to NS5B, bound to double-membrane vesicles (DMV) derived from the host endoplasmic reticulum (ER), constitute the viral replication machinery (5–7). This complex, by means of the RNA-dependent RNA polymerase activity of NS5B, replicates the viral genome (8, 9).

NS5A is a key multifunctional phosphoprotein (49 kDa) that is essential for HCV genome replication (10, 11) and is also involved in virion production (12). NS5A is known to interact with numerous viral and host proteins (4, 13–15). NS5A, which

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This article contains Table S1, Figs. S1–S21, and supporting information.

The NMR data reported in this paper has been submitted to the Biological Magnetic Resonance Data Bank under accession number 30037.

The atomic coordinates and structure factors (code 6HT4) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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⁷ The abbreviations used are: HCV, hepatitis C virus; CA, capsid protein; CHESPA, chemical shift projection analysis; CypA, cyclophilin A; CypIs, cyclophilin inhibitors; CsA, cyclosporin A; dPCA, dihedral principal component analysis; DAAs, direct-acting antivirals; DMV, double-membrane vesicles; ER, endoplasmic reticulum; GaMD, gaussian accelerated molecular dynamics; HTAs, host-targeted antivirals; HSQC, heteronuclear single quantum correlation; IDPs/IDRs, intrinsically disordered proteins (or regions); MD, molecular dynamics; NS, non-structural proteins; NS5A, non-structural protein 5A; NS5A-D1, domain 1 of NS5A; NS5A-D2, domain 2 of NS5A; NS5A-D3, domain 3 of NS5A; PepD2-WT, peptide corresponding to residues 304–323 of NS5A-D2; PPIase, peptidyl-prolyl *cis/trans* isomerase; PTMs, post-translational modifications; PW_fraction, fraction of the conformers that hold the structured PW-turn motif; PW-turn, short structural motif in NS5A-D2; TMSP, sodium 3-trimethylsilyl-[2,2,3,3-d₄]propionate; r.m.s., root mean square; RDC, residual dipolar coupling; dPCA, dihedral principal component analysis.

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is bound to the ER membrane via an N-terminal amphipathic helix (16), is composed by a folded cytoplasmic domain (NS5A-D1) (11) and two intrinsically disordered domains (NS5A-D2 and -D3) (17–19) (Fig. 1a). No enzymatic activity has been identified for any of these domains. Different homodimeric structures of NS5A-D1 (11, 20, 21) suggested it might be implicated in RNA-binding (22). Importantly, this domain is the molecular target for direct-acting antivirals (DAAs) against NS5A (23, 24). NS5A-D2 and -D3 are intrinsically disordered, and thus exist as a dynamic ensemble of conformers. NS5A-D2 is required for viral RNA replication (10), whereas NS5A-D3 is involved in viral particle production and assembly (12). We and others have shown that NS5A-D2 and -D3 interact with and are substrates of human cyclophilin A (CypA) (25–27), a peptidyl-prolyl *cis/trans* isomerase (PPIase) that is an essential host factor for HCV replication (28). Detailed analysis assigned the main CypA-binding site to a small region (about 20 amino acid residues) in NS5A-D2 that contains 5 strictly conserved residues across the seven HCV genotypes, among which are 3 proline residues (25, 29). An even shorter peculiar structural motif, called PW-turn ($^{314}\text{PXWA}^{317}$ in the Con1 strain, genotype 1b), which is essential for HCV RNA replication was identified in this region (30) (Fig. 1a). Disruption of the PW-turn weakens the molecular interaction between NS5A-D2 and CypA, but is required for efficient prolyl *cis/trans* isomerase activity of CypA toward the $^{313}\text{Met-Pro}^{314}$ bond, contrary to the other prolyl bonds of the domain (30). The interaction between NS5A-D2 and CypA can be inhibited by cyclosporin A (CsA) or its non-immunosuppressive analogs such as alisporivir, SCY-635, or NIM-811, or by other small-molecule cyclophilin inhibitors (31–34). These compounds thereby act as host-targeted antivirals (HTAs). NS5A hence can be equally targeted by DAAs (via NS5A-D1) or HTAs (via NS5A-D2 through its interaction with CypA). Of note, studies of HCV NS5A and CypA paved the way for the use of CypI for other viral infections such as human coronaviruses (e.g. MERS or SARS) that also require CypA for their replication (35).

The role(s) of CypA in HCV replication and the mechanism of action of cyclophilin inhibitors (CypIs) are complex and still not completely understood. CypIs bind host CypA in a single hydrophobic pocket that contains both its enzymatic PPIase and binding activities, and thereby disrupt both the molecular interaction between CypA and NS5A-D2 as well as the putative CypA PPIase activity toward selected prolyl bonds in NS5A-D2 (25, 36, 37). Therefore, all CypA catalytic mutants are also impaired in their binding properties. Several laboratories have demonstrated that CypIs inhibit the *de novo* formation of the DMVs, the HCV-induced membrane structures that hold the replication complex (38, 39). A remodeling of the ER in the presence of CypIs has also been observed in HCV-infected cells (40). Most of the identified CypI-resistance mutations, such as D320E, D320E,Y321N, and Y321H (28, 41, 42), are located in the immediate proximity of the PW-turn in NS5A-D2. These mutations confer a moderate resistance (~2–4-fold) to CypIs and hence a partial CypA independence, but do not abolish the interaction between CypA and NS5A-D2 (27, 43, 44). A better understanding of the functional role(s) of both CypA and NS5A, and specifically of the disordered domains of the latter,

in the HCV life cycle could shed light on the underlying molecular mechanisms of this resistance.

Intrinsically disordered proteins (or regions) (IDPs/IDRs) are functional despite not having a stable 3D structure (45, 46). They are best described as dynamic ensembles of interconverting conformers. Their biological functions are usually related to their capacity to interact with numerous partners, with a high specificity often related to low affinity (even if subnanomolar affinities have been reported (47)). As a consequence, they are often described as molecular hubs. IDPs/IDRs, despite any enzymatic activity, are nonetheless involved in cell signaling and regulatory processes, which can be physiological or pathological. Indeed, their implication has been demonstrated in numerous human diseases, including cancer, neurodegeneration, diabetes, and viral infections (48). IDPs/IDRs can interact with a biological partner while remaining disordered and form fuzzy complexes (49), but they can also establish interactions using short peptide motifs, including short linear motifs (50), molecular recognition features (51), or pre-structured motifs (52), and then fold upon binding. The biological functions of IDPs/IDRs are further regulated by alternative splicing and post-translational modifications (PTMs), including phosphorylation, ubiquitination, and glycosylation (53). These PTMs can modulate the structural and conformational properties of IDPs/IDRs and thereby break or promote interactions. IDPs are also enriched in proline residues (54), but whether these have any functional role has not yet been described in a conclusive manner. More recently, the concept of allostery has been expanded from its original paradigm (55) and allosteric regulations have been described in disordered protein (56, 57). It has been shown that allosteric perturbations (ligand binding, PTMs, mutations) can change the functional properties of IDPs/IDRS by remodeling their energy landscape or conformational ensemble (*i.e.* population shift). This extreme complexity allows IDPs/IDRs to exert a fine modulation of biological processes (58).

Here, we combine nuclear magnetic resonance (NMR) spectroscopy with molecular dynamics (MD) simulations to decipher the role of the proline residues on the structural disorder in HCV NS5A-D2, and link it to its functional consequences. The detailed conformational behavior of NS5A-D2 (JFH1 strain, genotype 2a) with its PW-turn structural motif centered on $^{310}\text{PAWA}^{313}$ confirms its pan-genotypic importance for HCV. Using the Trp 312 resonance as a probe, we show that the NS5A-D2 region encompassing the PW-turn exhibits a peculiar dynamic behavior whereby it may adopt, at least 2 conformational states, one structured with the PW-turn conformation fully formed and one totally disordered. These two states are in the fast-exchange NMR regime. Unexpectedly, we find that the *cis/trans* isomerization state of the 5 surrounding proline residues affects the ratio of conformers that adopt or not the PW-turn motif in the NS5A-D2 ensemble. Hence, this structural motif is allosterically regulated by proline *cis/trans* equilibria. CypA, through catalyzing the interconversion for distinct prolines, can thereby connect these different ensembles on a subsecond time scale. Importantly, we find that the CypI-resistance mutations in NS5A-D2 correspond to similar allosteric perturbations as they favor ensembles with a

decreased structured PW-turn population. Using a cell-based assay, we show that the population of the structured PW-turn motif in the ensemble directly correlates with HCV RNA replication efficiency. Our results reveal a complex mechanism in which CypA modulates NS5A-D2's function.

Results

NMR characterization of NS5A-D2 and its PW-turn motif

The ^1H , ^{15}N -HSQC NMR spectrum of NS5A-D2 of the HCV JFH1 strain (genotype 2a) (Fig. 1*b*) displays a narrow ^1H chemical shift dispersion that confirms the high level of intrinsic disorder in this domain (Fig. S1*a*) (18). The secondary structure propensity analysis, based on the ^{13}C chemical shifts, indicates the presence of several residual α -helices and extended regions in the N-terminal half (Fig. 1*c*). This is similar to the observations made on NS5A-D2 from HCV strains HC-J4 and Con1 (both genotype 1b) (30, 59). Comparison of the experimental ^1H and ^{15}N chemical shifts of NS5A-D2 (JFH1) with those predicted for a truly disordered protein (60) highlights unexpected values for the tryptophan 312 (Trp³¹²) residue in the C-terminal part of the domain (Fig. 1*d* and Figs. S1 and S2). A similar observation was made for the equivalent Trp³¹⁶ residue in NS5A-D2 of the HCV Con1 strain and was attributed to the presence of the PW-turn structural motif in the most conserved region of NS5A-D2 (30). The primary sequence of the PW-turn in the Con1 strain is $^{314}\text{PIWA}^{317}$, whereas it is $^{310}\text{PAWA}^{313}$ in JFH1. In this motif, all positions but the second are strictly conserved in all HCV genotypes. We used a 20-mer peptide (pepD2-WT) to obtain an atomic description of this region in NS5A-D2 (JFH1) (Fig. S3). All the NMR data (^1H , ^{15}N , ^{13}C chemical shifts, NOE contacts, as well $^3J_{\text{HN-H}\alpha}$ couplings) were used to calculate a NMR structural model of this PW-turn (Fig. 2 and Fig. S4*a*). The local r.m.s. deviation values suggest that beyond the core of the PW-turn (*i.e.* $^{310}\text{PAWA}^{313}$), where the ring of Pro³¹⁰ and the aromatic side chain of Trp³¹² are engaged in a CH/ π interaction (61), the residues at its C terminus also adopt a certain degree of order (Fig. 2*b*). The interaction of the PW-turn motif, and specifically the Trp³¹² residue, with the Tyr³¹⁷ side chain and the rings of Pro³¹⁹ and Pro³²⁰ (Fig. 2*c*) agree with a similar extension of the motif as described in the peptide when bound to the MOBKL1B protein (62). Conformations of pepD2-WT, free in solution (this study), are closely related to its structure in the crystallographic complex with MOBKL1B (Fig. S4*b*).

The plot of the ^1H , ^{15}N -HSQC peak intensity along the NS5A-D2 sequence reveals that the resonances in the N-terminal moiety are narrower than those in the C-terminal half (Fig. 1*e*). ^{15}N spin relaxation data on the full-length domain confirmed this, with higher R^2 values for the 304–321 region of NS5A-D2. Heteronuclear NOE values in this region are also clearly positive, whereas they are negative or close to zero for most residues in the N-terminal half of the fragment. We hence conclude that this region, encompassing the PW-turn and corresponding to the host CypA-binding site (25), is characterized by an increased rigidity while simultaneously experiencing exchange broadening (63) (Fig. 1, *f–h*). We next measured the residual dipolar couplings (RDC_{NH}) of the individual HN vec-

tors in NS5A-D2 using a partially oriented sample (Fig. 1*i*). The residues in the region 304–321 display RDC_{NH} values higher than expected for an IDR (64). The highest value (32.8 Hz) was observed for the Trp³¹² residue in the PW-turn motif. The RDC_{NH} values from the Trp³¹² and Trp³²⁵ side chains were 40 and 24 Hz, respectively (data not shown). Altogether, these data confirm that the CypA-binding site contains a structured PW-turn motif with nevertheless a peculiar structural dynamics.

We have previously reported that the I315G mutation in a peptide derived from NS5A-D2 of the Con1 strain precludes the presence of the PW-turn motif without making any change on the conserved residues. To investigate the functional role of the PW-turn motif in the JFH1 strain, we introduced the analogous mutation, A311G, in NS5A-D2. We found that this mutation also efficiently disrupts the PW-turn in NS5A-D2 because in the different NMR spectra acquired on this NS5A-D2 mutant, the proton amide and $^{13}\text{C}\alpha$ resonances of Trp³¹² and Ala³¹³ move toward their expected frequencies for a fully disordered region (Figs. S1*b* and S5). The NS5A-D2 A311G mutant hence provides a way to assess the structural and functional role(s) played by the PW-turn motif in the CypA-binding site.

Proline conformations induced a linear chemical shift pattern for Trp³¹²

Upon closer examination of the ^1H , ^{15}N -HSQC of NS5A-D2 WT, we identified up to six resonances that could be assigned to the same Trp³¹² residue (Fig. 3*a*). Based on the peak intensity, there are one major (W312_1) and five minor (W312_2 to W312_6) Trp³¹² resonances. As the Trp³¹² residue is surrounded by 5 different proline residues in NS5A-D2, we hypothesized that this conformational heterogeneity with slow exchange on the time scale imposed by the chemical shift differences between the individual resonances could correspond to the *cis/trans* equilibria of these individual proline residues. Evidence was found in the ^1H , ^{15}N , ^{13}C 3D experiments used for the assignment of the fragment. Starting from the proton amide W312_6 resonance and using the ^{13}C chemical shifts ($C\alpha$, $C\beta$, and CO), we connected it, in a sequential fashion, to a minor resonance of the Ala³¹¹ residue (A311_6), which is itself linked to a residue with ^{13}C chemical shifts typical of a *cis*-proline (62.4 and 34.3 ppm for $C\alpha$ and $C\beta$, respectively) (65) (Fig. S6). Thus, the minor W312_6 resonance originates from the *cis* conformation of the Pro³¹⁰ residue. Likewise, the other minor Trp³¹² resonances are also related to the *cis* conformation of proline residues. Indeed, in a ^1H , ^{15}N -heteronuclear *zz*-exchange experiment (66) in the presence of a catalytic amount of CypA, we identified exchange cross-peaks connecting each of the minor Trp³¹² resonances (W312_2 to W312_6) to the major one (W312_1) (Fig. 3*b*). To link each of the minor Trp³¹² resonances to a particular proline residue, we compared the ^1H , ^{15}N -HSQC spectra of NS5A-D2 Pro to Ala mutants (NS5A-D2 P306A, P310A, P315A, P319A, and P320A) with that of the WT construct (Figs. S7–S11) (67). In each mutant spectrum, a minor Trp³¹² resonance is missing. We therefore could unambiguously assign the minor Trp³¹² resonances in the ^1H , ^{15}N -HSQC of NS5A-D2 WT to subpopulations of the peptide with a single prolyl bond in the *cis* conformation (Fig.

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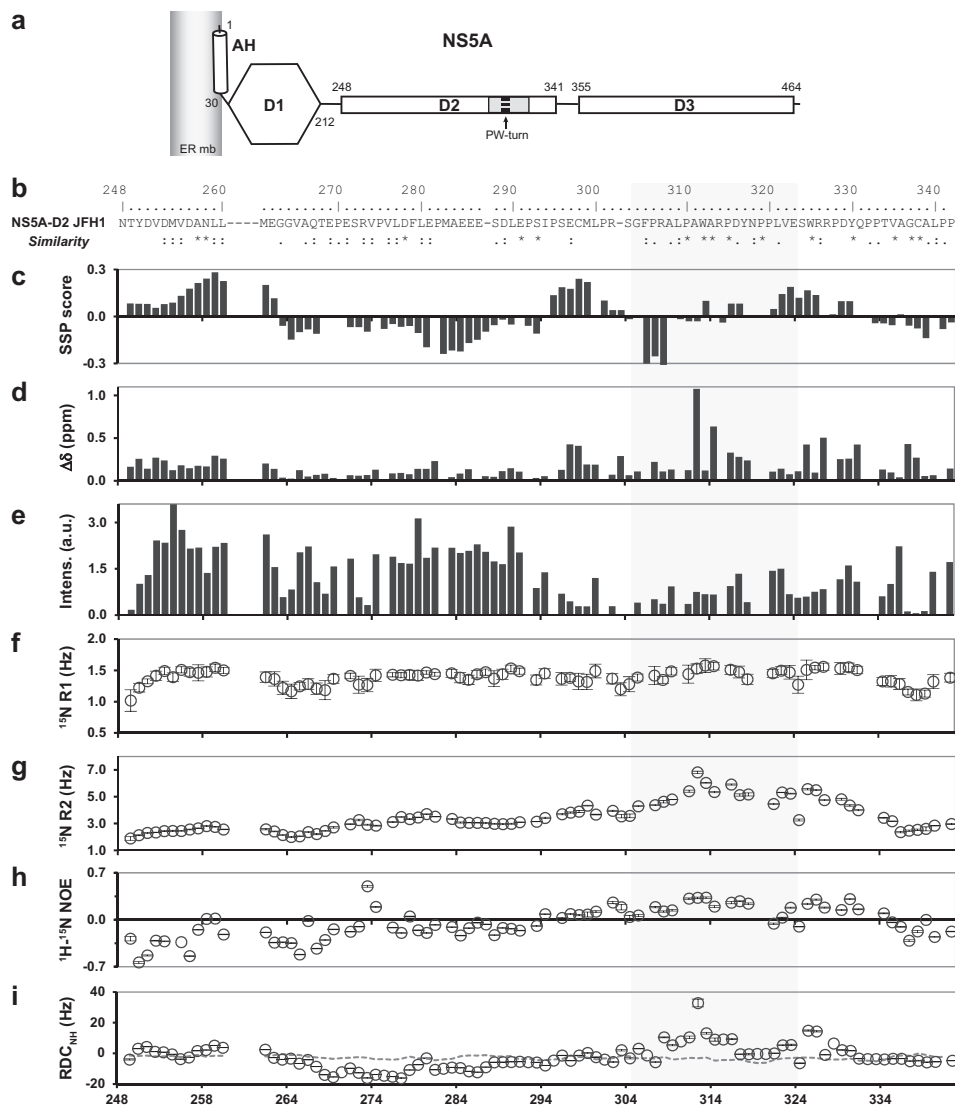


Figure 1. NMR structural dynamics of NS5A-D2. *a*, schematic representation of HCV NS5A protein anchored to the cytoplasmic side of the ER membrane via a N-terminal amphipathic helix (AH). Its folded domain 1 (NS5A-D1) is shown as a white hexagon, whereas its disordered domains 2 and 3 (NS5A-D2 and -D3) are represented by white rectangles. The CypA-binding site, which comprises the PW-turn motif (dashed black line), is shown as a gray area. *b*, NS5A-D2 amino acid sequence (residue 248–342) from HCV JFH1 strain (genotype 2a). Below is similarity deduced from the alignment of reference sequences from all confirmed HCV genotypes and subtypes. The light gray shaded region corresponds to the PepD2-WT peptide, which is also the CypA-binding site. *c*, secondary structure propensity analysis from experimental $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts of NS5A-D2. Positive and negative scores indicate helical tendencies and extended regions, respectively, whereas values close to 0 indicate a fully disordered state. *d*, deviation of experimental ^1H , ^{15}N -combined chemical shift values from neighbor-corrected IDP values calculated from the primary NS5A-D2 sequence. *e*, peak intensities in the ^1H , ^{15}N -HSQC spectrum, shown in Fig. S1a. *f–h*, longitudinal R1 (*f*) (Hz), transverse R2 (*g*) (Hz), and heteronuclear NOE (^1H , ^{15}N) (*h*) relaxation rates in NS5A-D2, measured at 600 MHz and 298 K. R1 and R2 were calculated by fitting peak heights in a series of spectra to a decaying exponential, using Sparky. The error bars indicate the standard error of the fit. For ^1H - ^{15}N NOE the error bars were calculated from the root sum square of (noise/signal) in the spectra with and without saturation. *i*, $^1\text{D}_{\text{NH}}$ dipolar couplings measured using 2D ^{15}N IPAP-HSQC experiments recorded both in isotropic and anisotropic conditions. The error bars represent experimental errors, calculated from the linewidths at half-height in the ^{15}N dimension and the signal to noise ratio.

3a). The W312_1 resonance thereby corresponds to the *trans* conformation of Pro³⁰⁶, Pro³¹⁰, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰.

All of these Trp³¹² resonances fall along a line and form a linear chemical shift pattern (Fig. 3). A similar spectral behavior was observed for the residue Ala³¹³ (Fig. S12), which is also part of the PW-turn motif. A linear pattern of chemical shifts was previously interpreted as a proof that this residue exists in (at least) two conformational states with distinct chemical shift environments in fast exchange on the NMR time scale (68). Even if the W312_6 and W312_1 resonances do not exactly match with the two pure states of this fast-exchange system, these resonances can be used as proxies for the two conforma-

tional states at the extremity of the linear chemical shift pattern. Considering the frequency difference between the W312_1 and W312_6 resonances ($\Delta\omega^1\text{H} \sim 420$ Hz, $\Delta\omega^{15}\text{N} \sim 300$ Hz), the time scale of this exchange is faster than 1–0.1 ms. Moreover, the observed line broadening or equivalent enhanced R² rates for this residue suggest that the exchange is close to this microsecond-millisecond time range.

Allosteric regulation of the PW-turn motif by proline *cis/trans* equilibria

To further characterize the PW-turn in each of these distinct conformers, we analyzed the same NMR parameters as for the

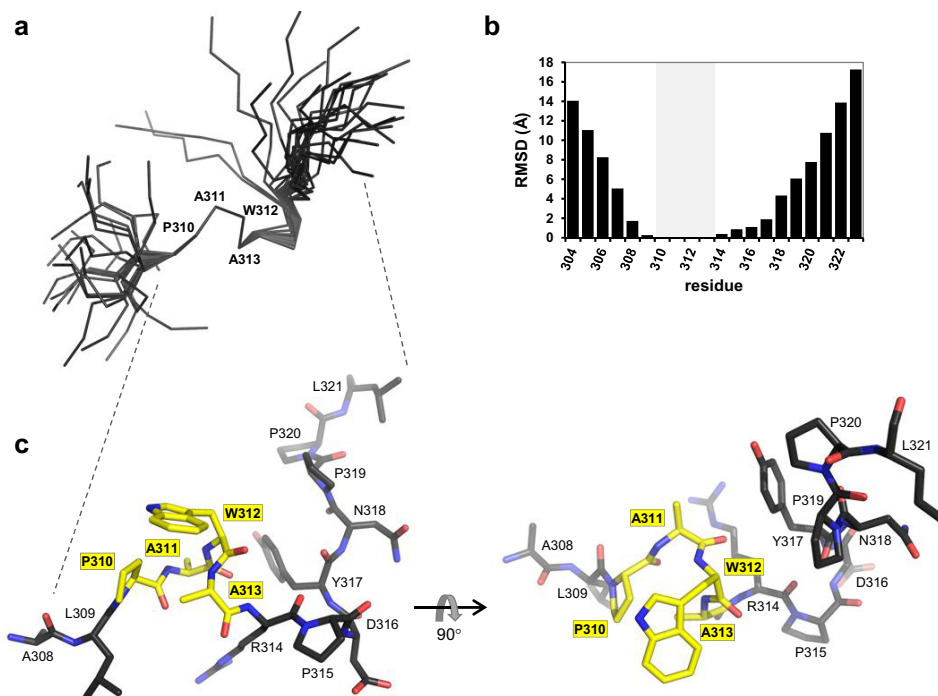


Figure 2. Structure of the PW-turn. *a*, superimposition of the 23 final NMR conformers of pepD2-WT (JFH1) (residues 304–323, GFPRALPAWARPDYNPPLVE), shown as C α traces. *b*, local r.m.s. deviation values (Å) for the backbone atoms (N, C α , and CO) of each residue in the final bundle of structures. The region shaded in light gray corresponds to the PW-turn motif (Pro³¹⁰ to Ala³¹³). *c*, structure of the PW-turn motif (in yellow) in stick representation. Figures were prepared using PyMOL (PyMOL Molecular Graphics System, version 1.8, Schrödinger, LLC).

major all-*trans* form for the distinct conformers: deviations between experimental ¹H,¹⁵N-combined chemical shift or ¹³C α chemical shift values and their expected neighbor-corrected random coil values, the RDC_{NH} for each of the Trp³¹² resonances and the difference between the proton chemical shift values of Trp³¹² H β 2 and H β 3, respectively. As a result, we firmly established a linear correlation between each of these structural NMR parameters and either the ¹H or ¹⁵N experimental chemical shift values of the Trp³¹² resonances (Fig. 4 and Fig. S13). The left-most point (W312_6) on the linear chemical shift pattern, corresponding to the *cis* conformer of Pro³¹⁰, is close to both the Trp³¹² resonance in the A311G mutant and to the expected position for random-coil region, whereas the experimental parameters used to derive the atomic structure of the structured PW-turn (Fig. 2) correspond to the all-*trans* right-most point (W312_1). The position of the Trp³¹² resonance along the linear chemical shift pattern corresponds to the population-weighted average between these two states. The folding-unfolding of the PW-turn structural motif in the disordered NS5A-D2 thus is allosterically regulated by the *cis/trans* conformation of 5 different proline residues, *i.e.* Pro³⁰⁶, Pro³¹⁰, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰. Adopting a *cis* conformation for any single proline residue reduces the structured population in the conformational ensemble. The impact of these *cis*-prolines on the PW-turn is not directly proportional to the distance to Trp³¹² in the primary sequence. The *cis*-Pro³¹⁹ and *cis*-Pro³²⁰, which are at a distance of 7 and 8 residues, respectively, have for example a more pronounced effect on the PW-turn than the *cis*-P306 that is at a distance of 6 residues (Fig. S14). Interestingly, the three proline residues having a major effect on the structured PW-turn content, *i.e.* Pro³¹⁰, Pro³¹⁵, and

Pro³¹⁹, respectively (Fig. 3 and Fig. S14), correspond to the ones having the most pronounced functional impact on HCV RNA replication (28).

CypA-inhibitor resistance mutations correspond to allosteric perturbations

The model of NS5A-D2 with a population-weighted average between a structured PW-turn motif and its disordered counterpart is further strengthened by the analysis of the NMR spectra of NS5A-D2 mutants. We examined the position of the major Trp³¹² resonance (*i.e.* W312_1) in the ¹H,¹⁵N-HSQC spectra of two NS5A-D2 mutants with CypA-inhibitor resistance mutations (D316E or D316E/Y317N (DEYN), respectively). These mutations confer to HCV a moderate resistance (~2–4-fold) to CypIs and a reduced CypA dependence (27, 41, 43, 44). Comparing them to the position of Trp³¹² in NS5A-D2 Pro to Ala mutants (P306A, P310A, P315A, P319A, and P320A) (Fig. 5) and the A311G mutant, in which the PW-turn motif is absent (see Fig. S1b), we find that the Trp³¹² peak in all these mutants displays a colinear chemical shift perturbation pattern (68) (Fig. 5a), which coincides with the one previously defined by the *cis* forms of individual prolines in NS5A-D2 WT (Fig. 4a). This further confirms the PW-turn as a dynamic, rapidly inter-converting ensemble wherein individual mutations directly influence the population of folded and unfolded conformers. The ¹H and ¹⁵N chemical shift values of the Trp³¹² resonance from the mutants correlate linearly with several structural NMR parameters (¹³C α , $\Delta\delta$ NH) (Fig. 5, b and c, and Fig. S15), and indicate that all but the P320A mutation reduce the population of the structured PW-turn motif in the dynamic ensemble (Fig. 5a). To verify whether the effect of the CypI-

Conformational regulation of NS5A-D2 by CypA

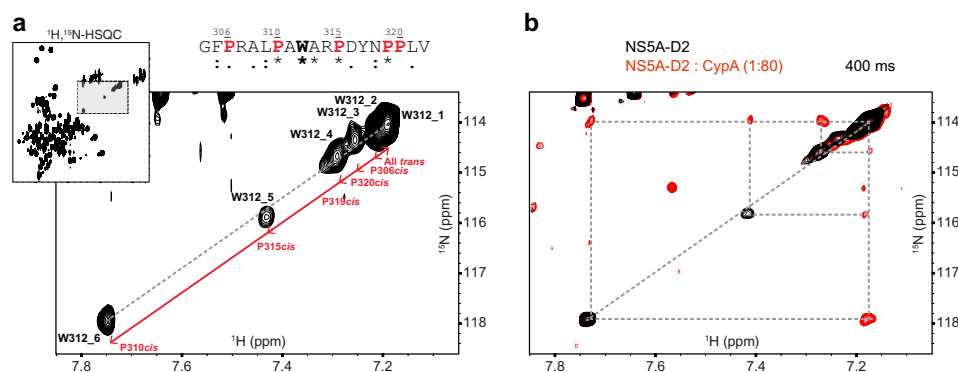


Figure 3. A linear chemical shift pattern for Trp³¹² and proline *cis/trans* equilibria in NS5A-D2. *a*, zoomed-in view of the Trp³¹²s resonances in the ¹H, ¹⁵N-HSQC spectrum of NS5A-D2 WT (box shaded in light gray in the upper left panel). A major (W312_1) and 5 minor (W312_2 to W312_6) NMR resonances were assigned to the Trp³¹² residue, which is surrounded by 5 proline residues in the NS5A-D2 sequence (see sequence at the top). Each minor Trp³¹² resonance arises from the *cis* conformation of one of these proline residues (indicated in red) and is in slow-exchange (red arrows), on the NMR time scale, with the main Trp³¹² resonance when all the surrounding proline residues are in *trans*. The linear chemical shift pattern is highlighted by a dashed gray line. *b*, ¹H, ¹⁵N-heteronuclear exchange spectra, recorded at 600 MHz, on ¹⁵N-NS5A-D2 samples (400 μM) alone (mixing times of 200 ms) (in black) or in the presence of catalytic amounts of unlabeled CypA (5 μM) (mixing time of 400 ms) (in red). Exchange peaks connecting each minor Trp³¹² resonances (W312_2 to W312_6) with the main one (W312_1) are highlighted by dashed gray boxes.

resistant mutants was direct rather than indirectly mediated through an altered proline *cis/trans* ratio, we explicitly measured the *cis/trans* ratio of each proline in the pepD2-WT or its D316E counterpart (Fig. 5*d* and Fig. S16). Only minor increases of the *cis* content was measured for Pro³¹⁵ and Pro³¹⁹, but the all-*trans* form remained the dominant fraction.

Our combined results demonstrate that the short structural PW-turn motif (³¹⁰PAWA³¹³) identified in NS5A-D2 is structurally coupled to a larger region encompassing the 304–323 residues, whereby this larger peptide can adopt a structured motif (the PW-turn) in equilibrium with a disordered conformer. Any perturbation in this region has its impact on this equilibrium (Fig. S17). The different amino acid substitutions (A311G, D316E, DEYN, P306A, P310A, P315A, P319A, and P320A) and the *cis*-proline conformation of Pro³⁰⁶, Pro³¹⁰, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰, all constitute a library of allosteric perturbations. We used the chemical shift projection analysis (CHESPA) method (69, 70) on the two linear chemical shift patterns described in Figs. 4*a* and 5*a*, respectively (Fig. 5*e*). To this end, W312_6 (disordered) and W312_1 (structured) have been considered as the extremities of the linear pattern. The projection angle ($\cos\theta$) defines the direction of the perturbation and also shows if the observed residue (here Trp³¹²) is affected by the perturbation through nearest-neighbor effects. All perturbations resulted in a $\cos\theta$ value of ~ 1 , which is expected for a linear pattern. The only exception is for the A311G mutation that has a stronger nearest-neighbor effect on the Trp³¹² resonance. The fractional shift (X) allows the quantification of the two states (here disordered/structured) in the presence of one of the perturbations (here, *cis*-Pro or mutations). The D316E and DEYN mutations, the *cis*-Pro³¹⁵ conformation, or the A311G mutation thereby correspond to ensembles wherein 82, 61, 55, or 7% of the conformers, respectively, adopt the structured PW-turn motif.

Molecular dynamics of the PW-turn

To validate our NMR-based conclusions by an independent approach, we performed Gaussian accelerated molecular dynamics (MD) simulations (~ 280 ns, 140,000 frames) on five

20-mer peptides (pepD2-WT, -D316E, -DEYN, -A311G, and -P315*cis*). For each simulation, a dihedral principal component analysis (dPCA) (71, 72) was performed on the backbone dihedral angles (ϕ and ψ) of residues 309 to 316, and a clustering of the structures was performed using the two first vectors of the dPCA (Fig. S18*a*). The percent of the time that each cluster is present was determined. Then, using our NMR structure as a reference, we probed the presence of the PW-turn motif in every cluster of each calculation. In the MD simulations, the PW-turn motif was found in 90, 73, 66, 65, and 2% of the conformers (called the PW_fraction) for pepD2-WT, pepD2-D316E, pepD2-DEYN, pepD2-P315*cis*, and pepD2-A311G, respectively (Fig. 6*a*). Ensemble average proton amide (¹H and ¹⁵N) chemical shift predictions, performed with ShiftX2 (73) on the MD structures, showed linear patterns for Trp³¹² and Ala³¹³, confirming the experimental observations (Fig. S18, *b* and *c*). There is a strong linear correlation between the PW_fractions from the MD simulations and the experimental ¹H or ¹⁵N chemical shifts of Trp³¹² in the HSQC spectra of NS5A-D2 (Fig. 6, *b* and *c*). We also found a linear correlation ($R^2 = 0.94$) between the MD PW_fractions and the fractional shift (X) from the CHESPA analysis (Fig. 6*d*), illustrating the validity of the model.

The interaction with CypA is modulated by the fraction of the structured PW-turn in NS5A-D2

We have previously shown that the disruption of the structured PW-turn motif weakens 3-fold the molecular interaction between CypA and NS5A-D2 (in the context of the Con1 strain) (30). As the CypA-inhibitor resistance mutation D316E reduces the fraction of the NS5A-D2 conformers that retain this structural element (Fig. 6), the molecular interaction between NS5A-D2 D316E and CypA was assessed. Using ¹⁵N-labeled CypA, NMR titration experiments were acquired with increasing amounts of unlabeled PepD2-WT, PepD2-D316E, or PepD2-A311G peptides (Fig. 7 and Fig. S19, *a–c*). The dissociation constants (K_D) were determined from the chemical shift perturbations of CypA induced by the addition of the peptides. The affinity between CypA and PepD2-WT ($K_D = 0.74$ mM),

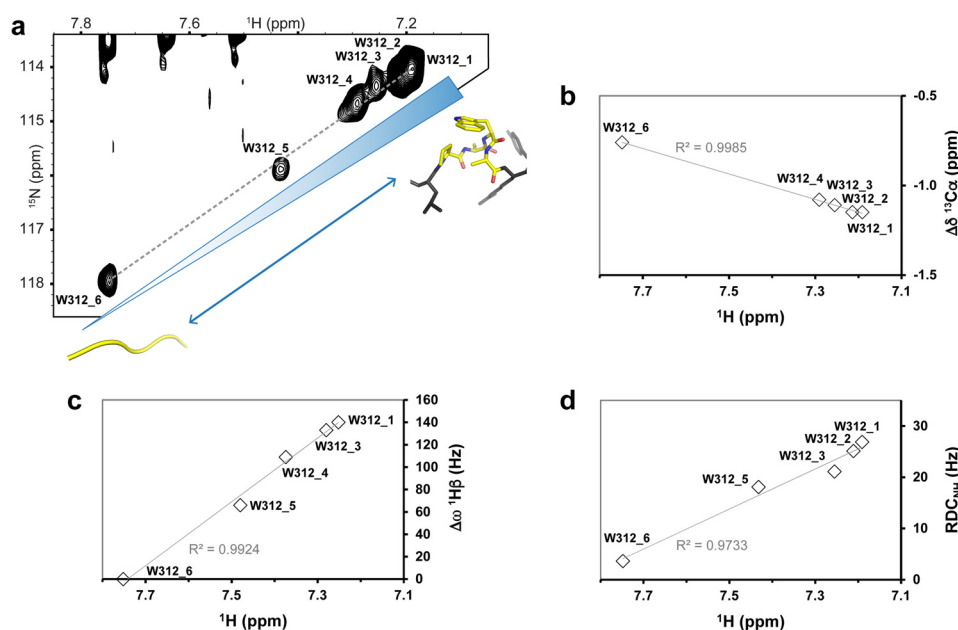


Figure 4. Allosteric regulation of the PW-turn. *a*, the position (*i.e.* its ^1H chemical shift) of each Trp 312 resonances along the linear pattern (highlighted by a dashed gray line) in the ^1H , ^{15}N -HSQC spectrum correlates with: the differences between experimental $^{13}\text{C}\alpha$ chemical shift (*b*) values and their expected neighbor-corrected random coil values; the differences (in ^1H ppm) between the two H β resonances of W312 (*c*) in a ^1H , ^1H -TOSCY experiment acquired on an unlabeled pepD2-WT (Fig. S13c); and also with its experimental NH residual dipolar coupling (*d*). There is a two-states fast-exchange between the structured PW-turn and its disorder counterpart (blue arrow in *a*). The position of the Trp 312 resonance is the population-weighted average of the pure structured and disordered states. The *cis/trans* conformation of each surrounding proline residues corresponds to an allosteric effector. The linear correlations with their corresponding correlation coefficients (R^2) are shown in gray.

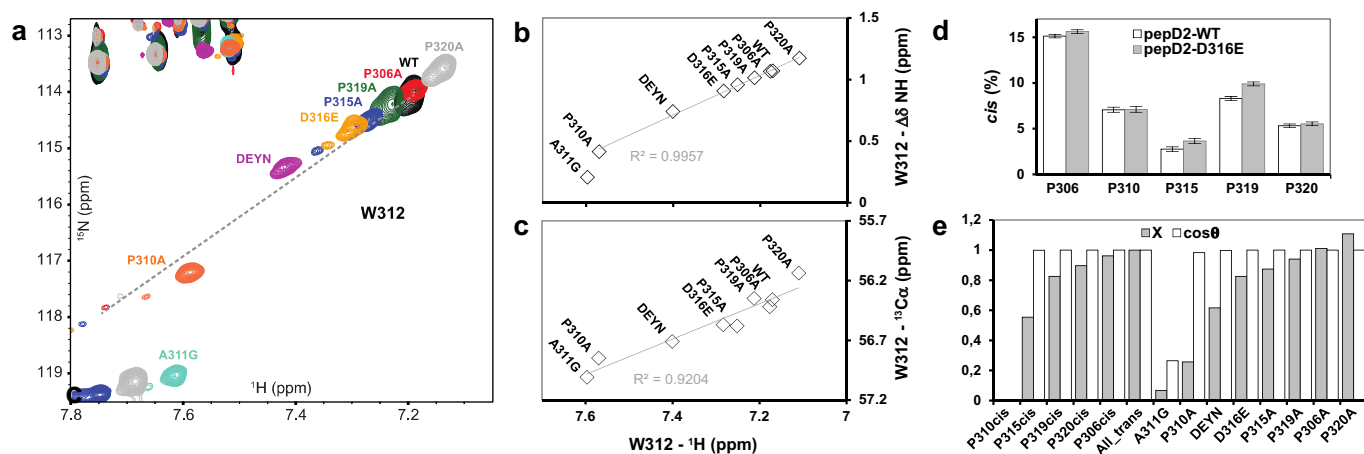


Figure 5. Allosteric perturbations of the PW-turn. *a*, overlay of the ^1H , ^{15}N -HSQC spectrum of NS5A-D2 WT (in black) with that of the mutants P306A (red), P319A (green), P315A (dark blue), D316E (orange), DEYN (D316E + Y317N, violet), P310A (salmon), A311G (light blue), and P320A (light gray), respectively. The spectra are centered on the Trp 312 resonance. The linear chemical shift pattern is highlighted by a dashed gray line exactly as in Figs. 3 and 4. *b* and *c*, linear correlations between the experimental Trp 312 ^1H chemical shift values and (*b*) the differences between experimental and expected neighbor-corrected random coil ^1H , ^{15}N -combined chemical shift values, or (*c*) experimental $^{13}\text{C}\alpha$ chemical shifts, respectively. The linear correlations with their corresponding correlation coefficients (R^2) are shown in gray. *d*, quantification of the *cis* conformation for each proline residues around Trp 312 , both in the pepD2-WT and pepD2-D316E peptides. The *cis* contents (in %) were calculated by using the peak heights of the H α -N resonances corresponding to the *trans* and *cis* proline conformers in ^1H , ^{15}N -H α (C α)N experiments recorded on ^{13}N , ^{13}C -doubly labeled peptides. Error bars were calculated based on the signal-to-noise ratio with uncertainties propagations. *e*, CHESPA analysis. Both the fractional structuration (X , gray bars) and the projection angle ($\cos\theta$, white bars) were calculated for the linear chemical shift patterns shown in Fig. 3 and in panel *a*, respectively. In both cases, the W312_1 (All_ *trans*) and W312_6 (P310cis) resonances of NS5A-D2 WT were taken as the extremities of the linear patterns.

which hold the structured PW-turn, is ~ 2 times higher than the one with the fully disordered PepD2-A311G peptide ($K_D = 1.37$ mM). These affinities are similar to the ones that we have previously measured between CypA and similar peptides derived from the HCV Con1 strain (0.53 and 1.38 mM, respectively) (30). With respect to the interaction between CypA and the peptide pepD2-D316E, an intermediate K_D value of 1.18 mM was measured. The affinities between CypA and the NS5A-D2-derived peptides, even being in the same order of magnitude, are correlated with the

fraction of the conformers that own the structured PW-turn motif in the NS5A-D2 ensemble, as determined by NMR spectroscopy and MD simulations (Fig. S19, *d* and *e*).

The fraction of structured PW-turn motif in NS5A-D2 ensemble tunes the HCV replication level

The PW-turn motif in NS5A-D2 is essential for HCV RNA replication (10, 30), but has the dynamic equilibrium between the structured and disordered states that we identified in this

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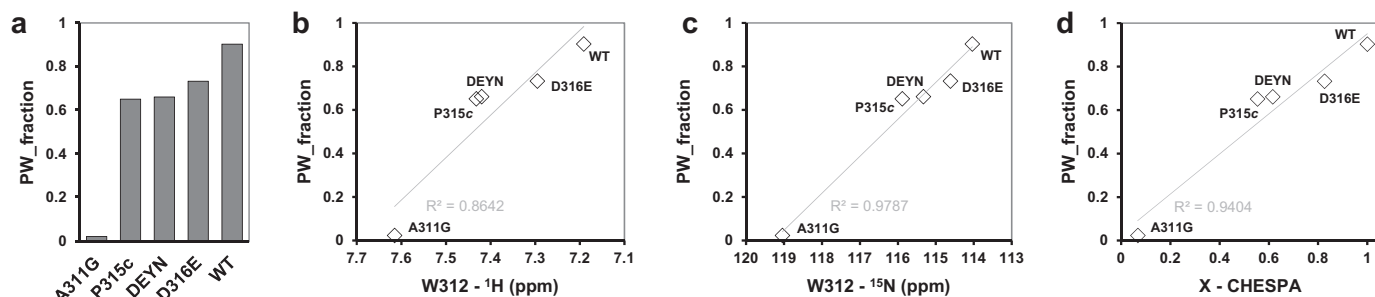


Figure 6. Molecular dynamics of the PW-turn. *a*, MD simulations were performed on peptides pepD2-WT (WT), pepD2-D316E (D316E), pepD2-DEYN (DEYN), pepD2-P315cis (P325c), and pepD2-A311G (A311G). The presence of the PW-turn motif (PW_fraction) in each simulation was quantified as described under “Experimental procedures,” using our NMR structural model as a reference. *b* and *c*, plots of the PW_fraction of each peptide, from MD simulations, versus the experimental Trp³¹² ¹H (*b*) or ¹⁵N (*c*) chemical shift. *d*, plot of the PW_fraction from MD simulations versus the fractional shift (X) from the NMR CHESPA analysis. The linear correlations with their respective correlation coefficients (R^2) are shown in gray.

study have any functional relevance? To address this question, we measured the replication of subgenomic HCV replicons (JFH1 strain) encoding a firefly luciferase gene. These replicon RNAs were transfected into Huh-7 cells and replication was determined by quantifying luciferase in lysates of cells that were harvested at different time points after transfection. We have earlier shown that luciferase activity is a direct measure of viral RNA replication with the 4-h value serving as baseline because it reflects transfection efficiency. To study the importance of the equilibrium shift of the PW-turn motif, we compared replication capacity of the wildtype (WT) replicon or replicon variants containing distinct mutations in NS5A-D2 (A311G, P310A, DEYN, and A311I) (Fig. 8*a*). The A311G substitution in NS5A reduced RNA replication to background levels as determined with the replication-dead replicon encoding an enzymatically inactive NS5B polymerase (mutant Δ GDD). By contrast, the replication efficiency of the A311I mutant was similar to that of the NS5A-WT consistent with the notion that the structural PW-turn motif in the disordered NS5A-D2 domain is essential for viral RNA replication. Indeed, in the HCV Con1 strain, position 311 corresponds to an isoleucine residue (30). Mutations P310A and DEYN in NS5A affected RNA replication, showing phenotypes intermediate between those of the WT and A311G mutant. Of note, we found a striking correlation between RNA replication efficiencies measured *in cellulo* and the NMR data acquired on purified NS5A-D2 *in vitro* (Fig. 8, *b* and *c*, and Fig. S20). Indeed, RNA replication levels of NS5A WT and its NS5A mutants correlate with the position (¹H and ¹⁵N) of the Trp³¹² resonance in their corresponding ¹H,¹⁵N-HSQC spectra. This strong correlation suggests that the structured PW-turn motif in the NS5A-D2 ensemble is required for robust HCV RNA replication, provided the CypA PPIase activity allows to reach, on a subsecond time scale, subensembles in which the structured PW-turn population is reduced. It means that the conformational equilibrium of the PW-turn motif in NS5A-D2 has to be finely regulated to be fully functional. Hence, our data also provide an explanation for the striking antiviral potency of CypIs.

Discussion

RNA replication, a central step in the HCV life cycle, requires the formation of a replication complex that includes the viral NS5B and NS5A proteins associated to membrane structures

(DMVs) (7, 74). NS5B with its RNA-dependent RNA-polymerase activity (8) is the catalytic core of this functional complex. In contrast, the absence of measurable enzymatic activity and the presence, alongside its first well-structured domain (11, 20, 21), of two intrinsically disordered domains (NS5A-D2 and -D3) (17–19), make the role of NS5A in the HCV replicase much less obvious. Human CypA, as an essential cellular protein required for the viral replication process (75), has been functionally linked to NS5A-D2 by the accumulation of CypI-resistance mutations in this disordered domain (41, 76), and by the identification of a physical interaction between these two proteins (25, 37). However, the role(s) of these two proteins in the replication of HCV remains elusive, which is in part because the PPIase activity and the binding properties of CypA cannot be uncoupled.

The precise role of human cyclophilins in a viral life cycle has been best studied in the context of HIV capsid (de)stabilization (77). CypA is incorporated into newly produced HIV virions through its interaction with a proline-rich loop (CypA-loop) in the viral capsid protein (CA) (78–80). Although the Pro⁹⁰ residue in CA is required for both the interaction with CypA and viral replication, the importance of other surrounding proline residues (Pro⁸⁵, Pro⁹³, and Pro⁹⁹) in viral replication was also pointed out (79). CypA displays *in vitro* catalytic PPIase activity toward the Gly⁸⁹–Pro⁹⁰ peptidyl bond in the CypA-loop of the CA protein in the intact HIV-1 virion (81), but the functional role of this activity remains uncertain. CA mutations such as A92E and G94D, obtained during HIV-1 passage in HeLa cells under CypA inhibition (82), allow these HIV-1 mutants to escape CypA dependence, without altering the interaction with CypA (83). However, the infectivity of these mutants drops by 90% in HeLa cells, which could be fully recovered upon CypA inhibition (83). Recently, it has been reported that the CypA-loop of the A92E and G94D CA mutants in the assembled capsid structures adopts comparable dynamics as the loop in the WT CA when bound to CypA (84). Hence, both selected resistance mutations and CypA binding seemingly lead to the same dynamic effect on this CA loop, but cannot be combined without the risk of overshooting the dynamical requirements for optimal infectivity. Along these lines, the rhesus monkey Trim5 α induces global attenuation of the capsid dynamics even beyond the CA loop and may ultimately promote its disassem-

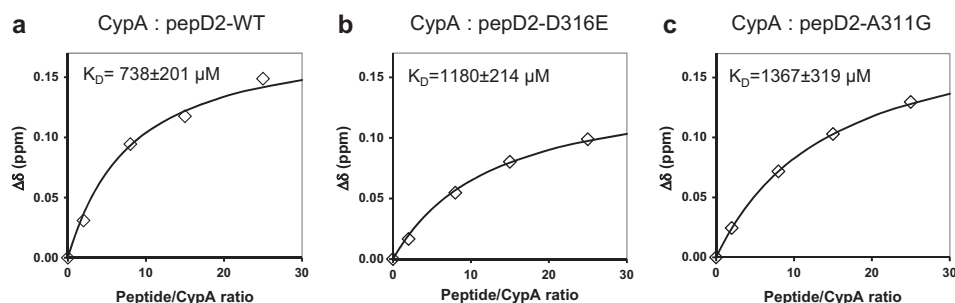


Figure 7. Interaction of CypA with NS5A-D2-derived peptides: pepD2-WT (a), pepD2-D316E (b), and pepD2-A311G (c). ^1H , ^{15}N -HSQC spectra of ^{15}N -CypA (0.1 mM) were acquired in the presence of increasing amounts of unlabeled NS5A-D2 derived peptides (0, 0.2, 0.8, 1.5, and 2.5 mM). The CypA ^1H , ^{15}N combined chemical shift perturbations ($\Delta\delta$, ppm) are plotted as a function of the peptide/CypA ratios. The K_D values correspond to the mean (\pm S.D.) calculated over five CypA resonances (Ile⁵⁶, Leu⁹⁸, Met¹⁰⁰, Ser¹¹⁰, and Gly¹²⁴) (Fig. S19).

bly (85). From these data on HIV-1 CA-CypA interaction, one can conclude that fine tuning of dynamical aspects of viral proteins (here CA) is an additional mean used by viruses to optimize infectivity.

Our present work on HCV NS5A-D2 and its relationship with CypA points toward the same direction. We detect the PW-turn structural motif in the mainly disordered NS5A-D2 domain from the HCV JFH-1 strain (Figs. 1 and 2), thereby confirming our previous results in the Con1 strain (30). Moreover, we show that the structural motif extends beyond the $^{310}\text{PAWA}^{313}$ sequence to include at least 4 more proline residues (Pro³⁰⁶, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰) (Fig. 3 and Fig. S17). However, this motif is not static. Using NMR chemical shifts as atomic resolution sensors, we demonstrate that the PW-turn motif exists as an equilibrium between two states, a structured state and a disordered state, which interconvert in fast exchange on the NMR time scale (Fig. 4). This equilibrium, probed via the Trp³¹² resonance, is modulated by the *cis/trans* equilibria of the 5 surrounding prolines (Pro³⁰⁶, Pro³¹⁰, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰). This translates into one major and 5 minor resonances for the Trp³¹² amide function that make a linear chemical shift pattern in the ^1H , ^{15}N -HSQC of NS5A-D2 WT (Figs. 4 and 5). The linear pattern is bordered on the structured side by the all-*trans* form used to derive the structure of the PW-turn (Fig. 3), and by the *cis*-Pro³¹⁰ form on the almost completely disordered side (Figs. 3 and 4). As these different equilibria are separated by the conformation (*trans* or *cis*) of distinct prolyl bonds, they are separated by the slow time scale of the spontaneous *cis/trans* isomerization of each of these bonds. CypA can lower this barrier through its PPIase activity, and thereby connect these different ensembles at a subsecond time scale (Fig. 3b) (25, 30). As such, the PPIase activity of CypA do not alter the folded and unfolded states of the PW-turn but rather allows fast reach to the NS5A-D2 subensembles in which the structured PW-turn population is reduced (Fig. S21). The CypI resistance mutations in NS5A-D2, D316E, or DEYN lead to a similar effect as they reduce the population of the structured PW-turn motif in the dynamic ensemble (Fig. 5 and Fig. S21). The folded conformer population, with all prolines in *trans*, drops from nearly $\sim 100\%$ in NS5A-D2 WT to 82% in the D316E mutant (*i.e.* the same population as in the *cis*-Pro³¹⁹ subensemble in the WT), and to 62% in the DEYN mutant (comparable with that of the *cis*-Pro³¹⁵ subensemble in the WT) (Figs. 3 and 5). Both mutations confer some CypA inde-

pendence, but lower the replication level when measured in cell lines where CypA is present (27, 41, 43, 44). Moreover, in a similar way to what was observed for the mutations in the CypA-loop of HIV-1 CA, the replication capacity of the DEYN HCV mutant has been shown to be higher when CypA was silenced (28). As the Trp³¹² chemical shift (^1H and ^{15}N) of the mutants correlates linearly with the RNA replication efficiency (\log_{10} scale) in a cell-based assay (Fig. 8), we conclude that CypA is fine tuning the dynamics of the PW-turn motif in NS5A-D2.

The relationships involving CypA/CA from HIV-1 or CypA/NS5A-D2 from HCV are strikingly similar. A short sequence involving a conserved proline residue constitutes the CypA-binding site (Gly⁸⁹-Pro⁹⁰ or $^{310}\text{PAWA}^{312}$, respectively); the residues C-terminal to this binding site trigger peculiar conformational properties and are involved in the replication efficiency of the viruses; CypI-resistance mutations conferring (partial) relief of the CypA-dependence are localized next to the CypA-binding site; and finally, CypA exerts a fine modulation of the dynamics of the viral proteins (the CypA-loop of CA or the PW-turn motif in NS5A-D2). HIV-1 and HCV seem to have evolved to use the host CypA as a fine-tuning rheostat, which allows them to keep their functional systems in a rather sharp optimal window. Finally, in the presence of the CypI-resistance mutations, CypA is detrimental as it over-attenuates the dynamics of the CypA-loop in HIV-1 CA or it over-reduces the fraction of the PW-turn in HCV NS5A.

Whereas we show that the fraction of the structured PW-turn motif in the NS5A-D2 conformational ensemble, which is allosterically regulated by both the *cis/trans* isomerization of 5 prolines residues (Pro³⁰⁶, Pro³¹⁰, Pro³¹⁵, Pro³¹⁹, and Pro³²⁰) and by CypI-resistance mutations, correlates with the HCV RNA replication efficiency (Fig. 8), the question of how a structured PW-turn motif in NS5A-D2 contributes to viral replication remains open. NS5A was shown to play a role in the formation of DMVs and functional replication complexes contained therein, through remodeling the ER membrane, and CypI could interfere with this role for NS5A. Alternatively, NS5A-D2 directly interacts with the dynamic molecular machine that is the NS5B RNA polymerase (86), and thereby might allosterically regulate its RNA binding (15) and/or enzymatic activity (87). Different conformational ensembles of the disordered NS5A-D2, with varying fractions of (un)folded PW-turn motif, might be required for

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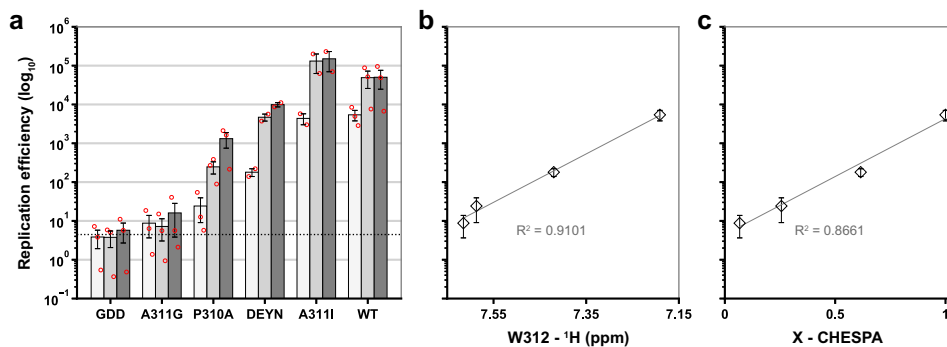


Figure 8. Functional relevance of the PW-turn. *a*, subgenomic JFH1 replicons (Luc-NS3–5B) containing or not (WT) single mutations (A311G, P310A, DEYN, or A311I) in NS5A or a defective polymerase inhibiting replication (Δ GDD) were electroporated into Huh-7 cells. The cells were lysed 4, 24, 48, and 72 h after transfection and luciferase activity (representing RNA replication) was measured. The data were normalized to their respective 4-h value reflecting transfection efficiency. Mean \pm S.E. of 3 independent experiments ($n = 3$) are shown (2 independent experiments for the A311I and DEYN mutants). The horizontal dashed line corresponds to the background level as determined with the Δ GDD replicon. *b* and *c*, plots of the RNA replication efficiencies (*i.e.* luciferase activity values, of the different subgenomic replicons in *a*) versus the experimental Trp³¹² ¹H chemical shift (*b*) or the fractional shift (X) from the NMR CHESPA analysis (*c*). The exponential correlations with their respective coefficients (R^2) are shown in gray.

these multiple functional roles. Our present data add further complexity to this picture, whereby allosteric regulation of the NS5A-D2 domain through its proline conformations might be modulated by the enzymatic function of the host factor CypA. Simultaneously, although, it shows that targeting the dynamical aspects of viral protein(s) can open new avenues in the discovery of antivirals.

Experimental procedures

Expression and purification of NS5A-D2 and CypA

The synthetic sequence coding for domain 2 of the HCV NS5A protein from JFH1 strain (GenBankTM accession number AB047639, genotype 2a) was introduced in the bacterial expression vector pT7.7 with a His₆ tag. The resulting recombinant domain 2 of HCV NS5A (NS5A-D2; residues 248–341) has extra M- and -LQHSHHHH extensions at the N and C termini, respectively. The NS5A-D2 mutants (A311G, P306A, P310A, P315A, P319A, P320A, C298S/C338S/M254C, C298S, C338S, D316E, D316E/Y317N) were generated by site-directed mutagenesis on the pT7-7-NS5A-D2 WT plasmid using the forward and reverse primers listed in Table S1. The expression, in *Escherichia coli* BL21(DE3), and purification of ¹⁵N (or ¹⁵N,¹³C)-labeled NS5A-D2 was performed as previously described (25, 30). The production and purification of both unlabeled and ¹⁵N-labeled CypA were performed as previously described by Hanouille and co-workers (25).

NS5A-D2–derived peptides

¹⁵N,¹³C-labeled peptides (PepD2-WT (GFPRALPAWARPDYNPPLVE), PepD2-A311G (GFPRALPGWARPDYNPPLVE), and PepD2-D316E (GFPRALPAWARPEYNPPLVE)) corresponding to residues 304–323 of NS5A-D2 JFH1 were produced as fusion proteins in *E. coli*, cleaved, and then purified as described previously (30). The resulting ¹⁵N,¹³C-labeled PepD2-WT, PepD2-A311G, and PepD2-D316E peptides thus contain an extra N-terminal proline residue resulting from the DP chemical-cleavage site. The unlabeled peptides (PepD2-WT, PepD2-A311G, and PepD2-D316E) were purchased from Genecust (Luxembourg).

Conservation of NS5A-D2 sequence among HCV genotypes

The NS5A-D2 sequence from the HCV JFH1 strain (AB047639, genotype 2a) is numbered as in the full-length NS5A protein. The amino acid repertoire was deduced from the ClustalW multiple alignments of 28 representative NS5A sequences from all confirmed HCV genotypes and subtypes (see the European HCV Database (88)) using the Network Protein Sequence Analysis webserver tools (89). Amino acids observed at a given position in less than two distinct sequences are not included. The degree of amino acid conservation at each position can be inferred from the extent of variability (with the observed amino acid listed in decreasing order of frequency from top to bottom) together with the similarity index according to ClustalW convention (asterisk, invariant; colon, highly similar; dot, similar).

NMR spectroscopy

All NMR experiments were performed at 298 K using Bruker Avance 600 MHz or 900 MHz NMR spectrometers, both equipped with a cryogenic triple resonance probe (Bruker, Karlsruhe, Germany). The proton chemical shifts were referenced using the methyl signal of TMSP (sodium 3-trimethylsilyl-[2,2,3,3-*d*₄]propionate) at 0 ppm. Spectra were processed and analyzed with the Bruker TopSpin software package 3.2. Data analysis, peak picking, and calculation of peak volumes were done with Sparky software (90).

Assignments of NS5A-D2 JFH1 (WT) backbone resonances were taken from Ref. 25 (BMRB accession code 16165). Two NMR datasets were acquired on each peptide (PepD2-WT, PepD2-A311G, and PepD2-D316E). The first set, which contains ¹H-¹H TOCSY, ¹H-¹H NOESY, and ¹H-¹⁵N HSQC spectra at 600 MHz were acquired on the unlabeled peptides at natural abundance. The second dataset was acquired on uniformly ¹⁵N,¹³C-labeled peptide and comprises ¹H-¹⁵N HSQC and three-dimensional HNCACB, HN(CO)CACB, HNCO, and HNHA spectra. Assignment of PepD2-WT, PepD2-A311G, and PepD2-D316E were performed both manually and using an in-house software based on the product plane approach (91).

The ^1H , ^{15}N -combined chemical shift perturbations were calculated using Equation 1, whereby $\Delta\delta$ (^1HN) and $\Delta\delta$ (^{15}N) are the chemical shift perturbations in the ^1H and ^{15}N dimensions, respectively. The normalization factor of 0.08 for the nitrogen frequency shift derives here from the ratio of the maximum proton frequency shift over the maximum nitrogen frequency shift.

$$\Delta\delta = |\Delta\delta(^1\text{HN})| + 0.08 \times |\Delta\delta(^{15}\text{N})| \quad (\text{Eq. 1})$$

Residual dipolar couplings measurements

The RDCs were collected on a 100 μM sample of ^{15}N NS5A-D2 aligned in a liquid crystalline medium consisting of 6.6% (w/v) polyoxyethylene 5-lauryl ether (C_{12}E_5) and 3% (w/v) 1-hexanol (Sigma), yielding a D_2O splitting of 35 Hz. $^1\text{D}_{\text{NH}}$ dipolar couplings were measured on a Bruker Avance III 900 MHz spectrometer equipped with a cryogenic triple resonance probe, using 2D ^{15}N IPAP-HSQC experiments (92), which allow the spin coupling measurements in the ^{15}N dimension. The difference between the couplings acquired either in isotropic or anisotropic media were calculated to get the RDCs.

Spin relaxation experiments

^{15}N R^1 , ^{15}N R^2 , and ^1H - ^{15}N NOE measurements were acquired at 600 MHz ^1H frequency. ^{15}N R^1 values were measured from spectra recorded with 10 different delays ($T = 10, 100, 200, 400, 600, 800, 1000, 1200, 1500,$ and 2000 ms). ^{15}N R^2 values were determined from spectra recorded with 11 different delays ($T = 15.77, 31.54, 47.31, 63.08, 78.85, 94.62, 126.16, 157.7, 189.24, 220.78,$ and 252.32 ms). The spin echo consisted of a train of ^{15}N hard π pulses (82 μs) separated by a 900- μs inter-pulse delay. In the ^{15}N R^1 and ^{15}N R^2 experiments, a relaxation delay of 1 s was applied. ^1H - ^{15}N NOE values were determined from spectra recorded either in the presence or absence of a proton pre-saturation period of 3 s, and a relaxation delay of 5 s.

NMR structure calculation

The NMR structure calculation was performed as previously described in Ref. 30. From the different NMR datasets acquired both on unlabeled and ^{15}N , ^{13}C -labeled peptide PepD2-WT (residues 304–323, JFH1), distance-based (NOEs) and backbone dihedral angle-based experimental restraints were derived. NOE intensities used as input for structure calculations were obtained from the NOESY spectrum recorded with a 400-ms mixing time. According to their intensity NOEs were classified in three categories, which were then converted into distance restraints (1.8–2.8 Å, 1.8–3.9 Å, and 1.8–5.0 Å). Protons without stereospecific assignments were treated as pseudo-atoms. From the ^1H , ^{15}N , and ^{13}C chemical shifts, dihedral angle constraints, calculated with Talos (93), were introduced. Peptide structures were generated from the experimental NOE distances and dihedral angles, using CNS (94) with the standard torsion angle molecular dynamics protocol, the standard force field and default parameter set. From the initial set of 100 structures that were calculated, with the dynamical annealing protocol to widely sample the conformational space, only structures with no distance restraint violations were retained. The 23

final selected structures, with the lowest energies, were compared by pairwise root mean square deviation over the backbone atom coordinates (N, C α , and C'). Ramachandran analysis performed on the final structures showed that 89, 11, and 0% of the residues were in favored, allowed, and outliers regions, respectively. The PyMOL software (PyMOL Molecular Graphics System, version 1.8; Schrödinger) was used for molecular graphics (95).

NMR PPIase assay

PPIase activity of CypA on NS5A-D2 WT was assessed using ^1H , ^{15}N z-exchange spectra (66), where the exchange was monitored on the basis of novel cross-peaks connecting a *trans* and *cis* peak. ^1H , ^{15}N z-exchange spectra, with a 200- or 400-ms mixing time, were recorded on a 600 MHz spectrometer equipped with a cryogenic triple resonance probe. Exchange spectra were acquired on a 400 μM ^{15}N -NS5A-D2 sample in the presence or the absence of 5 μM CypA in 30 mM NaH_2PO_4 / Na_2HPO_4 , pH 6.4, 30 mM NaCl, 1 mM DTT.

Chemical shift projection analysis (CHESPA)

The NMR chemical shift projection analysis was performed as described in Refs. 69 and 70, from the ^1H , ^{15}N -HSQC of NS5A-D2. We calculated $\cos\theta$, which represents the angle between vectors A (defined by the W312_6 and W312_1 peaks) and B (defined by the W312_6 peak and the Trp³¹² resonance from a mutant or corresponding to a minor form). Next, we calculated the fractional shift X, which corresponds to the projection of the vector B on the vector A. For the calculations, a scaling factor of 0.1 was applied to the ^{15}N chemical shift.

Molecular dynamics

Gaussian-accelerated molecular dynamics (GaMD) simulations (~280 ns) were performed with AMBER17 (96, 97) on five different peptides corresponding to the 304–323 residues in NS5A-D2 (pepD2-WT (WT), pepD2-D316E (D316E), pepD2-DEYN (DEYN), pepD2-P315*cis* (P325c), and pepD2-A311G (A311G)), and each trajectory was collected with ~140,000 frames. The detailed molecular dynamics protocol is described under the supporting information.

Briefly, the peptides were built using Tleap in AmberTools16 and all simulations were performed using pmemd.cuda of AMBER17 on graphics processing units P100 (98). Amber ff99SB*-ILDN force field (99, 100) was used in all simulations. The peptides were then solvated in a cubic water box of $75.5 \times 75.5 \times 75.5 \text{ \AA}^3$ pre-equilibrated TIP3P water molecules. The simulations were performed with 40 mM NaCl at 298 K, as in the experimental NMR studies. GaMD (101) simulations (~280 ns) were used to explore the conformational space of the peptides and the coordinates were saved every 2 ps. CPPTRAJ (1) was used to analyze r.m.s. deviations, secondary structure, dihedral torsions, and hydrogen bonds from the GaMD simulation trajectories. The sampled conformations of the peptides were analyzed using the dPCA method (71, 72) considering the backbone atoms of residues 309 to 316. The lowest energy conformations were identified by projecting the trajectories of the first two principal components onto a three-dimensional free energy (ΔG) (see Equation 2 below), in which R is the universal

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gas constant, T is the temperature, x , y , and z , are the calculated structural properties from the trajectory.

$$\Delta G = -RT \ln \left(\frac{P_{x,y,z}}{P_{\max}} \right) \quad (\text{Eq. 2})$$

Then, a clustering of the structures was performed using the two first vectors (PC1 and PC2) of the dPCA. For each MD simulation, the PyReweight toolkit was used to reweight the GaMD simulation to compute the free energy landscape from PCA components PC1 and PC2. The percent of the time that a particular cluster is present was determined. Next, using our NMR PepD2-WT structure as a reference, the presence of the structured PW-turn motif was probed in every cluster of each simulation. For clusters of each simulations, the centroid structures were fitted on the NMR reference structure using all atoms of residues 310–313. Then, backbone dihedral angles (ϕ and ψ) of residues 310 to 313 and χ_1 and χ_2 angles from Trp³¹² were measured using cpptraj in AMBER package. The presence of the structured PW-turn motif was assessed from the sum of squares of the differences between angles from the reference and centroid structures. Chemical shift predictions (^1H and ^{15}N) were performed with SHIFTX2 (73) from the MD simulations data. For each peptide, SHIFTX2 was used in the “ensemble mode” on all structures of each cluster. Then, from these predictions for a given peptide, a population-weighted average chemical shift value (^1H or ^{15}N) was calculated. The error bars represent the population-weighted standard deviations.

RNA replication assay

The protocol used for generation and electroporation of HCV RNAs has been described elsewhere (38). For transient replication assays, 400 μl of single cell suspensions of Huh-7 cells (10^7 cells/ml) were mixed with 5 μg *in vitro* transcribed subgenomic replicon RNA and transfected by electroporation. After transfection, cells were resuspended in 41 ml of complete Dulbecco's modified Eagle's medium, and 1.5 ml of the cell suspension was seeded in duplicate in each well of a 12-well plate. To measure luciferase activity, cells were washed with PBS 4, 24, 48, and 72 h after electroporation and lysed by addition of 350 μl of lysis buffer (0.1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO_4 , 4 mM EGTA, and 1 mM DTT). Lysates were immediately frozen at -70°C , and after thawing, 100 μl of the lysate was mixed with 360 μl of assay buffer (25 mM glycylglycine, 15 mM MgSO_4 , 4 mM EGTA, 1 mM DTT, 2 mM ATP, and 15 mM K_2PO_4 , pH 7.8). Luciferase activity was measured for 20 s in a luminometer (Lumat LB9507; Berthold, Freiburg, Germany) after addition of 200 μl of luciferin solution (200 mM luciferin, 25 mM glycylglycine, pH 8.0). Replication efficiency was calculated by normalizing values of the different time points to the respective value obtained at 4 h, which reflects transfection efficiency.

Interaction between CypA- and NS5A-D2– derived peptides

To study the interaction between CypA and PepD2-WT, PepD2-A311G, or PepD2-D316E, ^1H , ^{15}N -HSQC experiments were acquired on 400 μM ^{15}N -CypA with increasing amounts of unlabeled peptides (the molar ratios CypA:PepD2 were 1:2; 1:8;

1:15, and 1:25). The combined chemical shift perturbations following peptide additions were calculated using Equation 3, whereby $\Delta\delta$ (^1H N) and $\Delta\delta$ (^{15}N) are the chemical shift perturbations in the ^1H and ^{15}N dimensions, respectively.

$$\Delta\delta = |\Delta\delta(^1\text{H}\text{N})| + 0.2 \times |\Delta\delta(^{15}\text{N})| \quad (\text{Eq. 3})$$

For determination of dissociation constant (K_D), the combined chemical shift perturbations ($\delta\Delta$, ppm) were plotted as a function of the molar ratio [PepD2]/[^{15}N -CypA], and the resulting curve was fitted to Equation 4, which describes a 1:1 biomolecular interaction, and where $\Delta\delta$ is the measured chemical shift perturbation, $\Delta\delta_{\max}$ is the maximum value for this parameter, X corresponds to the ratio [PepD2]/[^{15}N -CypA], and [CypA] is the ^{15}N -CypA concentration.

$$\Delta\delta = \frac{\Delta\delta_{\max}}{2} \times \left(1 + X + \frac{K_D}{[\text{CypA}]} - \sqrt{\left(1 + X + \frac{K_D}{[\text{CypA}]} \right)^2 - 4 \times X} \right) \quad (\text{Eq. 4})$$

Accession numbers

NMR assignment of the peptide PepD2-WT (residues 304–323 of the domain 2 of NS5A, JFH1 strain) have been deposited in the Biological Magnetic Resonance Data Bank under accession code 30037. The NMR structure of the pepD2-WT (strain JFH-1) has been deposited in the Protein Data Bank (PDB) with code 6HT4.

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