Hepatitis C virus infection inhibits P-body granule formation in human livers

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Keywords: P-bodies; HCV; *in vivo*; liver

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Author's Contributions: GPV and CFC designed, performed experiments and analyzed data; CFC collected clinical data; CFC and LM set up conditions for the immunostaining of P-body components in formalin-fixed paraffin-embedded liver biopsies; XS provided technical assistance and acquired images for 3D reconstruction experiments; RM did the histopathological processing and diagnostic analysis of liver tissue samples; JD, SPP and XF supervised all aspects of this study, including design, execution, data analysis, interpretation and manuscript preparation; JD came up with the research idea; XF provided the patient cohort and the clinical samples; JD and GPV

wrote the manuscript; All authors contributed to the interpretation and discussion of the results, as well as to the revision of the manuscript.

Abstract

Background & Aims: Decoding the myriad of interactions that Hepatitis C virus (HCV) establishes with the infected cells is mandatory to obtain a complete understanding of HCV biology and its associated pathogenesis. We and others have previously identified in cell culture that HCV infection disrupts the formation of P-bodies. These are cytoplasmic RNA granules with key roles in post-transcriptional regulation of gene expression. Consequently, P-body disruption might have consequences beyond viral propagation. However, whether P-body disruption occurs also *in vivo* is unknown. Aim of this study was to address this important issue.

Methods: Formalin-fixed paraffin-embedded liver biopsies from 4 groups of patients (healthy donors, patients with non-virus related liver inflammation, HCV- and HBV-infected patients) were immunostained to detect DDX6 and Dcp1, two core P-body components. Changes in the localization of these proteins were assessed by confocal microscopy.

Results: HCV specifically inhibited P-body formation in hepatocytes from human livers regardless of viral genotype, inflammation grade or whether the infection was recent or long established. Importantly, this alteration was reversed once HCV is eliminated by therapy. Furthermore, *in vivo* an unexpected heterogeneity in P-body composition was observed that might reflect functional specializations.

Conclusions: This is the first comprehensive P-body analysis *in vivo* that links a pathogenic condition to P-body alterations. Given the role of P-bodies in cellular gene expression, their alteration should be considered to fully understand the complex HCV-associated pathologies.

Abstract word count: 229 words

Keywords: P-bodies; HCV; *in vivo*; liver

Introduction

Hepatitis C virus (HCV) remains a major threat for human health. Around 130-170 million people worldwide are chronically infected and at high risk to develop liver fibrosis, cirrhosis and hepatocellular carcinoma [1]. There is no vaccine against HCV and the recently approved treatments, although increasingly effective, are genotype specific, expensive, and present multiple side effects and contraindications [2, 3]. Thus, it is of interest to expand the therapy options against HCV. For this, it is necessary to gain a deeper knowledge of the HCV life cycle.

All steps of the HCV life cycle involve interactions between few HCV components and a myriad of host factors. Over the last years major advances have been made in the identification of such interactions using a robust HCV cell culture system that uses a virus strain isolated from a patient with fulminant hepatitis, a rare event in HCV infections [4], and Huh7 hepatoma cell lines [5]. However, although very useful, this is an artificial system that does not recapitulate the host environment that HCV faces in chronic infected livers. Moreover, Huh7 cells are transformed cells with an impaired innate antiviral response [6]. Consequently, *in vivo* studies in the infected human liver are essential to determine the relevance of identified host-HCV interactions.

Cell compartmentalization ensures the timely and quantitative provision of molecules in the cytoplasm of eukaryotic cells. These compartments include classical among others membrane organelles such as the endoplasmic reticulum and more recently identified membrane-less RNA granules named P-bodies. By using the HCV cell culture system we and others have shown that HCV not only manipulates host membranes but also P-bodies [7, 8]. P-bodies are involved in post-transcriptional regulation of gene expression. They are highly dynamic and contain translationally silent mRNAs together

with multiple proteins from the mRNA decay and silencing machineries [9, 10]. Once in P-bodies, mRNAs can be either degraded or stored for a later return into translation [11-13]. HCV hijacks the core P-body components PatL1, Lsm1-7 and DDX6 (also referred as Rck/p54), that are required for P-body formation, to efficiently translate and replicate its RNA genome [14]. This is associated with a significant reduction in the number of P-bodies within infected cells, presumably by keeping the required components away from participating in P-body formation since PatL1, Lsm1-7 and DDX6 expression is not changed [7, 8]. Alterations in P-body components and P-body formation might have consequences *in vivo* beyond HCV propagation. Indeed, such alterations not only have been connected to other viral infections but also to stress conditions and to cancer [15-19]. However, as for HCV, all these connections have been derived from indirect evidences and cell culture studies.

Here we show that HCV specifically decreases P-body abundance in hepatocytes from human livers. This is the first direct link *in vivo* between a pathogenic condition and P-body alterations.

Materials and Methods

Patients

A total of 55 patients were selected for this study, all of them attending the Hospital Clínic of Barcelona between 2002 and 2013. All patients provided written informed consent to use their liver samples. Our study was approved by the Ethics Committee of Hospital Clínic of Barcelona, in accordance with the guidelines set forth in the 1975 Declaration of Helsinki. Liver biopsies were performed from patients when clinically indicated. Aiming to assess changes in P-bodies during HCV infection, we studied 19 HCV chronically-infected immunocompetent patients with a liver biopsy done before

antiviral treatment (**Table 1**). Most of these patients showed typical histological features of chronic hepatitis with variable degree of periportal and/or lobular necroinflammation. As HCV-negative control group, 10 healthy donors for living donor liver transplantation (designated as healthy donors in the main text) were selected. To compare HCV infection with another viral hepatitis, we also enrolled 8 chronically HBV-infected immunocompetent patients with a liver biopsy performed before antiviral treatment. Most of the HBV-infected patients showed histological features of chronic hepatitis. In order to address a possible influence of inflammation *per se* on P-bodies, we selected 10 HCV- and HBV-negative liver transplant (LT) patients showing inflammatory changes at the liver biopsy due to non-viral causes, mainly transplant rejection. Most of the non-viral LT patients had portal lymphoid inflammatory infiltrates associated with variable lobular inflammation. The clinical and histological characteristics of the 3 control groups are summarized in **Supplementary Table 1**. To test the reversibility of P-body changes after HCV clearance, we also selected 3 patients who received antiviral treatment after LT and achieved a sustained virological response (demonstrated by negative PCR tests immediately after and at 6 months after treatment). In this group, liver biopsies were obtained before and after HCV clearance. To assess P-body changes in acute HCV infection we selected another 3 patients with hepatitis C recurrence after LT who were biopsied during acute phase hepatitis (0.5 and 6 months after LT). Colocalization studies with HCV core protein and DDX6 were performed in biopsies obtained from 2 patients with HCV recurrence after LT and very high viral load (7.7 and 8.0 $\log_{10}$ IU/mL, respectively).

Histopathological processing and diagnostic analysis of liver tissue samples

Tissue specimens were formalin-fixed, paraffin-embedded (FFPE) and stained with hematoxylin-eosin and Mason's trichrome as routinely done for diagnostic purposes in

the Pathology Department at the Hospital Clínic of Barcelona. Samples were analyzed by an expert liver pathologist (RM). Chronic viral hepatitis was graded and staged by the METAVIR score system [20]. Routine methods and scoring systems were used to characterize histopathological findings according to other specific clinical conditions (Banff recommendations for rejection, etc.; **Supplementary Table 1**).

Indirect immunofluorescence

Five-micrometer sections were cut from paraffin blocks and mounted onto charged slides. The slides were heated overnight at 37ºC and then subjected to antigen retrieval using the PT Link automated system (Dako, Glostrup, Denmark). Antigen retrieval conditions were high pH buffer, no preheat mode, target retrieval mode at 97ºC for 20 minutes. The sections were afterwards blocked with phosphate-buffered saline (PBS) / 10% goat serum (Jackson ImmunoResearch, PA, USA) during 30 minutes. Incubation with primary antibodies was performed at room temperature during 120 minutes. The primary antibodies used were 5 μg/mL rabbit polyclonal anti-DDX6/rck (p54) (MBL, Nagoya, Japan), 1.3 μg/mL mouse monoclonal IgG1 anti-DDX6 3D2 (Sigma-Aldrich, MO, USA), 0.8 μg/mL mouse monoclonal IgG2a anti-Dcp1A 3G4 (Abnova, Taipei, Taiwan) and 1 μg/mL mouse monoclonal IgG1 anti-core C7-50 (Santa Cruz Biotechnology, CA, USA). After incubation with the primary antibodies, sections were washed three times with PBS (5 minutes each) and incubated with the secondary antibody for 1 hour at room temperature. The secondary antibodies used were 2 μg/mL Alexa Fluor® 647 goat anti-rabbit IgG and Alexa Fluor® 568 goat anti-mouse IgG (Invitrogen, CA, USA). After the second incubation, 3 additional washes with PBS were performed and the slides were incubated again for 5 minutes with 1 μg/mL DAPI (Sigma-Aldrich, MO, USA) followed by three final washes with PBS and once with

deionized water. Finally, the samples were mounted with ProLong® Gold Antifade Reagent (Invitrogen, CA, USA).

Confocal imaging and data analysis

Images were acquired with a Leica TCS SP5 confocal microscope using a 63× 1.4-NA PL APO objective (Leica Microsystems GmbH). For each liver biopsy 10 different non-overlapping fields were analyzed. Portal spaces were systematically excluded, avoiding areas of high lymphocyte concentration or poor DAPI quality staining. In total, more than 200 hepatocytes were analyzed per biopsy. Image processing and analysis to obtain the number and size of DDX6- and Dcp1-containing P-bodies was performed with Fiji software [21]. Areas with liver sinusoids and/or lipofuscin aggregates were excluded from the analysis by masking them. The resulting images were processed by using a difference-of-Gaussians filter to enhance structures within the range of the two Gaussians (sigma 0.5 and 2). Filtered images were subsequently thresholded for P-body particles segmentation and counting. The number of hepatocytes per field was obtained by counting nuclei within a specified range in size (500-5000 pixels) and circularity (0.85-1.00) and final values for P-body counts per hepatocyte were obtained from relating the number of P-bodies in each image to the number of hepatocytes. In the indicated experiments, Z-stacks at the optimal Nyquist axial step size (0.13 µm) from at least 30 randomly selected cells under each condition were collected. Stacks were analyzed to obtain P-body numbers in a similar way as described above, yet using tools to process, threshold and count volumes instead of areas. To perform 3D reconstructions, Z-stacks were deconvolved using Huygens Essential 4.1 software (Scientific Volume Imaging BV, Hilversum, The Netherlands) and visualized with isosurfaces using Imaris 64X 7.6.4 software (Bitplane AG, Zurich, Switzerland).

Statistical analysis

Mann-Whitney *U* test was performed when comparing quantitative variables in unpaired groups. Wilcoxon signed-rank test was used to compare quantitative variables in paired groups. A two-tailed p-value of less than 0.05 was considered statistically significant. Data were analyzed with SPSS Statistics 20 (IBM) and Prism 5.01 (GraphPad) software.

Results

HCV infection specifically decreases P-body abundance in livers from chronically infected patients

We have previously reported that HCV utilizes P-body components to propagate and induce granule disruption in cell culture [7, 14]. These *in vitro* results encouraged us to investigate whether HCV infection affects P-body formation in the liver of chronically infected patients. For this, formalin-fixed paraffin-embedded liver biopsies from HCV-infected patients (n=19, **Table 1**) and healthy donors (n=10) were double immunostained to detect the two core P-body components DDX6 and Dcp1. P-body detection was assessed by confocal microscopy (**Fig. 1A**). Regardless of the infecting HCV genotype (1a, 1b, 2, 3 or 4) or the inflammation grade, the abundance of P-bodies containing DDX6 and Dcp1 were reduced by ~2- and ~10-fold, respectively, in hepatocytes from HCV-infected patients relative to hepatocytes from healthy donors (**Fig. 1B**). The number of Dcp1-positive P-bodies in the biopsies from healthy individuals exhibited a wide variation among samples that was narrowed down in the presence of HCV infection.

To determine whether the observed alterations were HCV-specific we included in our study liver biopsies from patients chronically infected with hepatitis B virus (HBV) (n=8) and from patients with non-virus related inflammatory changes (n=10). The first

group of patients allowed determining whether another virus infection that targets the liver could also lead to P-body disruption, while the second group allowed addressing the putative role of liver inflammation in P-body alterations. Importantly, no decrease in the number of P-bodies containing DDX6 was observed in liver biopsies from HBV-infected patients or with non-virus related inflammatory changes. Likewise, the values obtained for P-bodies containing Dcp1 in these patients were disperse and no significant differences were observed when compared to healthy donors (**Fig1. B**). Thus *in vivo* HCV infection specifically decreases P-body abundance.

The effect of HCV on P-body disruption occurs in both acute and chronic infections and is reversed once HCV is eliminated by therapy

To assess whether a reduced P-body abundance is reversibly linked to HCV infection, we longitudinally analysed P-bodies in liver biopsies from chronically infected patients before and after antiviral treatment (n=3), once a sustained virological response and thus viral clearance had been achieved. In all cases, the number of P-bodies per hepatocyte containing DDX6 or Dcp1 increased upon viral clearance, reaching levels comparable to the ones found in healthy donors (**Fig. 2**). Of note is that although the number of P-bodies per hepatocyte seemed patient specific, the ratio between P-body numbers obtained before and after antiviral treatment was similar among the three patients analysed.

To determine whether the HCV-induced changes in P-bodies from hepatocytes require a chronic state of the viral infection or also occurs in acute states, we analyzed biopsies from liver transplant patients with acute recurrent hepatitis C (n=3), which mimics the initial phase of the infection. In these biopsies the number of P-bodies that contained DDX6 or Dcp1 was similar to the ones found in chronically infected patients (**Fig. 2**).

Collectively, although the amount of samples was limited, these results support that HCV infection, acute or chronic, specifically decreases P-body abundance and that this decrease is reversed once HCV is eliminated by therapy.

In vivo, P-body granules in somatic cells are more heterogeneous than previously anticipated

The composition and formation of P-bodies in somatic cells *in vivo* is mostly unexplored since available studies have focused on cell culture systems [11, 12]. Interestingly, analyses of the liver images from confocal microscopy revealed unexpected peculiarities. There was no colocalization of DDX6 and Dcp1 in hepatocytes *in vivo* although such colocalization is commonly observed in hepatoma cell lines, like Huh 7.5, used to study HCV *in vitro* [7]. This lack of colocalization was hepatocyte-specific since parenchyma-infiltrating lymphocytes of the same individual showed a strong colocalization of DDX6 and Dcp1 (**Fig. 3**). Thus, *in vivo*, P-body granules seem to be more heterogeneous than previously anticipated *in vitro* and cell-type specific.

3D single cell analyses corroborates the P-body number decrease observed at a global level in HCV-infected livers

Confocal analysis allows the acquisition of single cell sections. To eliminate the possibility that the P-body changes in HCV chronically infected livers were due to alterations in P-body intracellular distribution, we carried out detailed single-cell 3D analyses. Liver biopsies from patients with severe HCV recurrence after liver transplantation were double immunostained for the HCV core protein and the P-body component DDX6. Double staining with the HCV core antigen ensured that only HCV-infected cells were analyzed. The use of biopsies from liver transplant patients with high

viral load and, therefore, high HCV antigen levels allowed overcoming the experimental limitations of the low abundance of HCV antigens in infected cells and the unfavorable imaging properties of the liver [22]. Z-stack images were obtained and P-body abundance and intracellular localization was evaluated and compared with that in hepatocytes from healthy donors. DDX6-containing P-bodies were reduced by ~2-fold in core-positive hepatocytes from HCV-infected patients relative to hepatocytes from healthy donors (**Fig. 4A**). This value parallels the one obtained with single stack analyses where infected and non-infected hepatocytes are indistinguishable (**Fig. 1B**). In line with this, when P-bodies were quantified in core-negative hepatocytes the average amount of DDX6-containing P-bodies per cell area was similar to the one in core-positive hepatocytes. In addition, as it has been observed previously [22], the 3D reconstruction of HCV-infected hepatocytes showed HCV core proteins forming ring-like structures that surround lipid droplets, the site of viral RNA encapsidation. Opposite to studies in cell culture [8], DDX6 did not colocalize with HCV capsid proteins (**Fig. 4B**).

Discussion

In vivo studies are essential to deeply understand the complex interaction of HCV with the infected hepatocyte and the associated pathogenesis. In this manuscript we report that HCV infection impairs P-body formation in hepatocytes from human livers regardless of viral genotype, inflammation grade or whether the infection is recent or long established. The observed alterations are HCV-specific since no significant P-body reduction was observed in chronically HBV-infected patients or in patients with non-viral related hepatic inflammatory changes. Furthermore, the number of P-bodies was restored once the virus is eliminated by therapy strengthening the requirement of the presence of HCV for P-body disruption to occur. Interestingly, the number of P-bodies

per hepatocyte was highly heterogeneous among individuals covering a wide range of values in healthy donors, HBV-infected, and non-viral related liver inflammation patients. Nonetheless, in HCV-infected patients this range was very narrow indicating that HCV is a decisive factor in P-body formation.

An unexpected observation in our study was the heterogeneous composition displayed by P-bodies *in vivo*. To our knowledge all previous P-body studies in somatic cells have been assessed in cell lines. In these studies DDX6 and Dcp1, two core P-body components widely-used to visualize P-bodies, colocalize [7]. However, in hepatocytes from both healthy donors and HCV-infected patients no colocalization was observed, while lymphocytes surrounding these hepatocytes showed clear colocalization patterns. The variable P-body composition among cell types might reflect different cell regulation requirements [23]. Currently, we do not know if this is a special feature of hepatocytes or is extended to other cell types. Another observation in conflict with previous cell culture data was the no colocalization of DDX6 with the viral core protein. Ariumi *et al.* determined that DDX6, among other P-body components, were hijacked to lipid droplets where they colocalize with the core protein from JFH1 HCV strain [8]. However, with Jc1 HCV strain we failed to observe this colocalization in cell culture [7]. These conflicting results might reflect differences in the dynamicity of core and DDX6 interactions. Finally, another interesting observation in hepatocytes is that DDX6-containing and Dcp1-containing P-bodies behave differently under certain conditions. In hepatocytes from HBV-infected patients the number of DDX6-containg P-bodies was significantly increased relative to hepatocytes from healthy donors. In contrast, hepatocytes from HBV-infected patients displayed a tendency to show lower Dcp1-containing P-body numbers when compared to healthy donors. Although the cause of these distinct behaviours remains unknown, they might reflect a specialization

in P-body granules. Together, the observed heterogeneity in P-body composition among cell types and within a single cell opens a new level of complexity that might reflect functional specializations and deserves further exploration.

The HCV-induced reduction in P-body numbers might have profound effects in the biology of the cell. P-body components are in a dynamic equilibrium between their granule and a soluble localization in the cytosol, thus a reduction in the number of P-bodies results in an increase of cytosolic P-body components [24-26]. As P-body components include multiple proteins that control decay and silencing of mRNAs, the transcriptome and translatome is expected to be deregulated and consequently gene expression altered. In chronic infections, HCV-induced reduction in P-body numbers would result in a long-term alteration of gene expression that might contribute to HCV-associated pathogenesis. In line with this, overexpression of the core P-body component DDX6 has been observed in hepatocellular carcinomas [18]. This overexpression is expected to alter the P-body numbers.

How and why HCV infection induces P-body disruption is not completely understood. Our data in hepatoma cell lines support the hypothesis that HCV disrupts P-bodies by hijacking or modifying core P-body components required for P-body formation [7, 8]. Lsm1-7, Pat1 and DDX6 are three core P-body components with a major role in decapping and decay of cellular mRNAs [27]. HCV needs these factors and redirects them for a role in the viral life cycle to promote HCV RNA translation and replication [14, 28]. At the same time, the subsequent P-body disruption might also benefit viral reproduction since P-bodies have been involved in innate immune responses [16]. Intriguingly, *in vivo*, the HCV-induced reduction in P-bodies was similar in core-positive and core-negative cells (**Fig. 4A**). Consequently, either very low and

undetectable HCV replication is enough to cause P-body disruption or HCV-infected cells induce P-body disruption of surrounding cells by unknown mechanisms. Interestingly, perturbed metabolic zonation in HCV-infected livers has been recently related to this last scenario [29].

In summary, this is the first comprehensive analysis of P-body granules *in vivo* and a direct demonstration of a link between a pathogenic condition and P-body alterations. Incorporating P-bodies into the mechanistic framework of multifaceted disease traits adds another level of complexity that should be considered to fully understand the molecular events causing HCV-induced liver disease and other serious diseases.

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Figures and Tables

Figure 1. HCV infection specifically decreases the number of P-bodies compared with healthy donors. Hepatic tissue was stained with antibodies to detect DDX6 (red) and Dcp1 (green). Nuclei were visualized using DAPI (blue). (A) Representative pictures from healthy donors and HCV-infected patients. Scale bar, 10µm. (B) Quantification of DDX6- and Dcp1-containing P-bodies in healthy donors (Healthy, n=10), HCV-infected (HCV, n=19), HBV-infected (HBV, n=8), and HCV-negative patients with liver inflammation (Inflam., n=10). Dots indicate the median value per biopsy, horizontal red lines indicate the median for each group of patients and vertical red lines the interquartile range. Statistical significance was tested with Mann-Whitney *U* test. **P* < 0.05; ***P* < 0.005; n.s., not significant.

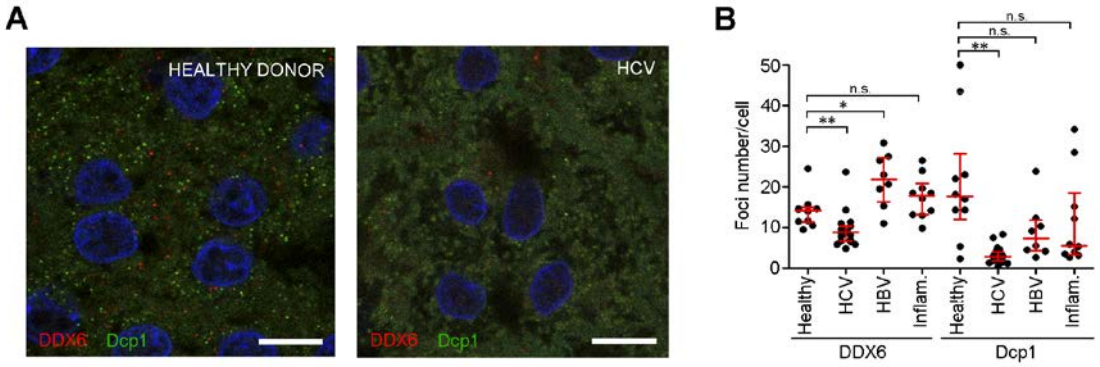
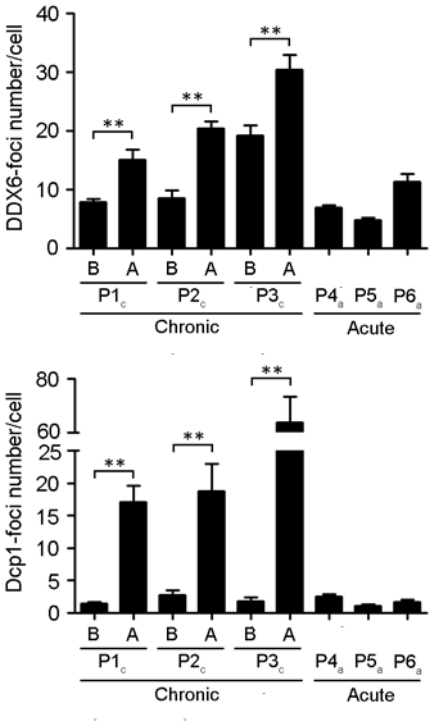


Figure 2. P-body disruption occurs in acute and chronic HCV infections and is reversed once HCV is eliminated. Hepatic tissue was stained as described for Fig. 1A and DDX6- and Dcp1-containing P-bodies were quantified in three HCV-chronic infected patients ($P1_c$, $P2_c$ and $P3_c$) before (B) and after (A) antiviral treatment, as well as in three HCV-acute infected patients ($P4_a$, $P5_a$ and $P6_a$). Displayed are the median values for each patient, error bars indicate the standard error of the mean. Statistical significance was tested with Wilcoxon test. ***P* < 0.005.



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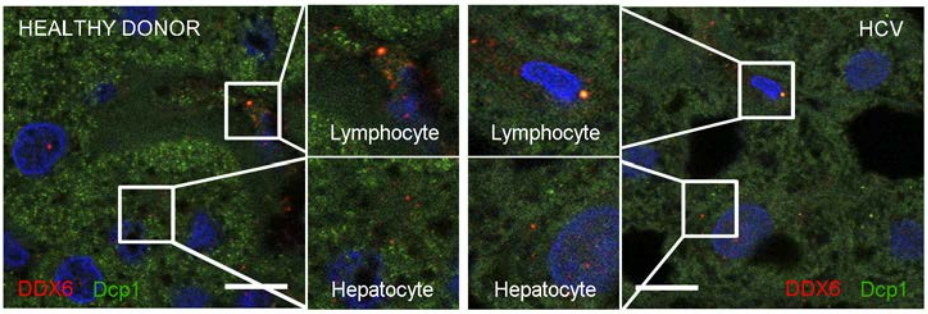
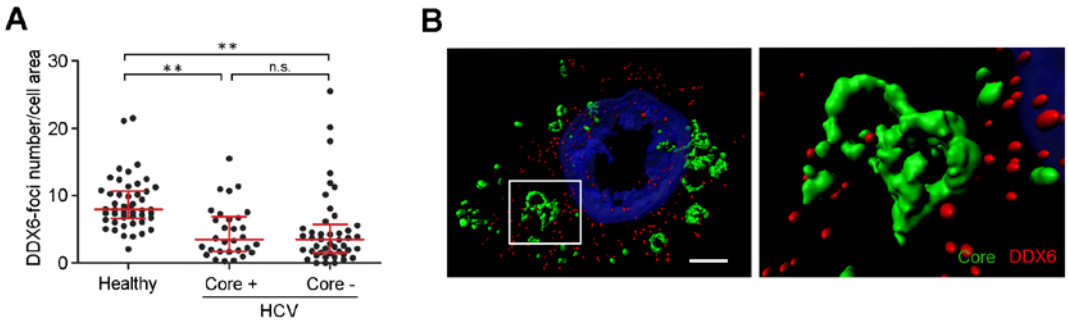
564 **Figure 3. Colocalization of DDX6 and Dcp1 in hepatocytes and parenchyma-**
565 **infiltrating lymphocytes.** Hepatic tissue was stained in healthy donors and HCV-
566 infected patients with antibodies to detect DDX6 (red) and Dcp1 (green). Nuclei were
567 visualized using DAPI (blue). Scale bar, 10µm. Indicated areas in the images are
568 magnified in the central columns.



569

570 **Figure 4. 3D single cell analysis validates DDX6-containing P-body decrease**
571 **observed at a global level.** Hepatic tissue was stained with antibodies to detect DDX6
572 (red) and the viral protein core (green). Nuclei were visualized using DAPI (blue). (A)

DDX6 P-body number was quantified at a single cell level along the Z-axis in healthy donors (Healthy, n=4) and in HCV core-positive (Core +) and core-negative (Core -) cells from HCV-infected patients (HCV, n=2). Horizontal red lines indicate the median for each group of patients and vertical red lines show interquartile range. (B) Z-stack images were 3D-segmented. Colocalization between both markers was assessed in HCV-infected patients. Statistical significance was tested with Mann-Whitney *U* test. ***P* < 0.005; n.s., not significant. Scale bar, 3 µm.



595 **Table 1. Baseline characteristics of immunocompetent patients with chronic**
596 **hepatitis C.** All patients (n= 19) were biopsied before treatment as clinically indicated
597 in order to assess liver injury and fibrosis stage (METAVIR grade and stage,
598 respectively). In this group, no specific association was found between P-bodies number
599 and clinical parameters.

600

Patient	Age *(years)*	Gender *(M/F)*	HCV Genotype	Viral Load *(log_{10} IU/mL)*	AST/ ALT *(IU/mL)*	METAVIR grade	METAVIR stage
P1	35	M	4	5.22	71/155	A2	F2
P2	36	M	1b	5.97	33/48	A0	F0
P3	54	F	1a	5.24	71/54	A1	F2
P4	45	M	1b	5.01	30/36	A0	F0
P5	55	M	1b	5.05	152/203	A1	F4
P6	65	F	1b	5.79	73/94	A1	F2
P7	20	F	1b	4.40	66/158	A1	F2
P8	37	M	1b	6.04	114/262	A1	F2
P9	43	F	1b	5.18	67/138	A1	F1
P10	33	M	3	5.77	40/64	A2	F4
P11	55	F	1b	6.03	73/60	A1	F1
P12	23	F	1b	3.04	31/69	A1	F0
P13	51	M	1b	6.33	39/70	A0	F1
P14	66	M	1b	5.72	227/322	A2	F3
P15	55	M	1a	6.31	73/121	A2	F2
P16	29	M	4	5.17	39/45	A0	F0
P17	52	M	1b	6.72	144/196	A2	F4
P18	46	F	4	5.42	60	A1	F2
P19	47	M	2	2.33	45	A1	F2

601 M/F, male/female; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

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606 **Supplementary Table 1. Clinical characteristics among the control groups: healthy**
607 **donors, HBV-infected and HCV/HBV-negative LT patients with liver**
608 **inflammation.** All patients were biopsied as clinically indicated.

609

Patient ID	Control group	Age *(years)*	Gender *(M/F)*	IS	AST/ALT *(IU/mL)*	METAVIR stage	Histopathogical diagnosis
P20	Healthy donors (n=10)	30	M	-	28/50	F0	Mild hemosiderosis
P21		30	M	-	21/18	F0	Mild hemosiderosis
P22		53	M	-	28/38	F0	Moderate hemosiderosis
P23		43	F	-	NA	F0	NAD
P24		54	F	-	NA/26	F0	Mild steatosis
P25		46	F	-	12/15	F0	NAD
P26		36	M	-	31/26	F0	Mild steatosis
P27		22	M	-	27/23	F0	Minimal changes
P28		28	M	-	25/33	F0	Mild hemosiderosis
P29		43	F	-	14/8	F0	NAD
P30	HBV (n=8)	27	M	-	231/574	F2	Suggestive of HBV infection
P31		40	M	-	31/44	F1	Suggestive of HBV infection
P32		49	F	-	53/60	F3	Suggestive of HBV infection
P33		37	M	-	48/68	F2	Compatible with HBV
P34		44	M	-	69/70	F3	Suggestive of HBV infection
P35		33	M	-	55/85	F2	Suggestive of HBV infection
P36		48	M	-	93/97	F3	Suggestive of HBV infection

P37		33	M	-	46/61	F1	Suggestive of HBV infection
P38	LT with inflammatory changes (n=10)	69	M	Other	52/73	F0	Steatohepatitis
P39		61	M	FK	11/25	F0	Rejection
P40		54	F	FK	22/20	F0	Unspecific inflammation
P41		31	F	CyS	55/215	F0	Rejection
P42		54	M	CyS	64/168	F1	Rejection
P43		59	M	FK	29/55	F0	Rejection
P44		38	M	FK	213/317	F0	Rejection
P45		69	M	FK	39/39	F0	Autoimmune hepatitis
P46		46	F	Other	43/53	F0	PBC recurrence
P47		67	M	CyS	100/88	F0	Rejection

610 LT, liver transplant; M/F, male/female; AST, aspartate aminotransferase; ALT, alanine aminotransferase;
611 IS, immunosuppression regimen; CyS, Cyclosporine A; FK, Tacrolimus; Other, mTOR inhibitor; PBC,
612 primary biliary cirrhosis; NAD, nothing abnormal detected.

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