Table 2. KEGG Pathway Functional Categories (Subclasses) Sorted by the Number of Enriched Pathways (≥3) Associated with One or More NS5A Interacting Bottlenecks

category	no. of enriched pathways	no. of bottlenecks	associated bottlenecks	KEGG pathways in the given category associated with most number of bottlenecks
infectious diseases	16	24	ACTB, AKT1, CDK1, CSNK2A1, CTNNB1, FLNA, FYN, GPS2, GRB2, GSK3B, HSPB1, JAK1, LCK, LYN, PIK3R1, PPP2CA, RAF1, SRC, STAT1, SYK, TBP, TGFBR1, TP53, TRAF2	"Epstein–Barr virus infection"; "HTLV-I infection"; "Hepatitis C"; "Hepatitis B"; "Measles"; "Influenza A"; "Herpes simplex infection"; "Tuberculosis"; "Toxoplasmosis"; "Chagas disease (American trypanosomiasis)"; "Bacterial invasion of epithelial cells"
cancers	16	19	AKT1, AXIN1, CDK1, CTNNB1, GRB2, GSK3B, HSP90AA1, JAK1, LYN, RAF1, SRC, STAT1, SYK, TBP, TGFBR1, THBS1, TP53, TRAF2	"Pathways in cancer"; "Viral carcinogenesis"; "Prostate cancer"; "Endometrial cancer"; "Colorectal cancer"; "Pancreatic cancer"; "Chronic myeloid leukemia"; "Non-small cell lung cancer"; "Glioma"; "Small cell lung cancer"; "Renal cell carcinoma"; "Melanoma"; "Acute myeloid leukemia"
immune system	10	16	ACTB, AKT1, CTNNB1, FYN, GRB2, GSK3B, HSP90AA1, LCK, LYN, PIK3R1, PIN1, RAF1, SRC, STAT1, SYK, TRAF	"Chemokine signaling pathway"; "T cell receptor signaling pathway"; "Fc epsilon RI signaling pathway"; "B cell receptor signaling pathway"; "Natural killer cell mediated cytotoxicity"; "Fc gamma R-mediated phagocytosis"
signal transduction	9	22	AKT1, AXIN1, CSKN1A1, CTNNB1, FLN, GRB2, GSK3B, HSP90AA1, HSPB1, JAK1, LCK, LYN, PIK3R1, PPP2CA, RAF1, SRC, STAT1, SYK, TGFBR1, THBS1, TP53, TRAF2	"PI3K-Akt signaling pathway"; "MAPK signaling pathway"; "Wnt signaling pathway"; "ErbB signaling pathway"; "VEGF signaling pathway"; "NF-kappa B signaling pathway"; "Jak-STAT signaling pathway"
nervous system	5	8	AKT1, GRB2, GSK3B, LYN, PIK3R1, PPP2CA, RAF1, TP53	"Neurotrophin signaling pathway"; "Long-term depression"; "Dopaminergic synapse"; "Long-term potentiation"
endocrine system	4	10	AKT1, CDK1, GRB2, GSK3B, HSP90AA1, PIK3R1, PLK1, RAF1, SRC, TRAF2	"Progesterone-mediated oocyte maturation"; "Insulin signaling pathway"; "GnRH signaling pathway"; "Adipocytokine signaling pathway"
cell growth and death	4	9	AKT1, CDK1, GSK3B, PIK3R1, PLK1, PPP2CA, THBS1, TP53, TRAF2	"Cell cycle"; "Apoptosis"; "p53 signaling pathway"; "Oocyte meiosis"
cell communication	3	14	ACTB, AKT1, CSNK2A1, CTNNB1, FLNA, FYN, GRB2, GSK3B, PIK3R1, PPP2CA, RAF1, SRC, TGFBR1, THBS1	"Focal adhesion"; "Tight junction"; "Adherens junction"
development	3	12	AKT1, PHL2, FYN, GRB2, GSK3B, JAK1, LCK, PIK3R1, STAT1, SYK, TGFBR1, THBS1	"Osteoclast differentiation"; "Axon guidance"; "Dorso-ventral axis formation"

Eight bottlenecks were mapped to the enriched KEGG pathway "Chemokine signaling pathway" ($p = 2.27 \times 10^{-10}$), which is consistent with the modulation of host interferon signaling by NS5A in HCV infection.[70] In addition, 7 bottlenecks each were mapped to "T cell receptor signaling pathway" ($p = 4.6 \times 10^{-24}$), "Fc epsilon RI signaling pathway" ($p = 2.86 \times 10^{-14}$) and "B cell receptor signaling pathway" ($p = 1.8 \times 10^{-14}$) and 6 bottlenecks were mapped to "Natural killer cell mediated cytotoxicity" ($p = 1.92 \times 10^{-12}$). Three bottlenecks (AKT1, PIK3R1 and STAT1) were also mapped to the enriched KEGG pathway "Toll-like receptor signaling pathway" ($p = 3.23 \times 10^{-7}$; Supporting Information, Tables S7a, S8a). Toll-like receptor 3 mediated chemokine and cytokine signaling plays an important role in the host immune response in HCV infection.[71] Therefore, NS5A interaction with bottlenecks, which function in various aspects of the host immune response, may significantly contribute to the perturbation of the host immune system in HCV pathogenesis.

Additionally, 32 of 132 NS5A interacting proteins examined in the present study, including 24 bottlenecks, were mapped to various pathways associated with the signal transduction and the endocrine system (Supporting Information, Tables S7a, S8a), many of which are implicated in HCV infection and HCC progression and are targets for molecular therapy in HCC.[22,72−74]

Eleven bottlenecks were mapped to the enriched KEGG pathway "PI3K-Akt signaling pathway" ($p = 2.2 \times 10^{-24}$; Supporting Information, Tables S7a, S8a), which is consistent with a previous study that NS5A stimulates the activation of PI3K-Akt pathway, which contributes to HCC in HCV infection.[75] Eight bottlenecks were mapped to the enriched KEGG pathway "MAPK signaling pathway" ($p = 2.4 \times 10^{-19}$; Supporting Information, Tables S7a, S8a). Elements of the MAPK signaling cascades are directly involved in the progression of HCV infection, particularly in association with HCV Core and E2 proteins,[22,24,76,77] thereby suggesting that NS5A interactions with the key facilitators of MAPK signaling in the host interactome may play an important role in regulating the reversible phosphorylation of NS5A and may contribute to the progression of HCV pathogenesis.

Bottlenecks AKT1, GRB2, GSK3B, PIK3R1 and RAF1 and many of their interactors were mapped to the enriched KEGG pathway "Insulin signaling pathway" ($p = 2.42 \times 10^{-13}$; Supporting Information, Tables S7a, S8a); these proteins are highlighted in Figure 2. Insulin signaling plays an important role in regulating glucose and lipid metabolism, and the disruption of this process may contribute to insulin resistance (IR). IR is linked with steatosis, fibrosis progression and poor interferon-α response in HCV infection.[78−80] Suppression of AKT1 and GSK3B activity in HCV infection disrupts glucose metabolism and contributes to IR.[81,82] Furthermore, PIK3R1 and NS5A interactor PIK3CB (Figure 2) are subunits of phosphatidylinositol 3-kinase (PI3K), which controls insulin secretion;[83] PI3K also facilitates the activation of the proto-oncogene beta-catenin (CTNNB1) by NS5A, which contributes to the development of HCC in HCV pathogenesis.[84] Previously, HCV Core protein has been directly implicated in the induction of IR in HCV infection,[85] while there is little evidence suggesting definitive links between NS5A and IR. Our observations, however, suggest that NS5A directly interacts with key regulators of insulin metabolism and may, therefore, play a major role in modulating HCV-induced IR and eventually HCC.

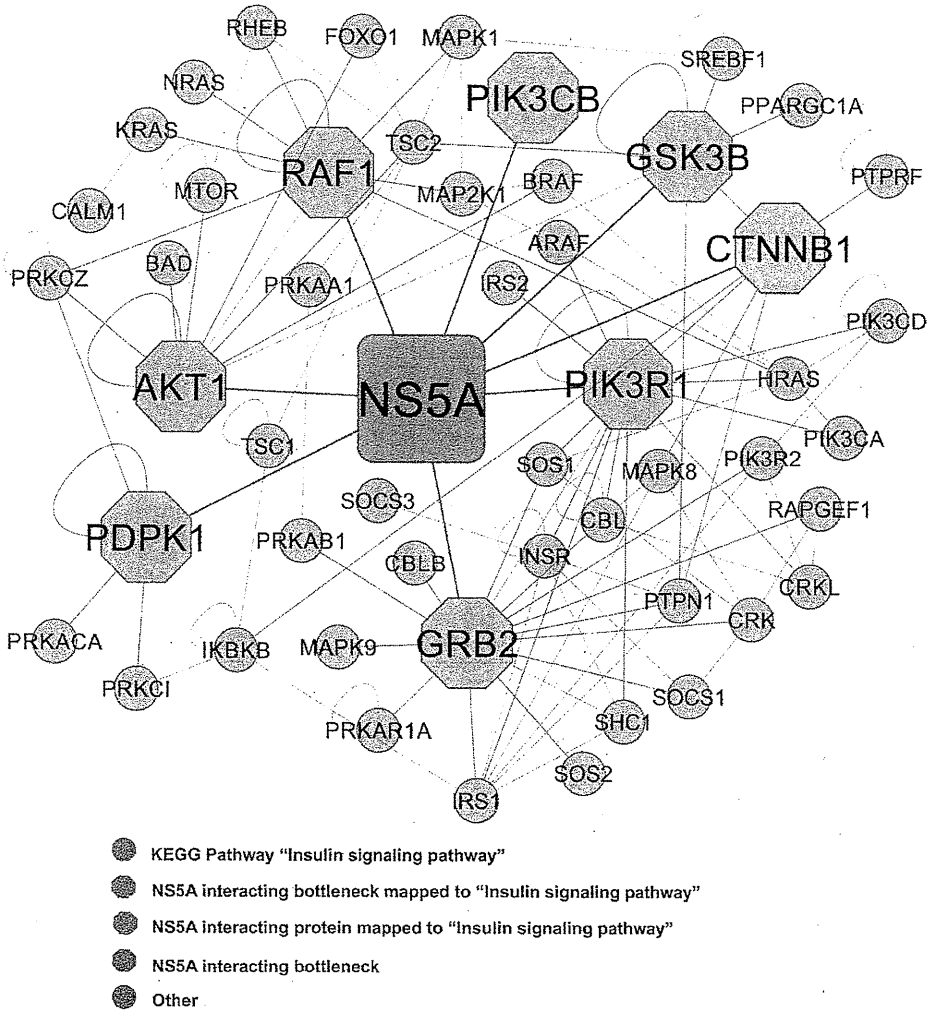


Figure 2. NS5A interacting bottlenecks and their interacting partners associated with the enriched KEGG pathway hsa04910: "Insulin signaling pathway".

Cell Adhesion and Communication

The perturbation of adherens and tight junction associated proteins has been implicated in HCV entry, cell–cell transmission and hepatoma migration in HCV infection.[86–88] In the NS5A infection network, eight bottlenecks (ACTB, AKT1, CSNK2A1, CTNNB1, FYN, PPP2CA, SRC and TGFBR1) were mapped to either or both of the enriched KEGG pathways "Adherens Junction" ($p = 1.03 \times 10^{-15}$) and "Tight junction" ($p = 1.19 \times 10^{-5}$), which are associated with cell adhesion junctions and cellular communication (Supporting Information, Tables S7a, S8a). CSNK2A1 is the catalytic (alpha) subunit of Casein Kinase II (CK2), which phosphorylates NS5A and regulates the production of infectious viral particles.[63] CTNNB1, a key component of cell-adhesion complexes, is positively regulated by CK2.[89] Furthermore, the activation of CTNNB1 by NS5A significantly contributes to HCC.[84] Taken together, our observations suggest that NS5A interactions with bottlenecks, which regulate cell–cell adhesion (CSNK2A1, CTNNB1) and cytoskeletal organization (ACTB), may significantly contribute to the progression of HCV life cycle and tumorigenesis in HCV pathogenesis.

Eleven bottlenecks were mapped to the enriched KEGG pathway "Focal Adhesion" ($p = 1.02 \times 10^{-17}$; Supporting Information, Tables S7a, S8a), thereby reiterating that focal adhesion is a major target of NS5A.[22] Focal adhesion regulates cell migration and adhesion, and some of its components were directly implicated in the regulation of HCV replication and propagation in our earlier study.[24] Our observations thus suggest that NS5A interactions with key components of the focal adhesion machinery may play important roles in the HCV lifecycle. For instance, NS5A interacts with bottleneck THBS1 (Thrombospondin-1), a glycoprotein, which was mapped to the KEGG "Focal Adhesion" pathway. THBS1 plays a key role in NS5A-mediated activation of the cytokine TGF-β1, which facilitates HCV replication and progressive liver fibrosis in HCV infection.[90] Our observations suggest that direct NS5A interactions with the bottlenecks THBS1 and TGFBR1 (TGF-β receptor 1; KEGG Pathway "Adherens Junction"), a key facilitator of TGF-β downstream signaling, may be crucial in facilitating HCV replication and tumorigenesis in HCV pathogenesis.

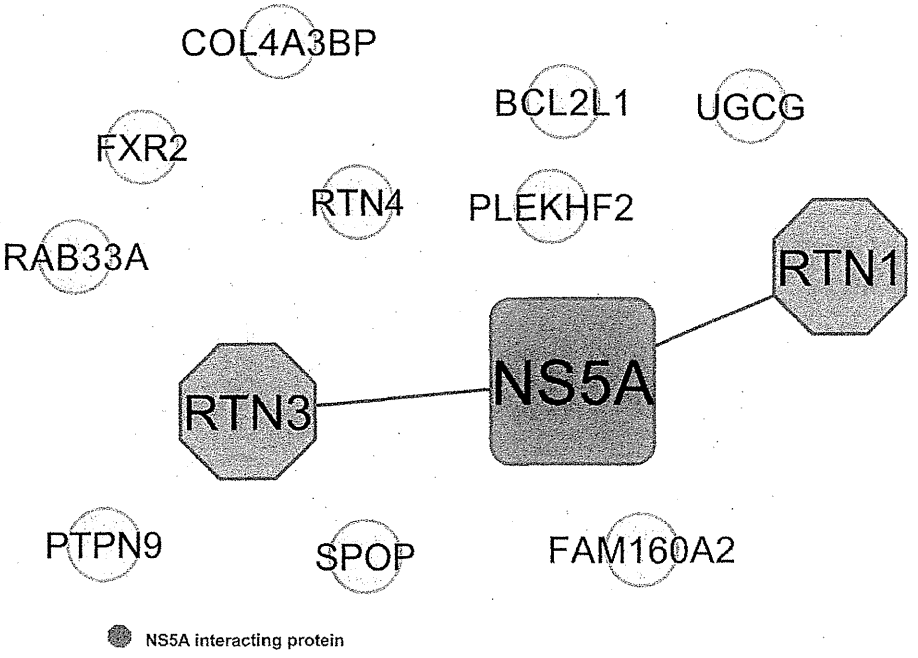


Figure 3. ER-localized host factors RTN1 and RTN3 were found to interact (blue edges) with NS5A in an Y2H screening of human liver cDNA library using NS5A as bait.

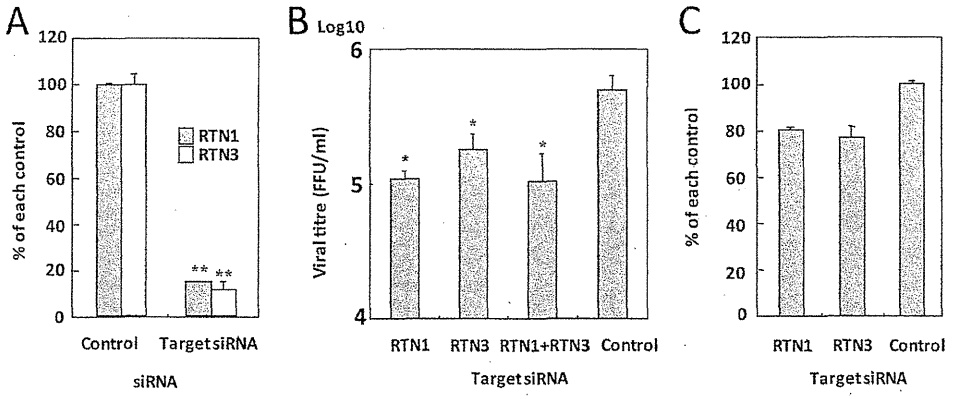


Figure 4. Effects of knockdown of RTN1 and RTN3 on HCV propagation and replication. Host factors RTN1 and RTN3 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a). The amounts of viral titer (B) and intracellular viral RNA (C) were estimated. Each value was represented as percentage of the cells transfected with the control siRNA. FFU: Focus-forming units; *: $p < 0.05$, **: $p < 0.01$.

Cellular Transport

Cellular factors associated with endocytic trafficking are key facilitators of the HCV life cycle, particularly HCV entry into the hepatic cells.[91−93] Endocytosis of the extracellular growth factor receptor (EGFR) in association with the cell surface glycoprotein CD81 plays a crucial role in HCV internalization and entry and is, therefore, an attractive target of anti-HCV strategies.[94] In the NS5A infection network, NS5A interactors ARAP1 and HSPA1A together with two bottlenecks (SRC, TGFBR1) were mapped to the enriched KEGG pathway "Endocytosis" ($p = 2.97 \times 10^{-8}$; Supporting Information, Tables S7a, S8a). ARAP1, a Golgi associated protein, negatively regulates EGFR trafficking, and decreased ARAP1 expression contributes to enhanced EGFR endocytosis.[95] Therefore, NS5A interaction with ARAP1 may facilitate EGFR internalization and thus viral entry in HCV infection.

NS5A Interacting Host Proteins RTN1 and RTN3 Function in HCV Propagation but Not Replication

Traditionally, viral and host proteins associated with the HCV lifecycle (internalization, replication, assembly and release) have been preferred targets in the anti-HCV studies. During infection, HCV localizes to the detergent-resistant membrane fraction (DRM) derived from the ER, where the viral replication and assembly take place.[4] Thus, of the novel interactions identified in our Y2H assay, we focused on two ER-localized host factors RTN1 and RTN3 (Figure 3). RTN1 and RTN3 belong to a group of proteins named Reticulons, which are integral to maintaining the shape and organization of the

ER and have been implicated in facilitating the replication of various positive-strand RNA viruses.[96−98] Furthermore, both RTN1 and RTN3 have been specifically detected in the very low density lipoprotein (VLDL) transport vesicle (VTV);[99] VTV is a key component of the VLDL secretory pathway, which plays an essential role in the production and the release of the infectious HCV particles.[100] Therefore, NS5A interactions with RTN1 and RTN3 suggested novel and potentially crucial roles of the two host proteins in the replication and/or release stages of the HCV lifecycle.

We performed cellular assays to assess the impact of RTN1 and RTN3 siRNA knockdowns on HCV replication and release. Since the HCV-production systems using the HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and the production of the infectious HCV particles, JFH1 was used to infect the Huh7OK1 cell line 24h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h postinfection, and the expression of each host protein was assessed by qRT-PCR (Figure 4A). The viral titer was significantly decreased by individual and double knockdowns of RTN1 and RTN3 (Figure 4B). However, RTN1 and RTN3 knockdowns had no effect on the intracellular viral RNA levels in the HCV infected cells (Figure 4C), suggesting that RTN1 and RTN3 regulate HCV propagation but not HCV replication.

CONCLUSIONS

We describe here our observations of PPIs between HCV NS5A and host proteins. By employing a multifold approach involving an experimental Y2H assay and literature mining, we derived a comprehensive set of experimentally determined binary interactions between NS5A and host proteins. We proceeded to map the combined NS5A−host interactions onto an overall interaction network, which comprised a repertoire of connections, which potentially enable NS5A to link up with and modulate the components of the host cellular networks. We then employed a network-based approach to understand the biological context of these connections in HCV pathogenesis with the help of the TargetMine data warehouse.

A functional analysis of the PPI networks highlighted NS5A interactions with several well connected host factors (hubs) and centrally located "bottlenecks" in the host cellular networks that function in cellular pathways associated with immune system and cell signaling, cellular adhesion and cell transport, cell growth and cell death and ER homeostasis among others. The "bottlenecks" include several proteins that were previously implicated in HCV pathogenesis, thereby suggesting that NS5A interactions with centrally connected host factors may enable the virus to influence strongly the host cellular processes in HCV infection. Notably, many bottlenecks were mapped to pathways associated with the infectious diseases induced by diverse bacterial and viral pathogens of the human host. These observations thus suggest the presence of some common themes underlying the onset of various human diseases associated with pathogenic infection in humans, a better understanding of which may be helpful in optimizing broad spectrum approaches to counteracting a wide range of pathogenic infections.

Cellular assays based on siRNA knockdowns in the HCV infected and replicon cells demonstrated RTN1 and RTN3, ER-localized NS5A interacting proteins, to be novel regulators of HCV propagation, but not replication, and thus promising novel candidates for anti-HCV therapy.

Our analysis therefore provides further insights into the role of NS5A−host interactions in HCV infection, a deeper understanding of which may aid in the identification of new clinically relevant targets for optimizing the therapeutic strategies to manipulate HCV−host interactions and thus more effectively combating HCV infection. Our analysis also emphasizes the importance of elaborate network-based computational approaches that integrate diverse biological data types in investigating host−pathogen interactions.

ASSOCIATED CONTENT

Supporting Information

Supporting methods, figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kenji@nibio.go.jp (K.M.); lokesh@nibio.go.jp (L.P.T.). Tel: +81-72-641-9890. Fax: +81-72-641-9881.

Author Contributions

¶L. P. Tripathi and H. Kambara contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by the Industrial Technology Research Grant Program in 2007 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and also by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

REFERENCES

(1) Dubuisson, J. Hepatitis C virus proteins. *World J. Gastroenterol.* **2007**, *13* (17), 2406−15.

(2) Moriishi, K.; Matsuura, Y. Host factors involved in the replication of hepatitis C virus. *Rev. Med. Virol.* **2007**, *17* (5), 343−54.

(3) Myrmel, H.; Ulvestad, E.; Asjo, B. The hepatitis C virus enigma. *APMIS* **2009**, *117* (5−6), 427−39.

(4) Tang, H.; Grise, H. Cellular and molecular biology of HCV infection and hepatitis. *Clin. Sci.* **2009**, *117* (2), 49−65.

(5) Pol, S.; Vallet-Pichard, A.; Corouge, M.; Mallet, V. O. Hepatitis C: epidemiology, diagnosis, natural history and therapy. *Contrib. Nephrol.* **2012**, *176*, 1−9.

(6) Kuiken, C.; Simmonds, P. Nomenclature and numbering of the hepatitis C virus. *Methods Mol. Biol.* **2009**, *510*, 33−53.

(7) Moradpour, D.; Penin, F.; Rice, C. M. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* **2007**, *5* (6), 453−63.

(8) Love, R. A.; Brodsky, O.; Hickey, M. J.; Wells, P. A.; Cronin, C. N. Crystal structure of a novel dimeric form of NS5A domain I protein from hepatitis C virus. *J. Virol.* **2009**, *83* (9), 4395−403.

(9) Yamasaki, L. H.; Arcuri, H. A.; Jardim, A. C.; Bittar, C.; de Carvalho-Mello, I. M.; Rahal, P. New insights regarding HCV-NS5A structure/function and indication of genotypic differences. *Virol. J.* **2012**, *9*, 14.

(10) Appel, N.; Zayas, M.; Miller, S.; Krijnse-Locker, J.; Schaller, T.; Friebe, P.; Kallis, S.; Engel, U.; Bartenschlager, R. Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. *PLoS Pathog.* **2008**, *4* (3), e1000035.

(11) Gale, M. J., Jr.; Korth, M. J.; Tang, N. M.; Tan, S. L.; Hopkins, D. A.; Dever, T. E.; Polyak, S. J.; Gretch, D. R.; Katze, M. G. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **1997**, *230* (2), 217–27.

(12) Ghosh, S.; Ahrens, W. A.; Phatak, S. U.; Hwang, S.; Schrum, L. W.; Bonkovsky, H. L. Association of filamin A and vimentin with hepatitis C virus proteins in infected human hepatocytes. *J. Viral Hepatitis* **2011**, *18* (10), e568–77.

(13) Gao, M.; Nettles, R. E.; Belema, M.; Snyder, L. B.; Nguyen, V. N.; Fridell, R. A.; Serrano-Wu, M. H.; Langley, D. R.; Sun, J. H.; O'Boyle, D. R., 2nd; Lemm, J. A.; Wang, C.; Knipe, J. O.; Chien, C.; Colonno, R. J.; Grasela, D. M.; Meanwell, N. A.; Hamann, L. G. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* **2010**, *465* (7294), 96–100.

(14) Lee, C. Discovery of hepatitis C virus NS5A inhibitors as a new class of anti-HCV therapy. *Arch. Pharmacal Res.* **2011**, *34* (9), 1403–7.

(15) Lemm, J. A.; O'Boyle, D., 2nd; Liu, M.; Nower, P. T.; Colonno, R.; Deshpande, M. S.; Snyder, L. B.; Martin, S. W.; St Laurent, D. R.; Serrano-Wu, M. H.; Romine, J. L.; Meanwell, N. A.; Gao, M. Identification of hepatitis C virus NS5A inhibitors. *J. Virol.* **2010**, *84* (1), 482–91.

(16) Lemon, S. M.; McKeating, J. A.; Pietschmann, T.; Frick, D. N.; Glenn, J. S.; Tellinghuisen, T. L.; Symons, J.; Furman, P. A. Development of novel therapies for hepatitis C. *Antiviral Res.* **2010**, *86* (1), 79–92.

(17) Fusco, D. N.; Chung, R. T. Novel therapies for hepatitis C: insights from the structure of the virus. *Annu. Rev. Med.* **2012**, *63*, 373–87.

(18) Buhler, S.; Bartenschlager, R. New targets for antiviral therapy of chronic hepatitis C. *Liver Int.* **2012**, *32* (Suppl 1), 9–16.

(19) Sarrazin, C.; Hezode, C.; Zeuzem, S.; Pawlotsky, J. M. Antiviral strategies in hepatitis C virus infection. *J. Hepatol.* **2012**, *56* (Suppl), S88–S100.

(20) Wang, S.; Wu, X.; Pan, T.; Song, W.; Wang, Y.; Zhang, F.; Yuan, Z. Viperin inhibits hepatitis C virus replication by interfering with binding of NS5A to host protein hVAP-33. *J. Gen. Virol.* **2012**, *93* (Pt1), 83–92.

(21) Durmus Tekir, S.; Cakir, T.; Ulgen, K. O. Infection strategies of bacterial and viral pathogens through pathogen-human protein-protein interactions. *Front. Microbiol.* **2012**, *3*, 46.

(22) de Chassey, B.; Navratil, V.; Tafforeau, L.; Hiet, M. S.; Aublin-Gex, A.; Agaugue, S.; Meiffren, G.; Pradezynski, F.; Faria, B. F.; Chantier, T.; Le Breton, M.; Pellet, J.; Davoust, N.; Mangeot, P. E.; Chaboud, A.; Penin, F.; Jacob, Y.; Vidalain, P. O.; Vidal, M.; Andre, P.; Rabourdin-Combe, C.; Lotteau, V. Hepatitis C virus infection protein network. *Mol. Syst. Biol.* **2008**, *4*, 230.

(23) Tan, S. L.; Ganji, G.; Paeper, B.; Proll, S.; Katze, M. G. Systems biology and the host response to viral infection. *Nat. Biotechnol.* **2007**, *25* (12), 1383–9.

(24) Tripathi, L. P.; Kataoka, C.; Taguwa, S.; Moriishi, K.; Mori, Y.; Matsuura, Y.; Mizuguchi, K. Network based analysis of hepatitis C virus Core and NS4B protein interactions. *Mol. BioSyst.* **2010**, *6* (12), 2539–53.

(25) Friedel, C. C.; Haas, J. Virus-host interactomes and global models of virus-infected cells. *Trends Microbiol.* **2011**, *19* (10), 501–8.

(26) Tafforeau, L.; Rabourdin-Combe, C.; Lotteau, V. Virus-human cell interactomes. *Methods Mol. Biol.* **2012**, *812*, 103–20.

(27) Aizaki, H.; Aoki, Y.; Harada, T.; Ishii, K.; Suzuki, T.; Nagamori, S.; Toda, G.; Matsuura, Y.; Miyamura, T. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* **1998**, *27* (2), 621–7.

(28) Hamamoto, I.; Nishimura, Y.; Okamoto, T.; Aizaki, H.; Liu, M.; Mori, Y.; Abe, T.; Suzuki, T.; Lai, M. M.; Miyamura, T.; Moriishi, K.; Matsuura, Y. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* **2005**, *79* (21), 13473–82.

(29) Rebholz-Schuhmann, D.; Kirsch, H.; Arregui, M.; Gaudan, S.; Riethoven, M.; Stoehr, P. EBIMed—text crunching to gather facts for proteins from Medline. *Bioinformatics* **2007**, *23* (2), e237–44.

(30) Rebholz-Schuhmann, D.; Arregui, M.; Gaudan, S.; Kirsch, H.; Jimeno, A. Text processing through Web services: calling Whatizit. *Bioinformatics* **2008**, *24* (2), 296–8.

(31) Stark, C.; Breitkreutz, B. J.; Reguly, T.; Boucher, L.; Breitkreutz, A.; Tyers, M. BioGRID: a general repository for interaction datasets. *Nucleic Acids Res.* **2006**, *34* (Database issue), D535–9.

(32) Turner, B.; Razick, S.; Turinsky, A. L.; Vlasblom, J.; Crowdy, E. K.; Cho, E.; Morrison, K.; Donaldson, I. M.; Wodak, S. J. iRefWeb: interactive analysis of consolidated protein interaction data and their supporting evidence. *Database* **2010**, *2010*, baq023.

(33) Chen, Y. A.; Tripathi, L. P.; Mizuguchi, K. TargetMine, an integrated data warehouse for candidate gene prioritisation and target discovery. *PLoS One* **2011**, *6* (3), e17844.

(34) Cline, M. S.; Smoot, M.; Cerami, E.; Kuchinsky, A.; Landys, N.; Workman, C.; Christmas, R.; Avila-Campilo, I.; Creech, M.; Gross, B.; Hanspers, K.; Isserlin, R.; Kelley, R.; Killcoyne, S.; Lotia, S.; Maere, S.; Morris, J.; Ono, K.; Pavlovic, V.; Pico, A. R.; Vailaya, A.; Wang, P. L.; Adler, A.; Conklin, B. R.; Hood, L.; Kuiper, M.; Sander, C.; Schmulevich, I.; Schwikowski, B.; Warner, G. J.; Ideker, T.; Bader, G. D. Integration of biological networks and gene expression data using Cytoscape. *Nat. Protoc.* **2007**, *2* (10), 2366–82.

(35) Smoot, M. E.; Ono, K.; Ruscheinski, J.; Wang, P. L.; Ideker, T. Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* **2011**, *27* (3), 431–2.

(36) Assenov, Y.; Ramirez, F.; Schelhorn, S. E.; Lengauer, T.; Albrecht, M. Computing topological parameters of biological networks. *Bioinformatics* **2008**, *24* (2), 282–4.

(37) Lees, J.; Yeats, C.; Perkins, J.; Sillitoe, I.; Rentzsch, R.; Dessailly, B. H.; Orengo, C. Gene3D: a domain-based resource for comparative genomics, functional annotation and protein network analysis. *Nucleic Acids Res.* **2012**, *40* (Database issue), D465–71.

(38) Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T.; Harris, M. A.; Hill, D. P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J. C.; Richardson, J. E.; Ringwald, M.; Rubin, G. M.; Sherlock, G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **2000**, *25* (1), 25–9.

(39) Aoki-Kinoshita, K. F.; Kanehisa, M. Gene annotation and pathway mapping in KEGG. *Methods Mol. Biol.* **2007**, *396*, 71–91.

(40) Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate—A practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* **1995**, *57* (1), 289–300.

(41) Noble, W. S. How does multiple testing correction work? *Nat. Biotechnol.* **2009**, *27* (12), 1135–7.

(42) Okamoto, K.; Mori, Y.; Komoda, Y.; Okamoto, T.; Okochi, M.; Takeda, M.; Suzuki, T.; Moriishi, K.; Matsuura, Y. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. *J. Virol.* **2008**, *82* (17), 8349–61.

(43) Okamoto, T.; Omori, H.; Kaname, Y.; Abe, T.; Nishimura, Y.; Suzuki, T.; Miyamura, T.; Yoshimori, T.; Moriishi, K.; Matsuura, Y. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J. Virol.* **2008**, *82* (7), 3480–9.

(44) Wakita, T.; Pietschmann, T.; Kato, T.; Date, T.; Miyamoto, M.; Zhao, Z.; Murthy, K.; Habermann, A.; Krausslich, H. G.; Mizokami, M.; Bartenschlager, R.; Liang, T. J. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **2005**, *11* (7), 791–6.

(45) Taguwa, S.; Kambara, H.; Omori, H.; Tani, H.; Abe, T.; Mori, Y.; Suzuki, T.; Yoshimori, T.; Moriishi, K.; Matsuura, Y. Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. *J. Virol.* **2009**, *83* (20), 10427–36.

(46) Kukihara, H.; Moriishi, K.; Taguwa, S.; Tani, H.; Abe, T.; Mori, Y.; Suzuki, T.; Fukuhara, T.; Taketomi, A.; Maehara, Y.; Matsuura, Y.

Human VAP-C negatively regulates hepatitis C virus propagation. *J. Virol.* **2009**, *83* (16), 7959–69.
(47) Nanda, S. K.; Herion, D.; Liang, T. J. The SH3 binding motif of HCV [corrected] NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. *Gastroenterology* **2006**, *130* (3), 794–809.
(48) Liu, A. W.; Cai, J.; Zhao, X. L.; Jiang, T. H.; He, T. F.; Fu, H. Q.; Zhu, M. H.; Zhang, S. H. ShRNA-targeted MAP4K4 inhibits hepatocellular carcinoma growth. *Clin. Cancer Res.* **2011**, *17* (4), 710–20.
(49) Woodhouse, S. D.; Narayan, R.; Latham, S.; Lee, S.; Antrobus, R.; Gangadharan, B.; Luo, S.; Schroth, G. P.; Klenerman, P.; Zitzmann, N. Transcriptome sequencing, microarray, and proteomic analyses reveal cellular and metabolic impact of hepatitis C virus infection in vitro. *Hepatology* **2010**, *52* (2), 443–53.
(50) MacPherson, J. I.; Sidders, B.; Wieland, S.; Zhong, J.; Targett-Adams, P.; Lohmann, V.; Backes, P.; Delpuech-Adams, O.; Chisari, F.; Lewis, M.; Parkinson, T.; Robertson, D. L. An integrated transcriptomic and meta-analysis of hepatoma cells reveals factors that influence susceptibility to HCV infection. *PLoS One* **2011**, *6* (10), e25584.
(51) Yamashita, T.; Honda, M.; Kaneko, S. Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection. *J. Gastroenterol. Hepatol.* **2011**, *26* (6), 960–4.
(52) Yu, H.; Kim, P. M.; Sprecher, E.; Trifonov, V.; Gerstein, M. The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics. *PLoS Comput. Biol.* **2007**, *3* (4), e59.
(53) Rasmussen, A. L.; Diamond, D. L.; McDermott, J. E.; Gao, X.; Metz, T. O.; Matzke, M. M.; Carter, V. S.; Belisle, S. E.; Korth, M. J.; Waters, K. M.; Smith, R. D.; Katze, M. G. Systems virology identifies a mitochondrial fatty acid oxidation enzyme, dodecenoyl coenzyme A delta isomerase, required for hepatitis C virus replication and likely pathogenesis. *J. Virol.* **2011**, *85* (22), 11646–54.
(54) Diamond, D. L.; Krasnoselsky, A. L.; Burnum, K. E.; Monroe, M. E.; Webb-Robertson, B. J.; McDermott, J. E.; Yeh, M. M.; Dzib, J. F.; Susnow, N.; Strom, S.; Proll, S. C.; Belisle, S. E.; Purdy, D. E.; Rasmussen, A. L.; Walters, K. A.; Jacobs, J. M.; Gritsenko, M. A.; Camp, D. G.; Bhattacharya, R.; Perkins, J. D.; Carithers, R. L., Jr.; Liou, I. W.; Larson, A. M.; Benecke, A.; Waters, K. M.; Smith, R. D.; Katze, M. G. Proteome and computational analyses reveal new insights into the mechanisms of hepatitis C virus-mediated liver disease posttransplantation. *Hepatology* **2012**, *56* (1), 28–38.
(55) He, Y.; Nakao, H.; Tan, S. L.; Polyak, S. J.; Neddermann, P.; Vijaysri, S.; Jacobs, B. L.; Katze, M. G. Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. *J. Virol.* **2002**, *76* (18), 9207–17.
(56) Jiang, Y. F.; He, B.; Li, N. P.; Ma, J.; Gong, G. Z.; Zhang, M. The oncogenic role of NS5A of hepatitis C virus is mediated by up-regulation of survivin gene expression in the hepatocellular cell through p53 and NF-kappaB pathways. *Cell Biol. Int.* **2011**, *35* (12), 1225–32.
(57) Pfannkuche, A.; Buther, K.; Karthe, J.; Poenisch, M.; Bartenschlager, R.; Trilling, M.; Hengel, H.; Willbold, D.; Haussinger, D.; Bode, J. G. c-Src is required for complex formation between the hepatitis C virus-encoded proteins NS5A and NS5B: a prerequisite for replication. *Hepatology* **2011**, *53* (4), 1127–36.
(58) Calderwood, M. A.; Venkatesan, K.; Xing, L.; Chase, M. R.; Vazquez, A.; Holthaus, A. M.; Ewence, A. E.; Li, N.; Hirozane-Kishikawa, T.; Hill, D. E.; Vidal, M.; Kieff, E.; Johannsen, E. Epstein-Barr virus and virus human protein interaction maps. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (18), 7606–11.
(59) Pichlmair, A.; Kandasamy, K.; Alvisi, G.; Mulhern, O.; Sacco, R.; Habjan, M.; Binder, M.; Stefanovic, A.; Eberle, C. A.; Goncalves, A.; Burckstummer, T.; Muller, A. C.; Fauster, A.; Holze, C.; Lindsten, K.; Goodbourn, S.; Kochs, G.; Weber, F.; Bartenschlager, R.; Bowie, A. G.; Bennett, K. L.; Colinge, J.; Superti-Furga, G. Viral immune modulators perturb the human molecular network by common and unique strategies. *Nature* **2012**, *487* (7408), 486–90.
(60) Huang, H.; Jedynak, B. M.; Bader, J. S. Where have all the interactions gone? Estimating the coverage of two-hybrid protein interaction maps. *PLoS Comput. Biol.* **2007**, *3* (11), e214.
(61) Nordle Gilliver, A.; Griffin, S.; Harris, M. Identification of a novel phosphorylation site in hepatitis C virus NS5A. *J. Gen. Virol.* **2010**, *91* (Pt 10), 2428–32.
(62) Qiu, D.; Lemm, J. A.; O'Boyle, D. R., 2nd; Sun, J. H.; Nower, P. T.; Nguyen, V.; Hamann, L. G.; Snyder, L. B.; Deon, D. H.; Ruediger, E.; Meanwell, N. A.; Belema, M.; Gao, M.; Fridell, R. A. The effects of NS5A inhibitors on NS5A phosphorylation, polyprotein processing and localization. *J. Gen. Virol.* **2011**, *92* (Pt11), 2502–11.
(63) Tellinghuisen, T. L.; Foss, K. L.; Treadaway, J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog.* **2008**, *4* (3), e1000032.
(64) Chen, K. C.; Wang, T. Y.; Chan, C. H. Associations between HIV and human pathways revealed by protein-protein interactions and correlated gene expression profiles. *PLoS One* **2012**, *7* (3), e34240.
(65) Rozenblatt-Rosen, O.; Deo, R. C.; Padi, M.; Adelmant, G.; Calderwood, M. A.; Rolland, T.; Grace, M.; Dricot, A.; Askenazi, M.; Tavares, M.; Pevzner, S. J.; Abderazzaq, F.; Byrdsong, D.; Carvunis, A. R.; Chen, A. A.; Cheng, J.; Correll, M.; Duarte, M.; Fan, C.; Feltkamp, M. C.; Ficarro, S. B.; Franchi, R.; Garg, B. K.; Gulbahce, N.; Hao, T.; Holthaus, A. M.; James, R.; Korkhin, A.; Litovchick, L.; Mar, J. C.; Pak, T. R.; Rabello, S.; Rubio, R.; Shen, Y.; Singh, S.; Spangle, J. M.; Tasan, M.; Wanamaker, S.; Webber, J. T.; Roecklein-Canfield, J.; Johannsen, E.; Barabasi, A. L.; Beroukhim, R.; Kieff, E.; Cusick, M. E.; Hill, D. E.; Munger, K.; Marto, J. A.; Quackenbush, J.; Roth, F. P.; DeCaprio, J. A.; Vidal, M. Interpreting cancer genomes using systematic host network perturbations by tumour virus proteins. *Nature* **2012**, *487* (7408), 491–5.
(66) Barnaba, V. Hepatitis C virus infection: a "liaison a trois" amongst the virus, the host, and chronic low-level inflammation for human survival. *J. Hepatol.* **2010**, *53* (4), 752–61.
(67) Hiroishi, K.; Ito, T.; Imawari, M. Immune responses in hepatitis C virus infection and mechanisms of hepatitis C virus persistence. *J. Gastroenterol. Hepatol.* **2008**, *23* (10), 1473–82.
(68) Kawai, T.; Akira, S. Toll-like receptor and RIG-I-like receptor signaling. *Ann. N. Y. Acad. Sci.* **2008**, *1143*, 1–20.
(69) Sklan, E. H.; Charuworn, P.; Pang, P. S.; Glenn, J. S. Mechanisms of HCV survival in the host. *Nat. Rev. Gastroenterol. Hepatol.* **2009**, *6* (4), 217–27.
(70) Kang, S. M.; Won, S. J.; Lee, G. H.; Lim, Y. S.; Hwang, S. B. Modulation of interferon signaling by hepatitis C virus non-structural 5A protein: implication of genotypic difference in interferon treatment. *FEBS Lett.* **2010**, *584* (18), 4069–76.
(71) Li, K.; Li, N. L.; Wei, D.; Pfeffer, S. R.; Fan, M.; Pfeffer, L. M. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology* **2012**, *55* (3), 666–75.
(72) Tanaka, S.; Arii, S. Molecularly targeted therapy for hepatocellular carcinoma. *Cancer Sci.* **2009**, *100* (1), 1–8.
(73) Tanaka, S.; Arii, S. Current status of molecularly targeted therapy for hepatocellular carcinoma: basic science. *Int. J. Clin. Oncol.* **2010**, *15* (3), 235–41.
(74) Villanueva, A.; Chiang, D. Y.; Newell, P.; Peix, J.; Thung, S.; Alsinet, C.; Tovar, V.; Roayaie, S.; Minguez, B.; Sole, M.; Battiston, C.; Van Laarhoven, S.; Fiel, M. I.; Di Feo, A.; Hoshida, Y.; Yea, S.; Toffanin, S.; Ramos, A.; Martignetti, J. A.; Mazzaferro, V.; Bruix, J.; Waxman, S.; Schwartz, M.; Meyerson, M.; Friedman, S. L.; Llovet, J. M. Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology* **2008**, *135* (6), 1972–83.
(75) Cheng, D.; Zhao, L.; Zhang, L.; Jiang, Y.; Tian, Y.; Xiao, X.; Gong, G. p53 controls hepatitis C virus non-structural protein 5A-mediated downregulation of GADD45alpha expression via the NF-kappaB and PI3K-Akt pathways. *J. Gen. Virol.* **2013**, *94* (Pt 2), 326–35.
(76) Tripathi, L. P.; Kambara, H.; Moriishi, K.; Morita, E.; Abe, T.; Mori, Y.; Chen, Y. A.; Matsuura, Y.; Mizuguchi, K. Proteomic analysis

of hepatitis C virus (HCV) core protein transfection and host regulator PA28gamma knockout in HCV pathogenesis: a network-based study. *J. Proteome Res.* **2012**, *11* (7), 3664–79.

(77) Zhao, L. J.; Zhao, P.; Chen, Q. L.; Ren, H.; Pan, W.; Qi, Z. T. Mitogen-activated protein kinase signalling pathways triggered by the hepatitis C virus envelope protein E2: implications for the prevention of infection. *Cell Proliferation* **2007**, *40* (4), 508–21.

(78) Basaranoglu, M.; Basaranoglu, G. Pathophysiology of insulin resistance and steatosis in patients with chronic viral hepatitis. *World J. Gastroenterol.* **2011**, *17* (36), 4055–62.

(79) Del Campo, J. A.; Romero-Gomez, M. Steatosis and insulin resistance in hepatitis C: a way out for the virus? *World J. Gastroenterol.* **2009**, *15* (40), 5014–9.

(80) Douglas, M. W.; George, J. Molecular mechanisms of insulin resistance in chronic hepatitis C. *World J. Gastroenterol.* **2009**, *15* (35), 4356–64.

(81) Das, G. C.; Hollinger, F. B. Molecular pathways for glucose homeostasis, insulin signaling and autophagy in hepatitis C virus induced insulin resistance in a cellular model. *Virology* **2012**, *434* (1), 5–17.

(82) Miyamoto, H.; Moriishi, K.; Moriya, K.; Murata, S.; Tanaka, K.; Suzuki, T.; Miyamura, T.; Koike, K.; Matsuura, Y. Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *J. Virol.* **2007**, *81* (4), 1727–35.

(83) Kaneko, K.; Ueki, K.; Takahashi, N.; Hashimoto, S.; Okamoto, M.; Awazawa, M.; Okazaki, Y.; Ohsugi, M.; Inabe, K.; Umehara, T.; Yoshida, M.; Kakei, M.; Kitamura, T.; Luo, J.; Kulkarni, R. N.; Kahn, C. R.; Kasai, H.; Cantley, L. C.; Kadowaki, T. Class IA phosphatidylinositol 3-kinase in pancreatic beta cells controls insulin secretion by multiple mechanisms. *Cell Metab.* **2010**, *12* (6), 619–32.

(84) Milward, A.; Mankouri, J.; Harris, M. Hepatitis C virus NS5A protein interacts with beta-catenin and stimulates its transcriptional activity in a phosphoinositide-3 kinase-dependent fashion. *J. Gen. Virol.* **2010**, *91* (Pt 2), 373–81.

(85) Alberstein, M.; Zornitzki, T.; Zick, Y.; Knobler, H. Hepatitis C core protein impairs insulin downstream signalling and regulatory role of IGFBP-1 expression. *J. Viral Hepatitis* **2012**, *19* (1), 65–71.

(86) Benedicto, I.; Molina-Jimenez, F.; Bartosch, B.; Cosset, F. L.; Lavillette, D.; Prieto, J.; Moreno-Otero, R.; Valenzuela-Fernandez, A.; Aldabe, R.; Lopez-Cabrera, M.; Majano, P. L. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. *J. Virol.* **2009**, *83* (16), 8012–20.

(87) Carloni, G.; Crema, A.; Valli, M. B.; Ponzetto, A.; Clementi, M. HCV infection by cell-to-cell transmission: choice or necessity? *Curr. Mol. Med.* **2012**, *12* (1), 83–95.

(88) Wilson, G. K.; Brimacombe, C. L.; Rowe, I. A.; Reynolds, G. M.; Fletcher, N. F.; Stamataki, Z.; Bhogal, R. H.; Simoes, M. L.; Ashcroft, M.; Afford, S. C.; Mitry, R. R.; Dhawan, A.; Mee, C. J.; Hubscher, S. G.; Balfe, P.; McKeating, J. A. A dual role for hypoxia inducible factor-1alpha in the hepatitis C virus lifecycle and hepatoma migration. *J. Hepatol.* **2012**, *56* (4), 803–9.

(89) Daugherty, R. L.; Gottardi, C. J. Phospho-regulation of beta-catenin adhesion and signaling functions. *Physiology* **2007**, *22*, 303–9.

(90) Presser, L. D.; Haskett, A.; Waris, G. Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-beta1: role of TGF-beta1 in HCV replication. *Virology* **2011**, *412* (2), 284–96.

(91) Berger, K. L.; Cooper, J. D.; Heaton, N. S.; Yoon, R.; Oakland, T. E.; Jordan, T. X.; Mateu, G.; Grakoui, A.; Randall, G. Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (18), 7577–82.

(92) Katsarou, K.; Lavdas, A. A.; Tsitoura, P.; Serti, E.; Markoulatos, P.; Mavromara, P.; Georgopoulou, U. Endocytosis of hepatitis C virus non-enveloped capsid-like particles induces MAPK-ERK1/2 signaling events. *Cell. Mol. Life Sci.* **2010**, *67*, 2491–506.

(93) Mankouri, J.; Griffin, S.; Harris, M. The hepatitis C virus non-structural protein NS5A alters the trafficking profile of the epidermal growth factor receptor. *Traffic* **2008**, *9* (9), 1497–509.

(94) Diao, J.; Pantua, H.; Ngu, H.; Komuves, L.; Diehl, L.; Schaefer, G.; Kapadia, S. B. Hepatitis C virus (HCV) induces epidermal growth factor receptor (EGFR) activation via CD81 binding for viral internalization and entry. *J. Virol.* **2012**, *86* (20), 10935–49.

(95) Yoon, H. Y.; Kales, S. C.; Luo, R.; Lipkowitz, S.; Randazzo, P. A. ARAP1 association with CIN85 affects epidermal growth factor receptor endocytic trafficking. *Biol. Cell* **2011**, *103* (4), 171–84.

(96) Katsarou, K.; Lavdas, A. A.; Tsitoura, P.; Serti, E.; Markoulatos, P.; Mavromara, P.; Georgopoulou, U. Endocytosis of hepatitis C virus non-enveloped capsid-like particles induces MAPK-ERK1/2 signaling events. *Cell. Mol. Life Sci.* **2010**, *67* (14), 2491–506.

(97) Diaz, A.; Ahlquist, P. Role of host reticulon proteins in rearranging membranes for positive-strand RNA virus replication. *Curr. Opin. Microbiol.* **2012**, *15* (4), 519–24.

(98) Diaz, A.; Wang, X.; Ahlquist, P. Membrane-shaping host reticulon proteins play crucial roles in viral RNA replication compartment formation and function. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (37), 16291–6.

(99) Rahim, A.; Nafi-valencia, E.; Siddiqi, S.; Basha, R.; Runyon, C. C.; Siddiqi, S. A. Proteomic analysis of the very low density lipoprotein (VLDL) transport vesicles. *J Proteomics* **2012**, *75* (7), 2225–35.

(100) Coller, K. E.; Heaton, N. S.; Berger, K. L.; Cooper, J. D.; Saunders, J. L.; Randall, G. Molecular determinants and dynamics of hepatitis C virus secretion. *PLoS Pathog.* **2012**, *8* (1), e1002466.

(101) Lai, C. K.; Jeng, K. S.; Machida, K.; Lai, M. M. Association of hepatitis C virus replication complexes with microtubules and actin filaments is dependent on the interaction of NS3 and NS5A. *J. Virol.* **2008**, *82* (17), 8838–48.

(102) Randall, G.; Panis, M.; Cooper, J. D.; Tellinghuisen, T. L.; Sukhodolets, K. E.; Pfeffer, S.; Landthaler, M.; Landgraf, P.; Kan, S.; Lindenbach, B. D.; Chien, M.; Weir, D. B.; Russo, J. J.; Ju, J.; Brownstein, M. J.; Sheridan, R.; Sander, C.; Zavolan, M.; Tuschl, T.; Rice, C. M. Cellular cofactors affecting hepatitis C virus infection and replication. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (31), 12884–9.

(103) Saxena, V.; Lai, C. K.; Chao, T. C.; Jeng, K. S.; Lai, M. M. Annexin A2 is involved in the formation of hepatitis C virus replication complex on the lipid raft. *J. Virol.* **2012**, *86* (8), 4139–50.

(104) Quintavalle, M.; Sambucini, S.; Summa, V.; Orsatti, L.; Talamo, F.; De Francesco, R.; Neddermann, P. Hepatitis C virus NS5A is a direct substrate of casein kinase I-alpha, a cellular kinase identified by inhibitor affinity chromatography using specific NS5A hyperphosphorylation inhibitors. *J. Biol. Chem.* **2007**, *282* (8), 5536–44.

(105) Ivanov, A. V.; Tunitskaya, V. L.; Ivanova, O. N.; Mitkevich, V. A.; Prassolov, V. S.; Makarov, A. A.; Kukhanova, M. K.; Kochetkov, S. N. Hepatitis C virus NS5A protein modulates template selection by the RNA polymerase in in vitro system. *FEBS Lett.* **2009**, *583* (2), 277–80.

(106) Park, C. Y.; Choi, S. H.; Kang, S. M.; Kang, J. I.; Ahn, B. Y.; Kim, H.; Jung, G.; Choi, K. Y.; Hwang, S. B. Nonstructural 5A protein activates beta-catenin signaling cascades: implication of hepatitis C virus-induced liver pathogenesis. *J. Hepatol.* **2009**, *51* (5), 853–64.

(107) Zhang, Z.; Harris, D.; Pandey, V. N. The FUSE binding protein is a cellular factor required for efficient replication of hepatitis C virus. *J. Virol.* **2008**, *82* (12), 5761–73.

(108) Chen, Y. J.; Chen, Y. H.; Chow, L. P.; Tsai, Y. H.; Chen, P. H.; Huang, C. Y.; Chen, W. T.; Hwang, L. H. Heat shock protein 72 is associated with the hepatitis C virus replicase complex and enhances viral RNA replication. *J. Biol. Chem.* **2010**, *285* (36), 28183–90.

(109) Choi, Y. W.; Tan, Y. J.; Lim, S. G.; Hong, W.; Goh, P. Y. Proteomic approach identifies HSP27 as an interacting partner of the hepatitis C virus NS5A protein. *Biochem. Biophys. Res. Commun.* **2004**, *318* (2), 514–9.

(110) Ahn, J.; Chung, K. S.; Kim, D. U.; Won, M.; Kim, L.; Kim, K. S.; Nam, M.; Choi, S. J.; Kim, H. C.; Yoon, M.; Chae, S. K.; Hoe, K. L. Systematic identification of hepatocellular proteins interacting with NS5A of the hepatitis C virus. *J. Biochem. Mol. Biol.* **2004**, *37* (6), 741–8.

(111) Amako, Y.; Sarkeshik, A.; Hotta, H.; Yates, J., 3rd; Siddiqui, A. Role of oxysterol binding protein in hepatitis C virus infection. *J. Virol.* **2009**, *83* (18), 9237–46.
(112) Lim, Y. S.; Tran, H. T.; Park, S. J.; Yim, S. A.; Hwang, S. B. Peptidyl-prolyl isomerase Pin1 is a cellular factor required for hepatitis C virus propagation. *J. Virol.* **2011**, *85* (17), 8777–88.
(113) Chen, Y. C.; Su, W. C.; Huang, J. Y.; Chao, T. C.; Jeng, K. S.; Machida, K.; Lai, M. M. Polo-like kinase 1 is involved in hepatitis C virus replication by hyperphosphorylating NS5A. *J. Virol.* **2010**, *84* (16), 7983–93.
(114) Waller, H.; Chatterji, U.; Gallay, P.; Parkinson, T.; Targett-Adams, P. The use of AlphaLISA technology to detect interaction between hepatitis C virus-encoded NS5A and cyclophilin A. *J. Virol. Methods* **2010**, *165* (2), 202–10.
(115) Chatterji, U.; Lim, P.; Bobardt, M. D.; Wieland, S.; Cordek, D. G.; Vuagniaux, G.; Chisari, F.; Cameron, C. E.; Targett-Adams, P.; Parkinson, T.; Gallay, P. A. HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A-cyclophilin A interaction to cyclophilin inhibitors. *J. Hepatol.* **2010**, *53* (1), 50–6.
(116) Georgopoulou, U.; Tsitoura, P.; Kalamvoki, M.; Mavromara, P. The protein phosphatase 2A represents a novel cellular target for hepatitis C virus NS5A protein. *Biochimie* **2006**, *88* (6), 651–62.
(117) Helbig, K. J.; Eyre, N. S.; Yip, E.; Narayana, S.; Li, K.; Fiches, G.; McCartney, E. M.; Jangra, R. K.; Lemon, S. M.; Beard, M. R. The antiviral protein viperin inhibits hepatitis C virus replication via interaction with nonstructural protein 5A. *Hepatology* **2011**, *54* (5), 1506–17.
(118) Kumthip, K.; Chusri, P.; Jilg, N.; Zhao, L.; Fusco, D. N.; Zhao, H.; Goto, K.; Cheng, D.; Schaefer, E. A.; Zhang, L.; Pantip, C.; Thongsawat, S.; O'Brien, A.; Peng, L. F.; Maneekarn, N.; Chung, R. T.; Lin, W. Hepatitis C virus NS5A disrupts STAT1 phosphorylation and suppresses type I interferon signaling. *J. Virol.* **2012**, *86* (16), 8581–91.
(119) Inubushi, S.; Nagano-Fujii, M.; Kitayama, K.; Tanaka, M.; An, C.; Yokozaki, H.; Yamamura, H.; Nuriya, H.; Kohara, M.; Sada, K.; Hotta, H. Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk. *J. Gen. Virol.* **2008**, *89* (Pt 5), 1231–42.

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

VIRAL HEPATITIS

Ca^{2+}/S100 proteins regulate HCV virus NS5A–FKBP8/FKBP38 interaction and HCV virus RNA replication

Joji Tani[1,*], Seiko Shimamoto[2,*], Kyoko Mori[3], Nobuyuki Kato[3], Kohji Moriishi[4], Yoshiharu Matsuura[5], Hiroshi Tokumitsu[2], Mitsumasa Tsuchiya[2], Tomohito Fujimoto[2], Kiyohito Kato[1], Hisaaki Miyoshi[1], Tsutomu Masaki[1] and Ryoji Kobayashi[2]

1 Department of Gastroenterology and Neurology, Kagawa University Faculty of Medicine, Kagawa, Japan
2 Department of Signal Transduction Sciences, Kagawa University Faculty of Medicine, Kagawa, Japan
3 Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
4 Department of Microbiology, Division of Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan
5 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Keywords
Ca^{2+}-binding proteins – HCV replicon harbouring cells – Hsp90 – ternary complex formation – tetratricopeptide repeat

Correspondence
Ryoji Kobayashi, MD, PhD, Department of Signal Transduction Sciences, Kagawa University Faculty of Medicine, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
Tel: +81-87-891-2249
Fax: +81-87-891-2249
e-mail: ryoji@med.kagawa-u.ac.jp

Received 29 September 2012
Accepted 22 February 2013

DOI:10.1111/liv.12151
Liver Int. 2013: 33: 1008–1018

Abstract

Background & Aim: FKBP8/FKBP38 is a unique FK506-binding protein with a C-terminal membrane anchor and localizes at the outer membranes of mitochondria and the endoplasmic reticulum. Similar to some immunophilins, such as FKBP51, FKBP52 and Cyclophilin 40, FKBP8/FKBP38 contain a putative Calmodulin-binding domain and a tetratricopeptide-repeat (TPR) domain for the binding of Hsp90. Both Hsp90 and the non-structural protein 5A (NS5A) of the hepatitis C virus (HCV) interact specifically with FKBP8/FKBP38 through its TPR domain, and the ternary complex formation plays a critical role in HCV RNA replication. The goal of this study is to evaluate that the host factor inhibits the ternary complex formation and the replication of HCV *in vitro* and *in vivo*. *Methods:* S100 proteins, FKBP38, FKBP8, HCV NS5A, Hsp90, and calmodulin were expressed in E.coli and purified. *In vitro* binding studies were performed by GST pull-down, S-tag pull-down and surface plasmon resonance analyses. The effect of S100 proteins on HCV replication was analysed by Western blotting using an HCV NS3 antibody following transfection of S100 proteins into the HCV replicon harbouring cell line (sO cells). *Results: In vitro* binding studies showed that S100A1, S100A2, S100A6, S100B and S100P directly interacted with FKBP8/FKBP38 in a Ca^{2+}-dependent manner and inhibited the FKBP8/FKBP38–Hsp90 and FKBP8/FKBP38–NS5A interactions. Furthermore, overexpression of S100A1, S100A2 and S100A6 in sO cells resulted in the efficient inhibition of HCV replication. *Conclusion:* The association of the S100 proteins with FKBP8/FKBP38 provides a novel Ca^{2+}-dependent regulatory role in HCV replication through the NS5A–host protein interaction.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, which frequently progresses to cirrhosis and hepatocellular carcinoma (1, 2). HCV represents a global public health problem, affecting approximately 170 million people worldwide, which is more than 3% of the world population (3, 4). HCV is a member of the positive strand RNA viruses, belongs to the family Flaviviridae, genus Hepacivirus, and contains a genome of approximately 9.6 kb in length, which encodes a large polyprotein precursor of approximately 3000 amino acids (5, 6). The polyprotein is cleaved by host and viral proteases to release the individual enzymes and proteins that mediate virus replication, assembly and release, producing viral structural proteins (Core, E1 and E2), a putative viropore protein (p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (5, 6). Importantly, the NS5A of HCV plays a critical role in HCV replication and is an attractive target for antiviral therapy of HCV infection (7). NS5A is a multifunctional 56–58 kDa serine phosphoprotein and interacts with a number of cellular proteins thereby affecting numerous host functions, including the modulation of signal transduction pathways, suppression of apoptosis and modulation of transcription (8–10). Recently, FK506-binding protein 8 (FKBP8)/FK506-binding protein 38 (FKBP38) was shown to interact with NS5A and to regulate HCV replication (11, 12),

*These authors contributed equally to this work.

suggesting that the immunophilins are promising therapies for chronic hepatitis C. FKBP8/FKBP38 binds to both NS5A and heat-shock protein 90 (Hsp90) through the tetratricopeptide-repeat (TPR) domain, and the ternary complex (FKBP8/FKBP38–Hsp90–NS5A) plays a critical role in HCV replication (11, 12). TPRs are loosely conserved 34 amino acid helix–turn–helix sequence motifs that mediate protein–protein interactions (13). This property enables TPR-containing proteins to function as scaffold proteins and allows them to be involved in a variety of cellular functions (14, 15).

The S100 protein family is composed of at least 25 members that share two EF-hand motifs, a 25–65% amino acid sequence homology and a molecular weight of 10–12 kDa (16–18).

Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hsp70/Hsp90-organizing protein (Hop), kinesin light chain (KLC) and Tom70 in a Ca^{2+}-dependent manner, leading to the dissociation of the Hsp90–Hop–Hsp70, KLC–JIP1 and Tom70–Hsps interactions *in vitro* and *in vivo* (19). Further studies have revealed an interaction of S100A1 and S100A2 bound to FK506-binding protein 52 (FKBP52) and cyclophilin 40 (Cyp40), which contain a TPR domain; in the presence of Ca^{2+}, this interaction led to the inhibition of the Cyp40–Hsp90 and FKBP52–Hsp90 interactions (20).

Because S100 proteins interact with TPR motifs (19, 20), we explored the potential role for S100 proteins in the regulation of the FKBP8/FKBP38–HSP90 and/or FKBP8/FKBP38–NS5A interactions and thereby the control of HCV replication. In this study, we demonstrate that Ca^{2+}/S100 proteins modulate replication of HCV via two different mechanisms. Firstly, S100A1, S100A2, S100A6, S100B and S100P interact with the TPR domain of FKBP8/FKBP38 and compete with Hsp90 binding to FKBP38. Secondly, these S100 proteins inhibit the interaction between NS5A and FKBP8/FKBP38. Using HCV replicon harbouring cells (sO cell: HCV O strain of genotype 1b) (21, 22), the overexpression of S100A1, S100A2 and S100A6 with A23187 treatment has shown a significant decrease in NS3 expression. These observations indicate that Ca^{2+}/S100 proteins could modulate HCV replication by inhibiting the interaction between FKBP8/FKBP38 and its binding partners.

Materials and methods

Materials

Phenyl–Sepharose and glutathione–Sepharose were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Nickel–nitrilotriacetic acid agarose was purchased from Qiagen. S-protein agarose was purchased from Merck. Antibodies were obtained as follows: anti-S100A1 (Novus Biologicals, Littleton, Colorado, USA), anti-S100A2 (ANOVA, Santa Clara, CA, USA), anti-S100A6 (Proteintech Group Inc., Chicago, IL, USA), Anti-HCVNS3 (Leica Microsystems, Wetzlar, Germany), anti-β-actin (BioVision, Milpitas, CA, USA) and horseradish peroxidase-conjugated antimouse or antirabbit IgG antibody (GE Healthcare). All other chemicals were obtained from standard commercial sources.

Plasmids

The following plasmids were previously described: pME18S-S100A1, -S100A2, -S100A6, pET-Calmodulin (pET-CaM), pET-CaM-Glutathione-S-transferase (pET-CaM-GST) and pET16b-Hsp90 (19, 20, 23, 24). Human FKBP38 and FKBP8 complementary DNAs (cDNAs) were purchased from Open Biosystems and subcloned into pET30a. For the expression of FKBP38 C-terminal deletion mutants, polymerase chain reaction (PCR) fragments encoding residues 1–166, −297, −315, −325 and −335 were also cloned into pET30a. To create a plasmid of the pET30a-FKBP38 carboxylate clamp mutant (K250E/R254E), the plasmid was generated using inverse PCR using pET30a-FKBP38 (1–335) as a template. pETUbHis-NS5A was kindly provided by Dr Craig E. Cameron (25). The sequence integrity of the all inserts was confirmed through automated sequence analysis (Applied Biosystems, Foster City, CA, USA).

Preparation of recombinant proteins

All recombinant proteins were produced in *Escherichia coli* strain BL21(DE3) or BL21(DE3) CodonPlus-RIL (Novagen, Darmstadt, Germany). S100 proteins (S100A1, S100A2, S100A4, S100A6, S100A10, S100A11, S100A12, S100B and S100P) were expressed and prepared as described previously (26, 27). Calmodulin (CaM) was prepared as described by Hayashi and colleagues (28). C-terminally GST-tagged CaM linked by a Gly_6 spacer (CaM-GST) was prepared as described (23, 24). A C-terminally His-tagged NS5A, lacking the N-terminal 32 amino acid residues of the membrane-anchoring region (NS5A-His), was expressed via the pET-ubiquitin expression system, in which the NS5A-His was fused with ubiquitin at the C-terminus and was cleaved by a ubiquitin-specific protease, Ubp1, in *E. coli* as described by Huang and colleagues (25). The His-tagged Hsp90 and NS5A were purified via the nickel–nitrilotriacetic acid (NTA)-agarose method according to the manufacturer's protocol. Protein expression of N-terminally His_6 and S-tagged FKBP38 (His_6-S-tag-FKBP38) and FKBP8 (His_6-S-tag-FKBP8) was induced with the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM and further incubation at 16°C for 4 h. The His_6-S-tag-FKBP38 mutants (1–166, −297, −315, −325) and the His_6-S-tag-FKBP8 mutants (1–382) were purified using NTA agarose. The His_6-S-tag-FKBP38 (1–335), His_6-S-tag-FKBP8 (1–392) and His_6-S-tag-FKBP38-K250E/R254E mutants were purified with CaM-GST-coupled glutathione–Sepharose

columns as follows. The bacterial pellet from 200 ml of culture was resuspended in 10 ml of Buffer A (20 mM Tris-HCl, 200 mM NaCl and 5 mM dithiothreitol, pH 7.5), lysed using ultrasonic disruption and centrifuged at 35 000 *g* for 30 min at 4°C. The purified CaM-GST (3 mg) and $CaCl_2$ (2 mM final concentration) were mixed with the supernatant. The resulting mixture was applied to a glutathione – Sepharose column (1 ml bed volume; GE Healthcare) and then the column was washed with 5 ml of Buffer A supplemented with 0.2 mM $CaCl_2$. His_6-S-tag-FKBP38 or His_6-S-tag-FKBP8 was eluted using Buffer A supplemented with 0.5 mM ethylene glycol tetraacetic acid (EGTA). The concentration and purity of the isolated proteins were determined with a Bradford assay (Bio-Rad, Hercules, CA, USA) and Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Pull-down assay

To assess the binding of S100 proteins, Hsp90 and NS5A to FKBP8/FKBP38, His_6-S-tag-FKBP8/FKBP38 and either S100 proteins, Hsp90 or NS5A, were mixed with S-protein-agarose beads (20 µl) in Buffer B (20 mM Tris-HCl, 150 mM NaCl and 0.02% Tween 20, pH 7.5) in the presence of 1 mM $CaCl_2$ or EGTA. The reaction mixtures (200 µl) were incubated for 60 min at 25°C. After the resin was washed three times with 1.0 ml of Buffer B, the resin was boiled in SDS-sample buffer (30 µl). Next, the samples were subjected to SDS-PAGE and visualized with Coomassie Blue staining. The details of the experimental conditions are described in the figure legends.

Surface Plasmon Resonance (SPR)

Binding kinetics were analysed using a SPR Biacore 2000 system (Biacore AB, Little Chalfont, Buckinghamshire, UK). CM5 research grade chips, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Biacore amine coupling kit) were used for the amine coupling of the recombinant N-terminally His_6 and S-tagged FKBP8 (His_6-S-tag-FKBP8) (4540RU) to the dextran surface of the CM5 chip. Recombinant S100A1, S100A2, S100A6, S100B, S100P and CaM (1.25 µM, 625 nM, 313 nM, 156 nM and 78 nM respectively) were injected over the sensor surface at a flow rate of 20 µl/min in HBS-P buffer (10 mM Hepes, 150 mM NaCl and 0.005% Surfactant P20, pH 7.4) containing 1 mM $CaCl_2$. The S100 proteins were allowed to interact with the surface of the sensor chip for 2.5 min, after which HBS-P buffer was injected over the sensor surface to monitor the dissociation of the S100 protein. At intervals based on the sample injection, an FKBP8-coupled sensor chip was regenerated with HBS-P buffer supplemented with 2.5 mM EGTA and 0.75% n-Octyl-ß-D-glucopyranoside. The response curves were prepared for fit using a subtraction of the signal generated simultaneously on the control flow cell. Biacore sensorgram curves were evaluated in BIA-evaluation 4.1 using a 1:2 binding model for the S100 proteins. A 1:1 Langmuir model was used for the CaM binding.

Cell culture, transfection and preparation of cell lysates

HuH-7-derived cells harbouring HCV replicon harbouring cells (sO cells) were maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 0.3 mg/ml G418 (Promega, Fitchburg, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified 5% CO_2 incubator at 37°C as described (21). Transient transfections were performed using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The sO cells (10 cm dish) were transfected with pME18S-S100A1, pME18S-S100A2 or pME18S-S100A6 (each 7 µg). After 8 h of incubation with the transfection reagents, the medium was changed and cells were grown until 100% confluent. Next, the cells were treated with or without A23187 (5 µM) for 6, 12 and 24 h and were then lysed via the addition of 1 ml of the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet-P40, 10% glycerol, pH 7.5), briefly sonicated and centrifuged at 15 000 *g* for 30 min at 4°C. The supernatants were subjected to SDS-PAGE, followed by Western blot analysis.

Results

Interaction between the members of the S100 protein family and FKBP38

Previously, we demonstrated that S100A1, S100A2 and S100A6 interacted with the TPR domains of Hop, Tom70, CyP40, FKBP52 and KLC in a Ca^{2+}-dependent manner and led to the disruption of the TPR protein –client protein interactions *in vitro* and *in vivo* (9, 20). Because S100 proteins interact with TPR motifs, we explored the potential role of S100 proteins in regulating the functions of FKBP8/FKBP38. Firstly, we examined the interaction of the S100 proteins with FKBP8/FKBP38. FKBP8/FKBP38, a TPR-containing non-canonical member of the immunosuppressive drug FK506-binding protein (FKBP) family, consists of four structural and functional domains. The FK506-binding domain is located in the N-terminal half, followed by a TPR domain, a putative CaM-binding site and a transmembrane domain. Human FKBP8 is identical to FKBP38 except for the extra 58 amino acid residues at the N-terminus. The domain structure organization of FKBP8/FKBP38 is shown in Figure 1a. Because our preliminary binding analyses demonstrated that the last 10 residues immediately upstream of the transmembrane domain (^{326}AWSIPWKWLF^{335}) in FKBP38, but not the putative CaM-binding site, compose the actual

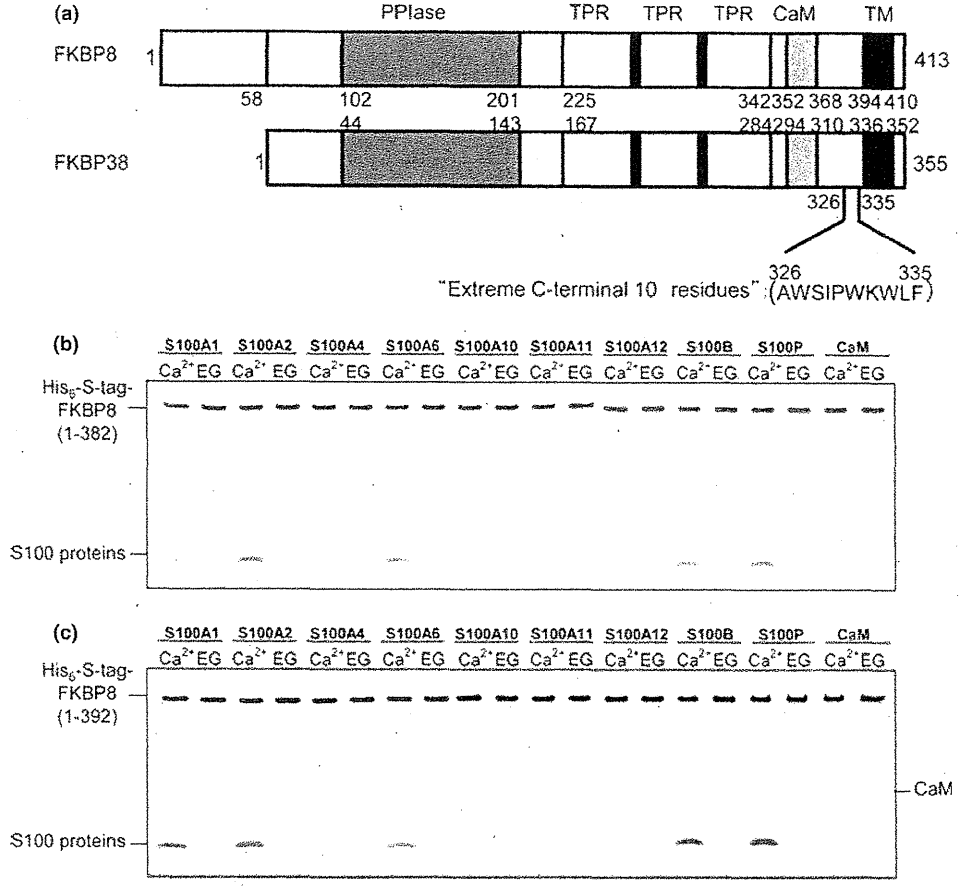
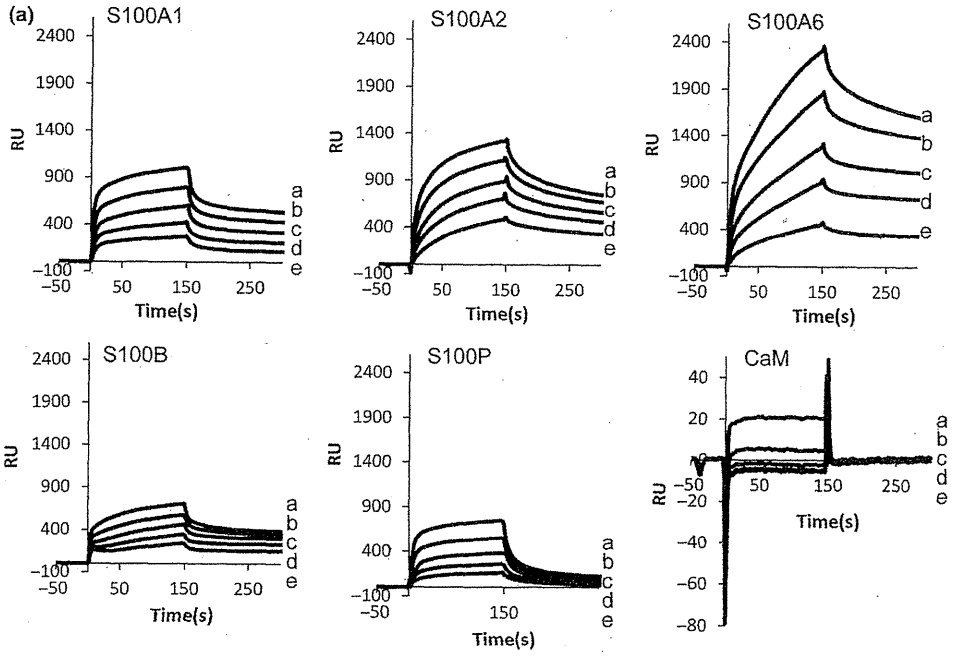


Fig. 1. Interaction of FKBP8 and S100 proteins. (a) Schematic representation of FKBP8 and FKBP38. The peptidyl prolyl *cis/trans* isomerases (PPIase), tetratricopeptide repeat (TPR), calmodulin-binding site (CaM), 'extreme C-terminal 10 residues' and transmembrane domain (TM) are shown. The number refers to the amino acid positions of FKBP8. (b) The S-tag pull-down assay was performed using His_6-S-tag-FKBP8 (1–382) and the S100 proteins. (c) The S-tag pull-down assay was performed using His_6-S-tag-FKBP8 (1–392) and the S100 proteins. The His_6-S-tag-FKBP8 (20 μg) and S100 proteins (20 μg) were incubated with S-protein agarose beads in the presence of either 1 mM $CaCl_2$ or EGTA for 1 h. Beads were washed and samples were separated with 10% Tricine–SDS-PAGE and stained with Coomassie Blue.

Ca^{2+}/CaM-binding site, we prepared a recombinant His_6-S-tag-FKBP8 (1–382) and a His_6-S-tag-FKBP8 (1–392). A precise analysis of the S100 proteins–FKBP38 binding will be described elsewhere (S. Shimamoto, H. Tokumitsu, M. Tsuchiya, Y. Kubota and R. Kobayashi, unpublished data). To test the direct binding of the S100 proteins to FKBP8, we performed a pull-down assay using the S100 proteins with His_6-S-tag-FKBP8 (1–382) or His_6-S-tag-FKBP8 (1–392) in the presence of 1 mM $CaCl_2$ or EGTA. The bound proteins were analysed using Tricine–SDS-PAGE. As shown in Figure 1b, c, S100A1, S100A2, S100A6, S100B and S100P bound strongly to His_6-S-tag-FKBP8 (1–382) or His_6-S-tag-FKBP8 (1–392) in a Ca^{2+}-dependent manner, whereas CaM did not bind to His_6-S-tag-FKBP8 (1–382). In addition, S100A4, S100A10 and S100A11 bound weakly to His_6-S-tag-FKBP8 (1–392).

To examine the real-time binding kinetics of S100A1, S100A2, S100A6, S100B, S100P and CaM to FKBP8, the recombinant His_6-S-tag-FKBP8 (1–392) was immobilized on a sensor chip surface and the protein complex formation was analysed using SPR (Fig. 2a). The binding curves of S100A1, S100A2, S100A6, S100B and S100P were fit to the 1:2 ligand-binding model, whereas the binding curve of CaM was fit to the 1:1 ligand-binding model. The association rate constant (Ka) of S100A1, S100B and S100P to the immobilized FKBP8 (1–392) is indistinguishable and the association of S100A6 and S100A2 occurred more slowly compared with that of other S100 proteins. This suggested an underlying interaction mechanism that was different among the S100 proteins (S100A1, S100B, S100P vs. S100A2, S100A6) (Fig. 2b). The binding concentration of the S100 proteins was measured using a sensorgram. The resonance unit (RU) correlates with the amount of analyte bound (1 RU = 1 pg/mm^2), and the amount of the S100 proteins and CaM binding to FKBP8 (1–392) were significantly different



(b) **The binding affinity of FKBP8 and S100 protein as analyzed by the Biacore**

Analyte	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/Ms)	kd2 (1/s)	KA1 (1/M)	KA2 (1/M)	KD1(M)	KD2 (M)
S100A1	1.08×10^{5}	0.98×10^{-4}	0.38×10^{5}	0.16×10^{-4}	1.11×10^{8}	0.2×10^{10}	9.03×10^{-9}	4.19×10^{-10}
S100A2	0.90×10^{5}	7.15×10^{-4}	0.61×10^{5}	0.08×10^{-4}	0.13×10^{8}	0.73×10^{10}	0.79×10^{-9}	1.37×10^{-10}
S100A6	0.68×10^{5}	5.31×10^{-4}	0.15×10^{5}	0.12×10^{-4}	1.29×10^{8}	0.13×10^{10}	7.76×10^{-9}	7.78×10^{-10}
S100B	5.47×10^{5}	1.17×10^{-4}	2.60×10^{5}	0.27×10^{-4}	4.68×10^{8}	0.97×10^{10}	2.14×10^{-9}	1.03×10^{-10}
S100P	1.21×10^{5}	1.60×10^{-4}	1.99×10^{5}	0.08×10^{-4}	0.76×10^{8}	2.45×10^{10}	0.13×10^{-9}	0.41×10^{-10}
CaM	0.36×10^{5}	0.10×10^{-4}			3.53×10^{8}		2.83×10^{-9}	

Fig. 2. Analysis of FKBP8 and S100 protein binding by *Surface Plasmon Resonance (SPR).* (a) Recombinant His_6-S-tag-FKBP8 was immobilized to the dextran surface of the CM5 chip in 20 mM ammonium acetate, pH 4.2, until 4540 response units (0.3 pmol) were bound and a stable base line was obtained. Recombinant S100A1, S100A2, S100A6, S100B, S100P, S100A12 and CaM were injected at various concentrations (a: 1.25 μM, b: 625 nM, c: 313 nM, d: 156 nM and e: 78 nM). The response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. (b) Biacore sensorgram curves were evaluated using BIAevaluation 3.0 using a 1:1 Langmuir model (for CaM) or 1:2 binding model (for the S100 proteins). The calculated kinetic parameters of S100 protein binding to FKBP8 were presented.

(S100A6 > S100A2 > S100A1 ≥ S100P > S100B >> CaM) (Fig. 2).

S100 protein binding to FKBP38

Previous co-immunoprecipitation studies indicated that both NS5A and Hsp90 bound to the TPR domain of FKBP8 and that the interaction between NS5A and FKBP8 did not affect complex formation with Hsp90 (11, 12). Hsp90 has been reported to bind to the two-carboxylate clamp positions within the TPR domain of FKBP8/FKBP38 through its EEVD motif (11, 29), whereas NS5A binds to the FKBP8-TPR through its Val/Ile^{121} residue (12). To define the binding domain of the S100 proteins in FKBP38, the various length FKBP38 and point mutants fused with S-tagged FKBP38 (1–166), FKBP38 (1–297), FKBP38 (1–315), FKBP38 (1–325), FKBP38 (1–335) and KR (where the

250 Lys, 254 Arg in the TPR domain were replaced with Ala) were constructed (Fig. 3a). Each mutant protein was incubated with S100A1, S100A2, S100A6, S100B, S100P, CaM, Hsp90 and NS5A. NS5A and all the S100 proteins bound to all the FKBP 38 truncation mutants and the KR mutant, but did not bind to FKBP38 (1–166) (Fig. 3b). These data indicate that the S100 proteins interact with the C-terminal portion [including TPR and putative CaM-binding domain) of FKBP38 and do not interact with the FK506-binding domain (i.e. FKBP38 (1–166)] (Fig. 3b). In contrast, the last 10 C-terminus residues of FKBP38 are required for the binding of CaM, and the two-carboxylate clamp is essential for the binding of Hsp90. Taken together, these observations prove the direct interaction of NS5A, Hsp90 and the S100 proteins with FKBP38 and imply the existence of similar, but not identical, binding sites of the S100 proteins, NS5A and Hsp90 within FKBP38.

S100 proteins interfere with the interactions of TPR-mediated FKBP8/FKBP38–HSP90 and FKBP8/FKBP38–NS5A

To study whether S100 proteins interfere with the binding of Hsp90 and NS5A to FKBP8/FKBP38, we performed competitive pull-down assays. Fixed amounts of His_6-S-tag-FKBP8/FKBP38 (25 μg) and Hsp90 (25 μg) were mixed with an increasing amount of the S100 proteins (0–50 μg), and a S-tag pull-down was performed (Fig. 4). SDS-PAGE gels demonstrate the displacement effect of the S100 proteins on the FKBP8–Hsp90 (Fig. 4a) and FKBP38–Hsp90 interactions (Fig. 4b). Furthermore, increasing the amount of the S100 proteins (S100A1, S100A2, S100A6, S100B and S100P) strongly inhibited the binding of His_6-S-tag-FKBP8/FKBP38 with Hsp90, whereas a weaker inhibition was detected with CaM. A significant inhibition was not observed with S100A12 as a negative control. To prove

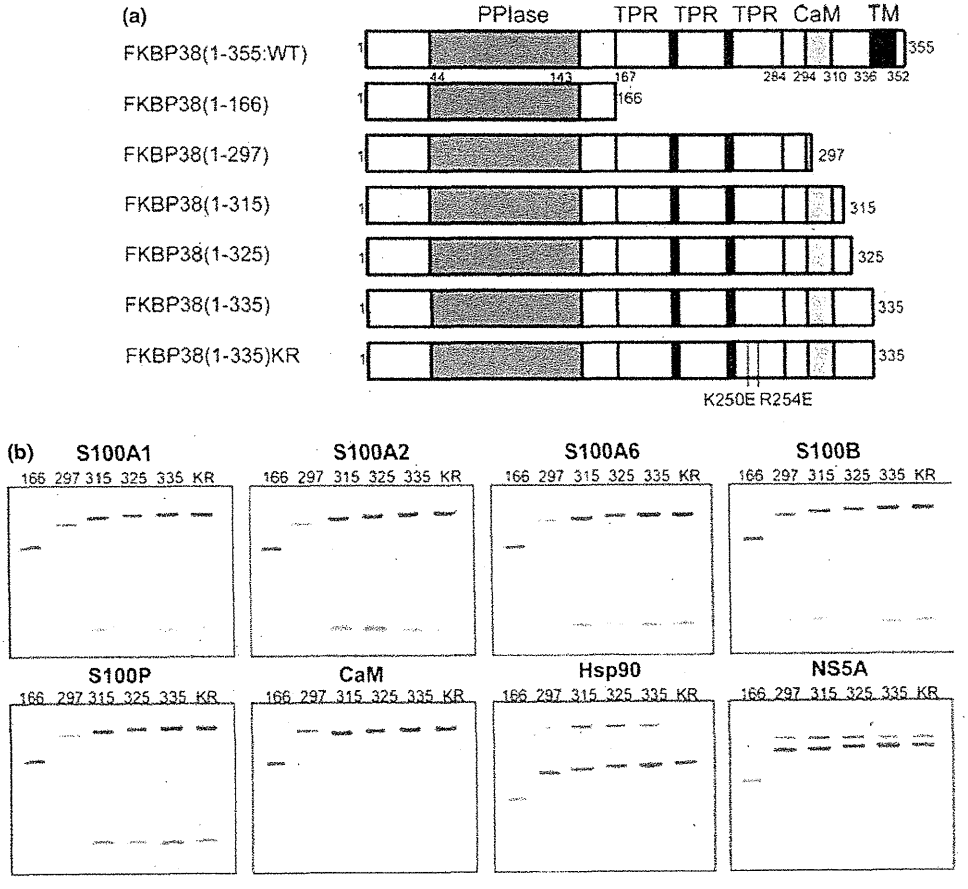


Fig. 3. Interaction of the FKBP8/FKBP38 mutants with the S100 proteins, Hsp90 and NS5A. (a) Schematic diagrams of the FKBP38 mutants. The number refers to the amino acid positions of FKBP38. The peptidyl prolyl *cis/trans* isomerases (PPIase), tetratricopeptide repeat (TPR), calmodulin-binding site (CaM) and transmembrane domain (TM) are shown. (b) The S-tag pull-down assay was performed using His_6-S-tag-FKBP38-WT and it's mutants. The His_6-S-tag-FKBP38 proteins (20 μg) and S100A1, S100A2, S100A6, S100B, S100P, CaM, Hsp90 or NS5A (25 μg each) were incubated with the S-protein agarose beads in the presence of 1 mM $CaCl_2$. Beads were washed and the eluted samples were analysed via a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

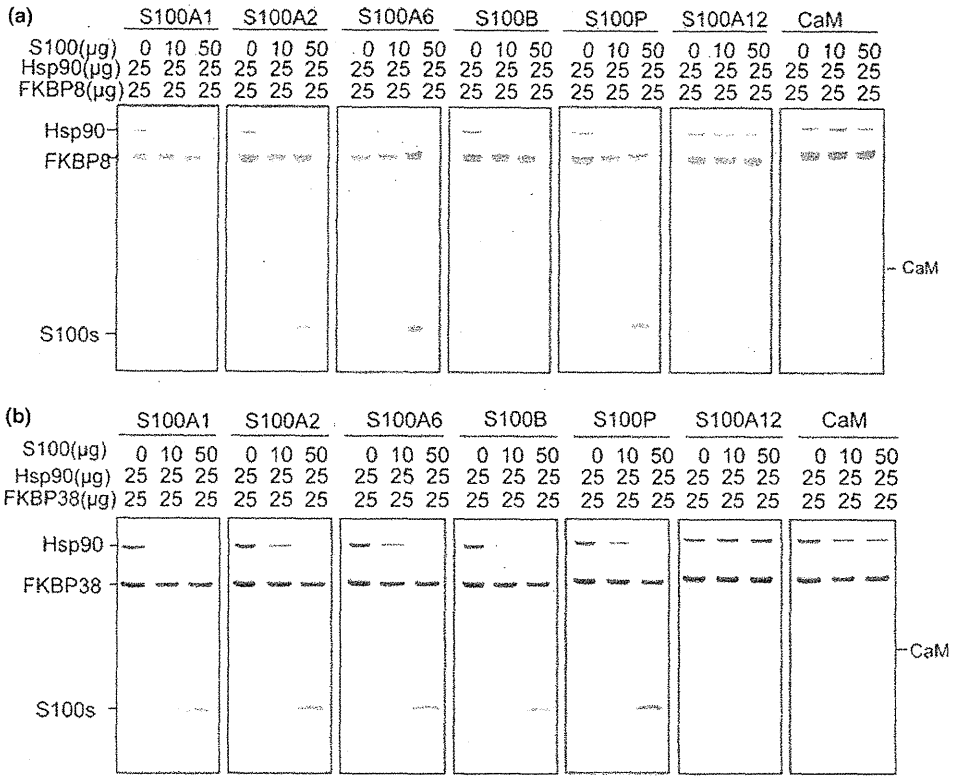


Fig. 4. S100 proteins competitively inhibit Hsp90 binding to FKBP8/FKBP38 *in vitro*. His_6-S-tag-FKBP8 (a, 25 μg) or His_6-S-tag-FKBP38 (b, 25 μg), Hsp90 (25 μg) and increasing amounts of the S100 proteins or CaM (0, 10 and 50 μg) were incubated with S-protein agarose beads in the presence of 1 mM $CaCl_2$ for 1 h. The S-tag pull-down assay was performed as described. Beads were washed and the eluted samples were analysed using a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

this further, a competitive binding assay was also performed with NS5A and His_6-S-tag-FKBP8/FKBP38 (Fig. 5). The addition of purified S100A1, S100A2, S100A6, S100B, S100P and CaM to the binding reactions substantially reduced the amount of NS5A retained on the immobilized His_6-S-tag-FKBP8 (Fig. 5a) and His_6-S-tag-FKBP38 (Fig. 5b). A significant competition was not observed with S100A12 (Figs 4 and 5).

S100 proteins have an inhibitory role in the replication of HCV RNA

The *in vitro* data described above suggest that Ca^{2+}/S100 proteins bind to the TPR domain of FKBP8/FKBP38, and lead to the inhibition of interactions of the FKBP8/FKBP38–Hsp90 and FKBP8/FKBP38–NS5A. Next, to examine whether these interactions are involved in HCV replication, the effect of the overexpression of the S100 protein in combination with ionophore treatment was studied in the HCV replicon harbouring cell line (sO cells) by measuring the amount of the NS3 protein (Fig. 6). We transfected the sO cells with S100A1 (Fig. 6a), S100A2 (Fig. 6b) and S100A6 (Fig. 6c). Following the transfection, the cells were treated with or without A23187 (5 μM) for 6, 12 and 24 h, and the cellular level of NS3 was examined via Western blot analysis using an anti-NS3 antibody. In the control replicon cells, the amount of NS3 did not change in the presence or absence of A23187 treatment. When the S100 proteins were overexpressed, the amount of NS3 was significantly decreased over the incubation period compared with the levels observed in the control cells (*lane 4* vs. *lane 2*). This suggested that increasing the concentration of intracellular Ca^{2+} stimulated the S100 proteins–FKBP8/FKBP38 complex formation, which inhibited the NS5A–FKBP8/FKBP38 and Hsp90–FKBP8/FKBP38 interactions and led to suppress the rate of NS3 production. Collectively, these results demonstrate that the S100 proteins may function as negative regulators of HCV replication in a Ca^{2+}-dependent manner.

Discussion

Hepatitis C virus replication occurs in the cytoplasm and is mediated by a membrane-associated replicase complex consisting of the NS3/NS4A, NS4B, NS5A and NS5B proteins and cellular proteins (host factors) (30).

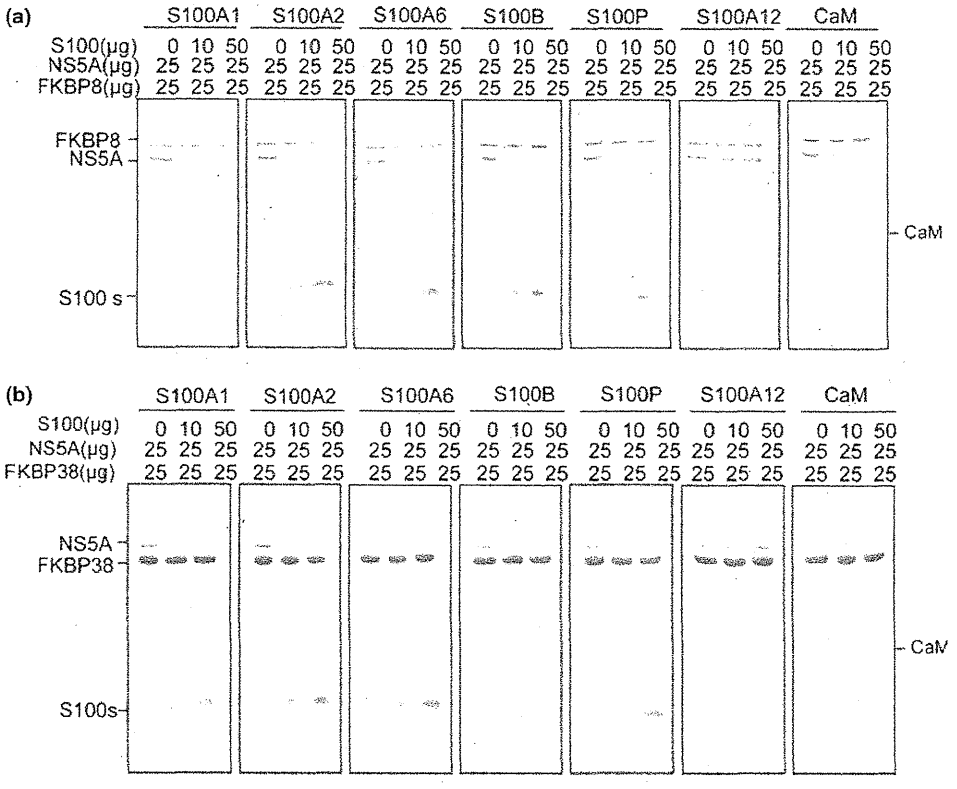


Fig. 5. S100 proteins competitively inhibit NS5A binding to FKBP8/FKBP38 *in vitro*. His_6-S-tag-FKBP8 (a, 25 μg) or His_6-S-tag-FKBP38 (b, 25 μg), NS5A (25 μg) and increasing amounts of the S100 proteins or CaM (0, 10 and 50 μg) were incubated with S-protein agarose beads in the presence of 1 mM $CaCl_2$. The S-tag pull-down assay was performed as described. Beads were washed and the eluted samples were analysed using a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

Importantly, the NS5A of HCV plays a critical role in HCV replication and is an attractive target for antiviral therapy of HCV infection (7).

NS5A is a multifunctional 56–58 kDa serine phosphoprotein and interacts with a number of cellular proteins thereby affecting numerous host functions, including the modulation of signal transduction pathways, suppression of apoptosis and modulation of transcription (8–10). Several cellular proteins, such as hVAP-A (31), hVAP-B (32), FKBP8 (11, 12), Hsp90 (11, 12), Hsp70 (33) and cyclophilin A, B (34, 35) are involved in the replication process of HCV. A recent report indicated that NS5A specifically interacts with FKBP8 via its TPR domain and recruits Hsp90 to the replicase complex; this complex formation is critical for the replication of HCV and thus geldanamycin was able to inhibit the RNA replication in a dose-dependent manner (11). *In vitro* pull-down assays revealed that geldanamycin inhibited the binding of FKBP8 to Hsp90 and/or NS5A domain I. In addition, the interaction between NS5A and FKBP38 disrupts FKBP38-mediated mTOR regulation (10). Moreover, NS5A inhibits apoptosis, potentially via the stabilization of the FKBP38–Hsp90 interactions (36).

TPR proteins are involved in many protein–protein interactions (14, 15); in particular, several cochaperones, including Hip, Hop and the cyclophilins, interact with Hsp70 or Hsp90 through TPR domains (37–40). The intracellular $Ca2^+$ signalling cascade is composed of many molecular components including a large family of EF-hand $Ca2^+$-binding proteins such as calmodulin (CaM), neuronal calcium sensor proteins and S100 proteins (41).

The S100 protein family is composed of at least 25 members that share two EF-hand motifs, a 25–65% amino acid sequence homology, and a molecular weight of 10–12 kDa (16–18).

S100 proteins are proposed to have intracellular and extracellular roles in the regulation of many cellular processes such as cell motility, cell-cycle progression, transcription, protein phosphorylation and tumour progression or suppression (16–18). Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hop, KLC and Tom70 in a Ca^{2+}-dependent manner, leading to the dissociation of the Hsp90-Hop-Hsp70, KLC-c-Jun N-terminal kinase-interacting protein-1 (JIP-1) and Tom70–Hsps interactions (19). Further studies revealed that the interaction

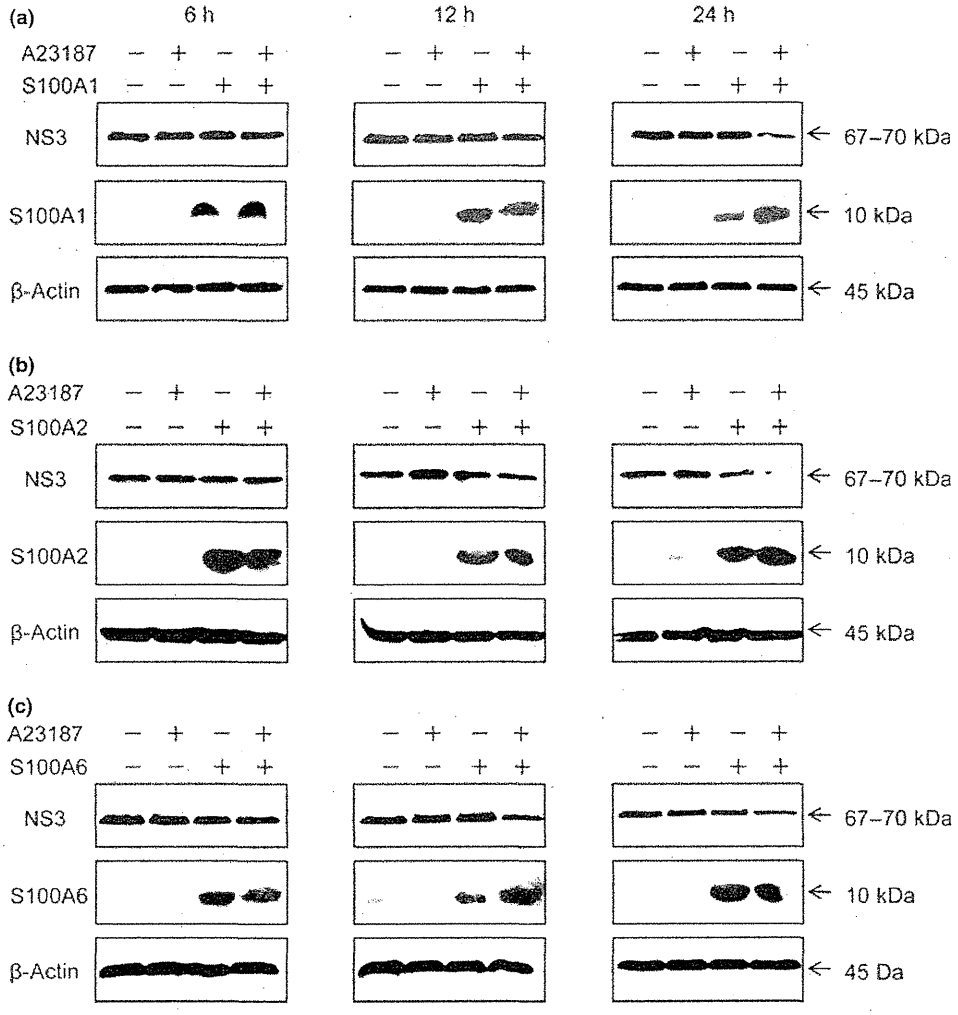


Fig. 6. S100 proteins suppress the cellular level of NS3 in HCV replicon harbouring cells (sO cells). HCV replicon harbouring cells (sO cells) were transiently transfected with (+) or without (–) S100A1 (a), S100A2 (b) and S100A6 (c) as indicated. After A23187 (5 μM) treatment, cell lysates were prepared at different time points (6, 12 and 24 h). The amount of NS3 and S100 proteins was analysed via Western blot analysis with the indicated antibodies. Equal amounts of lysates (20 μg of protein) were loaded in each lane for each time point. β-Actin was used as a control for equal loading.

of S100A1 and S100A2 with FKBP52 and Cyp40, which contain TPR domains, in the presence of Ca^{2+} led to the inhibition of the interactions of Cyp40–Hsp90 and FKBP52–Hsp90 (20).

In this study, we demonstrated that specific members of the S100 proteins, such as S100A1, S100A2, S100A6, S100B and S100P, specifically bind to the TPR domain of FKBP8/FKBP38 in a Ca^{2+}-dependent manner and lead to the inhibition of the Hsp90–FKBP8/FKBP38 interaction. The charged residues in the FKBP8/FKBP38-TPR domains are predicted to form the so-called two-carboxylate clamp form salt bridges with the EEVD of HSP90, and point mutations in the carboxylate clamp diminished the binding (11). Because S100 proteins interfered with the binding of Hsp90 to FKBP8/FKBP38, we anticipated that they bound to the amino acid residues composing the carboxylate clamp. However, S100 proteins bound to the alanine mutants of the carboxylate clamp, although Hsp90 binding was clearly inhibited (Fig. 3b). The results suggested that the mode of interaction of the S100 proteins with FKBP8/FKBP38 is different from the FKBP8/FKBP38–Hsp90 electrostatic interaction.

To explore further the interaction between FKBP8/FKBP38 and the S100 proteins, we investigated whether the S100 proteins inhibited the NS5A–FKBP8/FKBP38 interactions. Surprisingly, these S100 proteins effectively disrupted the NS5A–FKBP8/FKBP38 interactions. The binding site of S100 proteins may be close enough to physically interfere with the Hsp90 and NS5A binding to FKBP8/FKBP38. We have previously shown that the S100 proteins bind to TPR domains of Hop, KLC,

Tom70, Cyp40 and FKBP52, and result in the inhibition of the ligand–TPR protein interactions (19, 20). Notably, there is a preference in S100 protein binding among the TPR proteins. For example, S100A2 bound to KLC, FKBP52 and PP5 more tightly, whereas S100A6 preferably bound to Hop, Cyp40 and Tom70. The reason for the selectivity of S100 protein binding to TPR proteins is not fully understood. Currently, it is not possible to predict the residues that are important for the S100–TPR protein interactions.

Because the ternary complex formation of Hsp90, NS5A and FKBP8/FKBP38 is essential for the replication of HCV, it is important to determine whether the S100 proteins function as 'HCV-replication regulator' in intact cells. Using the HCV replicon harbouring cells (sO cells), the overexpression of S100A1, S100A2 and S100A6 in combination with ionomycin treatments showed a significant decrease in the protein level of NS3. The result suggests that the increase in intracellular Ca^{2+} by the treatment of ionomycin stimulated the binding of the S100 proteins and FKBP8/FKBP38, and inhibited the replication of HCV *in vivo*.

In conclusion, our results are the first to demonstrate that the association of the S100 proteins with FKBP8/FKBP38 provides a Ca^{2+}-dependent regulatory mechanism for the replication of HCV through the regulation of the formation of the NS5A–FKBP8/FKBP8–Hsp90 complex. These findings provide a new intracellular Ca^{2+}-signalling pathway via the interactions of the S100 protein–TPR motif.

Acknowledgements

We would like to thank Dr. Craig E Cameron (Pennsylvania State University) for kindly providing the HCV NS5A plasmids. This research is supported by the Kagawa University Characteristic Prior Research Fund for 2011 (to R.K.).

Disclosures

The authors disclose no competing interests

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41–52.
2. Thomson BJ, Finch RG. Hepatitis C virus infection. *Clin Microbiol Infect* 2005; **11**: 86–94.
3. Pawlotsky JM. Pathophysiology of hepatitis C virus infection and related liver disease. *Trends Microbiol* 2004; **12**: 96–102.
4. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 2005; **5**: 558–67.
5. Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM. Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 1993; **67**: 1385–95.
6. Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc Natl Acad Sci USA* 1991; **88**: 5547–51.
7. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 2004; **85**: 2485–502.
8. He Y, Nakao H, Tan SL, *et al.* Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. *J Virol* 2002; **76**: 9207–17.
9. Street A, Macdonald A, Crowder K, Harris M. The hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. *J Biol Chem* 2004; **279**: 12232–41.
10. Peng L, Liang D, Tong W, Li J, Yuan Z. Hepatitis C virus NS5A activates the mammalian target of rapamycin (mTOR) pathway, contributing to cell survival by disrupting the interaction between FK506-binding protein 38 (FKBP38) and mTOR. *J Biol Chem* 2010; **285**: 20870–81.
11. Okamoto T, Nishimura Y, Ichimura T, *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J* 2006; **25**: 5015–25.
12. Okamoto T, Omori H, Kaname Y, *et al.* A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J Virol* 2008; **82**: 3480–9.
13. Hirano T, Kinoshita N, Morikawa K, Yanagida M. Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. *Cell* 1990; **60**: 319–28.
14. D'Andrea LD, Regan L. TPR proteins: the versatile helix. *Trends Biochem Sci* 2003; **28**: 655–62.
15. Allan RK, Ratajczak T. Versatile TPR domains accommodate different modes of target protein recognition and function. *Cell Stress Chaperones* 2011; **16**: 353–67.
16. Donato R. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta* 1999; **1450**: 191–231.
17. Heizmann CW, Fritz G, Schafer BW. S100 proteins: structure, functions and pathology. *Front Biosci* 2002; **7**: d1356–68.
18. Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* 2001; **33**: 637–68.
19. Shimamoto S, Takata M, Tokuda M, *et al.* Interactions of S100A2 and S100A6 with the tetratricopeptide repeat proteins, Hsp90/Hsp70-organizing protein and kinesin light chain. *J Biol Chem* 2008; **283**: 28246–58.
20. Shimamoto S, Kubota Y, Tokumitsu H, Kobayashi R. S100 proteins regulate the interaction of Hsp90 with cyclophilin 40 and FKBP52 through their tetratricopeptide repeats. *FEBS Lett* 2010; **584**: 1119–25.
21. Ikeda M, Abe K, Dansako H, *et al.* Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* 2005; **329**: 1350–9.
22. Kato N, Sugiyama K, Namba K, *et al.* Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. *Biochem Biophys Res Commun* 2003; **306**: 756–66.
23. Yurimoto S, Hatano N, Tsuchiya M, *et al.* Identification and characterization of wolframin, the product of the wolfram syndrome gene (WFS1), as a novel calmodulin-binding protein. *Biochemistry* 2009; **48**: 3946–55.

24. Tokumitsu H, Hatano N, Tsuchiya M, *et al.* Identification and characterization of PRG-1 as a neuronal calmodulin-binding protein. *Biochem J* 2010; **431**: 81–91.
25. Huang L, Sineva EV, Hargittai MR, *et al.* Purification and characterization of hepatitis C virus non-structural protein 5A expressed in escherichia coli. *Protein Expr Purif* 2004; **37**: 144–53.
26. Okada M, Hatakeyama T, Itoh H, *et al.* S100A1 is a novel molecular chaperone and a member of the Hsp70/Hsp90 multichaperone complex. *J Biol Chem* 2004; **279**: 4221–33.
27. Yamashita K, Oyama Y, Shishibori T, *et al.* Purification of bovine S100A12 from recombinant escherichia coli. *Protein Expr Purif* 1999; **16**: 47–52.
28. Hayashi N, Izumi Y, Titani K, Matsushima N. The binding of myristoylated N-terminal nonapeptide from neuro-specific protein CAP-23/NAP-22 to calmodulin does not induce the globular structure observed for the calmodulin-nonmyristylated peptide complex. *Protein Sci* 2000; **9**: 1905–13.
29. Scheufler C, Brinker A, Bourenkov G, *et al.* Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* 2000; **101**: 199–210.
30. Moriishi K, Matsuura Y. Host factors involved in the replication of hepatitis C virus. *Rev Med Virol* 2007; **17**: 343–54.
31. Tu H, Gao L, Shi ST, *et al.* Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 1999; **263**: 30–41.
32. Hamamoto I, Nishimura Y, Okamoto T, *et al.* Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J Virol* 2005; **79**: 13473–82.
33. Gonzalez O, Fontanes V, Raychaudhuri S, *et al.* The heat shock protein inhibitor quercetin attenuates hepatitis C virus production. *Hepatology* 2009; **50**: 1756–64.
34. Watashi K, Ishii N, Hijikata M, *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111–22.
35. Nakagawa M, Sakamoto N, Tanabe Y, *et al.* Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. *Gastroenterology* 2005; **129**: 1031–41.
36. Simonin Y, Disson O, Lerat H, *et al.* Calpain activation by hepatitis C virus proteins inhibits the extrinsic apoptotic signaling pathway. *Hepatology* 2009; **50**: 1370–9.
37. Blatch GL, Lassle M. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *BioEssays* 1999; **21**: 932–9.
38. Nelson GM, Huffman H, Smith DF. Comparison of the carboxy-terminal DP-repeat region in the co-chaperones hop and hip. *Cell Stress Chaperones* 2003; **8**: 125–33.
39. Chen S, Smith DF. Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. *J Biol Chem* 1998; **273**: 35194–200.
40. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* 2003; **228**: 111–33.
41. Schafer BW, Heizmann CW. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci* 1996; **21**: 134–40.

Cell Host & Microbe

Antimicrobial Peptide LL-37 Produced by HSV-2-Infected Keratinocytes Enhances HIV Infection of Langerhans Cells

Youichi Ogawa,[1] Tatsuyoshi Kawamura,[1,*] Takamitsu Matsuzawa,[1] Rui Aoki,[1] Peter Gee,[4] Atsuya Yamashita,[2] Kohji Moriishi,[2] Kenshi Yamasaki,[3] Yoshio Koyanagi,[4] Andrew Blauvelt,[5] and Shinji Shimada[1]
[1]Department of Dermatology
[2]Department of Microbiology
Faculty of Medicine, University of Yamanashi, Yamanashi 409-3898, Japan
[3]Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[4]Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[5]Oregon Medical Research Center, Portland, OR 97223, USA
*Correspondence: tkawa@yamanashi.ac.jp
http://dx.doi.org/10.1016/j.chom.2012.12.002

SUMMARY

Herpes simplex virus (HSV)-2 shedding is associated with increased risk for sexually acquiring HIV. Because Langerhans cells (LCs), the mucosal epithelium resident dendritic cells, are suspected to be one of the initial target cell types infected by HIV following sexual exposure, we examined whether and how HSV-2 affects HIV infection of LCs. Although relatively few HSV-2/HIV-coinfected LCs were detected, HSV-2 dramatically enhanced the HIV susceptibility of LCs within skin explants. HSV-2 stimulated epithelial cell production of antimicrobial peptides (AMPs), including human β defensins and LL-37. LL-37 strongly upregulated the expression of HIV receptors in monocyte-derived LCs (mLCs), thereby enhancing their HIV susceptibility. Culture supernatants of epithelial cells infected with HSV-2 enhanced HIV susceptibility in mLCs, and this effect was abrogated by blocking LL-37 production. These data suggest that HSV-2 enhances sexual transmission of HIV by increasing HIV susceptibility of LCs via epithelial cell production of LL-37.

INTRODUCTION

Epidemiologic studies have indicated a strong association between the acquisition of HIV and other sexually transmitted diseases (STDs) (Galvin and Cohen, 2004). This link is especially evident in cases of genital ulcer diseases (GUDs), with a 2- to 11-fold increase in the rate of HIV acquisition in the presence of GUD (Cameron et al., 1989; Fleming and Wasserheit, 1999). It is widely recognized that herpes simplex virus type 2 (HSV-2) is a major cause of GUDs, and more than 50 epidemiologic studies have now indicated that HSV-2 shedding is associated with increased risk for acquiring HIV (Wald and Link, 2002). The risk ratio of HIV acquisition for a person with genital herpes is enhanced from 2 to 4 when compared with a person without genital herpes, and potentially 50% of new HIV infections are considered to be attributable or worsened by HSV-2 infection (Wald and Link, 2002).

During sexual transmission of HIV, virus crosses mucosal epithelium and is eventually transmitted to regional lymph nodes, where it establishes permanent infection. Many studies have shown that Langerhans cells (LCs) are one of the important initial cellular targets for HIV, and that this particular type of dendritic cell (DC) plays a crucial role in disseminating HIV (de Witte et al., 2007; Kawamura et al., 2005; Lederman et al., 2006; Shattock and Moore, 2003). LCs are present within genital skin (e.g., outer foreskin) and mucosal epithelium and, after contact with pathogens, readily emigrate from tissue to draining lymph nodes. Immature resident LCs express surface CD4 and CCR5, but not surface CXCR4 (Zaitseva et al., 1997). These LCs are readily infected ex vivo with R5 HIV, but not with X4 HIV (Kawamura et al., 2000, 2008; Reece et al., 1998; Zaitseva et al., 1997). These findings are consistent with previous epidemiologic observations, which have found that the majority of HIV strains isolated from newly infected patients are R5 HIV strains (Zhu et al., 1993). It has been reported that persons with *CCR5* homozygous defects are largely protected from sexually acquiring HIV (Liu et al., 1996).

Clinical trials performed over the last several years have shown that circumcision greatly reduces the probability of penile HIV transmission, suggesting that the foreskin is an important portal of HIV entry (Auvert et al., 2005; Bailey et al., 2007; Gray et al., 2007). Although the mechanism leading to protection remains undefined, several ex vivo experiments with foreskin explants have indicated that CD4 T lymphocytes and LCs within foreskin epidermis are initial target cells for HIV (Fahrbach et al., 2010; Ganor et al., 2010; Grivel et al., 2011; Zhou et al., 2011).

In primate models of simian immunodeficiency virus (SIV) infection, there is controversy regarding which cells in the genital mucosa are initially infected by SIV. Studies have demonstrated that the primary infected cells present in the lamina propria of the cervicovaginal mucosa 48–72 hr after intravaginal exposure to SIV are T cells or submucosal DCs, but not epithelial LCs (Spira et al., 1996; Zhang et al., 1999). When vaginal tissue was examined within 1 hr following vaginal inoculation, however, up to