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Proteome analysis of a human liver carcinoma cell line stably expressing hepatitis delta virus ribonucleoproteins

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ABSTRACT

Hepatitis delta virus (HDV) infects human hepatocytes already infected with the hepatitis B virus increasing about ten fold the risk of cirrhosis and fulminant hepatitis. The lack of an appropriate cell culture system capable of supporting virus replication has so far impaired the detailed investigation of the HDV biology including the identification of host factors involved in pathogenesis. Here, we made use of a HDV cDNA stably transfected cell line, Huh7-D12, in a proteomic approach to identify the changes in the protein expression profiles in human liver cells that arise as a consequence of HDV replication. Total protein extracts from Huh7-D12 cells and of the corresponding non transfected human liver carcinoma cell line, Huh7, were separated by 2-DE. Differentially expressed spots were identified by MALDI-TOF followed by database searching. We identified 23 differentially expressed proteins of which 15 were down regulated and 8 up regulated in Huh7-D12 cells. These proteins were found to be involved in different cellular pathways. The down regulation of the histone H1-binding protein and of triosephosphate isomerase was confirmed by Real time PCR, and the up regulation of the La protein and lamin A/C was validated by western blot.

1. Introduction

Hepatitis delta virus (HDV) is a satellite virus of the hepatitis B virus (HBV). HDV co-infection or super-infection of human hepatocytes already infected with the HBV greatly increases liver damage resulting in a 10 fold higher risk of cirrhosis and fulminant disease [1,2]. The two viruses are clinically associated since the outer envelope of the HDV consists of HBV surface antigens (HBsAgs) which are necessary for virus maturation and propagation of infection [3]. The HDV genome consists of a negative, circular, ssRNA molecule of about 1.7 kb. Replication of the virus genome is thought to occur via a rolling circle mechanism involving cellular RNA polymerase II. The resulting multimeric antigenomes are subsequently self cleaved and religated at precise monomeric intervals. The antigenomic RNA molecules then serve as template for a second round of replication and subsequent production of monomeric genomic RNA molecules by a similar mechanism [4].The HDV RNA contains a single ORF encoding for the only virus protein, the delta antigen (p24; S-HDAg). During replication an editing mechanism involving a cellular double-stranded RNA specific adenosine deaminase (ADAR 1) converts an amber stop codon UAG into a triptophan codon UGG extending the ORF by 19 aa, and resulting in the production of the large form of the delta antigen (p27; L-HDAg) [5]. Several functions have been assigned to S-HDAg and L-HDAg during

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the virus replication cycle. S-HDAg is thought to promote nuclear import of HDV ribonucleoproteins (RNPs) to the nucleus, to accelerate ribozyme activity, and to increase processivity during RNA transcription [6–8]. In contrast, L-HDAg was found to repress RNA replication and to be necessary for interaction with HBsAgs and virus packaging [9].

Despite the lack of a cell culture system capable of supporting HDV replication, considerable progress has been made towards the elucidation of the mechanisms of virus replication and pathogenesis. However, little is still known about the host factors involved in the different steps of HDV replication. The Delta interacting protein (DIPA), the double-stranded RNA-dependent protein kinase, nucleolin, the small form of adenosine deaminase (ADAR 1), RNA polymerase II, the negative elongation factor (NELF), glyceraldehyde 3-phosphate dehydrogenase (GADPH), and the polypirimidine tract-binding protein associated splicing factor (PSF), are the only proteins that were shown to interact *in vitro* either with the HDV antigens or RNA during the replication cycle [10–16].

The Huh7-D12 cell line has been previously used as a model system to study different aspects of the molecular and cellular biology of the HDV. This cell line was obtained by stable transfection of human liver carcinoma Huh7 cells with a plasmid containing a trimer of the HDV genomic cDNA [17]. The cDNA was synthesized from total liver RNA obtained from an infected woodchuck inoculated with HDV containing human serum. Three HDV expressing clones, D8, D12, and D14, were isolated and expanded. The clone D12 has been extensively characterized and was shown to constitutively express both delta antigens and replicate virus RNA [18,19]. Moreover, the molar ratio between HDAg and virus RNA in these clones was previously reported to be similar to that found in HDV particles released into the serum of experimentally infected woodchucks [20].

In a previous work, aimed to analyse the proteome of Huh7 cells transiently expressing HDV RNA and antigens separately, we were able to identify 32 differentially expressed proteins [21]. However, this strategy did not allow investigating the alterations in the cellular proteome due to the simultaneous expression of all the HDV specific components.

In this work, we made use of a proteomic approach to identify the changes in protein expression that arise in human liver cells as a consequence of synthesis of HDV RNPs. Total cellular protein extracts were prepared and separated by 2-DE. The spots corresponding to differentially expressed proteins were identified by Peptide Mass Fingerprint (PMF) using MALDI-TOF data. We identified 23 proteins of which 15 were down regulated and 8 were up regulated in Huh7-D12 cells when compared with Huh7 cells. The up regulation of lamin C and La proteins was confirmed by western blot analysis. The down regulation of the histone H1-binding protein (NASP) and of triosephosphate isomerase (TPI) was further validated by Real time PCR.

2. Materials and methods

2.1. Materials

For 2DE analysis, IPG DryStrips and IPG buffer were purchased from GE Healthcare. 30% Acrilamide/Bis solution and TEMED were obtained from BioRad. APS, Iodoacetamide, Thiourea, Brilliant Blue G, Urea, CHAPS, DTE and SDS were all purchased from Sigma. Tris was obtained from Merck.

Regarding mass spectrometry, DTT was purchased from GE Healthcare. NH_4HCO_3 and 2.5 DHB were obtained from Sigma. ACN LC-MS was purchased from Riedel-de-Häen. CHCA and peptide calibration mix 1 were from Laser Biolabs. Other chemicals were analytical grade products.

2.2. Cell culture

Two different cell lines were used in this study. The human hepatocellular carcinoma cell line (Huh7) and the correspondent HDV cDNA stably transfected cell line (Huh7-D12) that constitutively expresses virus ribonucleoproteins [18,19]. Both cell lines were cultured as monolayers in RPMI 1640 medium (Sigma) supplemented with 10% foetal bovine serum (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Immunofluorescence and in situ hybridization

In situ hybridization in whole cells was performed as earlier described [19]. Briefly, cells on coverslips were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS, for 10 min, at room temperature. After fixation, cells were sequentially treated with 50% formamide at 37 °C, for 5 min, followed by incubation wit 75% formamide at 70 °C, also for 5 min. Hybridization was performed using digoxigenin-11-dUTP (Roche) nick-translated plasmid pSVL(D3) as probe [22]. This plasmid contains a trimer of full-length genomic HDV cDNA. After hybridization, digoxigenin detection was performed using appropriate FITC-conjugated antibodies (Roche).

Immunofluorescence was performed on hybridized samples using a polyclonal antibody that recognizes both forms of the delta antigens (B3) [23], followed by incubation with an appropriate secondary antibody conjugated with Texas Red (Jackson Immunoresearch Laboratories).

Fluorescent labelled samples were analyzed under a laser scanning microscope Zeiss LSM 510 equipped with an Argon Ion laser (488 nm) to excite FITC fluorescence and a Helium–Neon laser (543 nm) to excite Texas Red fluorescence.

2.4. 2D electrophoresis

To prepare protein samples for 2DE, cells were first harvested, washed with PBS, and pelleted by centrifugation. The cell pellet was re-suspended in 10% TCA. Following precipitation, the protein samples were centrifuged at 12,000 g, for 5 min, and washed with 3 volumes of ice cold acetone, for 2 h at −20 °C. Subsequently, the precipitated proteins were centrifuged, as above, and stored at −80 °C until use. Finally, protein samples were re-suspended in IEF buffer (7 M Urea, 2 M Thioureia, 4% CHAPS, 0,5% IPG Buffer pH 3–11, 60 mM DTE and 0.002% Bromophenol Blue). To quantify protein samples a Bradford assay based kit (BioRad) was used.

For the first dimension, isoelectric focusing (IEF), approximately 200 μg of proteins, resuspended in IEF buffer, were loaded onto each precast 18 cm immobilized non-linear pH 3–11 (IPG) strip. The strips were rehydrated for 12 h, and IEF was

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carried out in an Ettan IPGphor Isoelectric Focusing System (GE Healthcare) until a total of 97,000–98,000 Vh was achieved.

After IEF separation the gel strips were incubated twice, for 15 min, with equilibration buffer (50 mM Tris–HCl pH 8,8, 6 M Urea, 30% Glicerol, 2% SDS, 2% DTE). Following equilibration, the strips were incubated twice with blocking buffer (50 mM Tris–HCl pH 8,8, 6 M Urea, 30% Glicerol, 2% SDS, 0.002% Bromophenol Blue, 2.5% Iodoacetemide) for 15 min, at room temperature. The second dimension SDS PAGE was carried out on 9% polyacrilamide gels in a Hoefer ES 600 Ruby apparatus (GE Healthcare). Protein separation was performed overnight, at 50 V, and at room temperature, until the bromophenol blue dye front reached the end of the gel.

2.5. Gel staining and image analysis

After electrophoresis the gels were stained using a Blue Silver staining protocol [24]. Images were acquired using a Sharp JX330 scanner and the gels were analysed using the Phoretix PG200 software (Nonlinear Dynamics). Five gel replicas were analysed for each sample. Only spots present in at least 4 of the gels were used to analyse expression differences.

Normalized spot volumes were used to compare the different samples. The Student's *t*-test ($p<0.05$) was used to analyse quantitative differences between Huh7 and Huh7-D12 protein gels. Spots displaying at least a two fold increase or decrease in protein expression were selected for identification by Peptide Mass Fingerprint.

2.6. Protein identification

Differentially expressed protein spots were excised, destained with 50% ACN and dried in a Speed Vac centrifuge. The polypeptides were reduced, alkylated, and digested with sequencing-grade modified trypsin (Promega) as described earlier [25]. After digestion, samples were desalted and concentrated using Gel Loader Tips (Eppendorf) filled with Poros R2 resin (Applied Biosystems). Peptides were directly eluted into the MALDI plate with 10 mg/mL CHCA in 50% acetonitrile and 0,1% (v/v) trifluoroacetic acid, and allowed to dry at open air.

The samples were analysed in a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems), and mass spectra were acquired using the Voyager control panel software v5.10. Spectra were analysed using the *m/z* program v2001.02.13 and contaminant peaks were removed using the Peakerasor program v1.76 (both from Genomic Solutions, UK). The remaining peaks were then matched with the theoretical peptide masses using MASCOT (Matrix Science, UK). The search was performed against a *Homo sapiens* database with a peptide tolerance of 100 ppm and a single missed cleavage allowed. Carbamidomethylation of cysteine and oxidation of methionine were, respectively, the fixed and variable possible amino acid modifications considered in the search procedure.

Significant homology scores achieved in MASCOT, significant sequence coverage values, and similarity between the protein molecular mass calculated from the gel and for the identified protein were the criteria utilized to accept the identification [26]. Evaluation of the presence of post-translational modifications was performed using the FindMod software (http://www.expasy.ch/sprot/findmod.html).

2.7. Computational analysis of protein interactions

To analyse possible interactions among the identified proteins, the IntAct database v 20071020 [27] was imported to the Cytoscape platform v 2.5.1 [28]. Differentially expressed proteins were selected using their accession number and marked as up regulated or down regulated proteins. The network analysis was performed by selecting the proteins that directly interacted with the previously selected proteins.

2.8. Isolation of total mRNA and cDNA synthesis

Extraction of mRNA was performed using the Oligotex Direct mRNA Mini kit (Qiagen) following the manufacturer's instructions. Prior to cDNA synthesis, samples were treated with DNase I using the DNase-free kit (Ambion, UK) also according to the manufacturer's instructions.

For cDNA synthesis, the Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas) was used. Reaction mixtures contained approximately 100 ng mRNA, 0.5 μg oligo(dT)$_{12–18}$ (Roche), 10 mM dNTP mix, 20 U RNase inhibitor (Roche), and 40 U MMuLV Reverse Transcriptase in reaction buffer (Fermentas). After 1 h incubation at 42 °C, reactions were stopped by heating at 70 °C, for 10 min.

Finally, cDNA samples were purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare).

2.9. Real time PCR

Real time PCR reactions were performed using the qPCR Core kit for SYBR® Green I (Eurogentec, Belgium) following the manufacturer's specifications. Typically, reaction mixtures contained 3.5 mM $MgCl_2$, 200 μM each dNTP, 300 nM of each primer, 0.025 U/μl of HotGoldStar enzyme, 0.6 μl of diluted SYBR® Green I, and reaction buffer in a final volume of 20 μL. The PCR program used for amplification was: 10 min at 95 °C, 40 cycles with 15 s at 95 °C, and 1 min at 60 °C. Reactions were performed in 96-well plates with optical caps (Applied Biosystems) in a GeneAmp® 5700 Sequence Detector System (Applied Biosystems). Each sample was assayed in triplicate and analysed with the GeneAmp® 5700 SDS v1.1 software and Microsoft Excel.

The $2^{-\Delta\Delta Ct}$ method earlier described [29] was used for relative quantification. The reference gene to which all the samples were compared with was β-2-Microglobulin (β2MG; Genbank accession number P61769). Primers for the reference and target genes were designed using the program Primer Express™ v1.5 and the bioinformatic tool Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). Melting temperature, GC content, secondary structure, and length were taken in consideration for primer design. The cDNA transcript sequences were obtained in Genbank database from NCBI. The primer sequences used in this work to amplify the β2MG, triosephosphate isomerase (TPI), and histone H1-binding protein (NASP) transcripts are displayed in Table 1.

2.10. Western blot

Western blot was essentially performed as previously described [19]. To prepare protein samples, Huh7 or Huh7-

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Table 1 – Sequences of primers used in Real time PCR

Target gene	Forward	Reverse
β2MG	5'-GGC TAT CCA GCG TAC TCC AA-3'	5'-TCA CAC GGC AGG CAT ACT C-3'
TPI	5'-GCA CTC AGA GAG AAG GCA TGT-3'	5'-CAA TGC AGG CGA TTA CTC CGA-3'
NASP	5'-GGA GTC TCA GCG TAG TGG GAA T-3'	5'-TCT ACT GGC AAT CAT GGA GAC TG-3'

D12 cells were washed with PBS, mixed with SDS sample buffer, and boiled.

Approximately 30 μg of total protein extracts were separated by SDS-PAGE on 12% polyacrilamide gels. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% low fat milk powder in PBS. Blots were then incubated for 1 h with primary antibodies, washed with PBS containing 0.1% Tween 20 and 2% low fat milk powder, and further incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (BioRad). Membrane development was performed using the Lumi-light^{Plus} western blotting kit (Roche). Finally, images were digitized using the ImageScanner (GE Healthcare), and analysed with Phoretix PG200 software (Nonlinear Dynamics). After background subtraction, the amount of each selected protein was normalized against the control housekeeping protein clathrin.

The following primary antibodies were used in this study: mouse monoclonal antibody against lamin C (Abcam, UK), mouse monoclonal antibody against the La protein [30], and rabbit polyclonal antibody against clathrin (Santa Cruz Biotechnology).

3. Results

3.1. *Detection of HDV RNA and antigens*

The human hepatoma cell line Huh7-D12 has been previously described [18,19]. This cell line was obtained by stable transfection of Huh7 cells with plasmid pSVL(D3) which contains three copies in tandem of full length genomic HDV cDNA. Huh7-D12 cells constitutively express S-HDAg and L-HDAg as well as virus genomic and antigenomic RNA [19]. To analyse the expression of HDV RNA and antigens in Huh7-D12 cells, the digoxigenin-labelled pSVL(D3) probe was used in *in situ* hybridization experiments. This probe hybridizes to both genomic and antigenomic virus RNA. Additionally, in the same experiments, the delta antigens were detected by immunofluorescence using a specific polyclonal antibody. Fig. 1 displays a typical example of the localization patterns in the nucleus of Huh7-D12 cells of both HDV RNA and proteins. The RNA is predominantly scattered throughout the nucleoplasm with accumulation in brighter foci which colocalize with the delta antigens. The latters can additionally be found in the nucleolus of some cells. Confirming previous findings, we also observed that only 10–15% of the cells, at a given time, express HDV RNPs (data not shown). Thus, the Huh7-D12 cell line constitutively expresses HDV RNPs.

3.2. *2-DE characterization of Huh7 and Huh7-D12 cells*

After establishing that the Huh7-D12 cell line expresses both HDV RNA and antigens, we proceeded to the comparative analysis of the proteome of human liver Huh7 cells and Huh7-D12 cells. To do this, we prepared total protein extracts and separated them by 2-DE. For the first dimension IEF, 200 μg of protein were loaded onto pH 3–11 NL IPG strips. The second dimension SDS PAGE was carried out on 9% polyacrilamide gels which were subsequently stained according to a Blue Silver staining protocol (see Fig. 2). Five gel replicas corresponding to five different cell culture protein samples were obtained for each of the Huh7 and Huh7-D12 cell lines. Spot detection and matching was performed using the Phoretix PG200 software to which gel images were previously uploaded. A total of 641±85 and 551±112 spots were detected on 2-DE gels for Huh7 cells and the Huh7-D12 cell line, respectively. Comparative analysis of Huh7 and Huh7-D12 gels showed that 24 proteins were differentially expressed by two fold or more ($p<0.05$). In Huh7-D12 cells 15 spots were found to correspond to down regulated proteins and 9 to over expressed proteins (Table 2).

3.3. *MS analysis and protein identification*

After separation by 2-DE the protein spots marked as differentially expressed were excised, digested with trypsin, and analysed by MALDI-TOF (typical examples of the obtained

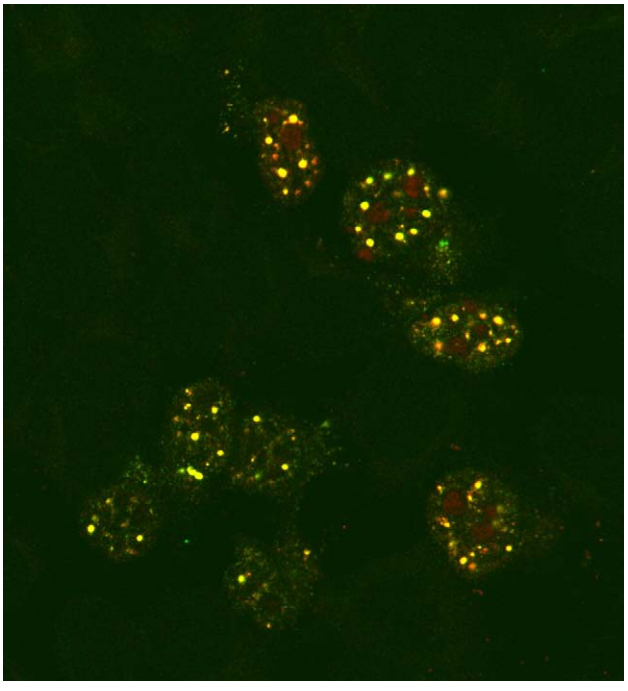

Fig. 1 – Visualization of HDV RNA and antigens in Huh7-D12 cells. Cells were first hybridized with plasmid pSVL(D3) (green) and immunolabeled with B3 antibody (red). Both delta RNA and antigens co-localize in bright foci (yellow). In some cells the delta antigens can additionally be detected in the nucleolus.

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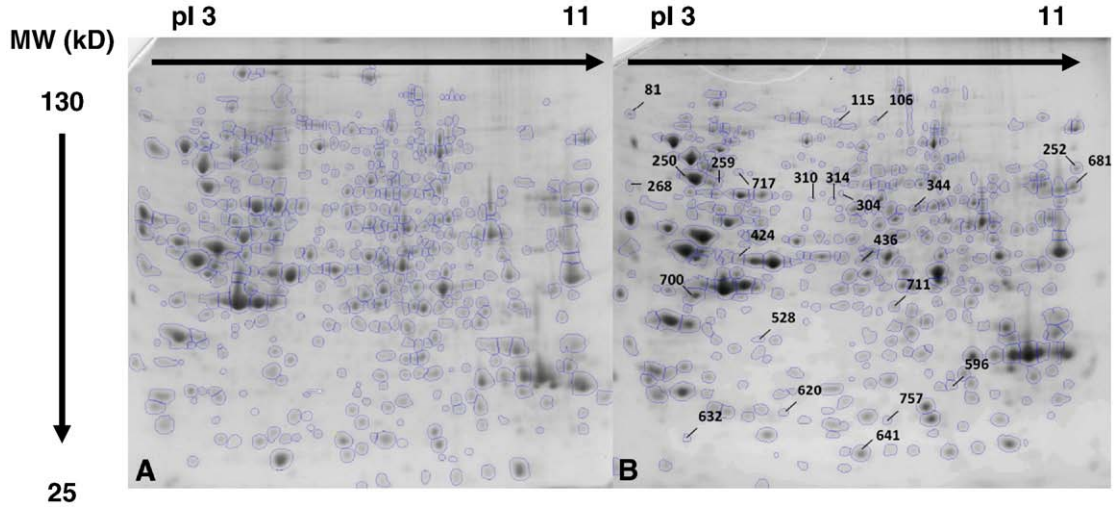

Fig. 2 – 2-DE analysis of proteins extracted from the Huh7 cell line (A) and from the Huh7-D12 cell line (B). Proteins were separated on pH 3–11 NL IPG strips followed by SDS-PAGE on a 9% polyacrilamide gel. The gels were stained using a Blue Silver staining protocol [24]. The identified proteins corresponding to each numbered spot are shown in Table 2

spectra are displayed on Fig. 3). Using a PMF strategy, we were able to identify 23 differentially expressed protein spots (Table 2) of which 15 corresponded to down regulated proteins and 8 to up regulated proteins in Huh7-D12 cells compared to Huh7 cells. Protein mixtures were not detected as a direct result from the protein sequence database or after a resubmission of the peptide masses not used for each protein identification. The non-matched subset of ions was re-evaluated for the presence of doubled missed cleavages using MASCOT. As a result, for 9 of the 23 identified proteins 2 miss cleavages were assigned during data base protein search. Additionally, using the FindMod software it was possible to predict the presence of already described post-translational modifications in 4 of the identified proteins: high mobility group box 1 [31], serum albumin [32], pyruvate carboxylase [33], and Sec1 family domain containing protein 1 [34,35].

Using the protein reference database (www.hprd.org) the identified proteins were grouped according to their biological function (Table 2). Most of these proteins were found to be involved in the regulation of the nucleic acid metabolism, regulation of cell growth and/or maintenance, and in energy pathways. Concerning cell metabolism and energy pathways, 4 proteins were found to be down regulated and 1 was up regulated in the Huh7-D12 cell line which expresses HDV RNPs. All 3 proteins involved in signal transduction and cell communication were down regulated in Huh7-D12 cells. In contrast, 3 out of 4 proteins involved in the regulation of nucleic acid metabolism were found to be over expressed in HDV RNPs expressing cells. Finally, in the group of proteins involved in cell growth and/or maintenance 3 were found to be up regulated and 3 were down regulated in Huh7-D12 cells when compared with the Huh7 cell line (see Table 2).

Following identification of the differentially expressed proteins we decided to perform an analysis of possible protein–protein interactions using the IntAct database and the Cytoscape platform. To construct the interaction network, the differentially expressed proteins were picked out followed by selection of the adjacent nodes. Overall, the network comprised 151 nodes and 379 edges (data not shown). This approach may provide additional information that is not detectable only by 2DE analysis. One of the observed predicted interactions concerns the triosephosphate isomerase, the histone H1 binding protein, and the polyadenylate binding protein which are down regulated in Huh7-D12 cells. The corresponding subnode is displayed on Fig. 4. These proteins are thought to interact through several protein kinases, one of which, the serine/threonine kinase SGK, is already described to be up regulated in human hepatocellular carcinomas [36]. Furthermore, it was reported that they can interact through the euchromatic histone methyltransferase SETDB1 which also interacts with the de novo DNA methyltransferases DNMT3A and DNMT3B promoting gene silencing in human cancers [37].

3.4. *Validation of proteomic results*

To validate proteomic results we choose the La protein, lamin C, histone H1-binding protein (NASP), and triosephosphate isomerase (TPI) since they are known to be involved in different metabolic pathways. Moreover, the TPI protein was previously found to be also down regulated as a consequence of transient expression of S-HDAg and L-HDAg, separately [21]. The analysis of the relative expression of the La protein and lamin C was performed by western blot. The internal housekeeping control in these experiments was clathrin. Total Huh7 and Huh7-D12 protein extracts were prepared and separated on 12% SDS-PAGE gels. The obtained blot images were analysed with the Phoretix PG200 software to determine the relative amounts of the proteins in each sample. The obtained results showed that the expression of the La protein and lamin C was 1,8 and 2,1 higher in Huh7-D12 cells (Fig. 5). These results are consistent with those obtained by analysis of the 2D proteome map where the expression of the La protein and lamin C was found to be 2.5 and 2.0 times higher in cells expressing HDV RNPs (Table 2).

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Table 2 – Proteins differentially expressed in Huh7-D12 cells when compared with the Huh7cell line

	SwissProt accession number	Protein	Differential expression	Spot	Norm. vol. (SD) Huh7	Norm. vol. (SD) Huh7-D12	Biological function
Huh7-D12	Q59H57	Fusion (involved in t(12;16) in malignant liposarcoma) isoform a variant [fragment]	−2.2	681	1.082 (0.420)	0.497 (0.175)	–
	Q16643	Drebrin 1	−2.4	81	1.082 (0.420)	0.497 (0.175)	Cell growth and/or maintenance
	P05787	Keratin 8	+2.0	424	0.119 (0.051)	0.246 (0.106)	Cell growth and/or maintenance
	P08727	Keratin, type I cytoskeletal 19	+3.6	700	0.220 (0.057)	0.804 (0.072)	Cell growth and/or maintenance
	Q5TCJ3	Lamin C	+2.0	344	0.085 (0.024)	0.173 (0.064)	Cell growth and/or maintenance
	Q6IBM5	Rho protein GDP-dissociation inhibior 1	−2.3	632	0.080 (0.029)	0.034 (0.016)	Cell growth and/or maintenance
	P18206	Vinculin	−2.6	115	0.129 (0.037)	0.050 (0.026)	Cell growth and/or maintenance
	Q9BWD1	Acetyl-CoA C-acetyltransferase	−2.8	711	0.287 (0.096)	0.104 (0.028)	Metabolism; Energy pathways
	P13798	Acylaminoacyl-peptidase	−3.1	717	0.073 (0.033)	0.023 (0.013)	Metabolism; Energy pathways
	Q13162	Probable Thiol-specific reductase/Peroxiredoxin 4	+3.2	620	0.036 (0.019)	0.113 (0.050)	Metabolism; Energy pathways
	P11498	Pyruvate carboxylase	−3.3	106	0.152 (0.052)	0.046 (0.014)	Metabolism; Energy pathways
	P60174	Triosephosphate isomerase	−3.2	757	0.104 (0.039)	0.033 (0.009)	Metabolism; Energy pathways
	P11021	GRP78/BiP	+2.1	250	1.086 (0.177)	2.231 (0.556)	Protein metabolism
	Q86U42	Polyadenylate binding protein II	−2.1	252	0.366 (0.128)	0.174 (0.057)	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
	Q5BKZ1	Zinc finger protein 326	+4.5	259	0.019 (0.014)	0.085 (0.049)	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
	Q53XJ4	Ribonuclear protein La	+2.5	436	0.045 (0.018)	0.111 (0.015)	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
	P09429	High mobility group box 1 (fragment)	+2.7	641	0.153 (0.059)	0.414 (0.078)	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
	Q5T626	Histone H1-binding protein	−2.5	268	0.108 (0.026)	0.044 (0.024)	Signal transduction; Cell communication
	P62873	Guanine nucleotide binding protein	−2.7	528	0.084 (0.026)	0.031 (0.016)	Signal transduction; Cell communication
	P63244	Guanine nucleotide-binding protein beta subunit 2-like 1	−2.8	596	0.118 (0.056)	0.043 (0.028)	Signal transduction; Cell communication
	Q8WVM8	Sec1 family domain-containing protein 1	−3.3	314	0.125 (0.033)	0.039 (0.016)	Transport
	P02768	Serum albumin	−2.4	304	0.081 (0.021)	0.034 (0.026)	Transport
	P02768	Serum albumin, chain A	−2.3	310	0.075 (0.026)	0.033 (0.016)	Transport

Score: The minimum MASCOT score for a probability less than 5% for the match to be a random event is 64.
Pep: Number of peptides submitted to search in protein databases/Number of peptides from experimental Peptide Mass Fingerprint whose masses match those from theoretical PMF determined from a known sequence.
Norm. vol.: Normalized spot volume.
SD: Standard deviation.
Sequence coverage: percentage of identified protein sequences covered by matched peptides.
[a] Calculated values using the PG200 software.
[b] Theoretical values.

To investigate the relative expression levels of NASP and TPI by Real time PCR, mRNA was extracted and used as template for further cDNA synthesis. The reference protein in these experiments was β-2-microglobulin. The obtained results are displayed in Fig. 6 and show that compared to Huh7 cells, the mRNA levels of the histone H1-binding protein and TPI are significantly lower in Huh7-D12 cells ($p<0.05$). The NASP mRNA expression level was 2.8 times lower in Huh7-D12 cells, a result consistent with the data obtained from proteome analysis, −2.5 times (Table 2). The relative quantification of the TPI mRNA level, however, showed that it was 108 times lower in HDV RNPs expressing cells. This result, however, is not

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Table 2 (*continued*)

MW (Da) [a]	pI [a]	MW (Da) [b]	pI [b]	Score	Pep	Sequence Coverage	p value
–	–	32,316	9.6	128	12/09	29	0.015
71,707	4.4	71,707	4.4	89	10/07	14	0.013
58,904	5.4	41,083	4.9	65	16/06	27	0.025
45,870	5.2	45,870	5.2	204	25/15	43	$2.58.10^{-8}$
57,575	6.8	65,153	6.4	239	28/21	41	0.01
–	–	23,119	5.0	73	11/05	27	0.012
124,161	5.51	124,161	5.5	90	27/14	13	0.002
41,783	6.3	41,783	6.3	70	08/05	17	0.007
82,210	5.3	82,210	5.3	100	10/08	12	0.012
30,759	5.9	30,749	5.9	108	35/10	40	0.01
130,309	6.4	130,309	6.4	212	29/22	24	0.004
26,807	6.5	26,807	6.5	120	14/08	39	0.037
72,185	5.03	72,185	5.0	226	29/21	33	0.003
58,709	9.3	58,709	9.3	221	28/21	37	0.028
74,634	5.3	65,654	5.1	77	07/05	17	0.019
64,538	6.2	46,979	6.7	99	20/10	25	$2.30\ 10^{-4}$
22,207	9.6	22,207	9.6	125	11/09	35	$1.31\ 10^{-4}$
–	–	49,003	4.4	113	17/10	23	0.003
39,684	5.58	38,020	5.6	93	21/08	29	0.004
–	–	35,380	7.6	66	18/06	23	0.046
69,949	5.7	72,704	5.9	83	10/07	15	$1.85\ 10^{-4}$
67,690	5.6	58,513	6.0	124	11/09	17	0.007
–	–	67,690	5.6	85	21/09	18	0.012

coincident with that obtained with the proteome analysis where the TPI protein was found to be less under expressed in Huh7-D12 cells, –3,2 times (Table 2). This discrepancy may be due to differences in the stability of this mRNA or in the efficiency of translation. Overall, these results are consistent with the proteome analysis.

4. Discussion

HDV is the smallest human pathogen known so far. Despite its simplicity, there is still a considerable lack of information concerning the host factors involved in HDV replication and

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pathogenesis. One of the main constraints in studying the biology of HDV infection is the lack of a robust cell culture system capable of supporting virus replication. The recently developed human embryonic kidney cell line (HEK293) expressing HDV RNA and antigens under tetracycline control [38], could represent an important tool to study a broad range of mechanisms involved in virus replication. However, this model may pose a number of constraints since HEK293 cells were previously reported to express neuronal specific antigens [39]. In this context, the human liver carcinoma cell line stably transfected with HDV cDNA (Huh7-D12) represents a well characterized and previously used system to investigate molecular and cellular mechanisms of HDV replication. This cell line was shown to constitutively express delta antigens and replicate virus RNA [18,19]. Moreover, the molar ratio between virus RNA and antigens in this cell line was also found to be similar to that observed in HDV particles of experimentally infected woodchucks [20]. To identify the changes in host cells protein expression that arise as a consequence of HDV replication, we performed a comparative analysis of the proteome of Huh7-D12 cells and of the corresponding non-transfected Huh7 cell line. Total cellular extracts were separated by 2-DE and differentially expressed proteins were identified by PMF. Overall, 24 proteins were found to be differentially expressed between the two cell lines. We were able to identify 23 of them, 15 of which were down regulated and 8 were up regulated in Huh7-D12 cells. These proteins could all be assigned to functional categories, namely the regulation of the cell metabolism and energy pathways, transport, regulation of protein metabolism, regulation of nucleic acid metabolism, signal transduction, and cell growth and maintenance.

Interestingly, 6 of the herein identified proteins were previously found to be differentially expressed in Huh7 cells transiently expressing HDV RNA or antigens separately [21]: zinc finger protein 326 in S-HDAg expressing cells, keratin type 1 cytoskeletal 19 in L-HDAg expressing cells, triosephosphate

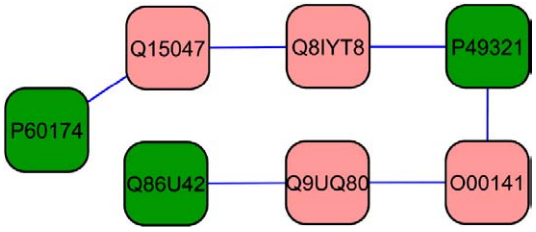

Fig. 4 – A sub network of protein–protein interactions observed in the Cytoscape platform. The IntAct database [27] was imported in the Cytoscape platform [28] and the predicted protein–protein interactions of the differentially expressed proteins were analyzed. This sub network includes TPI (P60174), histone H1-binding protein (P49321), and PABP (Q86U42). These proteins are down regulated in the presence of HDV RNPs.

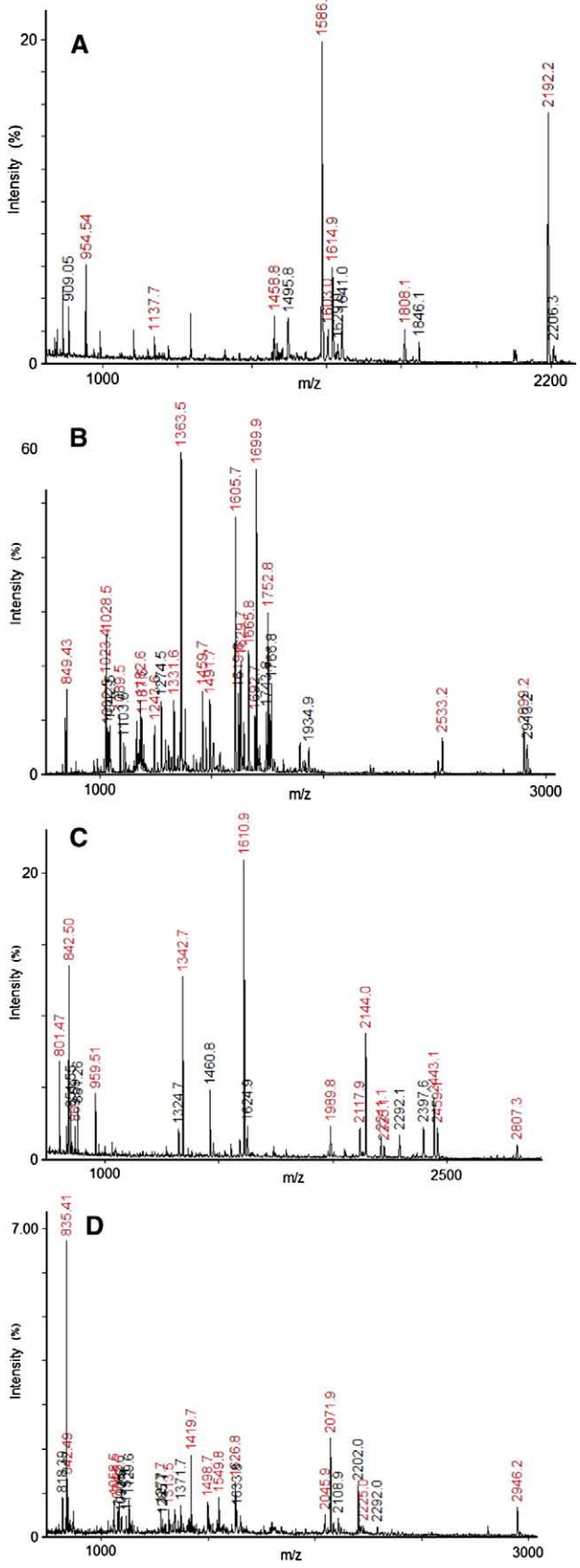

Fig. 3 – MALDI-TOF spectra obtained for the tryptic digest of triosephosphate isomerase (A), lamin C (B), histone H1 binding protein (C), and ribonuclear protein La (D), in spots 757, 344, 269, and 436, respectively. The peptide monoisotopic masses labeled in the spectra were used for search in a protein sequence database. The peptide masses coloured in red match the masses of peptides used for protein identification.

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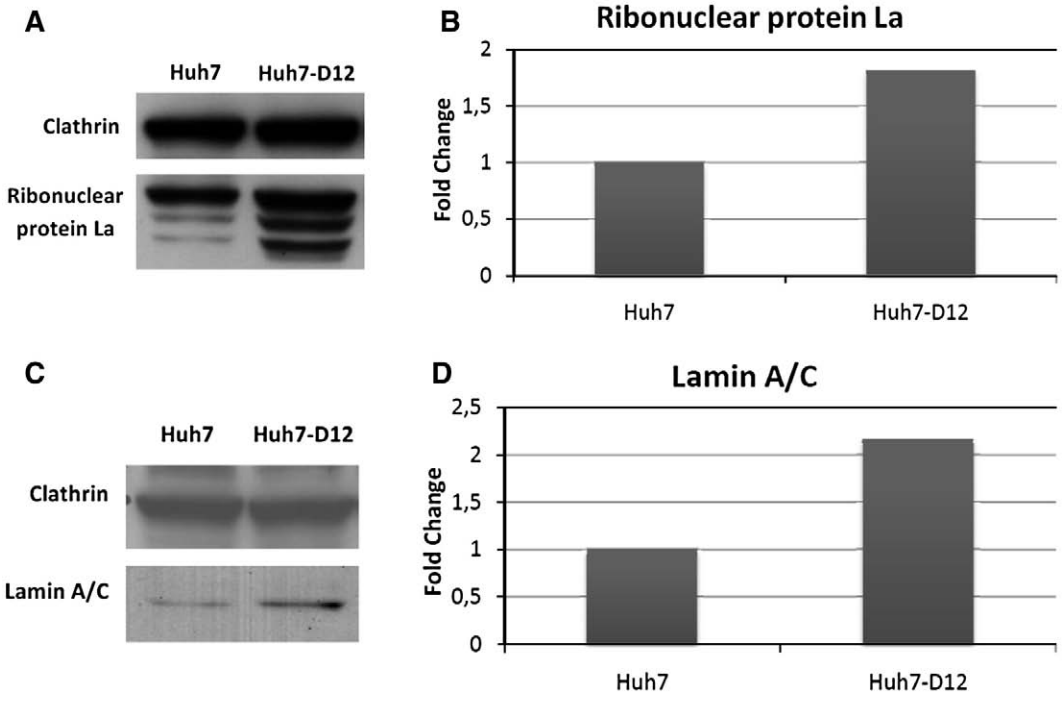

Fig. 5 – Western blot analysis of lamin C and La proteins in Huh7 and Huh7-D12 cells. Total cellular extracts were separated in 12% SDS-PAGE gels, electroblotted onto a nitrocellulose filter, and incubated with anti-La and anti-clathrin antibodies (A), and anti-lamin C and anti-clathrin antibodies (C) Images were analysed using the Phoretix PG200 software. Relative expression levels were obtained after normalization of lamin C and La spot volumes, respectively, against the housekeeping clathrin protein (B and D).

isomerase in both S-HDAg and L-HDAg expressing cells, and keratin 8, peroxiredoxin 4, and ribonuclear protein La in Huh7 cells expressing HDV antigenomic RNA. Moreover, with the exception of the ribonuclear protein La, the remaining 5 identified proteins displayed similar differences of expression levels in Huh7-D12 cells or transiently transfected Huh7 cells when compared with Huh7 cells alone. The observation that these proteins are differentially regulated as a consequence of expression of HDV RNPs or some of its components separately, may represent an additional evidence of a possible involvement in virus pathogenesis as further discussed below. In contrast, 17 proteins found to be differentially regulated in this study, were not reported in a previous work based on transient expression experiments of HDV RNAs and antigens, separately [21]. This may be due to the fact that expression of whole HDV RNPs may lead, at least partially, to the induction of a different set of changes in host protein synthesis than that observed when the different HDV components were expressed

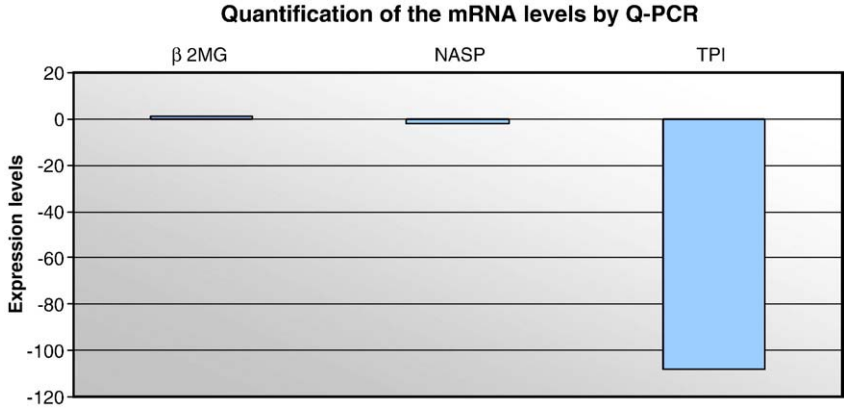

Fig. 6 – Real-time PCR analysis of the histone H1 binding protein (NASP) and triosephosphate isomerase (TPI) in Huh7 and Huh7-D12 cells. The vertical axis represents the fold change in relative gene expression levels: 2.8 fold for NASP and 108 fold for TPI. The β-2-microglobulin gene (β2MG) was used as an internal housekeeping control. Fold changes were determined as $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$ ($p < 0.05$).

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separately. In fact, it is well established that, for instance, S-HDAg and L-HDAg play distinct, and sometimes opposite roles during the virus replication cycle. Additionally, it is known that HDV RNA shares a number of secondary structure features with the RNA of plant viroids. Although speculative, it is possible that HDV RNA is also involved in the induction of gene silencing processes, a hallmark of several human neoplasias. RNA silencing in plants was reported to be a common feature during viroid infection [40].

HDV infection is associated with increased risk of cirrhosis and fulminant disease [1,2]. However, the detailed mechanisms of pathogenesis are still unclear. The ubiquitous nuclear high mobility group box-1 protein (HMGB 1) was one of the proteins found to be over expressed in Huh7-D12 cells. Over expression of HMGB 1 is associated with the proliferation and metastasis of various cancer types [41]. Furthermore, HMGB 1 was previously reported to be released by necrotic cells and promote neutrophilic hepatitis and tissue damage [42].

GRP78/BiP was also found to be over expressed in the presence of HDV RNPs. GRP78/BiP expression is known to be altered in response to several stress stimuli [43,44], including hepatitis C virus (HCV) E2 protein synthesis [44]. It was speculated that triggering of GRP78/BiP expression may be mediated by the formation of E2 protein aggregates during the folding process. HDAg aggregates are also detected in the nucleus of Huh7-D12 cells [19] and this could account for the observed stimulation of GRP78/BiP expression.

The down regulation of triosephosphate isomerase (TPI) was found, and confirmed by Real time PCR, in Huh7-D12 cells. This enzyme plays a role in the glycolysis pathway which is interconnected to lipid metabolism via triosephosphates [45]. Down regulation of TPI expression was also found in Huh7 cells bearing a hepatitis C virus replicon [46]. Furthermore, we observed that pyruvate carboxylase is also down regulated in Huh7-D12 cells. A lower availability of triosephosphate isomerase and pyruvate carboxylase may lead to an abnormal retention of lipids, a cellular pathology denominated steatosis. Microvesicular steatosis was reported to be an hallmark of fulminant hepatitis cases in Amazonian Indians [47]. However, further investigation is needed to clarify the putative role of these enzymes in the pathogenesis of HDV infected liver cells. In fact, the observed changes may not reflect a specific influence of HDV expression in host cells since both GRP78/BiP and TPI are among the most commonly found differentially expressed proteins in similar proteomic studies [48].

When performing a computational analysis of protein interactions we found a subnode involving NASP, TPI, and PABP2, all down regulated in Huh7-D12 cells. It was previously reported that NASP, TPI, and PABP2 can interact through the euchromatic histone methyltransferase SETDB1 which also interacts with de novo DNA methyltransferases DNMT3A and DNMT3B promoting gene silencing in human cancers [41]. Additionally, the proliferation associated protein 2G4, found in this sub network, is thought to be a RNA binding protein involved in the regulation of cellular growth [49]. This protein is associated with ribosomes and inhibits phosphorylation of the eukaryotic initiation factor 2a (eIF2a) [50]. The later was also found to be phosphorylated in response to several types of stress, namely by PKR. Interestingly, PKR was earlier found to be one of the cellular proteins that binds to viral RNA and to phosphorylate the S-HDAg [51]. Moreover, the proliferation associated protein 2G4 can also interact with the nucleolar phosphoprotein B23 [52], and this complex plays a critical role in cell proliferation and survival. The nucleolar phosphoprotein B23 was earlier described as one of the cellular proteins that interacts with HDAgs [53].

Up regulation of cytokeratins and down regulation of vinculin were detected in Huh7-D12 cells indicating a possible deregulation of cytoskeleton dynamics in these cells. However, the specific significance of these findings needs to be further investigated since keratins and vinculin were also reported to be among the most often identified proteins in 2DE-based proteomic studies [48].

Additionally, the over expression of lamin C, which belongs to the intermediate filament family of proteins was also observed in HDV expressing cells. This finding was validated by western blot. Interestingly, it was earlier reported that autoantibodies against lamin C were specifically associated with chronic hepatitis D [54]. However, the clinical and pathological relevance of this finding is unclear.

In an early proteome analysis of Huh7 cells transiently expressing S-HDAg, it was found that both PCNA and the Fen 1 endonuclease, two proteins involved in DNA replication, were down regulated [21]. Consistent with these data, we additionally report the down regulation of the NASP protein, a result that was confirmed by Real time PCR. This protein is thought to be involved in histone transport into nuclei and exchange with DNA, and is expressed in proliferating cells during S phase [55].

Replication of HDV RNA is thought to involve the redirection of cellular DNA-dependent RNA polymerase II [56]. The precise mechanism by which this enzyme recognizes a RNA template as well as the transcription factors involved in this process are still unknown. However, there is evidence of the possible involvement of a second cellular RNA polymerase, resistant to high doses of α-amanitin, in the synthesis of antigenomic RNA molecules [57]. In this context, the observation, confirmed by western blot, of the over expression of the La protein, known to bind to 3' UUU-OH tails of nascent RNA polymerase III transcripts [58], may support this view.

Poly(A)-binding proteins (PABPs) are a family of proteins that binds to poly(A) RNA and play multiple roles in the RNA metabolism. PABPs were shown to be cleaved during viral infections, namely enterovirus, and apoptosis [59]. In HDV stably expressing Huh7-D12 cells, PABP2 was found to be down regulated. This is consistent with the finding that HDV expressing cells display distinct cell growth and cell cycle parameters [38]. Ultimately, this deregulation of cell cycle control leads to apoptosis [38]. Apoptosis is a common event detected in several types of virus infected cells. Particularly, in chronic and acute liver diseases, persistent and massive apoptosis, respectively, are frequent observed features [60].

Finally, the Rho GDP dissociation inhibitor (GDI) and the guanine nucleotide-binding proteins, involved in signal transduction pathways, were found to be down regulated in Huh7-D12 cells. These proteins can regulate mitogen-activated protein kinase (MAPK) networks [61]. The use of signal transduction inhibitors has recently become an important therapeutic option for cancer treatment [62]. However, there is still a need for identification of aberrant signalling pathways

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characteristic of the different tumour types. The further detailed characterization of the alterations in signal transduction pathways that arise in HDV infected cells may provide new therapeutic alternatives.

In conclusion, this study provides evidence for a number of alterations in host protein synthesis that occur during expression of HDV RNPs. In some cases, namely lamin C and the enzymes involved in lipid metabolism, the detected protein expression and metabolic alterations seem to correlate with previous pathologic findings observed in both chronic and acute HDV patients, respectively. Furthermore, the observed changes are indicative of an overall deregulation of DNA replication and cell cycle control in liver cells, which may possibly lead to apoptosis and tissue damage.

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