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Identification of proteins that mediate the pro-viral functions of the interferon stimulated gene 15 in hepatitis C virus replication

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ABSTRACT

In previous studies we identified the interferon stimulated gene 15 (ISG15) as a pro-viral host factor in the pathogenesis of hepatitis C virus (HCV) infection. However, the functional link between ISG15 and the HCV replication cycle is not well understood. Aim of the present study was to functionally analyze the role of ISG15 and to identify possible HCV promoting effector molecules. Isg15 suppression was investigated in the murine subgenomic HCV replicon (MH1) transfected with Isg15-specific siRNA and in C57BL/6 mice intravenously injected with lipid nanoparticles (LNP)-formulated siRNA. Interestingly, the LNP-formulated siRNA led to hepatocyte-specific knockdown of Isg15 *in vivo*, which mediated a hypo-responsiveness to endogenous and exogenous interferon. A label free proteome analysis accompanied by western blot and quantitative RT-PCR techniques led to identification of five candidate proteins (Heterogeneous nuclear ribonucleoprotein A3 (HnrnpA3), Heterogeneous nuclear ribonucleoprotein K (HnrnpK), Hydroxymethylglutaryl-CoA synthase (Hmgcs1), Isocitrate dehydrogenase cytoplasmic (Idh1) and Thioredoxin domain-containing protein 5 (Txndc5)) that are either involved in lipid metabolism or belong to the family of Heterogeneous nuclear ribonucleoprotein (Hnrnp). All candidate proteins are likely to be associated with the HCV replication complex. Furthermore treatment with HnrnpK-specific siRNA directly suppressed HCV replication *in vitro*. Taken together these data suggest that targeting Isg15 may represent an attractive novel therapeutic option for the treatment of chronic HCV infection.

1. Introduction

170 million people worldwide are chronically infected with HCV which leads to the development of liver cirrhosis and hepatocellular carcinomas (Habersetzer et al., 2012). Standard treatment of HCV genotype 1-infected patients is the combination of pegylated interferon alpha (IFN-α), ribavirin and a protease inhibitor (Boceprevir/Telaprevir), which leads to viral clearance in approximately 75% of genotype 1-infected patients (Jacobson et al., 2011). However, this therapy is accompanied by adverse side effects (Habersetzer et al., 2012; Ramachandran et al., 2012) and the high daily pill burden may result in lower patient compliance. Non-response to standard IFN-based treatment can be predicted by hepatic expression levels of interferon stimulated genes (ISG) prior to treatment. Asselah et al. described significantly elevated ISG15 gene expression prior to treatment in the non-responding patients, suggesting an insufficient IFN response upon treatment (Asselah

Abbreviations: Acadm, acyl-coenzyme A dehydrogenase medium chain; HCV, hepatitis C virus; Herc5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; HnrnpA3, heterogeneous nuclear ribonucleoprotein A3; HnrnpK, heterogeneous nuclear ribonucleoprotein K; Hmgcs1, hydroxymethylglutaryl-CoA synthase; Ifn, interferon; Ifit3, interferon-induced protein with tetratricopeptide repeats 3; Ifitm3, interferon induced transmembrane protein 3; Isg15, interferon stimulated gene 15; Idh1, isocitrate dehydrogenase cytoplasmic; LNP, lipid nanoparticles; Mx2, myxovirus resistance protein 2; NPC, non-parenchymal liver cells; PolyI:C, polyinosinic:polycytidylic acid; PMH, primary murine hepatocytes; Psma6, proteasome subunit alpha type 6; Rsad2, radical S-adenosyl methionine domain containing 2; RnaseL, ribonuclease L; siRNA, small interfering RNA; Txndc5, thioredoxin domain-containing protein 5; Usp18, ubiquitin specific peptidase 18.

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et al., 2008) which is endorsed by Sarasin-Filipowicz et al. Their data support the hypothesis that activation of the endogenous IFN system during chronic HCV infection is not only ineffective in clearing the infection but also impairs the induction of ISGs in response to IFN-based therapies, suggesting a refractory state of the IFN signaling pathway (Sarasin-Filipowicz et al., 2008). Furthermore, ISG15 expression can be located in hepatocytes or non-parenchymal liver cells (NPC) predicting non-response or response to treatment, respectively (Chen et al., 2010a). ISG15 has been identified as a pro-viral host factor, which directly promotes HCV replication and additionally inhibits the IFN response (Chen et al., 2010b; Broering et al., 2010; Chua et al., 2009). Therefore, therapeutic strategies based on the suppression of ISG15 might overcome non-response to standard combination treatment in HCV-infected patients.

The use of efficient delivery tools facilitates to target only selective cell types with low doses of small interfering RNA (siRNA). This technique allows direct targeting of viral genes or host factors as a therapeutical approach and, thus, clinical trials are currently performed in several indications including HCV infection (Castanotto and Rossi, 2009; Sibley et al., 2010; Soutschek et al., 2004). Here we established an efficient, siRNA-based *in vivo* model, with a hepatocyte specific delivery system, which allows us to functionally analyze the role of Isg15 in the hepatic immune response *in vivo*. Using lipid nanoparticle (LNP)-formulated siRNAs, we demonstrate the possibility to silence genes selectively in hepatocytes, which are the largest population of liver cells and represent the target cells for HCV. We further show the involvement of Isg15 in hepatocellular processes *in vitro* and *in vivo* to find the enigmatic link to HCV replication.

2. Material and methods

2.1. Materials

The siRNAs formulated with lipid nanoparticles (LNPs) used for *in vivo* studies were provided by Roche Kulmbach GmbH (Kulmbach, Germany). The Interferon stimulated gene 15 (Isg15)-specific siRNA (target sequence 5'-CAGUGAUGCUAGUGGUACA-3') and the non-template control (siNC) (sequence 5'-CUUACGCUGAGUACUUCGA-3') are chemically modified at the ribose backbone to prevent nuclease driven degradation. An unmodified non-template control siRNA was used as immune activating control (siPositive-Control) (sequence 5′-CUACACAAAUCAGCGAUUUCCAUGU-3′). The siRNAs for *in vitro* studies were obtained from Qiagen (Hilden, Germany). Recombinant IFN-α (Inferax) was provided by Astellas Pharma (Munich, Germany). Polyinosine-polycytidylic acid (Poly-I:C) was obtained by Invivogen (Toulouse, France).

2.2. Culture of HCV subgenomic and full-length replicon cells

MH1 is a murine hepatoma derived cell line (Hepa1–6) harboring a subgenomic HCV genotype 1b replicon I_{377}/NS3–3′ (Zhu et al., 2003). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA), L-glutamine (PAA), penicillin/streptomycin (PAA) and genetecin (500 μg/ml) (PAA) at 37 °C and 5% CO_2.

Huh7.5 cells as well as the infectious plasmid clone for the HCV infectious cell culture system (JC1-luc) were provided by Ralf Bartenschlager and Thomas Pietschmann. Cell culture conditions, transfection and subsequent infection of Huh7.5 cells using this plasmid was performed as described elsewhere (Koutsoudakis et al., 2006). HCV replication was determined by the luciferase reporter gene assay (Bright-Glo™, Promega, Madison, WI, USA).

In vitro gene silencing experiments with MH1 and JC1-luc-expressing HuH7.5 cells were performed using 10 nM siRNA targeting the specific gene sequences (as indicated) and the non-template control using the HiPerFect transfection reagent according to the manufacturer's instructions (Qiagen).

2.3. Animals

C57BL/6 wild type mice were bred, at the University Hospital of Essen and kept under 12 h dark/light cycles. Mice were fed ad libidum and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health.

2.4. Isolation and culture of murine hepatocytes and NPC

Primary murine hepatocytes (PMH) were prepared as described previously (Wu et al., 2009). NPC suspension was prepared from the supernatants obtained after low-speed centrifugation of the hepatocytes-containing cell fraction. The purity of primary murine hepatocytes was routinely determined (data not shown).

2.5. Synthesis of siRNAs, manufacturing and characterization of LNPs

The synthesis for siRNA molecules is described as supplementary method. Manufacturing and characterization of LNPs have been described elsewhere (Broering et al., 2013).

2.6. RNA isolation and quantitative RT-PCR

Total RNA was isolated and purified using Qiazol™ solution (Qiagen, Hilden, Germany) and the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantitative RT-PCR was performed with the QuantiFast SYBR Green RT-PCR Kit (Qiagen) using 0.1–0.3 μg of total RNA with the primer sets presented in supplemental table ST1. The calculated copy numbers were normalized to the following housekeeping genes; β-Actin (*in vitro*) or Gapdh (*in vivo*).

2.7. Label free proteomics

Materials and methods for the label free proteomic study have been described previously (Poschmann et al., 2009; Megger et al., 2013; Perkins et al., 1999) and are described in detail as supplemental methods.

2.8. Western blot analysis

Western blot analysis was performed as described previously (Hilkens et al., 2003). Following antibodies were used: Idh1 (Cell signaling, Danvers, USA), Isg15 (Cell signaling), HnrnpK (Cell signaling), HnrnpA3 (Abcam), Txndc5 (Abcam), Hmgcs1 (Proteintech, Manchester, UK) and Gapdh (Cell signaling) was determined as loading control.

2.9. Statistical analysis

Data are expressed as mean ± SEM (standard error of mean). Differences between two groups were either determined by the Wilcoxon test or ANOVA analysis, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. LNP-formulated Isg15-specific siRNAs lead to hepatocyte specific gene knockdown and induce an immune response in NPC in vivo

The LNP formulation of Isg15-specific siRNA (siIsg15) as well as non-silencing control siRNA (siNC) were intravenously injected into C57BL/6 mice (4 μg/g bodyweight), animals were sacrificed at different time points after injection, RNA was extracted from liver tissue and Isg15 knockdown efficiency was determined by qRT-PCR. Hepatic knockdown of Isg15 became detectable 24 h after injection ($p < 0.0001$), reaching a maximum knockdown efficiency of approx. 80% ($p < 0.0001$) at day 2 post application. Isg15 was consistently suppressed for at least 10 days (Fig. 1A). To determine which liver cell types were targeted by LNP-formulated siRNAs, murine hepatocytes and a mix of NPC, including Kupffer cells, stellate cells and liver sinusoidal endothelial cells, were isolated 48 h after siRNA application, RNA was extracted and Isg15 expression was determined by qRT-PCR. As shown in Fig. 1B, suppression of Isg15 was restricted to hepatocytes with 72.6% ± 8.7 (mean ± SEM) knockdown efficiency. In contrast off-target effects, represented by induction of Ifn-β 6 h after injection of the siRNA (Fig. 1C), could only be shown for the non-parenchymal liver cell fraction. Here, LNP-formulated siRNAs led to significant up-regulation of Ifn-β in NPC ($p = 0.0003$). This kind of initial and transient off-target effect had been shown to be a sequence-dependent, TLR-mediated, immune response (Broering et al., 2013). The Isg15 knockdown in PMH could be verified by western blot analysis. The use of the siIsg15 led to a suppression of ISGylation and free Isg15, compared to the siNC. For additional comparison an immune-activation siRNA (siPositiveControl) had been used, which highly induced free Isg15 (15 kDa) and mediated a strong ISGylation pattern (Fig. 1D).

3.2. Isg15 knockdown enhances the responsiveness to exogenous and endogenous interferon in vivo

Further, we aimed to investigate the interferon-regulatory function of Isg15 for endogenous and exogenous interferons *in vivo*. Consensus IFN-α (Inferax), which had been tested for its reactivity in mice *in vivo* and *in vitro* (supplemental Fig. SF1), was administrated with 600 IU/g bodyweight 10 days after C57BL/6 mice were injected with LNP-formulated siIsg15 (4 μg/g bodyweight). An efficient Isg15 knockdown reaching 81.9% ± 2.0 (mean ± SEM; $p = 0.003$) occurred in total liver tissue, when compared to PBS (Fig. 2A). The injection of IFN induced a 7.0 ± 1.4 ($p = 0.0005$) fold induction in Isg15 expression, however it could be demonstrated that the siRNA-mediated Isg15 knockdown was efficient even under IFN treatment, reaching 94.3% ± 1.2 ($p = 0.0002$) when compared to PBS/IFN control and 60.0% ± 8.2 ($p = 0.0045$) when compared to the basal Isg15 expression, represented by the PBS-treated control (Fig. 2A). To determine the response to exogenous IFN the expression of Mx2 (myxovirus (influenza virus) resistance 2), Rsad2 (radical S-adenosyl methionine domain containing 2), Ifit3 (interferon-induced protein with tetratricopeptide repeats 3), RnaseL (ribonuclease L), Ifitm3 (interferon induced transmembrane protein 3), Herc5 (HECT and RLD domain containing E3 ubiquitin protein ligase 5) and Usp18 (ubiquitin specific peptidase 18) was determined by qRT-PCR. The expression of Mx2, Rsad2, Ifit3, Herc5 and Usp18, but not RnaseL and Ifitm3, was significantly induced by the IFN injection in the PBS control group. In combination with the Isg15 knockdown expression of all of these genes, except Usp18, was significantly enhanced (Fig. 2A). The expression of Usp18, an Isg15-specific peptidase and regulator of Jak-Stat signaling (Janus kinase/signal transducer and activator of transcription) (Randall et al., 2006), was significantly down regulated after the suppression of Isg15, in both control and IFN-treated animals (Fig. 2A).

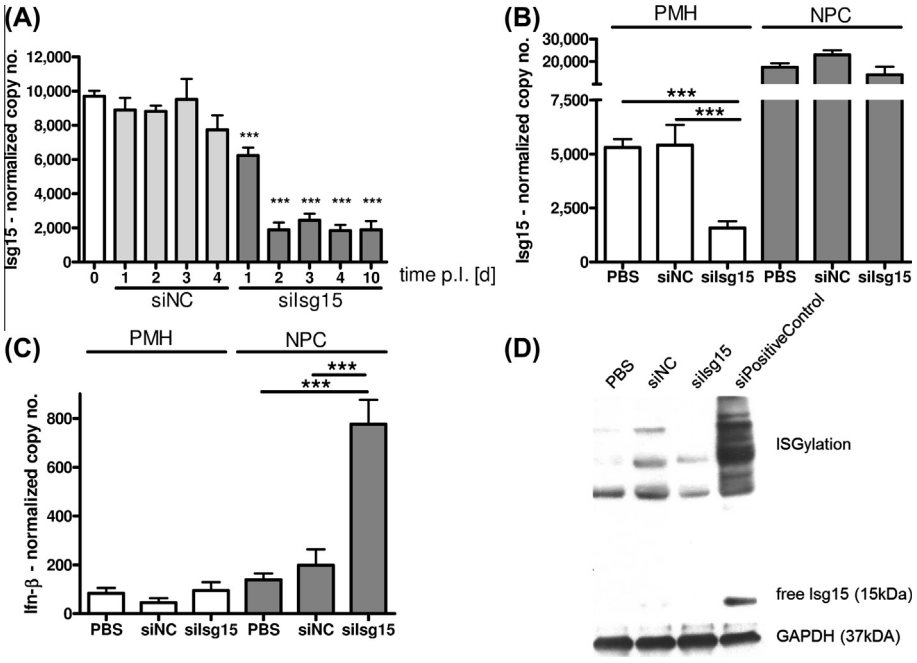

Fig. 1. Hepatocytes and NPCs showed differential responses to LNP-formulated siRNAs *in vivo*. 7-week old, male C57BL/6 mice received 200 μl of siRNA-LNP (4 μg/g bodyweight) specific for Isg15, a non-silencing control siRNA (siNC) and PBS via tail vein injection. Mice were sacrificed at different time point after injection. RNA from liver tissues was extracted and changes in gene expression of hepatic Isg15 was determined by quantitative RT-PCR (A). In addition, mice (3 animals per group) were sacrificed 48 h (B) or 6 h (C) after injection, primary murine hepatocytes (PMH) and non-parenchymal liver cells (NPC) were isolated, RNA was extracted and changes in gene expression of Isg15 (B) and Ifn-β (C) were determined by quantitative RT-PCR. Copy numbers were normalized to 100,000 copies of Gapdh (mean values ± SEM). Asterisks indicate significant results (***$p < 0.001$). Total proteins were extracted from hepatocytes, prepared 48 h after treatment with siRNAs and changes in free Isg15 and ISGylation were determined by Western blot analysis (D). An additional control (siPositiveControl) were used as immune-activating siRNA.

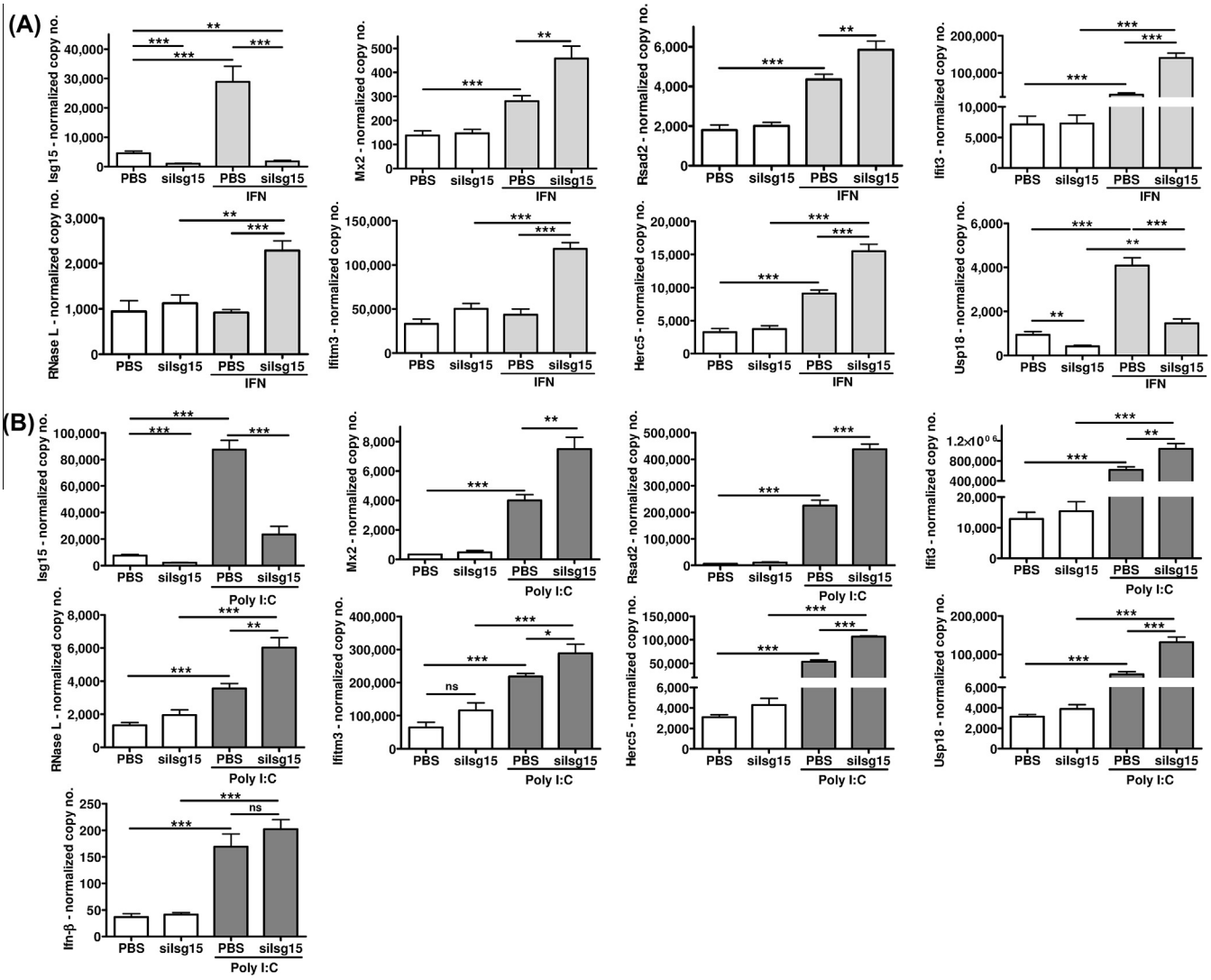

Fig. 2. Isg15 knockdown enhanced the response to exogeneous and endogenous IFN *in vivo*. 7-week old male C57BL/6 mice received 200 μl of Isg 15-specific siRNA-LNP (4 μg/g bodyweight) via tail vein injection. In addition these mice intravenously received IFN-α [600 IU/g bodyweight] (A) or Poly I:C (B) [4 μg/g bodyweight] (D–F, G) 10 days or 2 days after siRNA application, respectively (4 animals per group). Mice were sacrificed 6 h after second injection. RNA from liver tissue was extracted and determined by quantification RT-PCR. Copy numbers were normalized to 100,000 copies of Gapdh (mean values ± SEM). Asterisks indicate significant results ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

The next step was to determine the responses to endogenous Ifn *in vivo*. Therefore, the expression of Ifn-β and Isgs were analyzed, 6 h after tail vein injection of PolyI:C (4 μg/g bodyweight), in mice that received Isg15-specific siRNA formulation (4 μg/g bodyweight) 2d before. The Tlr3 ligand PolyI:C induced the production of endogenous Ifn-β in the liver (Fig. 2B), which did not differ between Isg15-suppressed mice and the control group ($p = 0.2786$). The injection of PolyI:C induced a 11.5 ± 0.9 ($p < 0.0001$) fold induction in Isg15 expression, determined for the PBS treatment. The siRNA-mediated knockdown of Isg15 under PolyI:C treatment reached 63.8% ± 10.9 ($p = 0.0008$) when compared to PBS/PolyI:C control (Fig. 2B). In contrast to exogenous IFN, PolyI:C treatment led to significant induction of Mx2, Rsad2, Ifit3, RnaseL, Ifitm3, Herc5 and Usp18 (Fig. 2B). However, the response to the endogenous Ifn was significantly enhanced in Isg15-suppressed animals as expression of all of these Isgs, including Usp18, were significantly elevated (Fig. 2B). Furthermore, the response to endogenous Ifn was considerable stronger than the response to the pharmacologically administrated interferon as shown in Fig. 2A.

3.3. Functional characterization of Isg15

Proteome analysis was performed to functionally analyze the Isg15 effect and to identify potential interaction partners, using the HCV replicon MH1, transfected with Isg15-specific siRNA (5 nM) for 24 h *in vitro* and primary murine hepatocytes, isolated from C57BL/6 mice which had been treated with LNP-formulated Isg15-specific siRNA for 48 h *in vivo*. The Isg15 knockdown efficiency in MH1 cells revealed 63.6% ($p < 0.001$) and led to 69.4% suppression in HCV replication ($p = 0.0071$) (supplemental Fig. SF2). In MH1 and PMH we analyzed the differential protein expression after siRNA-mediated suppression of Isg15 in comparison to a control group, which contained untreated controls and those treated with the control siRNA. The intersection of regulated proteins identified in both studies revealed a total of 7 equally regulated proteins (Fig. 3A), all of which were down regulated after Isg15 suppression. Gene descriptions and fold suppression of theses candidate proteins are given in Table 1.

Within the *in vitro* study, 613 regulated murine proteins (p- and q-value $\leqslant 0.05$, fold change $\geqslant 1.5$) were identified. In addition, the

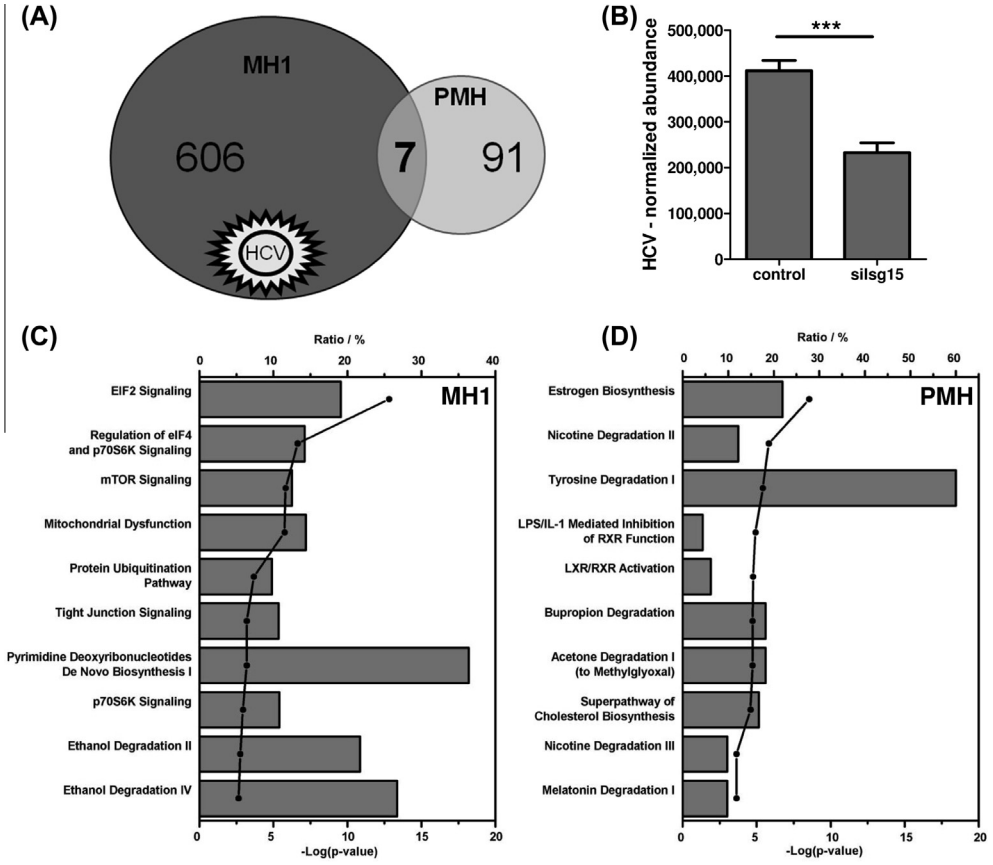

Fig. 3. Lable-free proteomics identified Isg15-regulated proteins. The venn diagram shows the overlap of seven equally regulated proteins and complements of the proteomic studies performed with Isg15-suppressed MH1 and primary murine hepatocytes (PMH), respectively (A). Suppression of HCV proteins in silsg15-treated MH1 cells (fold suppression 1.77, ANOVA $p = 0.00018$) could be measured, compared to the merged controls (untreated + siNC) (B). Canonical pathways associated to regulated proteins after Isg15 knockdown as detected in the MH1 *in vitro* (C) and PMH *in vivo* (D) studies, are given. Ratios (percentage of a pathway covered) are depicted as horizontal bars and corresponding values of −log(ANOVA *p*-values) as circles connected by a solid line.

Table 1
Proteins showing consistent regulation directions (down-regulation after Isg15 knockdown) *in vitro* and *in vivo*.

Gene ID	Gene name	Fold changes	
		MH1 *in vitro*	PMH *in vivo*
Idh1	Isocitrate dehydrogenase [NADP] cytoplasmic	1.57	Infinite
Acadm	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	1.65	Infinite
HnrnpK	Heterogeneous nuclear ribonucleoprotein K	1.59	3.69
HnrnpA3	Heterogeneous nuclear ribonucleoprotein A3	1.60	1.58
Hmgcs1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	2.62	4.22
Txndc5	Thioredoxin domain-containing protein 5	2.09	2.56
Psma6	Proteasome subunit alpha type-6	1.63	1.87

Infinite fold changes are due to the lack of corresponding peptide signals in the Isg15-suppressed group.

HCV polyprotein was also identified to be significantly down-regulated after Isg15 knockdown (fold suppression 1.77, ANOVA $p = 0.00018$) (Fig. 3B). The regulated mouse proteins were further analyzed using the IPA software. Here, 607 proteins were successfully annotated regarding their function and localization (supplemental data set SD1). As shown in Fig. 3C, pathway analysis of the proteins showing an altered expression after Isg15 knockdown revealed changes in ubiquitinylation, p70S6 kinase signaling and the Akt/mTor pathways. In contrast, label-free proteomics of the primary murine hepatocytes (PMH) obtained in the *in vivo* study identified 98 regulated proteins. In contrast to the pathways identified in the *in vitro* study, regulated proteins of the *in vivo* study belonged to metabolic signaling processes (Fig. 3D). Detailed information regarding protein types, functions, localizations and involved pathways can be found in supplemental data set SD2.

3.4. Validation of the Isg15-related candidates identified in the proteome studies

To validate the Isg15-dependent regulation of the seven candidate proteins, the murine HCV subgenomic hepatoma cell line MH1 was transfected with 5 nM siRNA (siNC, siIsg15). Total RNA and proteins were isolated 24 h after transfection. In addition, 7-week old, male C57BL/6 mice that received 200 µl of siRNA-LNP (4 µg/g bodyweight) via tail vein injection were sacrificed 48 h after injection, hepatocytes were isolated and RNA and total proteins were extracted. Western blot analysis of 30 µg total protein MH1 or PMH was performed (Fig. 4A) and reproduced the reduction of HnrnpK, Txndc5, HnrnpA3, Idh1 and Hmgcs1 in Isg15-suppressed cells. The light signals were quantified and the mean values of suppression were calculated (Table 2). Quantitative RT-PCR data also confirmed the significant reduction on transcriptional levels after Isg15 knockdown for Txndc5, Hmgcs1 and HnrnpA3 expression, in both systems MH1 cells (Fig. 4B) and PMH (Fig 4C).

All seven proteins were suppressed in HCV replicon cells by 10 nM of target-specific siRNAs to analyze possible direct effects on HCV replication. The knockdown efficiencies 24 h after transfection and the effects on HCV replication, determined 72 h after

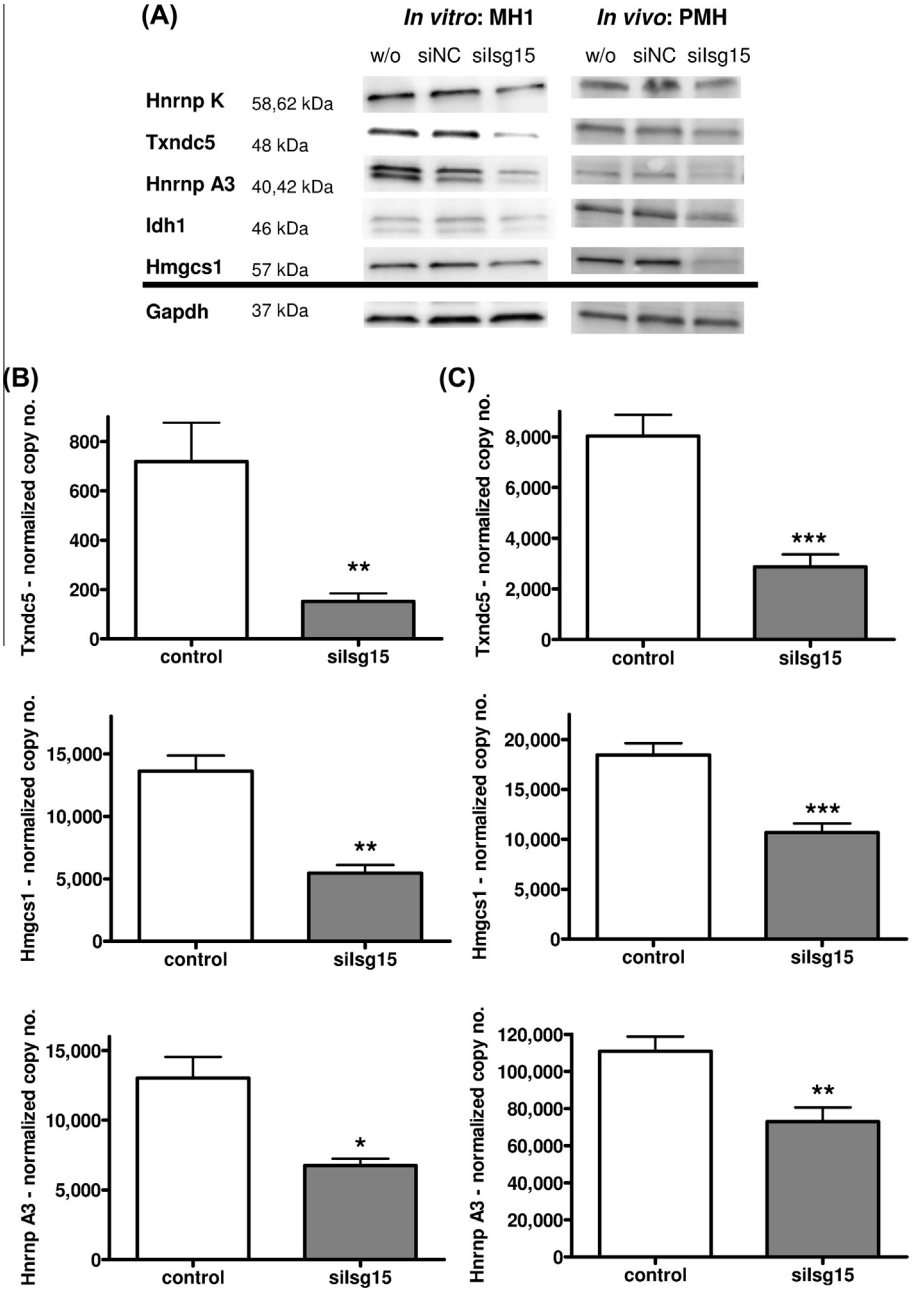

Fig. 4. Isg15-regulated proteins could be validated *in vivo* and *in vitro*. Murine hepatoma cell line MH1 harboring a subgenomic HCV replicon, was transfected with 5 nM siRNA (siNC, silsg15). After 24 h total RNA and proteins were isolated ($n = 4$). For the *in vivo* study 7 weeks old, male C57BL/6 mice received 200 µl of siRNA-LNP (4 µg/g) via tail vein injection (5 animals per group). Hepatocytes were prepared 48 h after injection and RNA and total proteins were extracted. Isg15 knockdown in MH1 and primary murine hepatocytes (PMH) led to reduction of HnrnpK, Txndc5, HnrnpA3, Idh1 and Hmgcs1 on protein level as shown by Western blot analysis (A). Quantitative RT-PCR data confirmed these data on transcriptional levels after Isg15 knockdown for three of the genes in PMH (B) and MH1 cells (C), compared to the merged controls (untreated + siNC). Copy numbers were normalized to 100,000 copies of β-Actin or Gapdh (mean values ± SEM). Asterisks indicate significant results ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Table 2
Protein suppression in Isg15-suppressed cells (MH1; PMH) determined by Western blot, normalized to Gapdh and compared to the control.

Gene	*In vitro*: MH1 Suppression [%] (mean ± SEM)	*In vivo*: PMH Suppression [%] (mean ± SEM)
HnrnpK	50.4 ± 1.6	39.4 ± 2.8
Txndc5	51.1 ± 2.6	40.0 ± 0.8
HnrnpA3	59.8 ± 22.9	52.0 ± 11.6
Idh1	53.6 ± 0.3	25.0 ± 5.6
Hmgcs1	40.5 ± 3.7	45.3 ± 20.0

Table 3
Knockdown efficiency of siRNAs and their HCV suppressive effects.

Target gene	Knockdown Efficancy [%] (mean ± SEM)	HCV suppression [%] (mean ± SEM)	*p*-Value
Isg15	**66.3 ± 3.7**	**69.4 ± 25.9**	**0.0001**
HnrnpA3	56.4 ± 5.1	15.4 ± 2.2	0.65
Psma6	76.6 ± 5.4	1.2 ± 0.3	0.73
HnrnpK	**72.9 ± 4.0**	**55.3 ± 2.5**	**0.0017**
Acadm	72.1 ± 8.3	18.2 ± 5.5	0.27
Hmgcs1	69.1 ± 4.7	4.6 ± 1.2	0.91
Idh1	74.3 ± 3.7	13.2 ± 3.3	0.41
Txndc5	73.7 ± 5.7	26.9 ± 4.9	0.18

Bold values indicate significant suppressive effect on HCV replication.

transfection, are given in Table 3. Here, siRNA-mediated knockdown of Isg15 and HnrnpK significantly suppressed HCV replication. This antiviral effect was also investigated in JC1-luc-expressing HuH7.5 cells. Accordingly, siRNA-mediated knockdown (10 nM) of ISG15 as well as HNRNP K in JC1-luc-infected HuH7.5 cells led to suppression of HCV replication determined by luciferase assay (supplemental Fig. SF3).

4. Discussion

The elevated expression of ISG15 in patients has two possible consequences: first, the hepatocytes do not further respond to exogenous IFN, as the ISG expression is already elevated (Chen et al., 2010a) and, thus, ISG15 functions as an attenuator of an efficient IFN response in these cells (Broering et al., 2010; Chua et al., 2009). Second, the HCV replication is directly promoted by ISG15, which has been described as a pro-viral factor in the HCV replication cycle (Broering et al., 2010; Chen et al., 2010b). We established an efficient *in vivo* knockdown model, in which a single injection of LNP-formulated siRNAs, specific for Isg15, led to hepatocyte-restricted suppression of Isg15, for at least 10 days. After hepatic suppression of Isg15 the animals showed a significantly higher sensitivity to endogenous and exogenous IFN *in vivo*, confirming previous *in vitro* results that demonstrate an IFN attenuating function of ISG15 (Chua et al., 2009; Broering et al., 2010). Interestingly, expression of Usp18, a regulator of Jak-Stat signaling, was down regulated after long term suppression of Isg15 *in vivo*. However, the suppression of Usp18 has also been described to enhance Ifn responsiveness (Randall et al., 2006). Functional analysis identified that the abundance of Txndc5, HrnnpA3, Hmgcs1, Idh1 and HnrnpK were reduced by the suppression of Isg15. These proteins can be clustered into two groups, one group plays a role in lipid metabolism, as Hmgcs1, Txndc5 and Idh1, the other group is represented by members of the Hnrnp family. These candidate proteins might mediate the pro-viral effect of Isg15 previously described for HCV replication (Broering et al., 2010).

The cellular lipid metabolism is indispensable for HCV entry, replication, assembly and release. The HCV core protein and the RNA replication complex are strongly associated with lipid droplets (LD), that are playing a role in production of viral particles and release (Coller et al., 2012). The viral replication and protein synthesis lead to ER (endoplasmic reticulum) stress (Tardif et al., 2002), which is lethal to the cell. HCV has to counteract this mechanism to prevent apoptosis. After Isg15 knockdown Txndc5 is reduced on the transcriptional and the protein level. Txndc5 belongs to thioredoxin family and is associated to regulation of ER stress (Jessop et al., 2009). Furthermore, the expression of Txndc5 is directly associated to hepatic fat content and plays a role in ApoB control and steatosis development in apoE knockout mice (Ramírez-Torres et al., 2012) which may also influence the HCV replication complex (Bartenschlager et al., 2011).

Hmgcs1 as well as Hmgcr (HMG CoA reductase) are part of the mevalonate pathway, which allocates products necessary for cholesterol synthesis (Nakamuta et al., 2009). It has been demonstrated, that Hmgcs1 possesses a prolonged expression in HCV infected humanized mice (Walters et al., 2006). Further, it has been shown that blocking the mevalonate pathway with Hmgcr inhibitors (statins) (Ye et al., 2003) or with the Hmgcs1 inhibitor ceestatin, leads to suppression of HCV replication (Delang et al., 2009; Peng et al., 2011). The siRNA-mediated knockdown of Hmgcs1 in our study had no direct effect on HCV replication; however, the Hmgcs1 knockdown has been described to suppress HCV replication in alternative HCV cell culture systems (OR6 cells and JFH1) (Peng et al., 2011).

Idh1, as well as the mevalonate pathway, is sterol-controlled and activated by sterol regulatory element binding proteins (Srebps) (Shechter et al., 2003). Srebps are the key enzymes of cholesterol and fatty-acid biosynthesis and work as cellular cholesterol sensors as part of the ER membrane (Xiao and Song, 2013). It has been shown that the establishment of HCV infection is inhibited by blocking the Srebp pathway (Olmstead et al., 2012). As Srebp-2, Isg15 regulates the Idh1 expression and may therefore have a similar effect on HCV replication.

The second cluster of regulated proteins, identified in this study, was the heterogenous nuclear ribonucleoprotein (Hnrnp) family. Hnrnps are RNA-binding proteins, that shuttle between the nucleus and the cytoplasm and have been identified to bind the HCV IRES (internal ribosom entry side) to support translation: HnrnpA1 (Kim et al., 2007), HnrnpD (Paek et al., 2008), HnrnpE2/PCBP2 (Fukushi et al., 2001; Wang et al., 2011), HnrnpL (Hwang et al., 2009) and HnrnpM (Yao et al., 2011). Some of these proteins additionally bind to the 3′UTR of HCV: HnrnpA1 (Kim et al., 2007), HnrnpE2/PCBP2 (Wang et al., 2011), HnrnpI/PTB (Chung and Kaplan, 1999), HnrnpL (Hwang et al., 2009) and HnrnpC (Gontarek et al., 1999). Thereby playing an important role in initiation and regulation of HCV RNA replication via the viral RNA-dependent-RNA-polymerase NS5b, which itself is directly interacting with HnrnpA1 as well (Kim et al., 2007). Furthermore, HnrnpE2 has been shown to be involved in HCV RNA circularization via binding the 3′ and the 5′ UTR and showed high homology to HnrnpK (Wang et al., 2011). The heterogeneous HnrnpK, which is known to interact with HCV core protein (Hsieh et al., 1998), was regulated by Isg15 on the translational level in the present study. Our data indicated that Isg15 knockdown suppressed the HnrnpK protein level *in vitro* and *in vivo*. In addition, siRNA-mediated suppression of HnrnpK led to reduction in HCV replication. Therefore, HnrnpK might be a direct mediator of the pro-viral Isg15 effect in HCV replication. Moreover, it has been shown that HnrnpK expression is enhanced by knockdown of the Isg15 protease USP18 (Duex et al., 2011). Another group has shown the USP18 knockdown results in increased ISG15 expression (Murray et al., 2011).

Another Isg15-regulated Hnrnp family member was HnrnpA3, which siRNA-mediated suppression, did not directly affected HCV replication, but it has been described to be associated with HnrnpM and HnrnpL (Papadopoulou et al., 2012), which have been identified as HCV IRES binding factors. Therefore, it is likely that HnrnpA3 interact with HCV RNA in the ribonucleoprotein complex.

The present study illustrates that suppression of Isg15 affected cellular processes that are differently involved in HCV replication. However, the molecular interaction needs to be further investigated. These data also showed that Isg15 regulates the expression of proteins that have been identified to directly affect the HCV replication. Therefore, the pro-viral effect of Isg15 in HCV replication might be mediated through these effector proteins. These findings are of high relevance for the development of novel therapeutic option for HCV patients, as the RNAi-mediated suppression of ISG15 might inhibit the HCV replication complex at different steps.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.10.009.

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