PPP2R5D Promotes Hepatitis C Virus Infection Through Binding to NS5B Protein

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Research

Abstract

Background: Hepatitis C virus (HCV) is an important human pathogen causing chronic hepatitis C, end-stage liver diseases, and hepatocellular carcinoma. The development of infectious HCV cell culture systems primarily relied on the replication enhancement effect of adaptive mutations. Although the mode of action may vary, those adaptive mutations could direct the study of virus-host interactions required for efficient virus infection. We previously identified a substitution D559G in NS5B (RNA dependent RNA polymerase) critical for the replication of HCV genomes. In this study, we set out to study whether D559G-NS5B specifically interacted with some host factors crucial for HCV infection.

Methods: Through mass spectrometry analysis of immunoprecipitation mixture of ectopically expressed wild-type and D559G-mutated NS5B, we identified candidate factors showing potential interactions with NS5B and D559G-NS5B. The requirement of selected host factor in HCV infection *in vitro* was demonstrated by gene knockout, overexpression, virus infection, and co-immunoprecipitation approaches.

Results: From the results of immunoprecipitation and mass spectrometry analysis, we selected protein phosphatase 2 regulatory subunit B'delta (PPP2R5D) for further characterization. Co-immunoprecipitation confirmed that both wild-type and D559G NS5B proteins interacted with PPP2R5D, but the interaction between D559G-NS5B and PPP2R5D was more efficient. Silencing of PPP2R5D decreased HCV infection, and knockout of PPP2R5D nearly eliminated HCV infection in Huh7.5 cells. Transient and stably overexpression of PPP2R5D in PPP2R5D-knockout cells restored HCV infection to a level close to that seen for wild-type Huh7.5 cells.

Conclusions: PPP2R5D is required for HCV infection in cultured hepatoma cells, and PPP2R5D may function through binding to HCV NS5B. The underlying mechanism of PPP2R5D in the complete HCV life cycle requires further investigation.

Introduction

Most hepatitis C virus (HCV) infections develop chronic hepatitis C, which increases the risk of developing fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). To date, ~71 million people are affected by HCV, with ~1.75 million new infections and ~0.7 million deaths annually (1). In recent years, treatment of hepatitis C has been revolutionized from interferon (IFN)-based to IFN-free direct-acting antivirals (DAAs) regimens, which significantly increases the cure rate up to 95%. Although great success was achieved in HCV therapy, challenges exist in HCV infection, which has included, but not limited to, the lack of an HCV vaccine, global low access to DAA therapy, unceasing progression to HCC and reinfection after the cure, the emergence of drug resistance, etc. Besides, the pathogenesis of HCV infection is incompletely understood.

HCV is a member of the *Hepacivirus* genus in the *Flaviviride* family. The positive RNA genome of 9.6 kb encodes a single polyprotein of ~3,010 amino acids, flanked by 5' and 3' untranslated regions (UTRs),

necessary for viral RNA replication, translation, and stability (2). 5'UTR contains a highly structured internal ribosomal entry site (IRES), which helps to initiate polyprotein translation. The polyprotein is further cleaved by both host and viral proteases to produce three structural (core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Structural proteins form HCV virions, while non-structural proteins are primarily responsible for RNA replication, translation, polyprotein processing, virus assembly, and release. (2).

HCV NS5B is an RNA dependent RNA polymerase (RdRp) required for viral RNA replication (2). Many host factors have been identified to be involved in and regulate HCV RNA replication by different mechanisms, such as disrupting viral proteins, RNA, or the replication complex (3, 4). In general, virus propagation in the cell displays a landscape of complex virus-host interaction, and uncovering host factors involved in the interactions is critical to understand the mechanism of HCV RNA replication and viral pathogenesis.

In developing infectious HCV recombinants and full-length clones, many cell culture adaptive mutations have been identified and demonstrated to initiate or enhance the viral RNA replication or virus production by accelerating the complete HCV life cycle (5, 6). We previously identified three key mutations, F1464L/A1672S/D2979G (LSG), that permitted the replication of full-length HCV clones of genotype 2a and 2b isolates (5–7) and the infectious clones of genotypes 1a, 2c, 3a and 6a (5, 6, 8–10). These studies demonstrate that the LSG mutations enhance HCV RNA replication and virus production. However, the mechanism of LSG function is still elusive. The "G" mutation is located in NS5B thumb domain and corresponding to the D559G of NS5B protein. The D559G change has been predicted to reduce the efficacy of HCV antivirals in a bioinformatics study (11). In this study, we investigated the functional role of NS5B D559G in the virus-host interaction and found that HCV NS5B interacted with cellular PPP2R5D protein, while D559G enhanced the interaction. Further, we demonstrated that PPP2R5D is required for HCV infection in cultured cells.

Materials And Methods

Plasmids and Reagents

Plasmids expressing NS5B-D559G was amplified from J6cc clone (6), and the wild-type NS5B (NS5B-WT) was made by changing 599D to 559G. The N-terminus of NS5B was fused with Flag-tag (NH$_2$-DYKDDDDK-COOH). The D559G (J6 strain nucleotide change A9277G) were introduced or eliminated by PCR. The NS5B-Flag sequences were ligated into the vector plasmid pcDNA3.1 (Addgene, USA). PPP2R5D gene was amplified from human hepatoma cell line Huh7.5 cells (generously provided by Dr. Charles Rice, Apath L.L.C. and Rockefeller University), and HA-tag (NH$_2$-YPYDVPDYA-COOH) was added at N- or C-terminus of PPP2R5D using fusion PCR, cloned into the pEGFP-C1 (Addgene) replacing the eGFP sequence, and designated HA/n-PPP2R5D or PPP2R5D-HA/c, respectively. Untagged PPP2R5D was also constructed. All of the constructs were confirmed by DNA sequencing (Sangon Biotech Company, China).

The antibodies used for western blotting included mouse monoclonal antibodies, anti-Core (ab2740; Abcam, UK), anti-NS3 (ab65407; Abcam), anti-Flag M2 (F3165; Sigma-Aldrich), anti-HA (sc-7392; Santa Cruz, USA), and anti-β-actin (ab8224; Abcam). The secondary antibodies were goat anti-mouse conjugated with horseradish peroxidase (HRP) (ProteinTech, China) or with Alexa Fluor 488 or Fluor 594 (Life Technologies, China). ECL^{TM} anti-mouse IgG and an HRP-linked whole antibody (GE Healthcare, UK) were used for the focus forming unit (FFU) assay.

Cell culture and transfection

Huh7.5 cells (Charles Rice, Apath, L.L.C., and Rockefeller University, USA) and HEK293T cells (CRL-11268; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (complete DMEM media) at 37°C with 5% CO_2. Transfection of HEK293T or Huh7.5 cells were performed by use of Lipofectamine 2000 (Invitrogen, USA) as per the manufacturers' instructions.

HCV RNA transfection, virus production and focus forming unit (FFU) assay

HCV RNA transfection, virus production and focus forming unit (FFU) assay were performed as previously described. (5, 6, 12). To determine the HCV titer, FFU assay was performed as previously described (6). Briefly, 6× 10^3 Huh7.5 cells were seeded in polylysine-coated 96-well plates for 24 h and then infected with serial dilutions of HCV for another 48 h. The cells were fixed and immunostained with the anti-HCV Core antibody C7-50 (Santa Cruz Biotechnology, USA). The percentage of HCV Core positive cells was enumerated under microscope.

HCV infection

Wild-type and PPP2R5D-KO Huh7.5 cells were seeded into 6-well plates (3×10^5 cells per well) and allowed to grow ~16 h before transfection of pC1-PPP2R5D for HCV infection validation. After 16 h of transfection, cells were infected with HCV at a MOI of 0.01 and allowed to grow for the next 48 h. HCV Core protein was detected by western blotting. Total RNA was extracted from the infected cells, and the level of HCV RNA was determined by qRT-PCR.

Generation of PPP2R5D knockdown and knockout cell lines

To generate PPP2R5D knockdown cells, lentivirus pLKO.1 vector (Addgene) was used to express PPP2R5D-targeting short hairpin RNA (shRNA). Two sets of shRNAs were designed: PPP2R5D-sh-F1 (5'-CCGGAGTCTGACTGAGCCGGTAATTCTCGAGAATTACCGGCTCAGTCAGACTTTTTTG-3') and PPP2R5D-sh-R1 (5'-AATTCAAAAAAGTCTGACTGAGCCGGTAATTCTCGAGAATTACCGGCTCAGTCAGACT-3'), PPP2R5D-sh-F2 (5'-CCGGCTTGCTCTCCTAGACCTATTTCTCGAGAAATAGGTCTAGGAGAGCAAGTTTTTG-3') and PPP2R5D-sh-R2 (5'-AATTCAAAAACTTGCTCTCCTAGACCTATTTCTCGAGAAATAGGTCTAGGAGAGCAAG-3'), as well as scramble control shRNAs. PPP2R5D-knockdown Huh7.5 cells were generated by lentivirus infection and puromycin (2 μg/ml) selection.

Knockout of PPP2R5D from Huh7.5 cells was done by CRISPR-Cas9 using sgRNAs as follows: PPP2R5D-1, 5'-CACCGGCTCCGGGCTTATATCCGT-3' and 5'-AAACACGGATATAAGCCCGGAGCC-3', PPP2R5D-2, 5'-CACCTAGCCGTGATGTTGTCACTG-3' and 5'-AAACCAGTGACAACATCACGGCTA-3', as well as control scramble sgRNA, 5'-CACCGACGGAGGCTAAGCGTCGCAA-3' and 5'-AAACTTGCGACGCTTAGCCTCCGTC-3'. Synthetic double-stranded DNA (dsDNA) fragments were cloned in pSpCas9 BB-2A-Puro PX459 (Addgene) (13). The PPP2R5D-KO cells were maintained in the presence of puromycin (1 µg/mL) and replenished every 2-3 days. The PPP2R5D disruption of knockout cell lines were confirmed by sequencing.

For complement expression of PPP2R5D, pC1-PPP2R5D were transfected into PPP2R5D-KO cells, followed by puromycin selection (2 µg/mL) for 72 h and further diluted to selected a monoclonal PPP2R5D-KO cell line that stably expressed PPP2R5D (designated PPP2R5D-Compl).

Immunoprecipitations

HEK 293T cells were co-transfected with D559G-NS5B and pC1-PPP2R5D, WT-NS5B and pC1-PPP2R5D, or each of constructs D559G-NS5B, WT-NS5B, and pC1-PPP2R5D using Lipofectamine 2000 (Life Technologies). For the immunoprecipitation (IP) experiments, 293T cells transfected with plasmids were lysed with 500 µl of IP lysis buffer [50 mM Tris-HCl (pH=7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1% cocktail protein inhibitors] and incubated on ice for 60 min. The cell lysates were clarified by centrifugation at 4°C and 12000 g for 10 min. The total protein concentration was determined using the BCA Protein Assay kit (GenStar, China). 450 µl was used for IP with the mouse anti-Flag or anti-HA antibody. Briefly, the 450 µl lysates were incubated with 5 µg of antibody with gentle rotation at 4°C for 2 h. For each IP experiment, 25 µl of protein A-agarose beads (Santa Cruz Biotechnology) were washed 3 times in IP lysis buffer and incubated with 5 µg of the anti-Flag antibody and lysates with rotation at 4°C overnight. The beads were washed 5 times (5 min/time with rotation) in IP lysis buffer, and finally, 60 µl of IP buffer was added before protein was eluted in 5× protein loading buffer for SDS-PAGE analysis.

Western blot

Total protein was loaded on 10% PAGE with 60-100 µg/lane, separated by SDS-PAGE procedure, and then transferred to a PVDF membrane (0.2 µm) (Bio-Rad, USA). The transferred membrane was blocked with 5% milk at room temperature for 1 h and incubated with primary antibodies at 4°C overnight. A secondary antibody was applied at room temperature for 2 h. Proteins were visualized with an ECL chemiluminescence kit (Proteintech, China) and OPTIMAX X-Ray Film Processor (PROTEC GmbH, Germany).

qRT-PCR

Total viral or cellular RNAs were extracted and quantified using Nanodrop-2000 (Thermo Fisher Scientific, USA), and 1 µg of RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo

Fisher Scientific). Real-time PCR was performed using TaqMan Universal master mix II (4440038, Applied Biosystems) or an ABI 7500 real-time PCR system (Applied Biosystems). Each reaction was performed in triplicates with TaqMan Gene Expression Master Mix (TAKARA, Japan). Levels of cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The mRNA of interest and HCV RNA were calculated by the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) and normalized to the level of GAPDH. The expression pattern was also analyzed by western blot using the HCV anti-Core antibody.

Statistical analysis

All the experiments were performed in triplicates and data are presented either from a representative experiment or as means standard errors of the means (SEM). Comparisons between groups were analyzed by Student's *t* test as indicated. GraphPad Prism software (version 8) was used to make graphs for different HCV infection experiments (GraphPad Software).

Results

Identification of cellular proteins binding to the mutated HCV NS5B

We previously identified the NS5B mutation D559G (D2979G in polyprotein, the "G" of LSG mutations) critical for the initiation of HCV replication and virus productions (5, 6) and here, we hypothesized that presence of D559G may have changed the interaction of NS5B with host proteins, which may facilitate HCV replication. To this end, we constructed wild-type (WT) and mutant D559G NS5B individually, with Flag tag at N-terminus (n-Flag), and confirmed its expression in 293T cells (Fig. 1A). As human hepatoma Huh7.5 cells are primarily used for HCV infections, n-Flag-NS5B-based immunoprecipitation (IP) was performed with the cell lysate of transfected Huh7.5 cells (Fig. 1B). After silver staining of PAGE-separated IP proteins, an extra protein band was visualized in the D559G-NS5B IP pellet mixture (Fig. 1B). The additional band of D559-NS5B, in parallel with the WT-NS5B staining gel at same position, were sliced and sent for mass spectrometry analysis. Several proteins were identified, which theoretically interacted with HCV NS5B. We selected to investigate the role of serine/threonine protein phosphatase 2A regulatory subunit delta isoform (PPP2R5D) in the HCV life cycle, since it was hit in D559G-NS5B only and had a relatively high score in mass spectrometry analysis. In addition, PPP2R5D was previously reported to suppress tumor development (14).

PPP2R5D interacted with NS5B

To confirm the interaction of PPP2R5D with NS5B in the cells, we performed co-immunoprecipitation (Co-IP) for HA-tagged PPP2R5D and Flag-tagged WT-NS5B, as well as HA-tagged PPP2R5D and Flag-tagged D559G-NS5B. The CO-IP experiment was performed with either anti-Flag or anti-HA antibodies to assure the authenticity of PPP2R5D-NS5B interactions (Fig. 2). The results of Co-IP experiment clearly showed that PPP2R5D interacted with WT-NS5B and D559G-NS5B. However, the interaction of PPP2R5D and D559G-NS5B was slightly stronger than that with WT-NS5B (Fig. 2A and B), consistent with the IP experiment, in which PPP2R5D was identified from D559G-NS5B-bound mixture (Fig. 1B). These results

confirmed that HCV NS5B interacted with PPP2R5D, while D559G enhanced the interaction. As D559G is necessary for HCV replication (6, 15), we hypothesized that PPP2R5D might play an essential role in HCV infection.

PPP2R5D was required for HCV infection in cultured cells

We initiated to examine whether PPP2R5D is required for HCV infection *in vitro*. We constructed two lentivirus-based shRNA targeting PPP2R5D, and both shPPP2R5D-1 and -2 reduced the mRNA levels of PPP2R5D by 50-60% in Huh7.5 cells (Fig. 3A). We infected the wild-type and PPP2R5D-knockdown Huh7.5 cells (PPP2R5D-KD) with genotype 2a recombinant J6$^{5'UTR-NS2}$/JFH1 (MOI=0.01) (12). The results showed that PPP2R5D-KD cells released HCV infectivity titers lower than the scramble shRNA-treated and wild-type Huh7.5 cells by ~55 folds (Fig. 3B), suggesting that PPP2R5D supported HCV infection.

To further validate the supportive role of PPP2R5D in HCV life cycle, we generated PPP2R5D-knockout Huh7.5 cells (PPP2R5D-KO) using CRISPR/Cas9 and confirmed the disruption of PPP2R5D gene by DNA sequencing; one nucleotide insertion was identified at the sgRNA-guide breakage region in PPP2R5D gene, which creates a frameshift mutation (Fig. 3C). The PPP2R5D-KO Huh7.5 cells were infected with HCV (MOI=0.01), and infection titer, as well as immunofluorescence, was performed to check HCV infection (Fig. 3D). The infection rate was reduced more than 95% in PPP2R5D-KO cells as compared with the wild-type cells (Fig. 3D).

To further investigate the effect of PPP2R5D in HCV infection, PPP2R5D was overexpressed in wild-type and PPP2R5D-KO Huh 7.5 cells (Fig. 3E). As expected, a very low HCV infection was detected in PPP2R5D-KO cells, while overexpression PPP2R5D restored HCV infection in PPP2R5D-KO cells to ~95% of that infection in wild-type and eGFP plasmid-transfected control Huh7.5 cells (Fig. 3E and F). Noting that overexpression of PPP2R5D or control eGFP in wild-type Huh7.5 cells resulted in ~20% reduction of HCV infection, indicating that plasmid transfection procedures affected partially HCV infection. The expression of PPP2R5D and HCV Core proteins was validated by western blotting, and HCV Core levels mirrored the immunofluorescence observed in the cultures (Fig. 3E and G).

PPP2R5D-KO cells complimentary expressing wild-type PPP2R5D rescued HCV infections

To further confirm the requirement of PPP2R5D for HCV infection *in vitro*, we selected a PPP2R5D-KO cell line stably expressing PPP2R5D. We constructed PPP2R5D-expressingg plasmid and transfected into PPP2R5D-KO cells (Fig. 4A). After puromycin selection, we obtained a stable PPP2R5D-KO cell line complementally expressing PPP2R5D and designated PPP2R5D-Compl (Fig. 4B). The complementation of PPP2R5D rescued HCV infection, as PPP2R5D-Compl produced HCV infectivity titers close to that of wild-type Huh7.5 cells (Fig. 4B). Together with the results of PPP2R5D knockout and transient transfections, these results strongly demonstrate that PPP2R5D was required for HCV infection in cultured cells.

Discussion

Here, we identified PPP2R5D as a host factor required HCV infection in Huh7.5 cells. PPP2R5D was first found as it differentially present in immunoprecipitation assay with D559G-mutant and WT NS5B protein of HCV. Then, we demonstrated that knockout of PPP2R5D eliminated HCV infection in Huh7.5 cells, while transiently and stably complemented PPP2R5D expression rescued HCV infections (Fig. 3 and Fig. 4). CO-IP experiments revealed that PPP2R5D directly interacted with HCV NS5B, and such interaction was slightly enhanced by D559G (Fig. 2). Although the mechanism of PPP2R5D in HCV infection had not been elucidated in this study, it is most likely that PPP2R5D promoted HCV infection through binding to NS5B. D559G mutation enhanced the binding of PPP2R5D with NS5B. These results may explain, at least in part, the enhancement effect of D559G in NS5B for HCV replication (6, 15).

PPP2R5D is a subunit of PP2A enzymes, which is involved in many regulating pathways and turn on or off gene expression by removing the phosphate group from proteins (16). Protein phosphatase 2A (PP2A) is a major and multifunctional serine/threonine-specific phosphatase consisting of structural subunit A, a regulatory subunit B, and a catalytic subunit C (17). PPP2R5D plays a major role in negative regulation of the PI3K/AKT signaling pathway, autism or other brain related disorders (17). PP2A have been evaluated in cancer progression and these group of proteins have shown the interaction with different HCV proteins (16, 18–20). A recent study demonstrated that PPP2R5 proteins interacted with HIV Vif protein and affected HIV pathogenesis (21). Here, we identified an important role of PPP2R5D in HCV infection warrants future studies to explore the underlying mechanism of PPP2R5D in the complete HCV life cycle, which will facilitate our understanding of HCV pathogenesis.

Conclusions

We have demonstrated that cellular PPP2R5D protein was required for HCV infection in hepatoma cells. PPP2R5D and HCV NS5B had a direct interaction, to which D559G showed an enhancement effect. This study discovers new host factor important for the HCV life cycle, facilitating the studies of virus-host interaction and pathogenesis of HCV.

List Of Abbreviations

Hepatitis C virus HCV

Hepatocellular carcinoma HCC

Interferon IFN

Direct-acting antivirals DAAs

Untranslated regions UTRs

Internal ribosomal entry site IRES

RNA dependent RNA polymerase RdRp

Non-structural NS

Wild-type NS5B NS5B-WT

Horseradish peroxidase HRP

Focus forming unit FFU

Dulbecco's modified Eagle's medium DMEM

Fetal bovine serum FBS

Short hairpin RNA shRNA

Double-stranded DNA dsDNA

Glyceraldehyde-3-phosphate dehydrogenase GAPDH

Comparative threshold cycle C_T

Standard errors of the means SEM

Serine/threonine protein phosphatase 2A regulatory subunit delta isoform PPP2R5D

co-immunoprecipitation Co-IP

PPP2R5D-knockdown Huh7.5 cells PPP2R5D-KD

Declarations

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Author contributions

Y.-P.L. and Y.Z. conceived and designed research M.I.A., N.L., Q.Z., C.H., H.C., T.W. performed research M.I.A., Y.-P.L., Y.Z. analyzed data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable. This is a pure *in vitro* study, no any animal or human data or tissue used.

Consent to publication

This is an in vitro study; all authors are consent to its publication.

References

1. Spearman CW, Dusheiko GM, Hellard M, Sonderup M. Hepatitis C. Lancet (London, England). 2019;394(10207):1451-66.

2. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nature Reviews Microbiology. 2007;5(6):453-63.

3. Li HC, Yang CH, Lo SY. Hepatitis C Viral Replication Complex. Viruses. 2021;13(3).

4. Li Q, Zhang YY, Chiu S, Hu Z, Lan KH, Cha H, et al. Integrative functional genomics of hepatitis C virus infection identifies host dependencies in complete viral replication cycle. PLoS pathogens. 2014;10(5):e1004163.

5. Li Y-P, Ramirez S, Mikkelsen L, Bukh J. Efficient Infectious Cell Culture Systems of the Hepatitis C Virus (HCV) Prototype Strains HCV-1 and H77. Journal of virology. 2014;89.

6. Li Y-P, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, Bukh J. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc Natl Acad Sci U S A. 2012;109(48):19757-62.

7. Ramirez S, Li Y-P, Jensen S, Pedersen J, Gottwein J, Bukh J. Highly Efficient Infectious Cell Culture of Three Hepatitis C Virus Genotype 2b Strains and Sensitivity to Lead Protease, Nonstructural Protein 5A, and Polymerase Inhibitors. Hepatology (Baltimore, Md). 2014;59.

8. Chen M, Zheng F, Yuan G, Duan X, Rong L, Liu J, et al. Development of an Infectious Cell Culture System for Hepatitis C Virus Genotype 6a Clinical Isolate Using a Novel Strategy and Its Sensitivity to Direct-Acting Antivirals. Frontiers in microbiology. 2018;9:2950-.

9. Pham LV, Ramirez S, Gottwein JM, Fahnøe U, Li YP, Pedersen J, et al. HCV Genotype 6a Escape From and Resistance to Velpatasvir, Pibrentasvir, and Sofosbuvir in Robust Infectious Cell Culture Models. Gastroenterology. 2018;154(8):2194-208.e12.

10. Ramirez S, Fernandez-Antunez C, Mikkelsen LS, Pedersen J, Li YP, Bukh J. Cell Culture Studies of the Efficacy and Barrier to Resistance of Sofosbuvir-Velpatasvir and Glecaprevir-Pibrentasvir against Hepatitis C Virus Genotypes 2a, 2b, and 2c. Antimicrob Agents Chemother. 2020;64(3).

11. Manjula S, Sivanandam M, Kumaradhas P. Probing the "fingers" domain binding pocket of Hepatitis C virus NS5B RdRp and D559G resistance mutation via molecular docking, molecular dynamics simulation and binding free energy calculations. J Biomol Struct Dyn. 2019;37(9):2440-56.

12. Li Y-P, Gottwein JM, Scheel TK, Jensen TB, Bukh J. MicroRNA-122 antagonism against hepatitis C virus genotypes 1-6 and reduced efficacy by host RNA insertion or mutations in the HCV 5' UTR. Proc Natl Acad Sci U S A. 2011;108(12):4991-6.

13. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157(6):1262-78.

14. Lambrecht C, Libbrecht L, Sagaert X, Pauwels P, Hoorne Y, Crowther J, et al. Loss of protein phosphatase 2A regulatory subunit B56δ promotes spontaneous tumorigenesis in vivo. Oncogene. 2018;37(4):544-52.

15. Li YP, Ramirez S, Gottwein JM, Scheel TK, Mikkelsen L, Purcell RH, et al. Robust full-length hepatitis C virus genotype 2a and 2b infectious cultures using mutations identified by a systematic approach applicable to patient strains. Proc Natl Acad Sci U S A. 2012;109(18):E1101-10.

16. O'Connor CM, Perl A, Leonard D, Sangodkar J, Narla G. Therapeutic targeting of PP2A. The International Journal of Biochemistry & Cell Biology. 2018;96:182-93.

17. Sandal P, Jong CJ, Merrill RA, Song J, Strack S. Protein phosphatase 2A - structure, function and role in neurodevelopmental disorders. Journal of cell science. 2021;134(13).

18. Duong FHT, Christen V, Berke JM, Penna SH, Moradpour D, Heim MH. Upregulation of protein phosphatase 2Ac by hepatitis C virus modulates NS3 helicase activity through inhibition of protein arginine methyltransferase 1. Journal of virology. 2005;79(24):15342-50.

19. Duong FHT, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. Gastroenterology. 2004;126(1):263-77.

20. 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.

21. Salamango DJ, Ikeda T, Moghadasi SA, Wang J, McCann JL, Serebrenik AA, et al. HIV-1 Vif Triggers Cell Cycle Arrest by Degrading Cellular PPP2R5 Phospho-regulators. Cell reports. 2019;29(5):1057-65.e4.
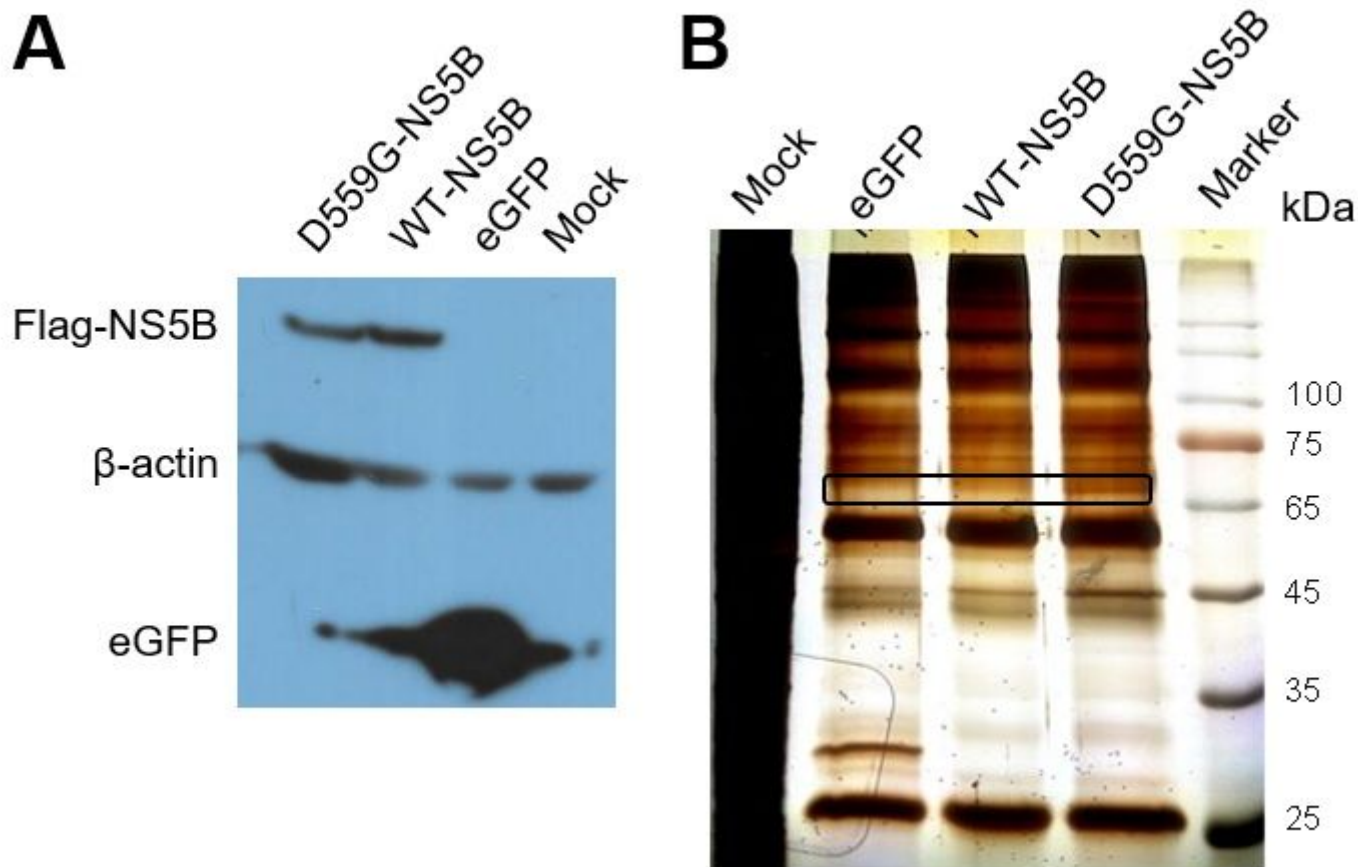
Figures

Figure 1

Ectopic expression and immunoprecipitation of D559G-NS5B and wild-type (WT)-NS5B. (A) Plasmids expressing Flag-tagged WT-NS5B and D559G-NS5B were transfected into 293T cells, and NS5B expression was detected by western blot using anti-Flag antibody 24 h post-transfection. Plasmid expressing eGFP was used as transfection control, and β-actin was detected as an internal control. (B) Silver staining of PAGE gel after separation of Flag-tagged NS5B-immunoprecipitated mixtures. D559G-NS5B showed an extra band (boxed), of which the slices from WT-NS5B and D559G-NS5B lanes were analyzed by mass spectrometry.
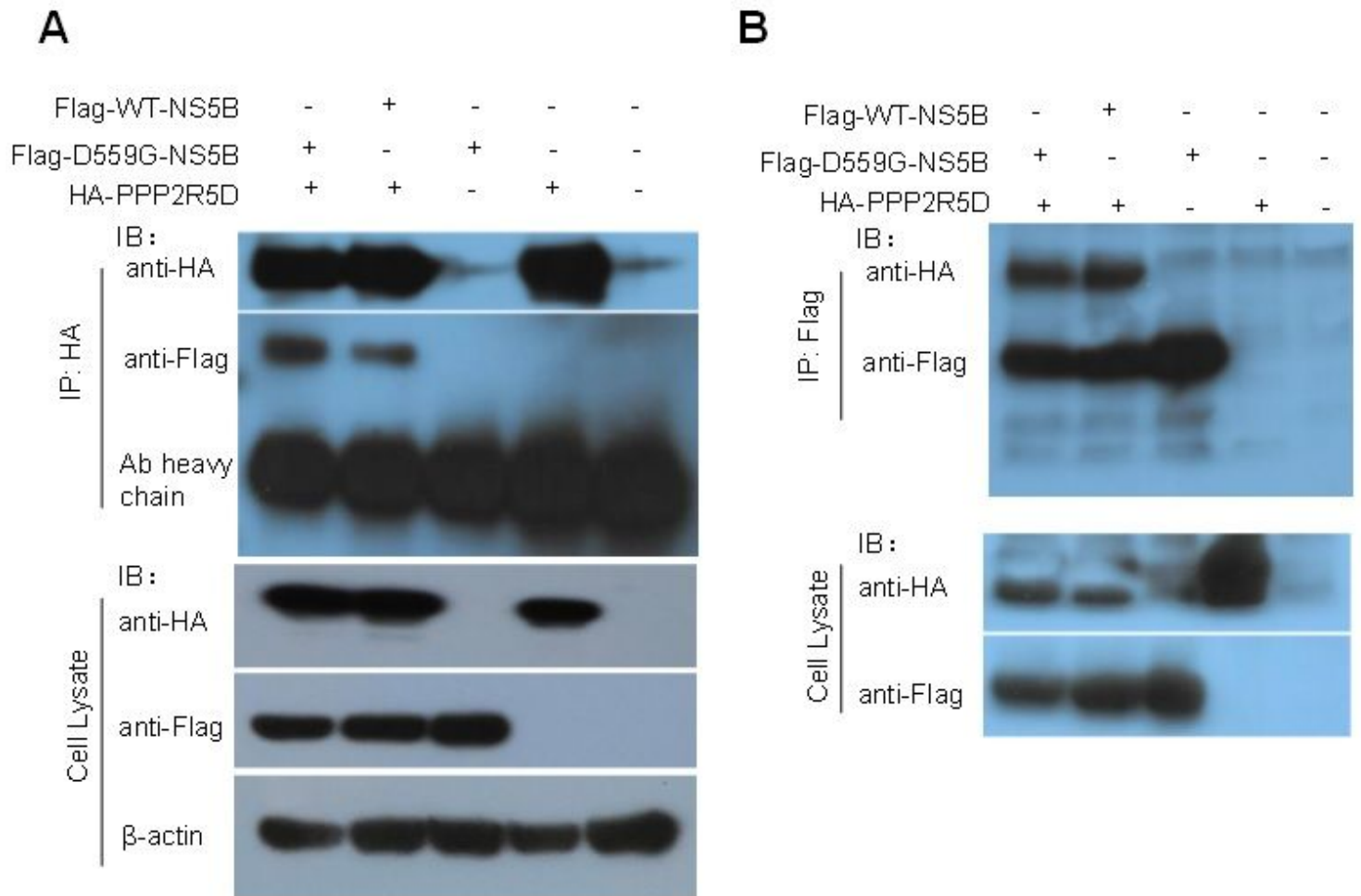
Figure 2

Co-immunoprecipitation (Co-IP) of HCV wild-type and D559G mutant NS5B with PPP2R5D. Each Flag-tagged WT-NS5B and D559G-NS5B was co-transfected with HA-tagged PPP2R5D into 293T cells, and the cells were lysed 48 h post-transfection and used for immunoprecipitation experiments. (A) Co-IP was performed using anti-HA antibody and immunoblotted with anti-HA or anti-Flag antibodies; precipitation of D559G-NS5B was more than WT-NS5B. (B) Co-IP was performed using anti-Flag antibody and immunoblotted with anti-HA or anti-Flag antibodies.
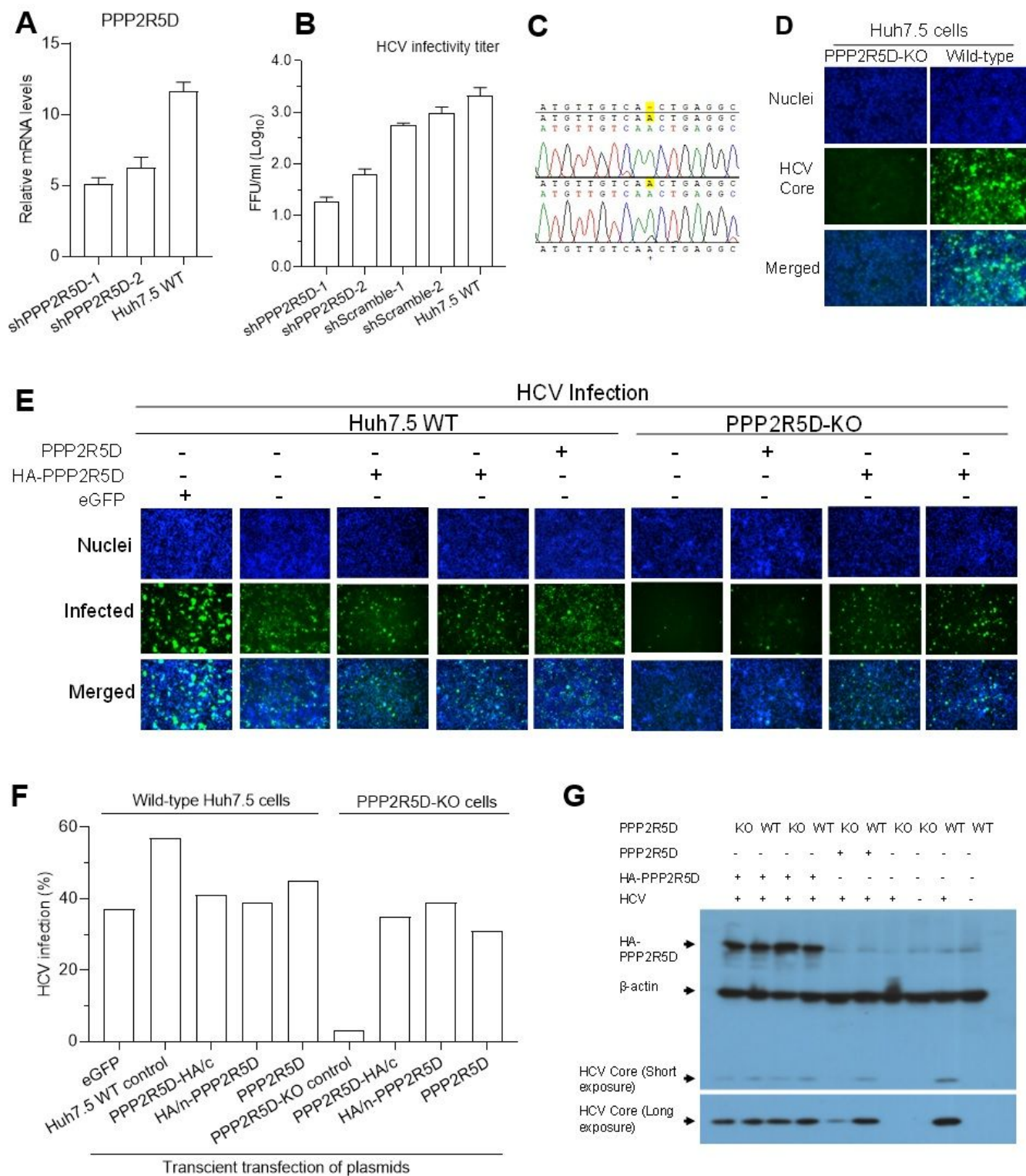
Figure 3

Knockdown and knockout of PPP2R5D inhibited HCV infection in Huh7.5 cells. (A) shRNAs targeting PPP2R5D (shPPP2R5D-1 and -2) suppressed the mRNA expression of PPP2R5D in Huh7.5 cells. the mRNA level of PPP2R5D in the shRNA-expressing Huh7.5 cells was determined by real-time RT-PCR. Mean plus-strand deviation (SD) is derived from three determinations. (B) HCV infectivity titers (FFU/ml, log10) were decreased in PPP2R5D-knockdown Huh7.5 cells. HCV infection of non-treated and control

shRNAs (shScramble-1 and -2)-treated Huh7.5 cells were performed in parallel. Culture supernatant was collected on day 7, and HCV infectivity titers were determined by FFU assay. For each infectivity titer, mean plus SD from three determinations is shown. (C) PPP2R5D disruption in puromycin-selected monoclonal cell line. A single clone of PPP2R5D-KO cells was populated and genomic DNA was sequenced; one nucleotide insertion was found in PPP2R5D gene. (D) HCV infection was dramatically decreased in PPP2R5D-KO cells, compared to Huh7.5 cells. (E) Transient expression of PPP2R5D restored HCV infection in PPP2R5D-KO cells. The wild-type and PPP2R5D-KO Huh7.5 cells were transfected with HA-tagged PPP2R5D or untagged PPP2R5D for 24 h, HCV infection was conducted with MOI of 0.01, and immunostaining of HCV Core was performed at 48 h post-infection. (F and G) HCV infection rate and western blot analysis of PPP2R5D and HCV Core in the transfected cell cultures shown in panel E.
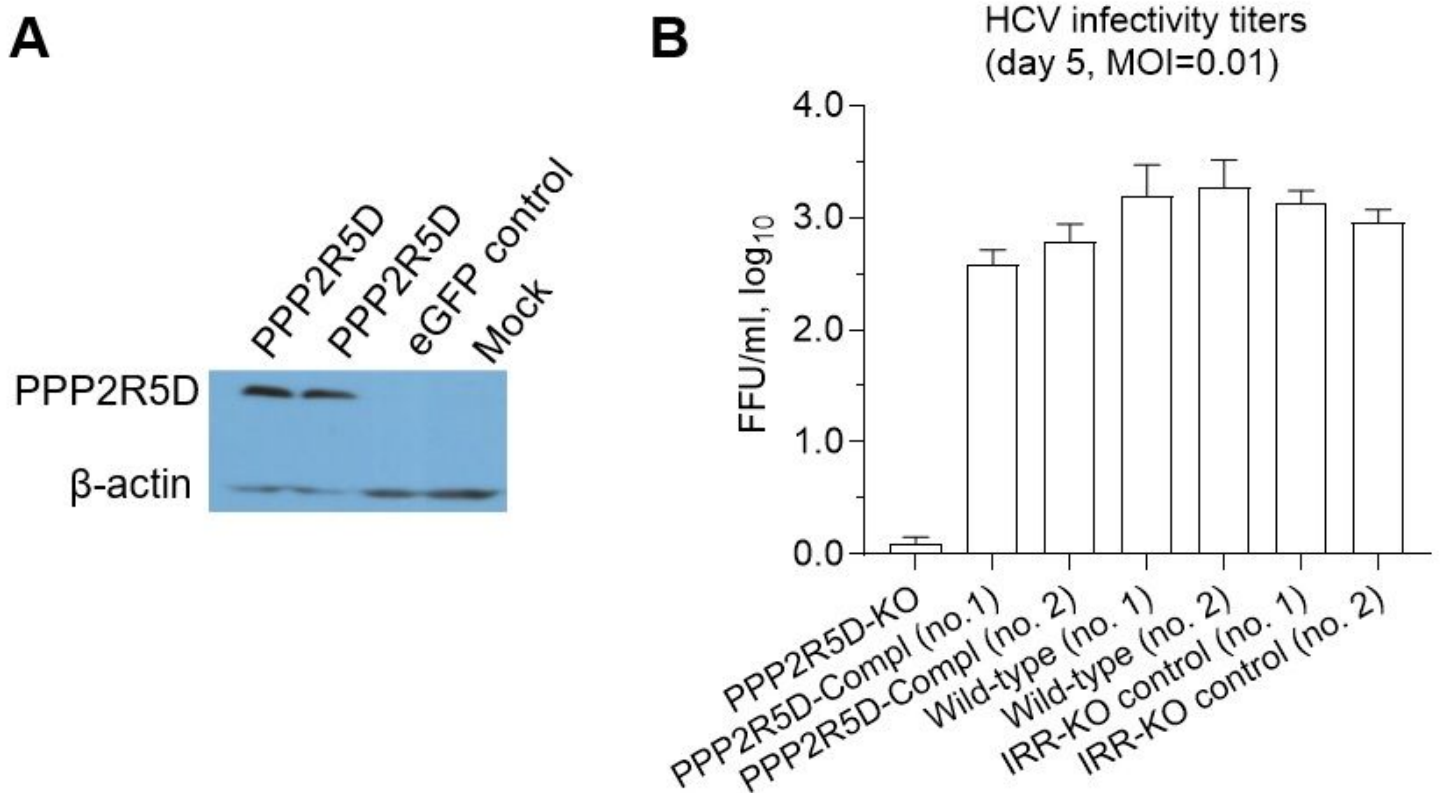

Figure 4

Stably complementation of PPP2R5D in PPP2R5D-KO cells rescued HCV infection (A) Plasmids expressing PPP2R5D were transfected into PPP2R5D-KO Huh7.5 cells (gene disruption shown in Fig. 3C), the transfected cells were then selected by the presence of puromycin (2 µg/mL), and the stable expression of PPP2R5D was detected by western blot (designated PPP2R5D-Compl). EGFP-expressing plasmid and non-transfected mock transfection were included. (B) The wild-type, PPP2R5D-Compl, and irrelative knockout control cells (IRR-KO) Huh7.5 cells were infected with HCV (MOI=0.01), and the culture supernatant was collected at day 5 for FFU assay, and mean plus SD of three determinations are shown. Data are reprehensive of two independent experiments.