

h-Prune as a novel binding protein of NS5A that regulates ERK1/2 activation

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Abstract Hepatitis C virus (HCV) non-structural 5A (NS5A) protein is associated with a wide variety of host signaling pathways by binding to C-terminal polyproline (PxxP) motifs of various proteins. In this study, we used yeast two-hybrid analysis and a GST pull-down assay to screen a novel NS5A interacting protein and elucidate the binding site and cellular signaling by focusing on recombinant human epidermal growth factor (rhEGF)-mediated ERK1/2 activation. Screening a liver cDNA library revealed that h-prune, a member of the DHH (Asp-His-His) protein superfamily, directly interacted with HCV NS5A C-terminus. In particular, a mutation of five proline amino acids to alanine in this region revealed that these two proteins produced strong interaction through this domain. It is known that h-prune possesses a highly conserved DHH motif, which has exopolyphosphatase activity that accelerates hydrolysis of inorganic polyphosphate. A time-chasing analysis after rhEGF treatment demonstrated that h-prune overexpression almost restored NS5A-mediated attenuation of ERK1/2 phosphorylation, but h-prune itself did not alter this signaling. Although the detailed

mechanisms need to be clarified, this study demonstrates that h-prune interacts directly with the PxxP motif of the HCV NS5A C-terminus and that this binding alters the rhEGF-mediated ERK1/2 signaling cascade in liver cells.

Keywords Extracellular signal-regulated kinases 1 and 2 · Hepatitis C virus · Non-structural 5A · Proline-rich motif · h-Prune

Introduction

Hepatitis C virus (HCV) is a small enveloped virus in the *Flaviviridae* family, which is most closely related to the pestiviruses, and which has a positive-sense single-stranded RNA genome. HCV RNA encodes a single large polyprotein of 3000 amino acids (Macdonald et al. 2003). Processing of this precursor polyprotein generates three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Among them, NS5A is a serine phosphoprotein (Yamauchi et al. 2015). Unlike other HCV proteins, the function of NS5A is not well understood.

C-terminal polyproline (PxxP) domain of NS5A is highly conserved throughout a range of HCV genotypes (Tan et al. 1999) and affects a wide variety of host cellular signaling proteins by binding to Src-homology (SH) 3 domains of the adaptor proteins, thus finally influencing immune invasion and carcinogenesis (Street et al. 2004). Also, NS5A inhibits phosphorylation of extracellular signal-regulated kinases (ERK) by activating epidermal growth factor (EGF) (Macdonald et al. 2003) and perturbs tumor suppressor protein p53 (Lan et al. 2002). Especially, NS5A regulates mitogenic and replication-related signaling pathways in HCV-infected hepatocytes, thereby regulating host cell growth.

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h-Prune is a human ortholog of *Drosophila prune*, which is originally identified as a gene involved in *Drosophila* eye development. The *Drosophila prune* eye color mutant lethally interacts with the *killer-of-prune* allele on the abnormal wing disk (*awd*) locus, which is the *Drosophila* homolog gene of the mammalian tumor metastasis gene *nm23* encoding a nucleotide diphosphate kinase. In addition, *prune* encodes GTPase-activating proteins (GAPs), which control Ras-like proteins (Hackstein 1992). The N-terminus of the h-prune protein contains DHH domain named after the characteristic Asp-His-His motif. The DHH domain is highly conserved within species and has phosphodiesterase and exopolyphosphatase activities (Tammenkoski et al. 2008). The h-prune C-terminus has a role in the interaction with glycogen synthase kinase-3 β and nm23-H1, which are known as suppressors of cancer metastasis (Reymond et al. 1999; Lacombe et al. 2000; Kobayashi et al. 2006). These two proteins bind simultaneously to different parts of h-prune, thereby regulating its cellular signaling activity. Moreover, overexpression of h-prune regulates cellular motility and metastasis in several solid tumors, such as prostate cancer (Carotenuto et al. 2015), breast cancer (Virgilio et al. 2012), and colorectal cancer (Muller et al. 2010). Physical interaction of this protein with nm23-H1 inhibits its anti-metastatic function in vivo (D'Angelo et al. 2004; Garzia et al. 2006). Taken together, these findings suggest that h-prune may play a negative role in the anti-metastatic activity of nm23-H1.

Inorganic polyphosphate (PolyP) is a biopolymer that has been detected in a variety of organisms from microorganisms to humans. Nuclear PolyP participates in the regulation of gene expression and the stress response. High-polymeric PolyP must be fragmented into shorter polymers to function in response to exopolyphosphatase or endopolyphosphatase. h-Prune is a member of the DHH protein superfamily and functions as a nucleotide phosphodiesterase and an exopolyphosphatase, thus supplying phosphate for DNA synthesis, ATP synthesis, and energy metabolism.

We report the identification of the NS5A-interacting protein, h-prune, and the effect of this protein in the NS5A-mediated ERK1/2 signaling pathway. The results clarify NS5A function in hepatocarcinoma.

Materials and methods

Plasmid construction

Full-length or partial NS5A constructs (NS5A-F, aa 1–447; NS5A-A, aa 1–150, NS5A-B, aa 1–300, NS5A-C, aa 300–447) were produced as reported previously (Ahn et al.

2004). Briefly, the polymerase chain reaction (PCR) was performed with the primer pairs indicated in Table 1, and cloned into the pHybLex/Zeo vector (Thermo Fisher Scientific, Rockford, IL, USA) using the linker sites at both ends (Fig. 1A). In addition, two PCR reactions were performed with a pair of PCR primers (Bioneer, Daejeon, Korea; NS5A-mut1/NS5A(447-R) and NS5A (1-F)/NS5A-mut2; Table 1) using pHybLex/Zeo-NS5A-F as a template to construct the pHybLex/Zeo-NS5A full-length (F)-mutant, in which five proline amino acids of the PxxPxR (class II) in NS5A were all mutated to alanine. After then, a bloc PCR was performed with the NS5A (1-F) and NS5A (447-R) primers using the two PCR fragments as a template. The resulting PCR fragment was treated with *Not* I and *Sal* I and ligated into the *Not* I/*Sal* I site of pHybLex/Zeo.

Yeast two-hybrid analysis

A yeast two-hybrid screening was performed as reported previously (Ahn et al. 2004) to search for host proteins that interact with HCV NS5A, following the protocol recommended by the manufacturer (Hybrid HunterTM; Thermo Fisher Scientific) with a minor modification. The entire or partial NS5A cDNA-coding region (genotype 1b, generously donated by Dr. Soichiro Miura, National Defense Medical College, Tokorozawa, Saitama, Japan) was fused in-frame with the Lex DNA-binding domain into the pHybLex/Zeo vector as bait for screening a liver cDNA library. In addition, full-length h-prune (liver cDNA library) was cloned into the yeast pYESTrp2 vector to construct the B42 activation domain (B42AD)/h-prune fusion protein as a prey plasmid (Table 1). Positive (pHybLex/Zeo-Fos and pYESTrp2-Jun) and negative (pHybLex/Zeo-lamin and pYESTrp2-Jun) control plasmid combinations were added during the analysis for validation. The pYESTrp2/h-prune and pHybLex/NS5A derivatives were cotransfected into the pEGY48/pSH18-34 yeast reporter strain using the standard lithium acetate method. Transformed cells were spread on synthetic dropout (SD)/zeocin (Zeo) agar plates [0.67 % (w/v) yeast nitrogen base without amino acids, 20 % (w/v) glucose, 2 % (w/v) agar powder, 10 \times dropout solution without uracil, tryptophan, histidine supplement, and 300 μ g/mL Zeo] for 5 days. Yeast cells on SD minimal plates were transferred to filter paper to assess β -galactosidase activity in a colony-lift filter assay. The filter was placed in liquid nitrogen for 30 s and incubated at room temperature in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄), containing 0.82 mM 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Then, the filter was placed at 30 $^{\circ}$ C and monitored for a color change to indicate expressed β -galactosidase activity.

Table 1 Oligonucleotides used in this study

Primer name	Primer sequence	Vector
A. PCR primers used in the construction of pHybLex/Zeo-NS5A-F, -F-A, -F-B, and -F-C		
NS5A (Full-F)	5'-GCGGCCGCTTTCCGGCTCGTGGCTAAGG-3'	a
NS5A (Full-R)	5'-GTCGACTCATTATCAGCAGCAGACGACGTCC-3'	a
NS5A (150-R)	5'-GCGGCCGCTTTTCACAGAATTGGATGGGGTGC-3'	a
NS5A (300-F)	5'-GCGGCCGCTTGGGAGATCCTGCGGAGAT-3'	a
NS5A (300-R)	5'-GTCGACTCATTACGCCGGAACGGATACTTCCC-3'	a
B. PCR primers used in the construction of pHybLex/Zeo-NS5A-F-mut		
NS5A (Full-F)	5'-GCGGCCGCTTTCCGGCTCGTGGCTAAGG-3'	a
NS5A (Full-R)	5'-GTCGACTCATTATCAGCAGCAGACGACGTCC-3'	a
NS5A-mut1	5'-ATCGTCCTCTTCCCTCCGTGCGGCTGCTATCGCTGGGGCCTTGTTAGGT G-3'	a
NS5A-mut2	5'-CACCTACCAAGCCCCAGCGATAGCAGCCGCACGGAGGAAGAGGACGAT-3'	a
C. PCR primers used in the construction of pYESTrp2, pMAL, and pCMV-Tag1-h-prune		
h-Prune-F	5'-CCGGAT CCATGGAGGACTACCTGCAGGGT-3'	b, c, d
h-Prune-R1	5'-CCCTCGAGCTTCTTGGACAGGGAGGC-3'	b, d
h-Prune-R2	5'-CCGTCGACAGCTTCTTGGACAGGGAGGC-3'	c

^a pHybLex/Zeo vector was used to construct pHybLex/Zeo-NS5A-F, -NS5A-F-A, -NS5A-F-B, and -NS5A-F-C

^b pYESTrp2 vector was used to create pYESTrp2/h-prune

^c pMAL vector was used to construct pMAL/h-prune

^d pCMV-Tag1 vector was used to produce pCMV-Tag1/h-prune

In vitro protein binding assay

We performed in vitro pull-down assays to confirm the existence of a protein–protein interaction between NS5A and h-prune. First, full-length NS5A and h-prune were cloned with the pGEX4T-1 (GE Healthcare Life Sciences, Wauwatosa, WI, USA) and pMAL-cR1 vectors (New England Biolabs, Ipswich, MA, USA), respectively (Ahn et al. 2004). The resulting plasmids were transformed into *Escherichia coli* (*E. coli*) strain DH5- α [(genotype: F⁻ ϕ 80dlacZAM15 Δ (*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_k⁻, m_k⁺) *phoA supE44* λ ⁻ *thi-1 gyrA96 relA1*)]. GST/NS5A and Mal/h-prune fusion protein expression was induced 5 h after adding 1 mM isopropyl- β -D-thiogalactopyranoside and the expression levels of each fusion protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. In detail, cells expressing GST/NS5A or the Mal/h-prune fusion proteins were harvested, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride and lysed by sonication in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The supernatant was clarified by centrifugation at 4 °C, the lysates expressing NS5A and the h-prune protein were mixed together, pull-downed using Glutathione Sepharose 4B (GE Healthcare Life Sciences), and reacted for 12 h at 4 °C. The Glutathione Sepharose 4B was washed out with PBS containing 1 %

Triton X-100 and phosphatase inhibitors to remove unbound non-specific proteins, and beads were collected.

Determination of exopolyphosphatase activity

Exopolyphosphatase activity was determined by the rate of P_i formation. h-Prune was cloned into the pET-28a(+) expression vector with a His tag and expressed using BL21 (DE3) (F⁻ *ompT hsdS*(r_B⁻ m_B⁻) *gal dcm* λ (DE3) pLysS (Cam^r) competent *E. coli* cells to obtain the protein for this assay. Expressed proteins were clarified by centrifugation. After removing the cell debris, the h-prune protein was concentrated with His-binding resin by overnight activation with Ni²⁺ at 4 °C, and 20 μ g of purified protein was mixed with 100 μ M inorganic polyphosphate 45 (polyP₄₅) in a reaction buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl₂, and 100 mM NaCl (Lorenz et al. 1997) and reacted for 30 min or 1 h at 37 °C (Eldarov et al. 2013). PolyP hydrolysates were subjected to PAGE on a 22 % polyacrylamide gel with 7 M urea, and the gels were stained with 0.05 % toluidine blue in water containing 25 % methanol and 1 % glycerol and then washed with distilled water (Mullan et al. 2002).

Cell lines

The Hep3B, Huh7, HepG2, and Chang hepatocarcinoma cell lines were cultured in high glucose Dulbecco's

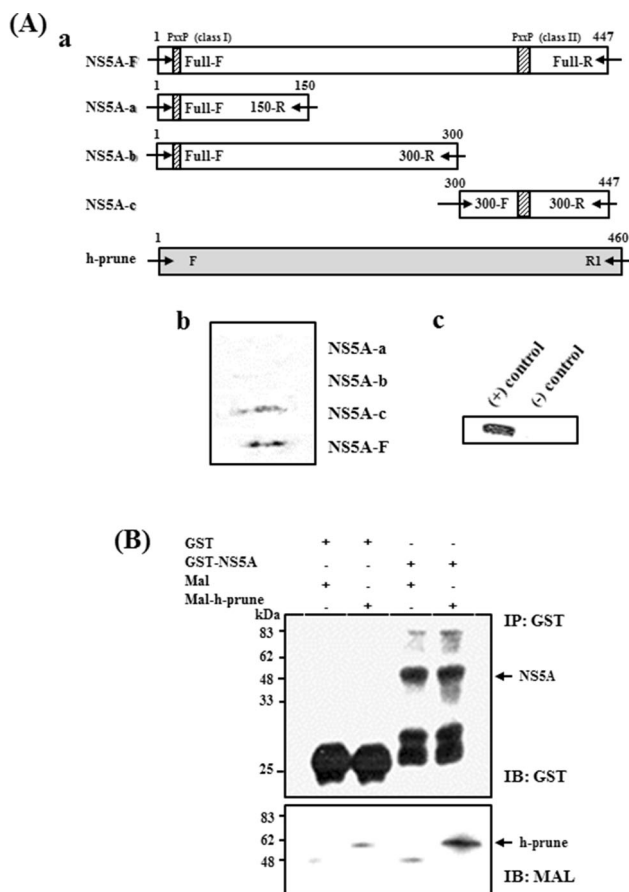


Fig. 1 Schematic representation of the full-length and the four different NS5A derivatives and determining the NS5A and h-prune binding site. **(A)** The DNA fragments of the NS5A derivatives were PCR-amplified and subcloned into pHybLex/Zeo (a). The four NS5A derivatives (designated NS5A-F, -a, -b, and -c) were tested to determine their suitability as baits for full-scale yeast two-hybrid screening in a colony-lift filter assay (b). The h-prune-binding fragments were NS5A-F and NS5A-c. pHybLex/Zeo-Fos2 and pYESTrp-Jun were cotransfected into the L40 yeast strain as a positive control [(+) control], and pHybLex/Zeo-Lamin was transfected alone for the negative control [(-) control] in the colony-lift filter assay (c), unless specified elsewhere. **(B)** GST pull-down assay with GST (control) and GST-NS5A (pull-down) of yeast extracts expressing h-prune with the MAL tag. NS5A directly interacted with h-prune

modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. A stable cell line expressing NS5A was constructed using 500 µg/mL G418. The mammalian cell lines were incubated at 37 °C in a 5 % CO₂ incubator.

RNA isolation

The hepatocarcinoma cell lines were washed once with ice-cold PBS. After aspirating the PBS, the cells were treated with 1 mL TRIzol (Thermo Fisher Scientific) and the plate was scraped briefly. After pipetting, the cell lysates were

transferred to 1.5 mL Eppendorf tubes, 250 µL chloroform was added, the tube was shaken vigorously for about 15 s, and incubated at room temperature for 5 min. After centrifugation at 12,000×g for 15 min at 4 °C, the aqueous phase containing RNA was transferred to a new tube, and the RNA was precipitated by mixing with an equal volume of isopropyl alcohol. The tubes were incubated for 12 h at -20 °C. The supernatant was centrifuged at 12,000 rpm for 20 min at 4 °C. After aspirating the supernatant, the remaining pellet was washed once with 75 % cold ethanol in diethylpyrocarbonate (DEPC)-treated H₂O, the ethanol was aspirated, and the samples were dried. The pellets were re-dissolved in DEPC-treated water, added to the same volume of phenol, inverted slowly, and centrifuged at 12,000 rpm for 2 min. The supernatants were transferred to a new tube, and two-fold volumes of ethanol and 1 M sodium acetate were added. The supernatants were centrifuged at 12,000 rpm at 4 °C and re-dissolved in DEPC water.

Northern blot analysis

Isolated RNAs were mixed with 10 × formaldehyde gel-loading buffer (50 % glycerol, 10 mM EDTA, 0.25 % bromophenol blue, and 0.25 % xylene cyanol FF), electrophoresed on a formaldehyde gel containing 1.2 % agarose, and the separated RNA was transferred to a Hybond N⁺ membrane (Thermo Fisher Scientific) and cross-linked using UV light. Saline sodium citrate (SSC) (0.15 M NaCl in 15 mM sodium citrate, pH 7.0) was used as the transfer buffer. DNA probes with sequences (TAGTGCAATATATATGGATTGGAG) were end-labeled with [γ-³²P] CTP. The membranes were pre-hybridized with Rapid-Hyb buffer (GE Healthcare Life Sciences) for 2 h at 65 °C and then hybridized to radiolabeled probes for 2 h at the same temperature. The hybridized blots were washed in 6 × SSC for 20–30 min at room temperature and were washed 2 times in 2 × SSC with 0.1 % SDS for 20–30 min at 42 °C, one time in 0.1 × SSC with 0.1 % SDS for 20–30 min at 42 °C, and then exposed to X-ray film (FUJIFILM Medical Systems, Stamford, CT, USA) for 24 h at -70 °C. Finally, the signals were detected using an automatic X-ray film processor.

Immunoprecipitation

NS5A and h-prune were cloned with pCMV/Tag1 with a FLAG tag (Dr. Cho Rok Jung, Biomedical Translational Research Center, KRIBB, Daejeon, Korea) and pCMV/Tag1 with a Myc tag, respectively, to assay protein binding and each cloned plasmid was expressed in the Huh7 hepatocarcinoma cell line. In brief, Huh7 cells were

transfected with pCMV-Tag1/FLAG-NS5A and pCMV-Tag1/Myc-h-prune using a calcium phosphate transfection kit (Thermo Fisher Scientific). After transfection, NS5A and h-prune were expressed for 24 h. The cells were lysed by sonication in RIPA buffer, the supernatants were clarified by centrifugation at 4 °C, and cell debris was removed. The supernatants were incubated with anti-FLAG antibody (1 µg/mL) (Sigma, St. Louis, MO, USA) for 12 h at 4 °C, 20 µL (1:2 slurry) of protein agarose A beads were added (Sigma), and incubated for another 4 h at 4 °C. The protein agarose A beads were washed out five times with lysis buffer to remove unbound non-specific protein. All reactions were performed under ice-cold conditions.

Western blot analysis

Bound proteins were released by boiling in SDS sample buffer containing 62.5 mM Tris/HCl pH 6.8, 2 % SDS, 0.002 % bromophenol blue, 0.7135 M β-mercaptoethanol, and 10 % glycerol, resolved by SDS-PAGE, and transferred to a PVDF membranes (Pall, Port Washington, NY, USA). After incubation with horseradish peroxidase-conjugated antibodies, the blots were visualized using an enhanced chemiluminescence system (ECL Western blotting detection reagent; GE Healthcare). The GST/NS5A and Mal/h-prune fusion proteins were detected with the GST polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and the MBP antibody (New England Biolabs).

Determination of Erk 1/2 phosphorylation

The pCMV-Tag1/h-prune was transfected into Huh7 cells, the Huh7 cells were overexpressed with FLAG-NS5A using Lipofectamine reagent (Thermo Fisher Scientific), and incubated for 24 h. The transfected cells were starved with 0.1 % FBS/DMEM for 12 h, stimulated with 20 ng/mL recombinant human EGF (rhEGF) (Cell Signaling Technology), and chased. The cells were lysed by sonication with lysis buffer and a Western blot analysis was performed. Erk1/2 phosphorylation was detected with a phospho ERK1/2 antibody (Cell Signaling Technology). β-actin, NS5A, and h-prune expression were detected with FLAG and Myc antibodies (Sigma), respectively.

Results

Identification of the h-prune interacting domain in NS5A

We previously detected hepatocellular proteins interacting with HCV NS5A using a yeast two-hybrid system (Ahn

et al. 2004). Through further study with a human liver cDNA prey-library for yeast two-hybrid screening, we presently identified, a hepatocellular protein, h-prune, that interacted with the HCV NS5A (Fig. 1A-a). h-Prune is a member of the DHH protein superfamily that functions as a nucleotide phosphodiesterase and an exopolyphosphatase, thus being involved in the progression of cancer metastasis through negative regulation of the metastasis suppressor nucleoside diphosphate kinase A (Dooley et al. 1994).). To define the interacting portion of h-prune and NS5A, we created four different NS5A derivatives as baits; full-length NS5A (NS5A-f, aa 1–447) and its three different derivatives, denoted as NS5A-a (aa 1–150), -b (aa 1–300), and -c (aa 300–447) (Fig. 1A-a). As shown in (Fig. 1A-b), the full-length h-prune construct strongly interacted with the NS5A full-length (NS5A-F) construct and the NS5A-C-terminal fragment (NS5A-c). However, the NS5A-a and -b fragments did not bind to h-prune. This was an interesting finding because NS5A-a also contains the proline-rich PxxP (class I) motif (Fig. 1A-b), suggesting that the specific PxxP motif (class II) in NS5A may be associated with h-prune binding. Moreover, a pull-down assay demonstrated that Mal/h-prune fusion protein interacted directly with the GST/NS5A protein (Fig. 1B).

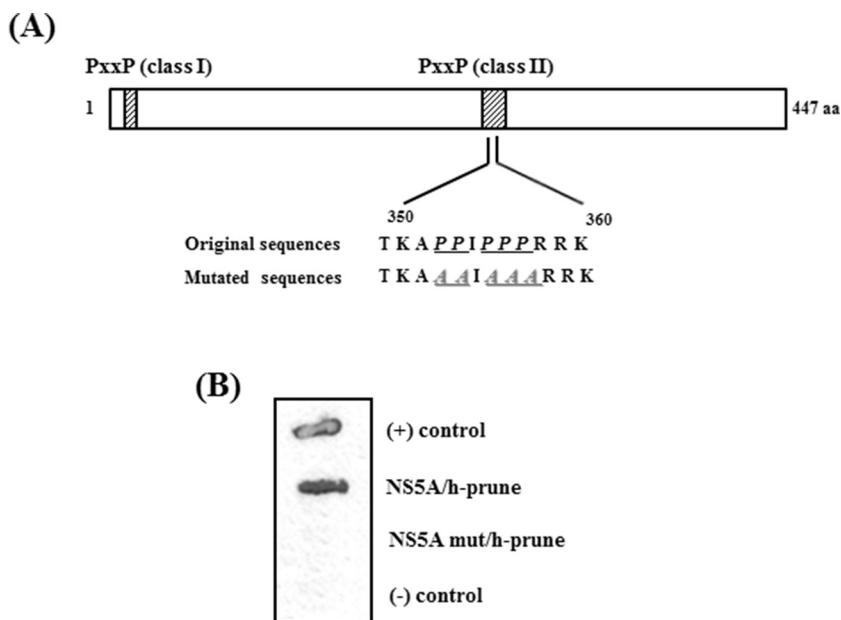
The class II proline-rich SH3-binding motif of HCV NS5A is the h-prune binding domain

The NS5A proline-rich motif is highly conserved among HCV species and plays an important role in various NS5A-mediated signaling functions. Therefore, we examined if a mutation in this motif alters the interaction between these two proteins. The proline amino acids of the class II PxxP motif in full-length NS5A were mutated to alanine using a PCR method (Ahn et al. 2004) (Fig. 2A) and the interactions were verified by a colony filter-lift assay. As shown in (Fig. 2B), the NS5A mutant (NS5A-mut) did not bind to h-prune contrary to the case of normal NS5A, indicating that the PxxP region of the NS5A-c fragment is responsible for mutual interactions of the two proteins.

h-Prune consensus sequences in different species and their exopolyphosphatase activity

From previous results, we found a new protein interacting with a specific proline-rich motif of NS5A. As mentioned above, h-prune is a well-conserved protein in many species. As previously reported by another group (Aravind and Koonin, 1998), a NCBI blast homology search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) showed that h-prune has similarities with exopolyphosphatases from *Schizosaccharomyces pombe* and *D. melanogaster*. In particular, four representative motifs (designated motif I, II, III, and IV)

Fig. 2 Domain analysis of NS5A-c interacting with h-prune. **(A)** The class II PxxP domain of the NS5A-c portion. Five proline amino acids in the NS5A-c PxxP motif were mutated to alanine. **(B)** A colony-lift filter assay with NS5A-F or NS5A-F-mut, and the full-length h-prune protein. NS5A-F bound to the h-prune protein but not to NS5A-F-mut. pHybLex/Zeo-Fos2 and pYESTrp-Jun were used for the positive control [(+) control], and pHybLex/Zeo-Lamin alone was transfected into the L 40 yeast strain for the negative control [(-) control]



and their five invariant aspartate residues, which are characteristic of the DHH phosphoesterase family, were conserved (Fig. 3A), asterisks with black box and gray square box). As described above, these conserved signature regions were involved in h-prune exopolyphosphatase activity. Exopolyphosphatases cleave the inorganic polyphosphatase by Pi. Therefore, we tested h-prune-mediated exopolyphosphatase activity by measuring the time-chasing hydrolysis of PolyP using purified h-prune. As indicated on the PolyP PAGE-gel stained with toluidine blue to show the metachromatic shift in phosphate (Mullan et al. 2002), the 20 µg h-prune protein reacted with 100 µM PolyP (type 45) for 1 h increased degradation of PolyP compared to that of the control, showing a decrease in PolyP chain length (Fig. 3B).

h-Prune in liver cells directly interacts with NS5A

Since the present results showed that h-prune binds to HCV NS5A. We next examined which liver cell line expressed h-prune to demonstrate the role of h-prune in liver cells. The yeast two-hybrid assay of HCV NS5A was performed with a liver cDNA library and expression was confirmed by Northern blot analysis in the Hep3B, Huh7, HepG2, and Chang liver cell lines. h-Prune was expressed in all liver cell lines, showing the highest expression in Huh-7 cells (Fig. 4A). Next, we performed co-immunoprecipitation to determine if the two proteins directly interacted. Therefore, FLAG-tagged NS5A and Myc-tagged h-prune were constructed, and Western blots revealed that h-prune and NS5A bound together in Huh-7 cells (Fig. 4B), indicating that h-prune may have some role in liver cells through interaction with NS5A.

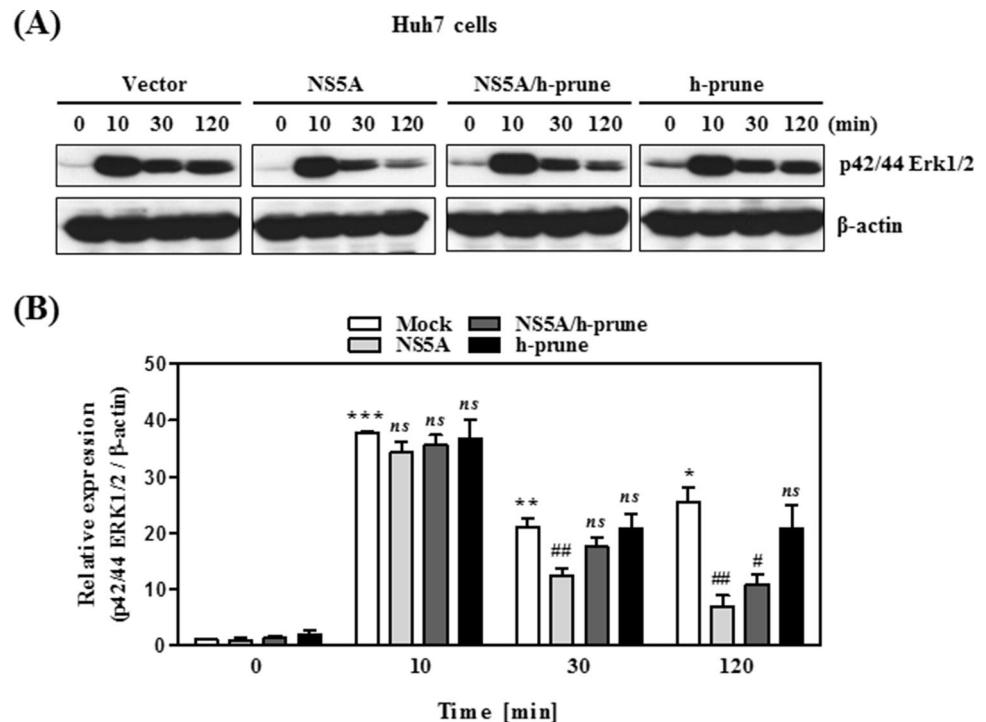
h-Prune restores NS5A-induced inhibition of Erk1/2 phosphorylation

NS5A influences negative phosphorylation of ERK1/2 in HCV-infected cells (Tan et al. 1999). Therefore, we further examined if h-prune influences the NS5A-mediated change in ERK1/2 signaling. rhEGF (20 ng/mL) dramatically increased ERK1/2 phosphorylation, which decreased over time, showing maximal activation 10 min after treatment (Fig. 5, vector). As reported previously (Tan et al. 1999), Western blot analysis demonstrated that NS5A transfection noticeably attenuated rhEGF-induced phosphorylation of ERK1/2 in Huh-7 cells (Fig. 5, NS5A). However, overexpressing h-prune in NS5A-transfected cells almost completely restored the reduced ERK1/2 phosphorylation following rhEGF treatment (Fig. 5, NS5A/h-prune), although h-prune itself did not significantly change this signal (Fig. 5, h-prune).

Discussion

HCV is a small and enveloped Hepacivirus in the family *Flaviviridae* that causes hepatocellular carcinoma as well as chronic liver disease and cirrhosis. Thus, HCV infection is the leading indicator for liver transplantation (Kim 2002). The HCV genome encodes a polyprotein precursor of approximately 3,000 residues (Bartenschlager and Lohmann 2000), consisting of 10 distinct virion proteins, including four structural proteins (core, E1, E2, and p7) and six non-structural proteins (NS2, NS3, NS5A, NS4B, NS5A, and NS5B) (Bartenschlager et al. 1994).

Fig. 5 h-Prune restores NS5A-mediated attenuation of Erk1/2 signaling. **(A)** Western blots for phospho-ERK1/2. Huh7 cells transfected with FLAG-NS5A and Myc-h-prune were treated with 20 ng/mL rhEGF and immunoblotted with anti-phospho ERK1/2 antibody. **(B)** Bars denote the density ratio of phospho-ERK1/2 relative to β -actin. All ratio values were normalized to the WT ratio at time 0 and defined as 1 ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. WT 0 time; $\#P < 0.05$, $\#\#P < 0.01$, *ns* (not significant) versus mock at each time point; mean \pm SE, $n = 4$ experiments)



et al. 2012; Reeves et al. 2012; Schweneker et al. 2012; Wang et al. 2012). Therefore, inhibition of the ERK signal in NS5A-overexpressing liver cells may impair HCV replication by attenuating susceptible cell survival and proliferation.

Several reports have suggested that HCV triggers the release of various cytokines including alpha interferon (IFN- α) and interleukin-6, which are important in the initial antiviral immune defense, thereby restricting viral replication. However, reports on the effect of the ERK signal pathway are controversial in view of the pathway's roles in viral infection and IFN- α signaling. For example, the HCV protein induces activation of the ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathways in transgenic mice (Tsutsumi et al. 2003), whereas HCV NS5A inhibits transcription factor-activating protein-1 function by perturbing the ERK pathway (Macdonald et al. 2003). Viruses use various strategies for infection, and ERK signaling may be affected at various stages, which needs to be clarified.

The h-prune found by us to be a NS5A-interacting protein promotes metastasis by binding and inactivating the anti-metastatic protein nm23-H1, but its role in liver cells has not been reported. Presently, while h-prune overexpression itself did not affect rhEGF-mediated ERK1/2 activity, co-expression of h-prune and NS5A rescued the decreased ERK1/2 signal by NS5A overexpression. Independent of cell mortality regulation through phosphodiesterase activity of h-prune, the protein helps survival of liver cells infected with HCV NS5A.

Inorganic polyphosphate is used as an energy source in mammalian systems and is involved in tumor metastasis (Galasso and Zollo 2009), apoptosis (Hernandez-Ruiz et al. 2006), and cell proliferation (Wang et al. 2003). For example, PolyP activates mammalian target of rapamycin (mTOR) signaling and *S. cerevisiae* *PPX1*, the human *h-prune* ortholog, inhibits phosphorylation of 4E-BP1 by mTOR (Wang et al. 2003). Therefore, h-prune may also downregulate 4E-BP1 as in *PPX1*. For this reason, we may need to examine how the interaction between h-prune and HCV NS5A is coordinated with other signaling pathways, such as mTOR.

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References

- Ahn J, Chung KS, Kim DU, Won M, Kim L, Kim KS, Nam M, Choi SJ, Kim HC, Yoon M, Chae SK, Hoe KL (2004) Systematic identification of hepatocellular proteins interacting with NS5A of the hepatitis C virus. *J Biochem Mol Biol* 37:741–748
- Aravind L, Koonin EV (1998) A novel family of predicted phosphoesterases includes *Drosophila* prune protein and bacterial RecJ exonuclease. *Trends Biochem Sci* 23:17–19
- Bartenschlager R, Lohmann V (2000) Replication of hepatitis C virus. *J Gen Virol* 81:1631–1648
- Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H (1994) Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J Virol* 68:5045–5055
- Carotenuto M, de Antonellis P, Chiarolla CM, Attanasio C, Damiani V, Boffa I, Aiese N, Pedone E, Accordi B, Basso G, Navas L,

- Imbimbo C, Zollo M (2015) A therapeutic approach to treat prostate cancer by targeting Nm23-H1/h-prune interaction. *Naunyn Schmiedeberg Arch Pharmacol* 388:257–269
- Chang TH, Chen SR, Yu CY, Lin YS, Chen YS, Kubota T, Matsuoka M, Lin YL (2012) Dengue virus serotype 2 blocks extracellular signal-regulated kinase and nuclear factor-kappaB activation to downregulate cytokine production. *PLoS ONE* 7:e41635
- Chuluunbaatar U, Roller R, Mohr I (2012) Suppression of extracellular signal-regulated kinase activity in herpes simplex virus 1-infected cells by the Us3 protein kinase. *J Virol* 86:7771–7776
- D'Angelo A, Garzia L, Andre A, Carotenuto P, Aglio V, Guardiola O, Arrigoni G, Cossu A, Palmieri G, Aravind L, Zollo M (2004) Prune cAMP phosphodiesterase binds nm23-H1 and promotes cancer metastasis. *Cancer Cell* 5:137–149
- Dooley S, Seib T, Engel M, Theisinger B, Janz H, Piontek K, Zang KD, Welter C (1994) Isolation and characterization of the human genomic locus coding for the putative metastasis control gene nm23-H1. *Hum Genet* 93:63–66
- Eldarov MA, Baranov MV, Dumina MV, Shgun AA, Andreeva NA, Trilisenko LV, Kulakovskaya TV, Rysanova LP, Kulaev IS (2013) Polyphosphates and exopolyphosphatase activities in the yeast *Saccharomyces cerevisiae* under overexpression of homologous and heterologous PPN1 genes. *Biochemistry (Mosc)* 78:946–953
- Galasso A, Zollo M (2009) The Nm23-H1-h-prune complex in cellular physiology: a 'tip of the iceberg' protein network perspective. *Mol Cell Biochem* 329:149–159
- Garzia L, Roma C, Tata N, Pagnozzi D, Pucci P, Zollo M (2006) h-Prune-nm23-H1 protein complex and correlation to pathways in cancer metastasis. *J Bioenergy Biomembr* 38:205–213
- Hackstein JH (1992) The lethal prune/Killer-of-prune interaction of *Drosophila* causes a syndrome resembling human neurofibromatosis (NF1). *Eur J Cell Biol* 58:429–444
- Hernandez-Ruiz L, Gonzalez-Garcia I, Castro C, Brieve JA, Ruiz FA (2006) Inorganic polyphosphate and specific induction of apoptosis in human plasma cells. *Haematologica* 91:1180–1186
- Jacque JM, Mann A, Enslin H, Sharova N, Brichacek B, Davis RJ, Stevenson M (1998) Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase. *EMBO J* 17:2607–2618
- Kim WR (2002) The burden of hepatitis C in the United States. *Hepatology* 36:S30–S34
- Kobayashi T, Hino S, Oue N, Asahara T, Zollo M, Yasui W, Kikuchi A (2006) Glycogen synthase kinase 3 and h-prune regulate cell migration by modulating focal adhesions. *Mol Cell Biol* 26:898–911
- Lacombe ML, Milon L, Munier A, Mehus JG, Lambeth DO (2000) The human Nm23/nucleoside diphosphate kinases. *J Bioenerg Biomembr* 32:247–258
- Lan KH, Sheu ML, Hwang SJ, Yen SH, Chen SY, Wu JC, Wang YJ, Kato N, Omata M, Chang FY, Lee SD (2002) HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene* 21:4801–4811
- Liu X, Li Q, Dowdell K, Fischer ER, Cohen JI (2012) Varicella-Zoster virus ORF12 protein triggers phosphorylation of ERK1/2 and inhibits apoptosis. *J Virol* 86:3143–3151
- Lorenz B, Munkner J, Oliveira MP, Leitao JM, Muller WE, Schroder HC (1997) A novel method for determination of inorganic polyphosphates using the fluorescent dye fura-2. *Anal Biochem* 246:176–184
- Macdonald A, Crowder K, Street A, McCormick C, Saksela K, Harris M (2003) The hepatitis C virus non-structural NS5A protein inhibits activating protein-1 function by perturbing ras-ERK pathway signaling. *J Biol Chem* 278:17775–17784
- Mullan A, Quinn JP, McGrath JW (2002) A nonradioactive method for the assay of polyphosphate kinase activity and its application in the study of polyphosphate metabolism in *Burkholderia cepacia*. *Anal Biochem* 308:294–299
- Muller T, Stein U, Poletti A, Garzia L, Rothley M, Plaumann D, Thiele W, Bauer M, Galasso A, Schlag P, Pankratz M, Zollo M, Sleeman JP (2010) ASAP1 promotes tumor cell motility and invasiveness, stimulates metastasis formation in vivo, and correlates with poor survival in colorectal cancer patients. *Oncogene* 29:2393–2403
- Pawson T (1995) Protein modules and signalling networks. *Nature* 373:573–580
- Reeves MB, Breidenstein A, Compton T (2012) Human cytomegalovirus activation of ERK and myeloid cell leukemia-1 protein correlates with survival of latently infected cells. *Proc Natl Acad Sci USA* 109:588–593
- Reymond A, Volorio S, Merla G, Al-Maghteh M, Zuffardi O, Bulfone A, Ballabio A, Zollo M (1999) Evidence for interaction between human PRUNE and nm23-H1 NDPKinase. *Oncogene* 18:7244–7252
- Schweneker M, Lukassen S, Spath M, Wolferstatter M, Babel E, Brinkmann K, Wielert U, Chaplin P, Suter M, Hausmann J (2012) The vaccinia virus O1 protein is required for sustained activation of extracellular signal-regulated kinase 1/2 and promotes viral virulence. *J Virol* 86:2323–2336
- Street A, Macdonald A, Crowder K, Harris M (2004) The Hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. *J Biol Chem* 279:12232–12241
- Tammenkoski M, Koivula K, Cusanelli E, Zollo M, Steegborn C, Baykov AA, Lahti R (2008) Human metastasis regulator protein H-prune is a short-chain exopolyphosphatase. *Biochemistry* 47:9707–9713
- Tan SL, Nakao H, He Y, Vijaysri S, Neddermann P, Jacobs BL, Mayer BJ, Katze MG (1999) NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. *Proc Natl Acad Sci USA* 96:5533–5538
- Tanji Y, Kaneko T, Satoh S, Shimotohno K (1995) Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* 69:3980–3986
- Tsutsumi T, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, Matsuura Y, Koike K, Miyamura T (2003) Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 38:820–828
- Virgilio A, Spano D, Esposito V, Di Dato V, Citarella G, Marino N, Maffia V, De Martino D, De Antonellis P, Galeone A, Zollo M (2012) Novel pyrimidopyrimidine derivatives for inhibition of cellular proliferation and motility induced by h-prune in breast cancer. *Eur J Med Chem* 57:41–50
- Wang L, Fraley CD, Faridi J, Kornberg A, Roth RA (2003) Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. *Proc Natl Acad Sci USA* 100:11249–11254
- Wang B, Zhang H, Zhu M, Luo Z, Peng Y (2012) MEK1-ERKs signal cascade is required for the replication of Enterovirus 71 (EV71). *Antiviral Res* 93:110–117
- Yamauchi S, Takeuchi K, Chihara K, Sun X, Honjoh C, Yoshiki H, Hotta H, Sada K (2015) Hepatitis C Virus Particle Assembly Involves Phosphorylation of NS5A by the c-Abl Tyrosine Kinase. *J Biol Chem* 290:21857–21864