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(54) Title: NOVEL TARGETS FOR THERAPIES OF HCV INFECTIONS

(57) Abstract: The present invention relates to eight newly identified human proteins that specifically interact with hepatitis C virus protein NS5A, and their use as novel targets for treatment of infections and diseases caused by Hepatitis C.



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NOVEL TARGETS FOR THERAPIES OF HCV INFECTIONS

The present invention relates to eight newly identified human proteins that specifically interact with hepatitis C virus protein NS5A, and their use as novel targets for treatment of infections and diseases caused by hepatitis C.

5 The hepatitis C virus (HCV) is a major human pathogen, infecting an estimated 170 million persons worldwide. A substantial fraction of these HCV infected individuals develop serious progressive liver disease such as chronic hepatitis, cirrhosis, liver failure and hepatocellular carcinoma. HCV is a positive-stranded RNA virus and belongs to the *Flaviviridae* family. All members of the *Flaviviridae* family have enveloped virions that contain a positive stranded RNA genome encoding all known virus-specific proteins via translation of a single, uninterrupted,
10 open reading frame. Considerable heterogeneity is found within the nucleotide and encoded amino acid sequence throughout the HCV genome. At least six major genotypes have been characterized, and more than 50 subtypes have been described.

The single strand HCV RNA genome encodes a single large polyprotein (about 3000 amino acids) in the following gene order: NH₂-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-
15 COOH. In infected cells, this precursor polyprotein is cleaved at multiple sites by host and viral proteases to produce the structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins of the virion. Among the non-structural proteins, NS5A is a phosphoprotein critical for the HCV life cycle, playing an important role in viral RNA replication, modulation of cell signaling pathways, interferon response, pathogenesis and
20 apoptosis regulation. The mature NS5A protein is composed of approximately 447 amino acids and is generated by the action of the viral NS3/4A serine protease. NS5A influences key cellular processes and viral RNA replication by interacting with host signaling proteins. The host proteins that interact with NS5A are valuable drug targets, but to date not all of these interaction proteins are known.

25 Currently, one of the most effective HCV treatments uses a combination of α -interferon and ribavirin, leading to sustained efficacy in only about 40% of patients. Thus, a substantial fraction of patients do not have a sustained reduction in viral load. Moreover, the treatment is

cumbersome and sometimes has debilitating and severe side effects and many patients do not durably respond to treatment.

Thus, there is a continuing need to develop effective therapeutics for treatment of HCV infection.

- 5 The technical problem underlying the present invention is the provision of novel targets useful for treatment of HCV infection. The technical problem is solved by provision of the embodiments characterized in the claims.

- The present invention is directed to novel targets for the medical intervention against hepatitis C virus (HCV) infections and HCV-related diseases in mammals, in particular humans. The
- 10 invention provides the identity of eight new specific NS5A-interacting human proteins, in particular of two human cellular protein kinases responsible for NS5A phosphorylation and optimal HCV replication. These novel specific NS5A-interacting human proteins can be used as targets of novel therapeutic protocols for the treatment and/or prevention of infections and diseases caused by HCV and for the identification and development of new HCV antiviral agents.
- 15 More specifically, with the aim of identifying novel host proteins that interact with hepatitis C virus protein NS5A, the inventors have applied an innovative Immuno-Competitive Capture Mass Spectrometry (ICC-MS) method to allow systematic investigation of protein-protein interaction. ICC-MS, which introduces a pre-competition step between free and capturing
- 20 antibody followed by a comprehensive statistical analysis of MS signal, drastically increases the specificity of classical immunoprecipitation (IP). The inventors applied ICC-MS to the interactome analysis of the viral non-structural protein 5A (NS5A), a key regulatory protein of Hepatitis C virus (HCV), in a cellular HCV replication system. As a result, eight new specific NS5A-interacting proteins along with two previously validated partners (PI4KIII α and hVAP-33) were identified. These eight new specific NS5A-interacting proteins are: **NP1L4, PKHG2,**
- 25 **FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.**

Follow up validation experiments revealed that LATS1 and LATS2, two closely related human protein kinases are novel host kinases responsible for NS5A phosphorylation and optimal HCV replication.

All the identified specific NS5A-interacting proteins represent potential targets for novel antiviral intervention. Accordingly, in one aspect the present invention provides these protein kinases as targets for antiviral therapies against HCV infection and HCV-related diseases.

Therefore in one embodiment of the invention, a method for preventing Hepatitis C virus (HCV) infection of a cell is provided, comprising a step of contacting the cell with an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5**.

In a preferred embodiment, a method for preventing Hepatitis C virus (HCV) infection of a cell is provided, comprising a step of contacting the cell with an agent that inhibits the activity of at least one NS5A binding protein selected from **LATS1 and / or LATS2**. In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7)**.

In one embodiment of the invention, a method for treating a Hepatitis C virus (HCV) infection of a cell is provided, comprising a step of contacting the cell with an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5**.

In a preferred embodiment, a method for treating Hepatitis C virus (HCV) infection of a cell is provided, comprising a step of contacting the cell with an agent that inhibits the activity of at least one NS5A binding protein selected from **LATS1 and / or LATS2**. In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7)**.

In one embodiment of the invention, a method for preventing Hepatitis C virus (HCV) infection in a subject is provided, comprising administering to a subject an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5**.

In a preferred embodiment, a method for preventing Hepatitis C virus (HCV) infection in a subject is provided, comprising administering to a subject an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from **LATS1 and / or LATS2**. In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7)**.

In one embodiment of the invention, a method for treating a Hepatitis C virus (HCV) infection of a patient is provided, comprising administering to a patient infected with HCV an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.**

In a preferred embodiment, a method for treating Hepatitis C virus (HCV) infection of a patient is provided, comprising administering to a patient infected with HCV an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from **LATS1 and / or LATS2.** In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7).**

The agent of the methods of the invention is selected from the group consisting of small molecules, monoclonal antibodies, polyclonal antibodies, kinase inhibitor and any combination thereof.

In another embodiment a pharmaceutical composition is provided comprising at least one pharmaceutically acceptable carrier or excipient and an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.**

In a preferred embodiment, a pharmaceutical composition is provided comprising at least one pharmaceutically acceptable carrier or excipient and an effective amount of an agent that inhibits the activity of at least one NS5A binding protein selected from **LATS1 and / or LATS2.** In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7).**

In yet another embodiment a method for identifying a potential HCV anti-viral agent is provided, comprising the steps of: (a) contacting in vitro a cell that expresses at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.**; and (b) determining the activity of said NS5A binding protein, wherein a candidate compound is identified as a potential HCV anti-viral agent if the activity determined in step (b) is lower than the activity of said protein in the absence of the candidate compound.

In a preferred embodiment, a method for identifying a potential HCV anti-viral agent is provided, comprising the steps of: (a) contacting in vitro a cell that expresses at least one NS5A binding protein selected from **LATS1 and / or LATS2**; and (b) determining the activity of said NS5A binding protein, wherein a candidate compound is identified as a potential HCV anti-viral agent if the activity determined in step (b) is lower than the activity of said protein in the absence of the candidate compound. In one embodiment, the NS5A binding protein is selected from **LATS1 (SEQ ID. NO 6) and / or LATS2 (SEQ ID. NO 7)**.

In another aspect the present invention provides methods for the identification of compounds useful for the prevention and/or treatment of HCV infections and/or HCV-related diseases. Specifically, these methods involve contacting a biological system (e.g., a cell) that expresses or can express at least one NS5A binding protein selected from the group of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5** with a candidate compound and determining the activity of said NS5A binding protein or a factor that is representative of the activity of said NS5A binding protein.

A candidate compound is identified as a potential HCV anti-viral agent (i.e., a compound potentially useful for treating and/or preventing infections or diseases caused by HCV) if the activity of the NS5A binding protein is lower in the presence of the candidate compound than in the absence of the candidate compound. Alternatively, a candidate compound is identified as a potential HCV anti-viral agent if the factor representative of the activity of the NS5A binding protein selected from the group of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5** is different (lower or higher depending on the relationship between the factor and the activity) in the presence of the candidate compound and in the absence of the candidate compound.

In a preferred embodiment the present invention provides methods for the identification of compounds useful for the prevention and/or treatment of HCV infections and/or HCV-related diseases comprising contacting a biological system (e.g., a cell) that expresses or can express the **LATS1 and/ or the LATS2** protein kinase with a candidate compound and determining the activity of said protein kinase or a factor that is representative of the activity of said protein kinase.

A candidate compound is identified as a potential HCV anti-viral agent (i.e., a compound potentially useful for treating and/or preventing infections or diseases caused by HCV) if the activity of the LATS1 and/ or the LATS2 protein kinase is lower in the presence of the candidate compound than in the absence of the candidate compound. Alternatively, a candidate
5 compound is identified as a potential HCV anti-viral agent if the factor representative of the LATS1 and/ or the LATS2 protein kinase is different (lower or higher depending on the relationship between the factor and the activity) in the presence of the candidate compound and in the absence of the candidate compound.

10 In certain embodiments, the methods of the invention are used to screen individual candidate compounds. In other embodiments, the methods of the invention are used to screen libraries of candidate compounds. A candidate compound may belong to any of a wide variety of family of molecules. In certain embodiments, the candidate compound is selected from the group consisting of small molecules, monoclonal antibodies, polyclonal antibodies, protein kinase
15 inhibitors and any combination thereof.

The candidate compound of the methods disclosed above is selected from the group consisting of small molecules, monoclonal antibodies, polyclonal antibodies, kinase inhibitor, and any combination thereof.

In another embodiment there is provided at least one NS5A binding protein selected from the
20 group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5** for use as a target for treatment of Hepatitis C. In a preferred embodiment there is provided LATS1 and / or LATS2 for use as a target for treatment of Hepatitis C.

In another embodiment there is provided use of an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1,**
25 **UBP19, LATS1, LATS2, PGAM5** for treatment of Hepatitis C. In a preferred embodiment there is provided an agent that inhibits the activity of LATS1 and / or LATS2 for use in treatment of Hepatitis C.

The term "NS5A binding protein" relates to the new proteins identified in the present invention, which were shown to interact with NS5A. In particular: **NP1L4, PKHG2, FBW1B, NP1L1,**
30 **UBP19, LATS1, LATS2, PGAM5.** In one embodiment, the NS5A binding protein is selected from the group consisting of **NP1L4 (SEQ ID. NO 1) , PKHG2 (SEQ ID. NO 2), FBW1B**

(SEQ ID. NO 3), NP1L1 (SEQ ID. NO 4), UBP19 (SEQ ID. NO 5), LATS1 (SEQ ID. NO 6), LATS2 (SEQ ID. NO 7), PGAM5 (SEQ ID. NO 8).

The terms "kinase" and "protein kinase" are used herein interchangeably. They refer to an enzyme that catalyzes the transfer of a phosphate group from a nucleoside triphosphate to certain amino acid residues of another molecule (herein called "substrate" or "kinase substrate") that is involved in a signaling pathway. The phosphate group may be transferred, for example, from an ATP or GTP (adenosine or guanine triphosphate) molecule. Kinases may be transmembrane or intracellular proteins. Eukaryotic protein kinases are characterized by the sequence of a contiguous stretch of approximately 250 amino acids that constitutes the catalytic (kinase) domain. Kinases may be tyrosine kinases, serine/threonine kinases, histidine kinases, or dual-specificity kinases.

The terms "kinase activity" and "activity of a kinase" are used herein interchangeably and refer to the ability of a protein kinase to catalyze the phosphorylation of certain amino acid residues of a substrate molecule.

The term "inhibitor of kinase activity", when used in reference to a compound, refers to the ability of the compound to inhibit (e.g., fully suppress or partially decrease) the ability of a protein kinase to catalyze the transfer of a phosphate group from a nucleoside triphosphate to certain amino acid residues of a substrate molecule. In the practice of the present invention, inhibition of kinase activity may be achieved by any of a wide variety of mechanisms. However, irrespective of the mechanism, kinase activity inhibition results in the reduction of the ability of the kinase to catalyze the phosphorylation of its substrate(s). Thus, by "inhibition" is meant that the level of phosphorylation of the substrate is reduced at least 50% after incubation in the presence of the compound, for example in an assay of the invention. Preferably, the level of phosphorylation of the substrate is reduced at least 90% by the compound. More preferably, the level of phosphorylation of the substrate is reduced at least 95% by the compound. A candidate compound that induces such a decrease in the level of phosphorylation of a substrate molecule in a kinase assay of the invention is "identified" as an inhibitor of the activity of the kinase. Thus, in certain embodiments, an "inhibitor of kinase activity" is a compound that is/has been identified by a screening method of the invention as inhibiting/suppressing the activity of a given kinase.

The terms "HCV-related disease" and "HCV-associated disease" are herein used interchangeably. They refer to any disease or disorder known or suspected to be associated with and/or directly or

indirectly caused by HCV. HCV-related (or HCV-associated) diseases include, but are not limited to, a wide variety of liver diseases, such as subclinical carrier state of acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The terms include symptoms and side effects of any HCV infection, including latent, persistent and sub-clinical infections, whether or not the infection is clinically apparent.

The term "treatment" is used herein to characterize a method or process that is aimed at (1) delaying or preventing the onset of a disease or condition (e.g., HCV infection or HCV-related disease); (2) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease or condition; (3) bringing about amelioration of the symptoms of the disease or condition; or (4) curing the disease or condition. A treatment may be administered prior to the onset of the disease or condition, for a prophylactic or preventing action.

Alternatively or additionally, a treatment may be administered after initiation of the disease, for a therapeutic action.

A "pharmaceutical composition" is defined herein as comprising an effective amount of at least one biologically active ingredient (e.g., a protein kinase inhibitor) and at least one pharmaceutically acceptable carrier or excipient.

The term "preventing, inhibiting or blocking HCV infection" when used in reference to an agent (e.g., a protein kinase inhibitor), means reducing the amount of HCV genetic information introduced into a susceptible cell or susceptible cell population as compared to the amount that would be introduced in the absence of the agent.

An "individual," "subject," or "patient" is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, a mammal is a human.

An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

Short description of the Figures

Figure 1 Overview of the label-free quantitative Immuno-Competitive Capture Mass Spectrometry (ICC-MS) experimental procedure for the detection of protein interactors. Cell lysates are pre-treated with increasing concentrations of free NS5A antibody, and individually incubated with the same antibody immobilized on agarose matrix. The eluted fractions are separated by SDS-PAGE, proteins digested with trypsin, and extracted peptides analyzed by LC-MS. Quantified proteins on the basis of their corresponding peptides' extracted ion chromatograms (EICs) are subjected to a comprehensive statistical analysis highlighting concentration-dependent signal decrease of the specific interactors.

Figure 2 Concentration dependent decrease of the NS5A capture by applying ICC-MS. **(a)** NS5A capture from Huh-7 cells including a stable Con1 (genotype 1b) replicon pre-incubated with 0, 0.6, 1, 1.7, 2.9, 5, 8.5, 14.5, 24.6, 41.9, 71.2, and 120 µg/ml of free NS5A antibody was probed by western blot analysis. NS5A IP was gradually decreased due to pre-incubation of the cell lysates with increasing concentration of the free form of the antibody. **(b)** NS5A from each immunoprecipitates was identified and quantified by LC-MS. The protein intensity was plotted on the y axis and the free NS5A antibody concentrations on the x-axis. The relative abundances for technical replicates (red for technical replicate A, blue for technical replicate B) are highly similar, indicating a low technical variance and very good repeatability of the process.

Figure 3 Decrease of relative protein abundance of some interesting proteins vs. concentration of free anti-NS5A antibody. The relative abundances for technical replicates (red for technical replicate A, blue for technical replicate B) are highly similar, indicating a low technical variance and very good repeatability of the process.

Figure 4 *In vitro* NS5A phosphorylation by LATS2. **(a)** Phosphoprotein (Pro-Q Diamond) and **(b)** total protein (SYPRO) staining of the SDS-PAGE separated reaction mixtures. Two recombinant forms of the protein, NS5A domain 1 (residues 33-202) and NS5A 32 (residues 33-447) were used, and detected at ca. 20 kDa and 50 kDa, respectively. Phosphorylation was clearly detected after 20 min treatment with the kinase. **(c)** Relative quantitation of Ser71 phosphorylation by LC-MS. Ratio between phosphorylated and un-phosphorylated NGSMRIVGPK peptide EICs are represented.

Figure 5 Replication capacity of mutant transient replicon Con1-NS5A-S71A. The replication capacity of the mutant transient replicon Con1-NS5A-S71A was expressed as its normalized replication efficiency compared to that of the reference strain Con1 that is set at a value of 100 %. Average and standard deviation were calculated from 4 independent experiments.

5 **Figure 6** Effect of siRNA knock down on HCV replicon replication.

Figure 7 NS5A immunoprecipitation in Huh7Con1 cell extracts.

Examples:

1. ONLINE METHODS

Cell culture and lysates preparation. Huh-7 cells stably expressing Con-1 genotype 1b replicon (Huh-7 2209-23) were maintained in DMEM (with GlutaMAX) containing antibiotics (500 µg/ml penicillin-streptomycin, 500 µg/ml G418, all Invitrogen) and 10% fetal bovine serum (Sigma) as previously described (Lohmann, V., Hoffmann, S., Herian, U., Penin, F., Bartenschlager, R. Viral and Cellular Determinants of Hepatitis C Virus RNA Replication in Cell Culture. *Journal of Virology* 77, 3007-3019 (2003).). Harvested replicon cells were re-suspended in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40, and protease and phosphatase inhibitor tablets (Complete and PhosStop, Roche Applied Science). After 15 min on ice, lysates were cleared by centrifugation at 1,500 ×g for 10 min at 4 °C, and protein concentration was estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce).

Immunoprecipitation, competition experiment, and in-gel digestion. Anti-NS5A clone 7-D4 was used for western blot detection from (Thermo Fischer Scientific) and anti-NS5A clone H26 (epitope between residues 228 and 278) for IP and competition experiments (Abcam). The antibody was desalted on Zeba columns (Pierce) following manufacturer's instructions and then coupled to Affigel 10 agarose beads (BioRad) for 3 h at 4 °C. Unreacted binding sites were blocked by the addition of 0.2 M ethanolamine, beads washed four times with cold PBS, and stored at 4 °C. Beads (20 µl) were incubated overnight with cell lysate (260 µg protein per condition) at 4 °C, washed four times with lysis buffer, and eluted with SDS sample buffer.

For competition experiments, lysates were pre-incubated with increasing amounts of free anti-NS5A antibody (0, 0.06, 0.1, 0.17, 0.29, 0.5, 0.85, 1.45, 2.46, 4.19, 7.15, and 12 µg) for 3 h (100 µl of final volume) before being treated with immobilized anti-NS5A resin for 3 h (all at 4 °C).

Eluates were separated on a 4-20% Tris-Gly SDS-PAGE by migrating the gel half its length, and proteins stained with colloidal blue. Four bands spanning from 20 to 150 kDa (named 20, 40, 80, and 140 kDa, respectively) were cut and in-gel trypsin digested following standard procedures.

LC-MS/MS analyses, data processing, and protein quantitation. Each sample was analyzed in duplicate with a nanoflow Easy-nLC system (Proxeon) connected to an LTQ-Orbitrap Velos (ThermoFisher Scientific). Peptides were concentrated on an AQUA C18 trap (100 $\mu\text{m} \times 10 \text{ mm}$, Phenomenex) before being separated on a ReproSil-Pur C18-AQ (75 $\mu\text{m} \times 200 \text{ mm}$, 3 μm particle size, 120 Å, Dr. Maisch GmbH) analytical column using a 50 min gradient of 0-35% acetonitrile (with 0.6% acetic acid) at 250 nl/min. Acquisitions were cycled between full scan at 60,000 resolution (at m/z 400) in the Orbitrap and 10 data-dependent collision-induced dissociation (CID) scans in the ion trap. Ions were selected only with assigned charge states $>1+$, and then excluded for 30 s. MS-raw files were processed converted into -dta files using Extract-MSn (version 1.0.0.8) and data searched using Sequest (version 27.0, revision 12, both ThermoFisher Scientific). A database consisting of the human part of the SwissProt (June 2009, 34,275 entries, including splice variants) augmented with the five viral non-structural proteins from the Con1 replicon NS3P, NS4a, NS4B, NS5A, and NS5B was used. Searching parameters were trypsin (full) as an enzyme, one missed-cleavage, and a mass tolerance of 10 ppm and 1.0 Da for precursor and fragment ions, respectively. Oxidized methionines (+15.9949 Da), phosphorylated serine, threonine, and tyrosines (+79.9663 Da) were set as differential, while carbamidomethylated cysteines (+57.0215 Da) were set as static modifications. The spectral false discovery rate (specFDR) was restricted to 2.5% by performing a target-decoy search (Elias, J., Gygi, SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature methods* **4**, 207-214 (2007). Peptides were assigned to their parent proteins, and each combination of parent proteins was defined as a protein quantification group.

Label-free quantitation was performed using RefinerMS (version 5.2.6, Genedata). After loading and pre-processing of the MS files, peaks were recognized, retention times aligned, and extracted ion chromatograms (EICs) were generated. Samples of each respective antibody concentration and each gel band were normalized using the quantile-quantile approach (Smyth, G., Speed, T. Normalization of cDNA microarray data. *Methods (San Diego, Calif)* **31**, 265-273 (2003). The peptide sequences were assigned to peaks found by RefinerMS via their scan IDs, peaks with no or ambiguous identification were removed. In order to control quality, the log2-

transformed quantitation data were subjected to principal component analysis (PCA). Scores of the first four principal components were correlated with antibody concentration and process parameters, such as gel lane band, and LC-MS run number. Samples outside the 99% confidence interval, as determined by Mahalanobis distance evaluation based on the scores of the first two principal components, were considered as outliers and removed from further analysis. Protein quantitation was performed by summarizing peptide EICs using the median polish procedure (Holder 2001), in analogy to the robust multichip analysis (RMA) method applied in the microarray field (Irizarry, Hobbs et al. 2003).

Statistical analysis of dose response. We hypothesized that good binders would have (much)

higher relative abundance in samples treated with low antibody concentrations than in samples treated with low antibody concentrations. As it is not known a priori at which concentration point the relative protein concentration will start to decrease, the splitting point between groups of high and low concentrations of antibody is shifted along the antibody concentration axis. The assignment of samples to a set of high concentration or low antibody concentrations defines a "contrast", and since the series of concentrations allows multiple splitting points, a series of contrasts can be summarized in a contrast matrix. Then, two concepts were combined: 1) Stewart and Ruberg (Stewart & Ruberg, S. J. (2000) Detecting dose response with contrasts. Stat. Med. 19, 913-921) and later Bretz & Hothorn (Bretz, F., and Hothorn, L. A. (2001) Testing dose-response relationships with a priori unknown, possibly nonmonotone shapes. J. Biopharm. Stat. 11, 193-207) proposed the use of contrast tests for detecting dose-response relationships, which numerically applies also to problem. 2) Wu and Kuster (Wu, Z., Doondeea, J. B., Moghaddas Gholami, A., Janning, M. C., Lemeer, S., Kramer, K., Eccles, S. A., Gollin, S. M., Grenman, R., Walch, A., Feller, S. M., and Kuster, B. (2011) Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. Mol. Cell. Proteomics.) elaborated the benefits of the moderated linear model from the limma package (Smyth, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3) in Bioconductor on label free mass spectrometry data generated similar to ours. Both concepts were combined by applying the contrast tests in a moderated linear model, resulting in a series of T values for each protein quantitation group. As outlined by Bretz and Hothorn, the maximum T statistic from such a series is determined per protein quantitation group. P-values were obtained based on the algorithm for step-down maxT adjusted p-Values with 1000 permutations based on Ge 2003 (Ge Y., D. S., Speed T.P. (2003) Resampling-based multiple testing for microarray data hypothesis. Test. 12, 1-44), which

originates from Westfall and Young ((Westfall P.H., Y. S. S. (ed) (1993) Resampling-based Multiple Testing); Algorithm 4.1, p116-117). It was modified with respect to the one-sidedness of the statistic by not choosing $|T|$ but T . Visualizations and any computations are performed with R 2.10.1 (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0).

In vitro NS5A phosphorylation. Recombinant NS5A Δ 32 (BK strain, residues 33-447) was expressed in *E. coli* and isolated from the soluble fraction as reported (Huang, L. *et al.*

Purification and characterization of hepatitis C virus non-structural protein 5A expressed in *Escherichia coli*. *Protein expression and purification* **37**, 144-153 (2004)). The purified protein (400 ng) was pre-mixed with active human LATS2 (residues 480-1088, SignalChem) in kinase activity buffer (12.5 mM β -glycerol phosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT in 25 mM MOPS pH 7.2), reaction initiated by the addition of 1 mM ATP (15 μ l reaction volume), and further incubated at 30 °C for 0, 20, 40, 60 and 80 min. Reaction was stopped by the addition of SDS sample buffer, proteins separated by SDS-PAGE, stained with Pro-Q Diamond and SYPRO (Invitrogen), or processed for LC-MS analysis as described above.

Transient replicon assay. Transient wild type replicon Con1 (expressing firefly luciferase reporter gene, Lohmann, V., Hoffmann, S., Herian, U., Penin, F., Bartenschlager, R. Viral and Cellular Determinants of Hepatitis C Virus RNA Replication in Cell Culture. *Journal of Virology* **77**, 3007-3019 (2003)) was used to introduce the S71A mutation in the NS5A region using Quick Change site-directed mutagenesis kit following manufacturers' instructions (Stratagene). The introduced mutation was confirmed by DNA sequencing. Lunet Huh7 cells (4×10^6) were transfected with 10 μ g of in vitro transcribed HCV transient subgenomic replicon RNAs6 (WT or NS5A mutant). The normalized replication capacity was determined by taking the ratio of the luciferase signal at 96/4 h post-transfection. The replication capacity of the replicon containing the NS5A-S71A mutant was expressed as its normalized replication efficiency compared to that of the reference replicons that is set at 100. Average and standard deviation were calculated from four independent experiments.

siRNA knock down experiments. All siRNAs used in the study were obtained from Integrated DNA Technologies, Inc. Transfection of siRNA in the HCV replicon cells was performed using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol. Briefly, 5,000 replicon cells, seeded in a 96-well plate 24 h pre-siRNA transfection, were transfected in duplicate with 1 pmol of siRNA (or 1pmol per siRNA in the siRNA dual combination

experiments). Renilla luciferase signal was read 72 h post-siRNA transfection using the Renilla Luciferase Assay System (Promega) following the manufacturer's protocol. Renilla luciferase signal of each gene-specific siRNA was normalized to the one obtained using negative control siRNAs. Average and standard deviation were calculated from at least three independent experiments.

HCV NS5A Immuno-Competitive Capture (ICC) in Huh-7 replicon cells. The ICC methodology is based on a pre-incubation of cell lysates with increasing concentrations of free antibody, prior to IP with the same antibody immobilized on agarose beads (**Fig. 1**). Because the free antibody will bind and take up more of its target protein in the successive samples, fewer will remain in the mixture for the IP antibody. In theory, specific interactors should show the same behavior, however, with different abundances depending on the stoichiometries. We applied this new strategy to the analysis of the NS5A interactome in an HCV subgenomic replicon system. This *in vitro* model expresses the non-structural HCV proteins NS3, NS4B, NS5A and NS5B and encompasses all steps involved in viral RNA replication (Lohmann, V., Hoffmann, S., Herian, U., Penin, F., Bartenschlager, R. Viral and Cellular Determinants of Hepatitis C Virus RNA Replication in Cell Culture. *Journal of Virology* 77, 3007-3019 (2003)). The HCV NS5A protein was selectively and efficiently pulled down by immobilized NS5A antibody from a stable Con1 (genotype 1b) replicon cell line derived from human hepatoma Huh-7 cells (**Fig. 7**).

The cell lysate was divided into equal aliquots and pre-incubated with increasing concentration of free anti-NS5A antibody. Initially, the concentration range of the free antibody to be used during the competition experiment was determined. The NS5A present in the elution fractions were probed by immuno-blotting (**Fig. 2a**). As a result, a near-complete disappearance of the signal corresponding to the IP-ed NS5A protein was observed at 120 µg/ml, indicating a complete reaction of the target protein with the free anti-NS5A antibody. Expanding to 12 concentrations of free antibody with an equal dilution factor of 1.6, a dose-dependent inhibition of NS5A protein capture by the immobilized antibody was observed. The largest drop of signal occurred between 1.7 and 2.9 µg/ml of free NS5A antibody (**Fig. 2a**). In order to validate the approach, the same samples were analyzed by nanoflow liquid-chromatography-tandem mass spectrometry (nanoLC-MS/MS). The resulting curve obtained for NS5A based on the MS signals showed an excellent correlation with the immune-blot signals (**Fig. 2b**). Again, the largest drop of the NS5A signal was detected at the exact same concentrations of free NS5A antibody, that is

between 1.7 and 2.9 $\mu\text{g/ml}$, confirming that the label-free quantitative MS was a reliable readout for our approach.

Identification of HCV NS5A interactors using ICC-MS approach

After we had validated the ICC-MS approach at the NS5A protein target level, we applied it toward identification of specific NS5A co-immuno-precipitated proteins based on the hypothesis that any specific NS5A binder co-precipitating with NS5A should have approximately similar displacement curve profile as NS5A itself in the presence of increasing concentrations of competitor antibody.

On average about around 771 protein quantitation groups were identified as co-immuno-precipitating with NS5A without any filtering step (**data not shown**). A statistical model (Bretz, F., Hothorn, LA. Testing dose-response relationships with a priori unknown, possibly nonmonotone shapes. Journal of Biopharmaceutical Statistics 11, 193-207 (2001) and Wu, Z. e. a. Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. Mol Cell Proteomics 10 (2011)) was applied on the mass spectrometry signals to derive concentration-dependent signal decrease of specific binders: besides NS5A, 1 viral protein (NS3) and 10 Huh7 proteins (NP1L4, PKHG2, FBW1B, NP1L1, PI4Ka, UBP19, VAPA, LATS1, LATS2, PGAM5) were specifically displaced by the free antibody with an adjusted p-Value below 5% (**Table 1, Fig. 3**).

Table 1: Statistics of proteins that are significantly decreasing in their relative abundance with increasing free antibody concentration. T value. pAdj: p Value adjusted by the Westfall-Young approach. Signal reduction: signal difference between the highest and the lowest concentration.

Protein	Gene name	p (adjusted)	Signal reduction	Number of signals	Different sequences
NS5A		6.65E-06	99%	41	13
NP1L4	NAP1L4	6.67E-06	95%	9	6
PKHG2	PLEKHG2	6.70E-06	99%	1	1
FBW1B	FBXW11	3.16E-04	99%	1	1
NP1L1	NAP1L1	7.99E-04	90%	10	5
PI4KA	PI4KA	3.12E-03	99%	10	7
UBP19	USP19	3.41E-03	99%	18	11
VAPA	VAPA	5.65E-03	86%	7	2
LATS1	LATS1	1.80E-02	99%	10	7
LATS2	LATS2	2.00E-02	99%	4	4
PGAM5	PGAM5	3.47E-02	43%	1	1
NS3		7.02E-02	52%	13	5

Table 2 HCV NS5A interacting proteins identified

Huh protein- Symbol	Description	Seq ID No	Sequence
NAP1L4	nucleosome assembly protein 1- like 4	1	MADHSFSDGVPSDSVEAAKNASNTEKLTQVMQNPRVLAALQERLDN VPHTPSSYIETLPKAVKRRINALKQLQVRCAHIEAKFYEEVHDLERK YAALYQPLFDKRREFITGDVEPTDAESEWHSENEEEKLAGDMKSKV VVTEKAAATAEEPDPKGIPEFWFTIFRNVDMLSELVQEYDEPILKHL QDIKVKFSDPGQPMSEVLEFHFEPNDYFTNSVLTKTYKMKSEPDKAD PFSFEGPEIVDCDGCITDWKKGNVTVKTIKKKQKHKGRTVTRITK QVPNESFFNFFNPLKASGDGESLDEDESEFTLASDFEIGHFFRERIVP RAVLYFTGEAIEDDDNFEEGEGEGEELEGEDEGEDDAEINPKV
PKHG2	pleckstrin homology domain containing, family G (with RhoGef domain) member 2	2	MPEGAQGLSLSKPSPSLGCGRGEVDCDGTVCETRTPAAPTMASPR GSGSSTSLSTVGSEGDPAAGPTPACSASRPEPLPGPPIRLHLSVGI PGSARPSRLERVAREIVETERAYVRDLRSIVEDYLGPLLDGGVLGLS VEQVGTLFANIEDIYEFSSSELLEDLENSSSAGGIAECFVQRSEDFDI YTLYCMNYPSSLALLRELSLSPPAALWLQERQAQLRHSPLPLQSFLK PVQRIKYLHLLQELGKHWAEGPGTGGREMVEEAIVSMTAVAWYIND MKRKQEHAARLQEVQRRLLGGWTGPELSAFGELVLEGAFRGGGGGGPR LRGGERLLFLFSRMLLVAKRRGLEITYKGIHFCNLSVSESPRDPLG FKVSDLTIPKHRHLLQAKNQEEKRLWIHCLQRLFFENHPASIPAKAK QVLLNSLHCAPKSKPVLEPLTPPLGSPRPRDARSFTPGRNTAPSP GPSVIRRGRRQSEPVKDPYVMFPQNAKPGFKHAGSEGELYPPESQPP VSGSAPPEDLEDAGPPTLDPSGTSITEEILELLNQRLRDPGPPSTHD IPKFPGDSQVPGDSETLTFQALPSRDSSEEEEEEGLEMDERGPSP LHVLEGLESSIAAEMPSICLTKIPDVPNLPEIPSRCEIPEGSRLPS LSDISDVFEMPCLPAIPSVNTPSLSSTPTLSCDSWLQGPLQEPAEA PATRRELFSGSNPGKLGEPPSGGKAGPEEDEEGVSFTDFQPQDVTQH QGFPDELAFRSCSEIRSAWQALEQQQLARPGFPEPLLILEDSDLGGD SGSGKAGAPSSERTASRVRELARLYSERIQQMORAETRASANAPRRR PRVLAQPQPSPCLPQEQAEPLLPAGHVLVCELAFPLTCAQESVPL GPAVWVQAAIPLSKQGGSPDGQGLHVSNLPKQDLPGIHVSAATLLPE QGGSRHVQAPAATPLPKQEGPLHLQVPALTTFSQGHPEIQVPATTP LPEHRSHMVIAPSTAFCEQGHCAIHVPTTPALPKEICSDFTVSV TTPVPKQEGHLDSESPNTIPLTKQGGSRDVQGPDPVCSQPIQPLSWH GSSLDPPQPGDTPPLPCHLPDLQIPGTSPLPAHGSHLDHRI PANAP LSLSQELPDTQVPATTPPLPQVLTDIWVQALPTSPKQGSPLDIQGP AAAPPLPEPSLTDQVQKLTPSLEQKSLIDAHVPAATPLPERGGS LD IQGLSPTPVQTTMVL SKPGGSLASHVARLESSDLTPPHSPPPSSRQL LGPNAALSRYLAASYISQSLARRQPGGGAPAAARGSWSSAPTSRA SSPPPQPQPPPPPARRLSYATTVNIHVGGGRLRPAKAQVRLNHPAL LASTQESMGLHRAQGAPDAPFHM
FBW1B	F-box and WD repeat domain containing 11	3	MEPDSVIEDKTIELMISNGTSSVIVSRKRPSEGN YQKEKDLCKYFD QWSESDQVEFVEHLISRMCHYQHGHI NSYLKPMQLRDFITALPEQGL DHIAENILSYLDARSLCAAELVCKEWQRVISEGMLWKKLIERMVRTD PLWKGLSERRGWDQYLFKNRPTDGPPNSFYRSLYPKIIQDIETIESN WRCGRHNLQRIQCRSENSKGVYCLQYDDEKIIISGLRDN SIKIWDKTS LECLKVLTGHTGSVLC LQYDERVIVTGSSDSTVRVWDVNTGEVLNTL IHHNEAVLHLRF SNGLMVTCSKDRSIAVWDMASATDITLRRVLV GHR AAVNVVDFDDKYIVSASGDRTIK VVSTSTCEFVRTLNHGKRGIA CLQ YRDRLVVSGSSDNTIRLWDIECGACLRVLEGHEELVRCIRFDNKRIV SGAYDGKIKVWDLQAALDPRAPASTLCRLTLVEHSGRVFRLQFDEFQ IISSSHDDTILIWDFLNVPPSAQNETRSPSRITYTYSR

Huh protein- Symbol	Description	Seq ID No	Sequence
NP1L1	nucleosome assembly protein 1- like 1	4	SKEQSELDQDLDDVEEVEEEETGEETKLKARQLTVQMMQNPQILAAAL QERLDGLVETPTGYIESLPRVVKRRVNALKNLQVKCAQIEAKFYEEV HDLERKYAVLYQPLFDKRFEIINAIYEPTEECEWKPDDEDEISEEL KEKAKIEDEKKDEEKEDPKGIPEFWLTVFKNVDLLSDMVQEHDPEIL KHLKDIKVKFSDAGQPMFVLEFHFEPNEYFTNEVLTKTYRMRSEPD DSDPFSFDGPEIMGCTGCQIDWKKGKNVTLKTIKKKQKHKGRGTVRT VTKTVSNDSSFNFAPPEVPESGDLDDDAEAILAADFEIGHFLRERI IPRSVLYFTGEAIEDDDDDYDEEGEEADEEGEEEGDEENDPDYDPKK DQNPAECKQQ
USP19	ubiquitin specific peptidase 19	5	MSGGASATGPRRGPPGLEDTTSKKKQKDRANQESKDGDPKGSASTP QEEQTKEELLLDWRQSAEEVIVKLRVGVGPLQLEDVDAFTDTCVV RFAGGQQWGGVFYAEIKSSCAKVQTRKGSLLHLTLPKKVPMLTWPSL LKKPLGTQELVPLRCQENGQELSPIALEPGPEPHRAKQEARNQKRA QGRGEVGAGAGPGAQAGPSAKRAVHLCRGPEGDGSRDDPGPRGDAPP FVADPATQVEADEQLCIPPLNSQTCLLGSEENLAPLAGEKAVPPGND PVSPAMVRSRNPBKDDCAKEEMAVAADAATLVDEPESMVNLAFVKND SYEKGPDSSVVHVYVKEICRDTSRVLFREQDFTLIFQTRDGNFLRLH PGCGPHTTFRWQVKLRNLIEPEQCTFCFTASRIDICLRKRQSQRWGG LEAPAARGAVGGAKVAVPTGPTPLDSTPPGGAPHPLTGQEEARAVEK DKSKARSEDGLDSVATRTPMHVTPKPETHLASPKPTCMVPPMPHS PVSGDSVEEEEEEEKVCLPGFTGLVNLGNTCFMNSVIQSLSNREL RDFHHRDSFEAEINYNPLGTGGRLAIGFAVLLRALWKGTHHAFQPS KLKAI VASKASQFTGYAQHDAQEFMAFLLDGLHEDLNRIQNKPYTET VDSGRPDEVVAEEAWQRHKMRNDSFIVDLFQGGYKSKLVCPVCAKV SITFDPFLYLPVPLPQKQKVLVVFYFAREPHSKPIKFLVSVSKENST ASEVLDLSLSQSVHVKPENLRLAEVINKRFHRVFLPSHSLDTPVSPSDT LLCFELLSSSELAKERVVVLEVQQRQVPSVPI SKAACQRKQQSEDE KLKRCTRCYRVGYCNQLCQKTHWPDHKGLCRPENIGYPFLVSVPASR LTYARLAQLLEGYARYSVSVFQPPFQPGRMALESQSPGCTTLLSTGS LEAGDSERDPIQPPELQLVTPMAEGDTGLPRVWAAPDRGPVPSTSGI SSEMLASGPIEVGSLPAGERVSRPEAAVPGYQHPSEAMNAHTPQFFI YKIDSSNREQRLEDKGDTPLELGDDCSLALVWRNNERLQEFVLVASK ELECAEDPGSAGEAARAGHFTLDQCLNLFTRPEVLAPEEAWYCPQCK QHREASKQLLLWRLPNVLIVQLKRFSFRSFIWRDKINDLVEFPVRNL DLSKFCIGQKEEQLP SYDLYAVINHYGGMIGGHYTACARLPNDRSSQ RSDVGWRLFDDSTVTTVDESQVVTRYAYVLFYRRRNSPVERPPRAGH SEHHPDLGPAAEAAASQASRIWQEEAEEEPVPEGSGPLGPWGPQDW VGPLPRGPTTPDEGLRYFVLGTVAALVALVLNVFYPLVSQSRWR
LATS1	WARTS protein kinase	6	MKRSEKPEGYRQMRPKTFPASNYTVSSRQMLQEIRESLRNLSPKPSDA AKAEHNMSKMSTEDPRQVRNPPKFGTHHKALQEIRNSLLPFANETNS SRSTSEVNPQMLQDLQAAGFDEDMVIALQKTNNRSIEAAIEFISKM SYQDPRREQMAAAAARPINASMKPGNVQQSVNRKQSWKGSKESLVPQ RHGPPLGESVAYHSESPNSQTDVGRPLSGSGISAFVQAHPKNGQVRN PPPPQVRSVTPPPPPRGQTPPPRGTPPPPSWEPNSQTKRYSGNME YVISRISPVPPGAWQEGYPPPLNTSPMNPQNQQRGISSVPVGRQP IIMQSSSKFNFP SGRPGMQNGTGQTD FMIHQNVVPAGTVNRQPPPPY PLTAANGQSPSALQTGGSAAPSSYTNGSIPQSMMPVNRNSHNMELYN ISVPGLQTNWPQSSSAPAQSSPSSGHEIPTWQPNIPVRSNSFNPLG NRASHSANSQPSATTVTAITPAPIQQPVKSMRVLKPELQTALAPTHP SWIPQPIQTVQPSPFPEGTASNVTVMPPVAEAPNYQGGPPPPYPKHL HQNPSPVPPYESISKPSKEDQPSLPKEDESEKSYENVDSGDKEKKQIT TSPITVRKNKKDEERRESRIQSYSPQAFKFFMEQHVENVLKSHQQL HRKKQLENEMMRVGLSQDAQDQMRKMLCQKESNYIRLKRKMDKSMF VKIKTLGIGAFGEVCLARKVDTKALYATKTLRKKDVLRLNQVAHVKA

Huh protein-Symbol	Description	Seq ID No	Sequence
			ERDILAEADNEWVVRLYYSFQDKDNLYFVMDYIPGGDMMSLLIRMGIFPESLARFYIAELTCAVESVHKMGFIHRDIKPDNILIDRDGHIKLTDFGLCTGFRWTHDSKYYQSGDHPRQDSMDFSNEWGDPSSCRCGDRCLKPLERRAARQHQRCLAHSVLGTPNYIAPEVLLRTGYTQLCDWWSVGVILFEMLVGQPPFLAQTPLETQMKVINWQTSLHIPQAKLSPEASDLIIKLCRGPEDRLGKNGADEIKAHPFFKTIDFSSDLRQQSASYIPKITHPTDTSNFDVPDPAKLWSDNEEENVNDTLNGWYKNGKHPEHAFYEFTFRFFDDNGYPYNYPKPIEYEYINSQGSEQQSEDDQNTGSEIKNRDLVYV
LATS2	kinase phosphorylated during mitosis protein large tumor suppressor homolog 2	7	MRPKTFPATTYSGNSRQRLQEIREGLKQPSKSSVQGLPAGPNSDTSLDKAVLGSKDATRQQQQMRATPKFGPYQKALREIRYSLLPFANESGTSAAAEVNRQMLQELVNAGCDQEMAGRALKQTGSRSEAALEYISKMGYLDPRNEQIVRVIKQTSFGKGLMPTPVTRRPSFEGTGDSFASYHQLSGTPYEGPSFGADGPTALEEMPRPYVDYLFPGVGPHGPHQHHPKGYGASVEAAGAHFPLQGAHYGRPHLLVPGEPLGYGVQSRSPSFQSKTPPETGGYASLPKGGQGGPPGAGLAFPPPAAGLYVPHPHHKQAGPAAHQHLHVLGSRSQVFASDSPQSLLTPSRNSLNVDLYELGSTSVQQWPAATLARDSLQKPGLEAPRAHVAFRPDCPVPSRTNSFNHQPRGPPGKAEPSLPAPNTVTAVTAAHILHPVKSVRVLRLPEPQTAVGSPHPAWVPAPAPAPAPAAEGLDAKEEHALALGGAGAFPLDVEYGGPDRRCPPPPYPKHLRLRSKSEQYDLDSLCAQMEQSLRAGPNEPEGGDKSRKSAKGDKGGKDKKQIQTSFVPVRKNSRDEEKRESRIKSYSPYAFKFFMEQHVENVIKTYQQKVNRLQLEQEMAKAGLCEAEQEQMRKILYQKESNYNRLKRAKMDKSMFVKIKTLGIGAFGEVCLACKVDTHALYAMKTLRKDVDLNRNQVAHVKAERDILAEADNEWVVKLYYSFQDKDSLYFVMDYIPGGDMMSLLIRMEVFPEHLARFYIAELTLAIESVHKMGFIHRDIKPDNILIDLGDGHIKLTDFGLCTGFRWTHNSKYYQKGSVHRQDSMEPSDLWDDVSNCRCGDRCLKTLEQRARKQHQRCLAHSVLGTPNYIAPEVLLRKGYTQLCDWWSVGVILFEMLVGQPPFLAPTPTETQLKVINWENTLHIPAQVKLSPEARDLITKLCCSADHRLGRNGADDLKAHPFFSAIDFSSDIRKQPAFYVPTISHPMDTSNFDPVDEESPWNDASEGSTKAWDTLTSPNNKHPEHAFYEFTFRFFDDNGYPFRCPKPSGAEASQAESSDLESSDLVDQTEGCQPVYV
PGAM5	phosphoglycerate mutase family member 5	8	MAFRQALQLAACLAGGSAAVLFSAVAVGKPRAGGDAEPRPAEPPAWAGGARPGPGVWDPNWDREPLSLINVRKRNVESGEEELASKLDHYKAKATRHIFLIRHSQYHVDGSLEKDRTLTPLGREQAELTGLRLASLGLKFNKIVHSSMTRAIETTDIIISRHLPGVCKVSTDLLREGAPIEPDPVSHWKPEAVQYYEDGARIEAAFRNYIHRADARQEEDSYEIFICHANVIRYIVCSIPPLLSAGDFVLLGS

Validation of LATS1 & 2 kinases as novel NS5A interacting partners

Among the list of newly identified candidate interacting partners, two members of the nuclear Dbf2-related (NDR) family of AGC kinases (Pearce, L. R., Komander, D. & Alessi, D. R. The nuts and bolts of AGC protein kinases. *Nature reviews* **11**, 9-22 (2010)), LATS1 and LATS2, were highlighted. In particular, these two Ser/Thr kinases have been shown to bind and

phosphorylate the specific substrate consensus sequence His-X-Arg/His/Lys-X-X-Ser/Thr (Zhao, B. *et al.* Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development* **21**, 2747-2761 (2007) and Lei, Q. Y. *et al.* TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway *Molecular and cellular biology* **28**, 2426-2436 (2008)). This particular amino acid sequence motif is present in domain 1 of NS5A (His65 to Ser71) and is highly conserved across all viral genotypes. To test whether NS5A is indeed a substrate of the LATS kinases, we performed *in vitro* phosphorylation using purified recombinant NS5A derived from *E. coli* with catalytically active recombinant LATS2. For this, two forms of the proteins were used, namely NS5A domain 1 spanning residues 33-202, and the truncated version NS5A Δ 32 (residues 33-447). Minimal basal phosphorylation was detected for the two forms using phosphoprotein staining (**Fig. 4a**). The level of phosphorylated NS5A increased within minutes of incubation with the kinase, suggesting NS5A to be a substrate of LATS2.

To further pinpoint NS5A phosphorylation sites, the same experiment was repeated using full length recombinant NS5A, and samples analyzed by LC-MS. For this particular part, the reaction was monitored over 80 min. As a result, NS5A was detected with 32 unique peptides (55% sequence coverage), the phosphopeptide NGSMRIVGPK was clearly identified and the phosphorylation site unambiguously localized to Ser71 (**data not shown**). Relative quantitation of the phosphorylation stoichiometry was performed by comparing the peak areas of both the unmodified and the modified peptide (**Fig. 4c**). As can be observed from the kinetics, phosphorylated NGSMRIVGPK was approximately nine times more abundant than its unmodified counterpart after 80 min of reaction. Four additional sites were identified on NS5A, but as their respective phosphorylation stoichiometry ratios were considerably smaller (in the range of 0.02) and also not contained in the sequence motif recognized by the kinase, they were considered as LATS2 unspecific (data not shown).

Phosphorylation of NS5A is known to modulate its protein interaction, as well as affecting HCV RNA replication (Evans, M. J., Rice, C. M. & Goff, S. P. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 13038-13043 (2004)). To investigate the role of NS5A phosphorylation at residue 71 in the HCV RNA replication, a point mutation (Ser-to-Ala) at this position in the wild type transient genotype 1b Con1 replicon was engineered by site directed mutagenesis. Replicon RNAs were transiently

transfected into Lunet Huh-7 cells and their replication levels were measured as previously described (Le Pogam, S. *et al.* In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. *Virology* **351**, 349-359 (2006)). As shown in Figure 5, the replication capacity of Ser71Ala mutant replicon was impaired, with a
 5 replication capacity of 14% compared to the wild type.

siRNA molecules targeting LATS1 and LATS2 genes were used to evaluate the potential role of the LATS proteins in the HCV replication cycle. As controls, a siRNA targeting the Con1 HCV replicon sequence (located in the NS4B gene, see Randall, G., Grakoui, A., Rice, CM. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs.

10 *Proceedings of the National Academy of Sciences of the United States America* **1000**, 235-240 (2003)) and an siRNA targeting the PI4 kinase gene (Berger, K. e. a. Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication.

Proceedings of the National Academy of Sciences of the United States America 106, 7577-7582 (2009).), known to be important for the HCV replication, were used. As previously reported, the

15 siRNAs targeting either Con1 or PI4 kinase decreased efficiently the renilla luciferase signal (94% and 80% inhibition respectively, Figure 6a). One of the 10 siRNAs targeting LATS1 and one of the 3 siRNAs targeting LATS2 tested in the study decreased the renilla luciferase signal with 53% and 37% inhibition respectively (Figure 6a). Real-time PCR was used to evaluate the level of knockdown for each siRNA used in the study (figure 6b). Con1, PI4K and LATS2-1
 20 siRNAs inhibited the mRNA level of their respective targets by 90 %, 82 %, 81 % respectively whereas LATS1-7 siRNA inhibited the LATS1 mRNA level by 54 %.

Claims

1. A method for preventing Hepatitis C virus (HCV) infection of a cell comprising a step of contacting the cell with an agent that inhibits the activity of at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1,**
5 **LATS2, PGAM5.**
2. The method according to claim 1, wherein the agent inhibits the activity of **LATS1 and / or LATS2**
3. The method according to claim 1 or 2, wherein the agent is selected from the group consisting
10 of small molecules, monoclonal antibodies, polyclonal antibodies, kinase inhibitor, and any combination thereof.
4. A pharmaceutical composition comprising at least one pharmaceutically acceptable carrier or excipient and an effective amount of an agent that inhibits the activity of at least one NS5A
15 binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.**
5. The pharmaceutical composition of claim 4, wherein the agent inhibits the activity of **LATS1 and / or LATS2.**
20
6. A method for identifying a potential HCV anti-viral agent comprising the steps of: (a) contacting in vitro a cell that expresses at least one NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B, NP1L1, UBP19, LATS1, LATS2, PGAM5.;**
25 and (b) determining the activity of said NS5A binding protein, wherein a candidate compound is identified as a potential HCV anti-viral agent if the activity determined in step (b) is lower than the activity of said protein in the absence of the candidate compound.
7. The method of claim 6, wherein the cell expresses LATS1 and/or LATS2
8. The method according to claim 6 or 7, wherein the candidate compound is selected from the
30 group consisting of small molecules, monoclonal antibodies, polyclonal antibodies, kinase inhibitor, and any combination thereof.
- 9 A NS5A binding protein selected from the group consisting of **NP1L4, PKHG2, FBW1B,**
35 **NP1L1, UBP19, LATS1, LATS2, PGAM5** for use as a target for treatment and/ or prevention of a Hepatitis C infection.
10. Use according to claim 9, wherein the NS5A binding protein is LATS1 and / or LATS2.

Fig. 1

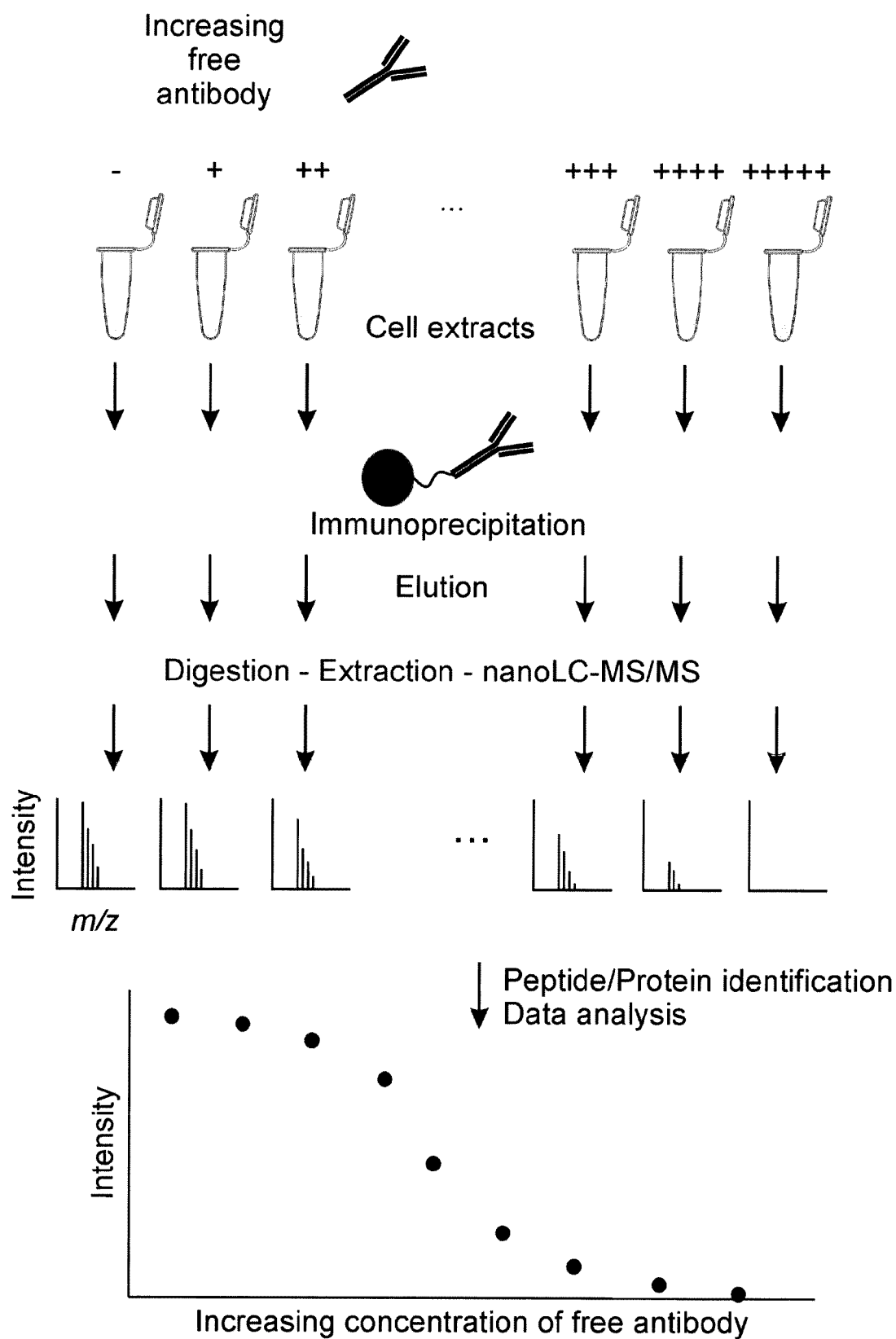


Fig. 2

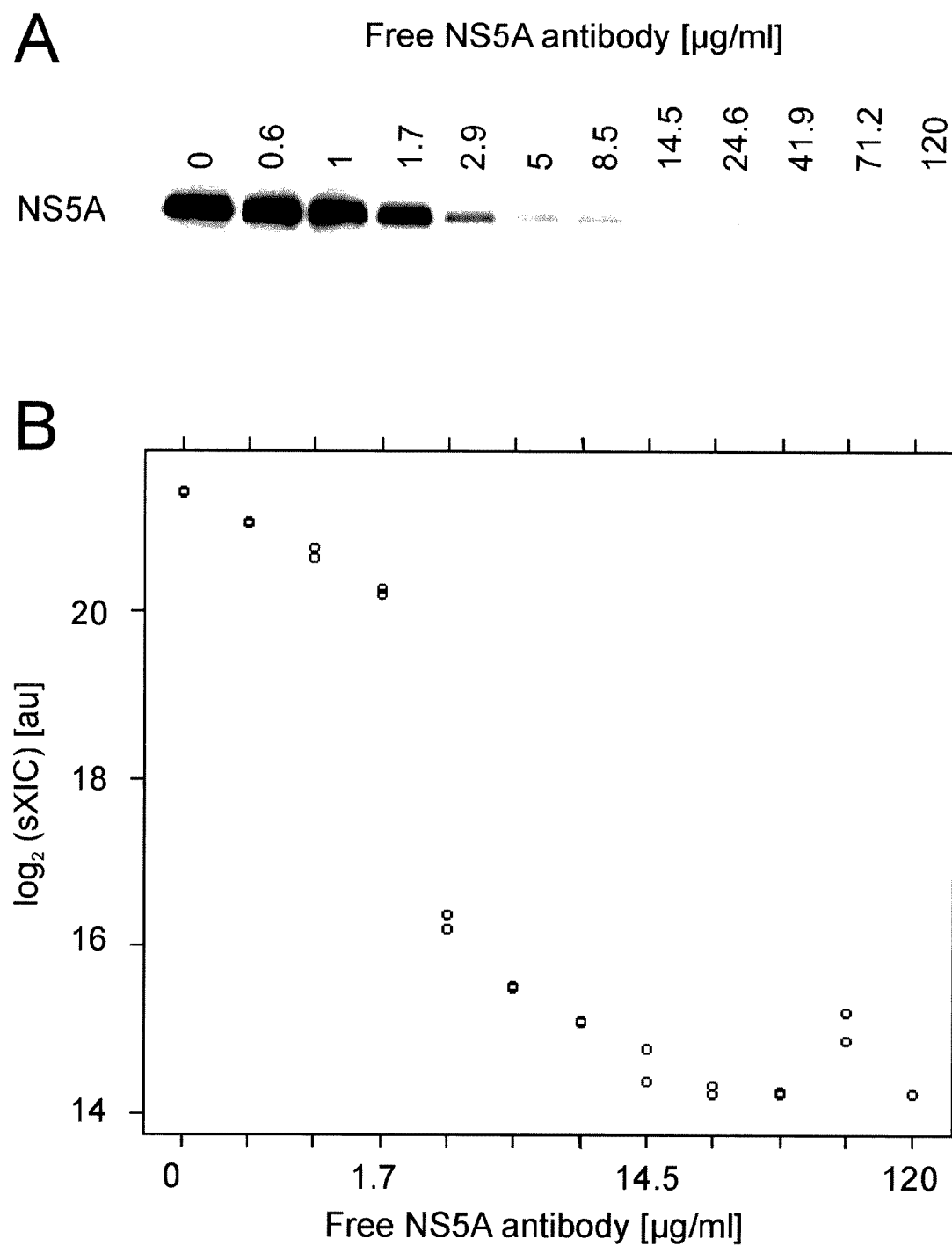


Fig. 3

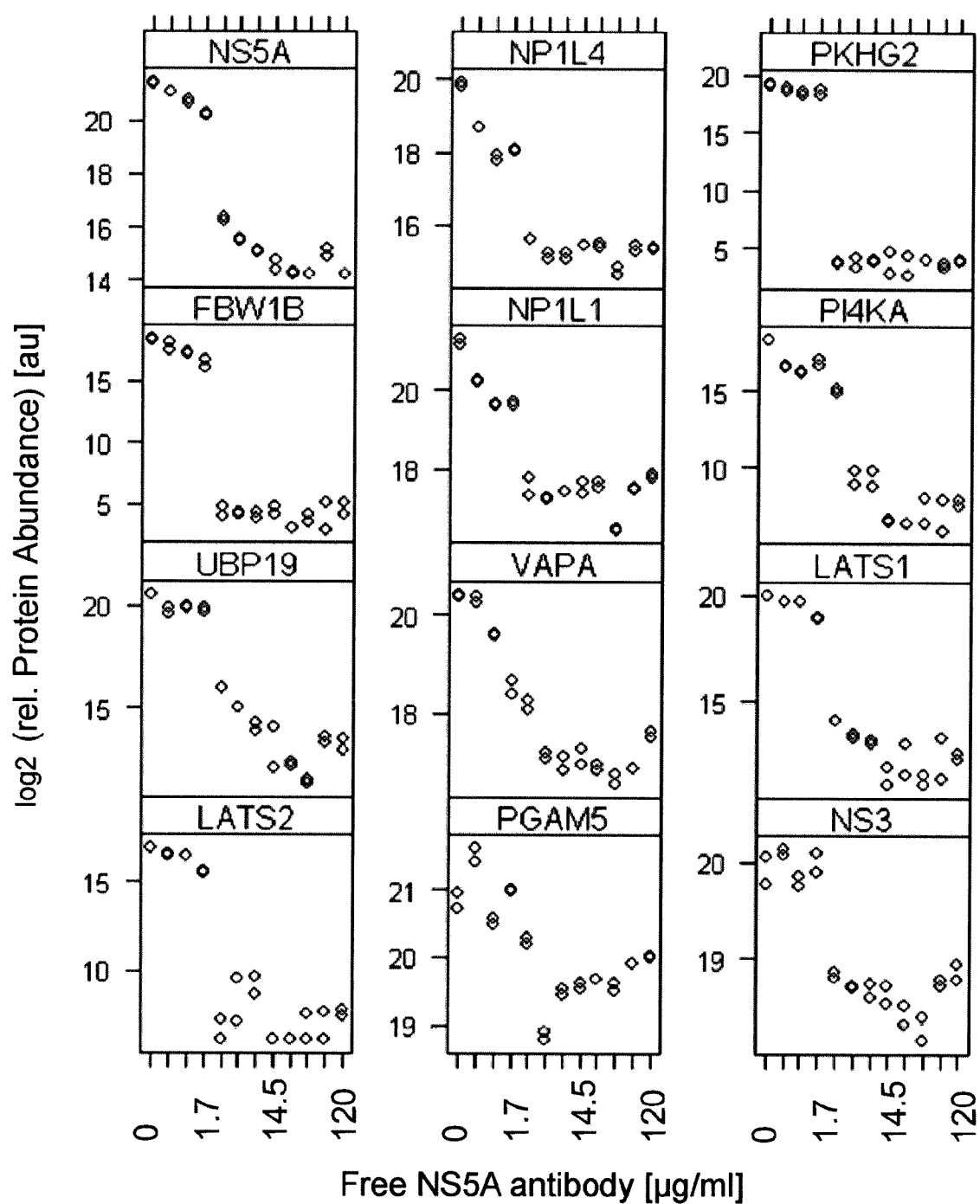


Fig. 4 a)

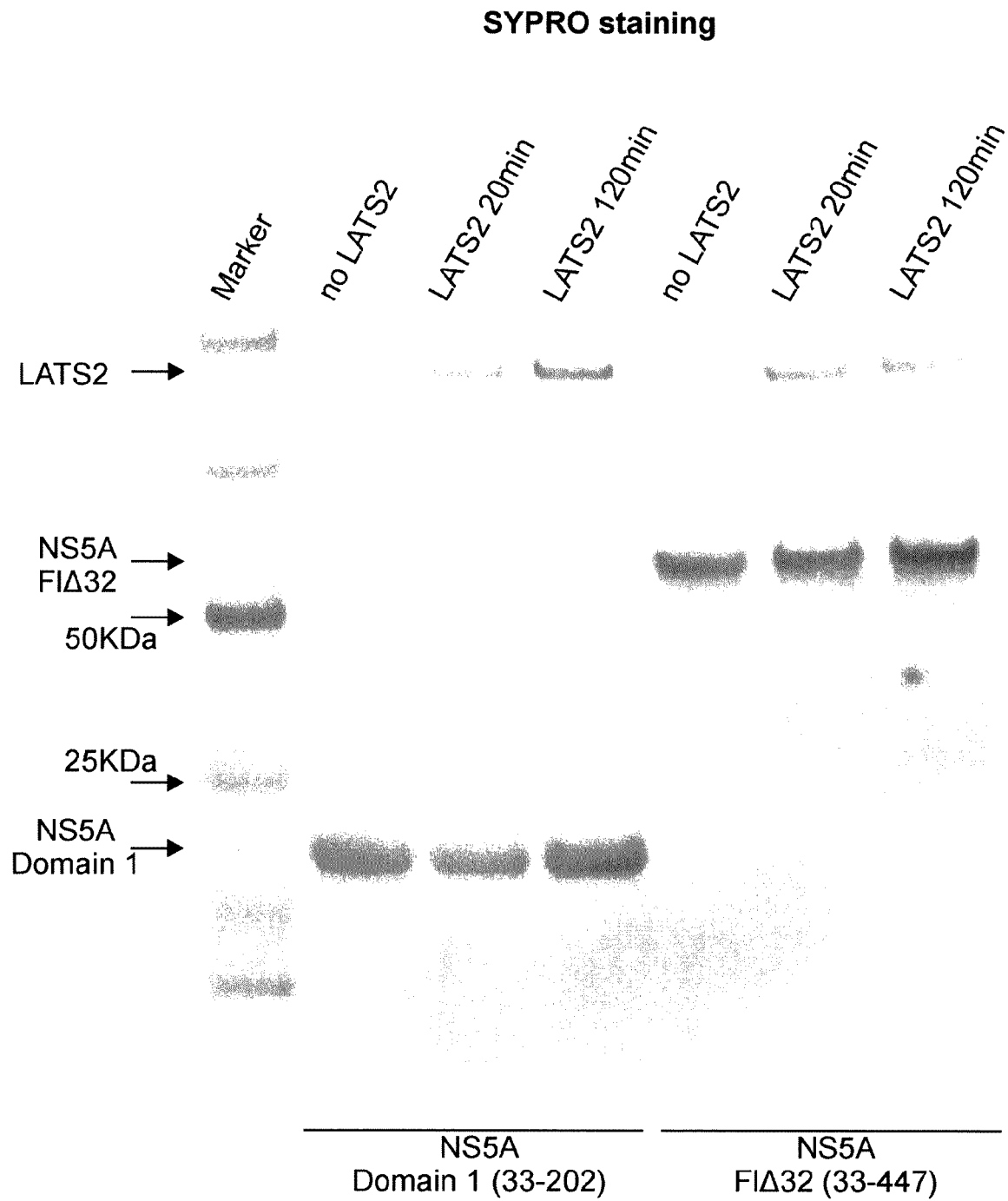


Fig. 4 b)

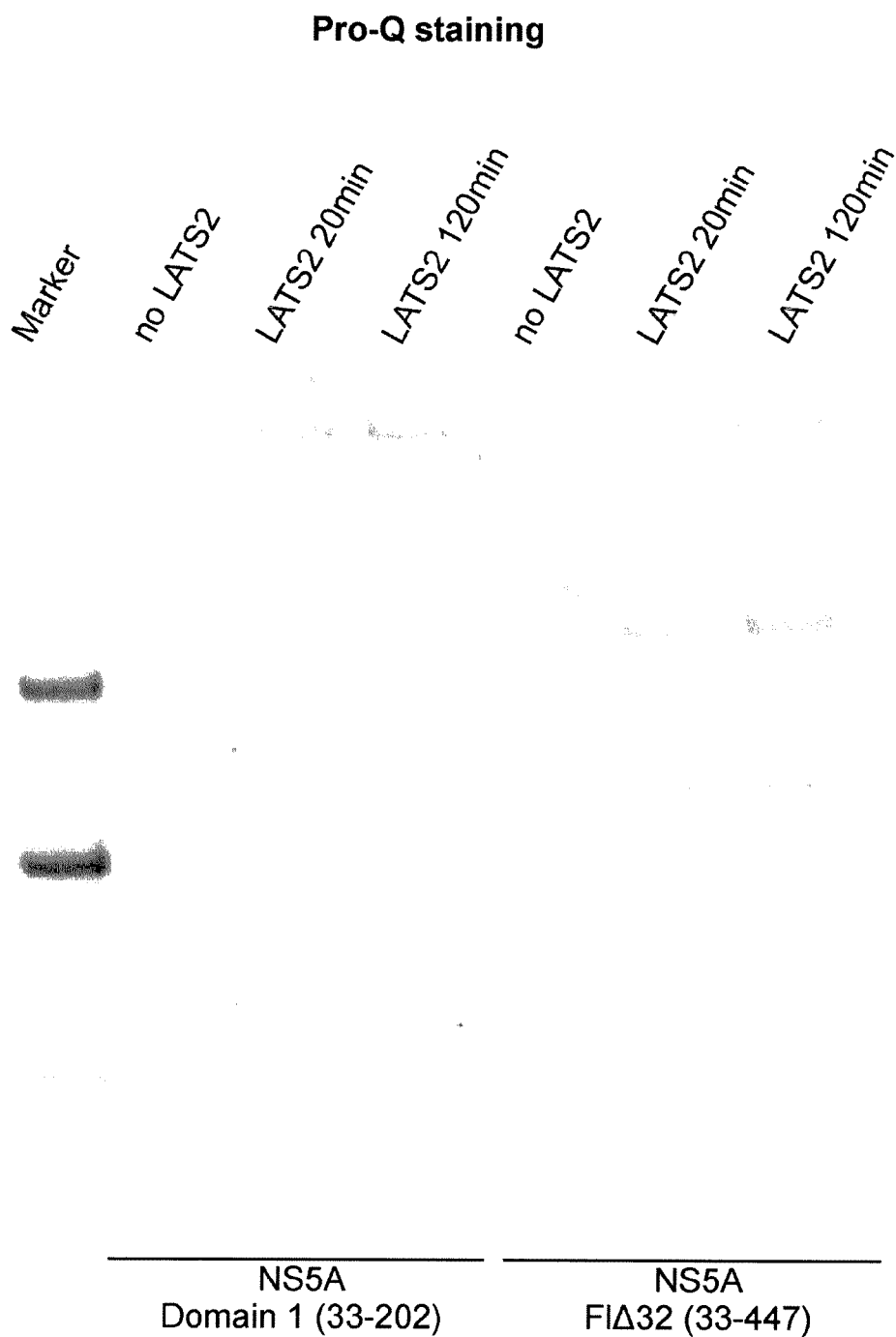


Fig. 4 c)

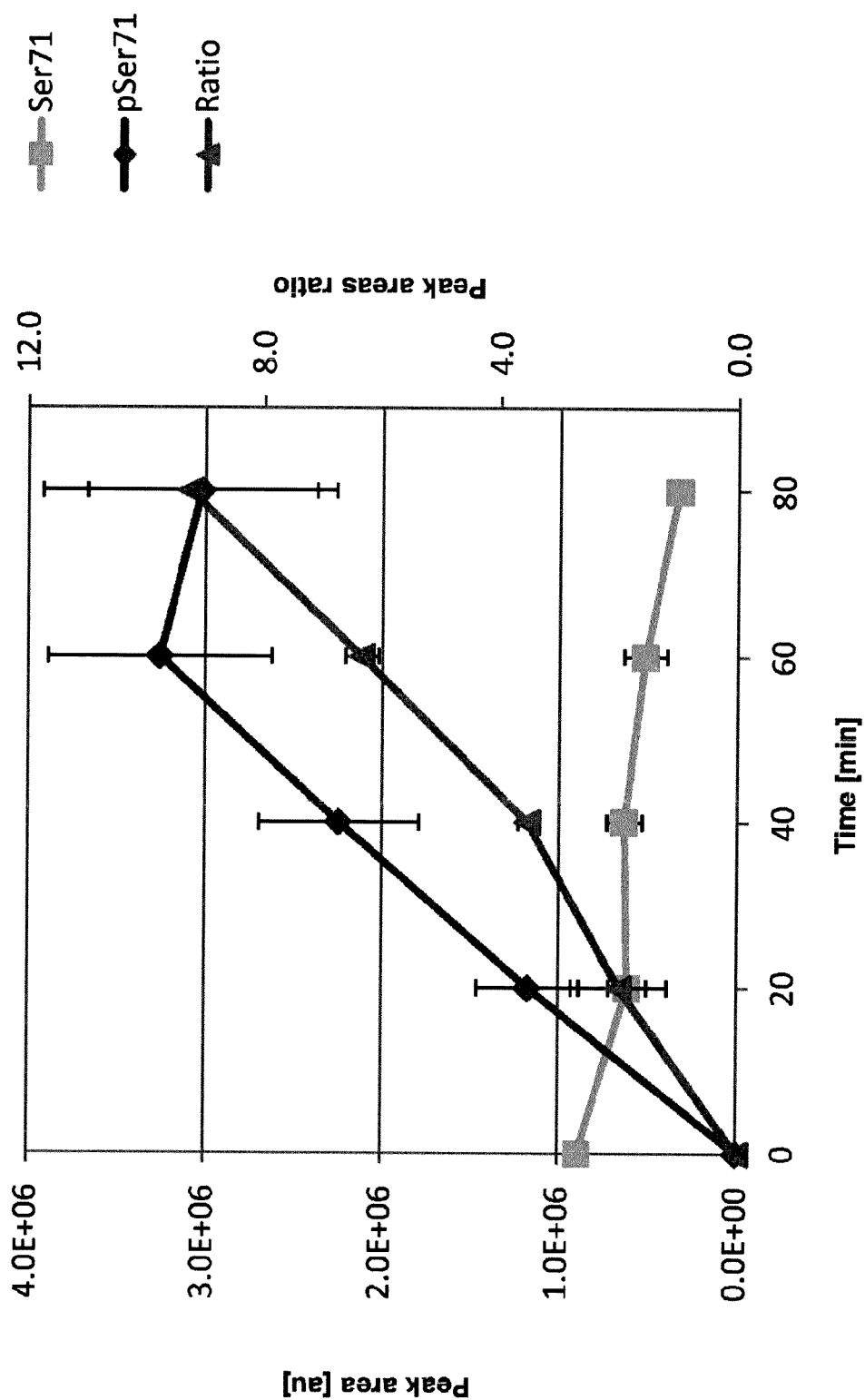


Fig. 5

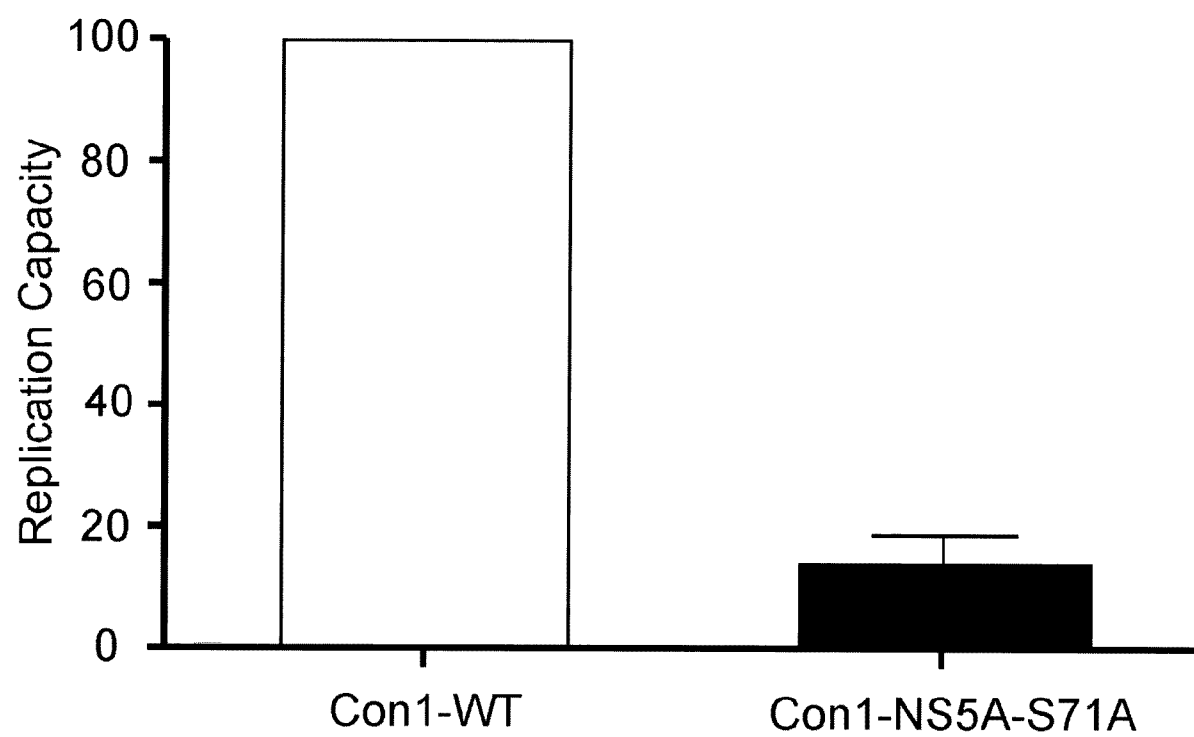


Fig. 6 a)

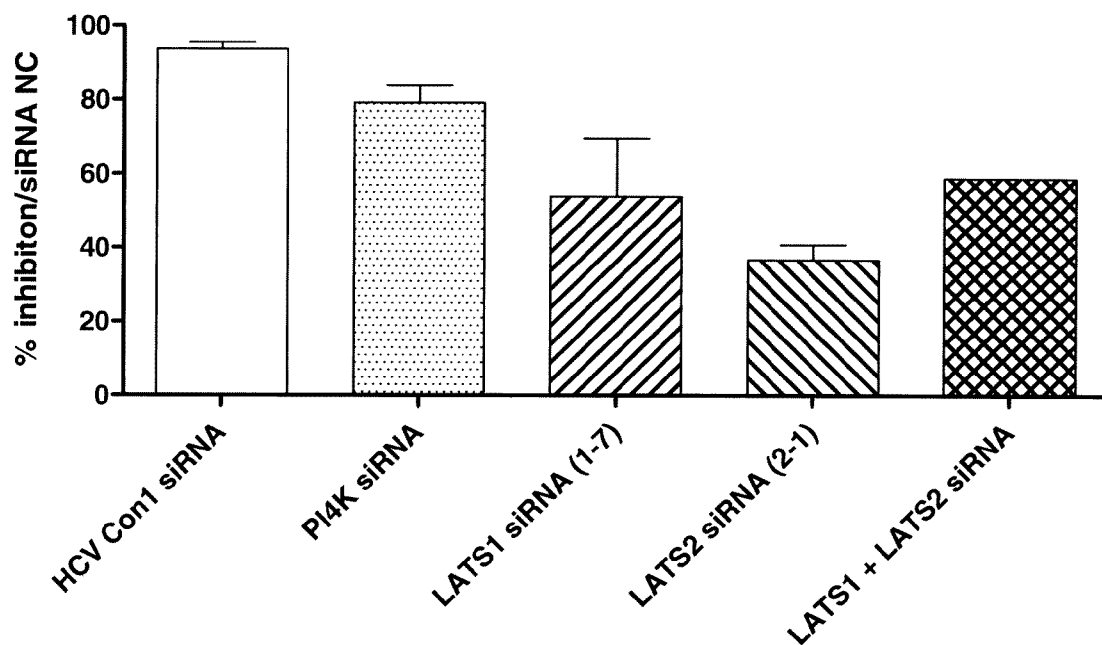


Fig. 6 b)

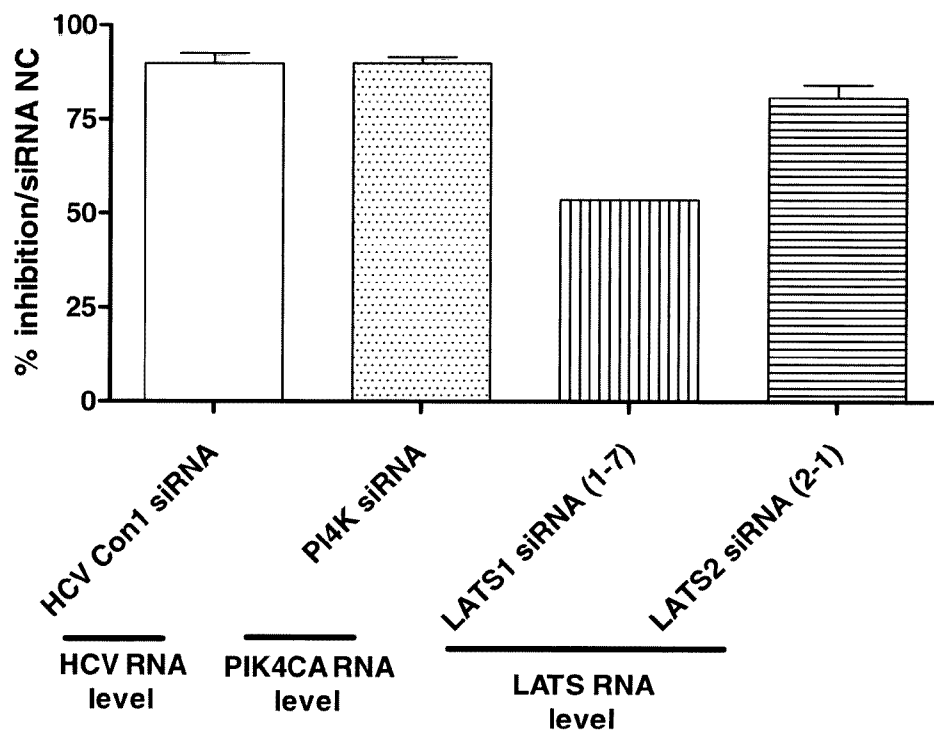
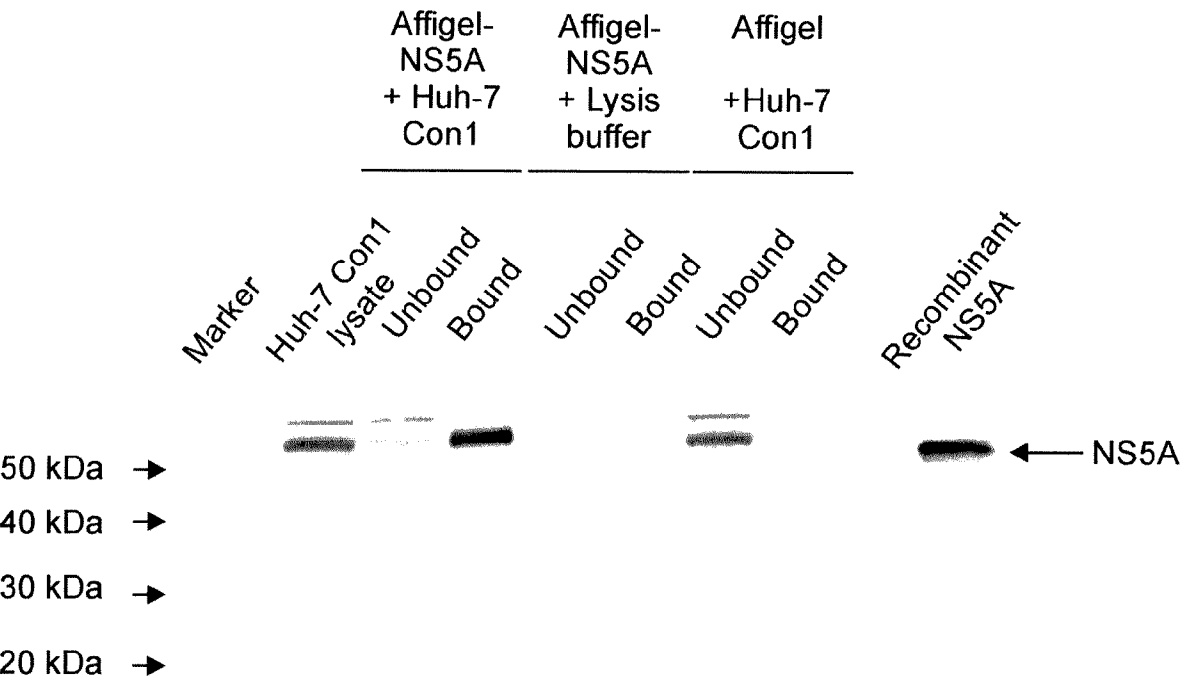


Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2013/068583

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. (means)
- ☐ on paper
- ☒ in electronic form
- b. (time)
- ☒ in the international application as filed
- ☐ together with the international application in electronic form
- ☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068583

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 A61P31/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RANDALL G ET AL: "Cellular cofactors affecting hepatitis C virus infection and replication", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES - PNAS, NATIONAL ACADEMY OF SCIENCES, US, vol. 104, no. 31, 31 July 2007 (2007-07-31), pages 12884-12889, XP002532652, ISSN: 0027-8424, DOI: 10.1073/PNAS.0704894104 abstract; figures; tables	1-10
Y	WO 2007/058384 A1 (UNIV OSAKA [JP]; MATSUURA YOSHIHARU [JP]; MORIISHI KOHJI [JP]; OKAMOTO) 24 May 2007 (2007-05-24) claims ----- -/-	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

5 December 2013

Date of mailing of the international search report

02/01/2014

Name and mailing address of the ISA/

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Langer, Astrid

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068583

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JULIE A LEMM ET AL: "Identification of Hepatitis C Virus NS5A Inhibitors", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 84, no. 1, 1 January 2010 (2010-01-01), pages 482-491, XP002639563, ISSN: 0022-538X, DOI: 10.1128/JVI.01360-09 [retrieved on 2009-10-07] abstract	1-10
X	----- KIM DONG SEON ET AL: "Identification of novel phosphorylation modification sites in human proteins that originated after the human-chimpanzee divergence", BIOINFORMATICS (OXFORD), vol. 27, no. 18, September 2011 (2011-09), pages 2494-2501, XP002717460, abstract; tables	9
Y	----- EMERY AMY ET AL: "A novel cell-based, high-content assay for phosphorylation of Lats2 by Aurora A.", JOURNAL OF BIOMOLECULAR SCREENING SEP 2011, vol. 16, no. 8, September 2011 (2011-09), pages 925-931, XP002717461, ISSN: 1552-454X	1-10
Y	abstract	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/068583

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007058384	A1	24-05-2007	NONE
