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Proteomic analysis of human hepatoma cells expressing methionine adenosyltransferase I/III[☆]:

Characterization of DDX3X as a target of S-adenosylmethionine

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Abstract

Methionine adenosyltransferase I/III (MATI/III) synthesizes S-adenosylmethionine (SAM) in quiescent hepatocytes. Its activity is compromised in most liver diseases including liver cancer. Since SAM is a driver of hepatocytes fate we have studied the effect of re-expressing *MAT1A* in hepatoma Huh7 cells using proteomics. *MAT1A* expression leads to SAM levels close to those found in quiescent hepatocytes and induced apoptosis. Normalization of intracellular SAM induced alteration of 128 proteins identified by 2D-DIGE and gel-free methods, accounting for deregulation of central cellular functions including apoptosis, cell proliferation and survival. Human Dead-box protein 3 (DDX3X), a RNA helicase regulating RNA splicing, export, transcription and translation was down-regulated upon *MAT1A* expression. Our data support the regulation of DDX3X levels by SAM in a concentration and time dependent manner. Consistently, DDX3X arises as a primary target of SAM and a principal intermediate of its antitumoral effect. Based on the parallelism between SAM and DDX3X along the progression of liver disorders, and the results reported here, it is tempting to suggest that reduced SAM in the liver may lead to DDX3X up-regulation contributing to the pathogenic process and that replenishment of SAM might prove to have beneficial effects, at least in part by reducing DDX3X levels. This article is part of a Special Issue entitled: Proteomics: The clinical link.

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Keywords

S-adenosylmethionine; Hepatocellular carcinoma; DDX3X; 2D-DIGE; Mass spectrometry; Proteomics

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with a global annual incidence of close to 1 million cases [1] and an estimated 600,000 deaths per year [2]. Major causes of HCC are currently known, including infection with hepatitis B (HBV) and C (HCV) viruses, ingestion of aflatoxin contaminated food and alcohol abuse [3]. Other less prevalent risk factors have also been described such as iron or copper deposition or non-alcoholic steatohepatitis (NASH) [4]. Although the identification of the main risk factors and the routine screening of population at risk may lead to the early diagnosis of HCC, the prognosis of the affected patients is poor mainly due to the aggressiveness of the lesions at the time when the disease is detected and also to the lack of effective therapies. Therefore, a detailed definition of the mechanisms associated with the neoplastic transformation of hepatocytes, leading to the identification of molecular targets for early detection and treatment, are of great interest and one of the priorities of clinical hepatology.

Since its discovery in 1953 [5] an increasing number of studies provide evidences supporting the central function of S-adenosylmethionine (SAM) in cell biology. Along with ATP, SAM is one of the metabolites most frequently used in intermediary metabolism as the main alkylating agent in living cells that participates in the methylation of a plethora of biomolecules through the transference of its methyl group to acceptors in reactions catalyzed by methyltransferases. Additionally, SAM is involved in the biosynthesis of different metabolites such as polyamines, biotin, and, in the liver, it is an intermediary metabolite in the synthesis of cysteine and glutathione [6–8]. Methionine adenosyltransferase (MAT) catalyzes the only known SAM biosynthetic reaction using methionine and ATP as substrates [5,9]. In mammalian cells three distinct MAT isoforms are known [10–13], which are distributed in a tissue specific manner [14]. MATI/III, products of gene *MAT1A*, predominate in adult liver [15] while MATII, encoded by gene *MAT2A* is common to the rest of the tissues, as well as fetal liver, but is progressively replaced by MATI/III during development [16,17]. Although all three MAT forms share 85% of the aminoacidic sequence [18,19], they differ in their kinetic and regulatory properties [14]. While MATI/III are activated by the substrates methionine and ATP [20], MATII is inhibited by the product of the reaction, SAM [17]. Consistently, cells expressing *MAT1A* have higher SAM levels than cells expressing *MAT2A* [21]. SAM levels must be precisely tuned in the liver to preserve its normal function; a decrease or an increment of SAM content promotes loss of liver function and development of liver diseases including HCC [22,23]. Data have been accumulated indicating that expression of *MAT1A* and normal MATI/III activity can be considered as markers of normal differentiated liver. Inactivation of MATI/III occurs in hepatitis, cirrhosis as well as in many animal models of liver injury [9,24,25]. The central role of SAM in hepatocytes and its implication in the pathogenesis of liver disorders is

further underlined by its protective effect to liver insults [9,25], its capacity to increase survival of patients with alcoholic liver cirrhosis [26] and its preventive effect to HCC onset in rodents [27]. Cellular content of SAM seems to be related to the differentiation status of the hepatocyte [14]. In this regard, quiescent hepatocytes display higher SAM content than proliferating hepatocytes [14] resulting from a switch in gene expression from *MAT1A* to *MAT2A* [21,28–31]. Reduced intracellular SAM might represent an advantage for malignant degeneration of the liver by preventing apoptosis [32], favouring angiogenesis in cancer cells [27] and promoting proliferation [14,31]. Consistent with this idea, overexpression of *MAT1A* in liver cancer cells reduces tumorigenesis both, in vitro and in vivo, according to mechanisms explored by transcriptomic analyses [28,33]. To further understand the mechanisms by which restoring SAM concentration in human HCC Huh7 cells to values close to normal hepatocytes might interfere with the transformed phenotype, we have performed a comprehensive proteomic analysis combining 2D-DIGE and gel free approaches. Our findings suggest that the RNA related protein DDX3X is a pivotal intermediate that is a primary target of SAM.

2. Materials and methods

2.1. Construction of *MAT1A* expression vector

Human *MAT1A* full-length cDNA (NM_000429) was cloned according to the manufacturer's instructions into vector pCR-II-TOPO (Invitrogen) by using the TOPOTA Cloning Kit (Invitrogen). Forward 5'-ATGAATGGACCGGTGGATGGC-3' and reverse primers 5'-CTAAAATACAAGCTTCCTGGGAAC-3' were used for PCR amplification. Hygromycin resistance gene from pSwitch (Invitrogen) was obtained from digestion with *Sma*I and *Nae*I and cloned into *Sma*I pIRES (Clontech) plasmid obtaining pIRES-Hyg. *MAT1A* was obtained from pCR-II-TOPO-MAT1A by digestion with *Spe*I and *Xho*I subsequently cloned into pIRES-Hyg on *Nhe*I-*Xho*I sites obtaining expression vector pMAT1A-Hyg. EGFP gene from pEGFP (Clontech) was digested with *Xba*I and cloned into *Nhe*I site of pIRES-Hyg obtaining pEGFP-Hyg. Sequence analysis and restriction digest was performed to detect and confirm orientation. The coding sequence of pMAT1A vector is shown in Supplemental Fig. 1.

2.2. Transfection of Huh7 cells with *MAT1A* expression vector

Huh7 cells were obtained from JCRB Genebank Japan, HepG2 and Hep3B from ATCC, all cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% L-glutamine/penicillin/streptomycin. Transient transfections were performed according to Lipofectamine 2000 (Invitrogen) manufacturer's guidelines and standard techniques. Huh7 cells (3.75×10^6) were plated in 10-cm Petri dishes overnight. Then, 24 μ g of either *MAT1A* or EGFP control vector and 60 μ l of Lipofectamine 2000 (Invitrogen) were suspended in 1.5 ml of Opti-MEMI, mixed, and added to Huh7 cells. The medium was changed after 6 h. Confirmation of successful transfection was achieved by documenting the presence of EGFP protein by fluorescence and MAT protein as described below. Only cells showing more than 90% transfection were used for further analysis (Supplemental Fig. 1).

2.3. Western blot analysis

Western blotting was performed following standard protocols (Amersham) using primary polyclonal rabbit anti-human MAT1A antibody (1:10000) from our lab. Antibodies for active caspase 3, p-ERK, p-AKT, p-NF- κ B p65, p-C-RAF Ser338, p-C-RAF Ser259, total ERK, total AKT, total NF- κ B p65, total C-RAF and DDX3X were purchased from Cell Signaling Technology. Antibodies KDEL and β -ACTIN were from Abcam, H4 from Upstate and PI3K p110 from Santa Cruz Biotechnology. Equal amounts of protein (15 μ g) were resolved in 12.5% SDS-PAGE gels. Proteins were electrophoretically transferred onto nitrocellulose membranes for 45 min at 120 V. Membranes were probed with primary antibodies at 1:1000 dilution in 5% BSA or 5% non-fat milk, depending on the primary antibody used. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000), the immunoreactivity was visualized by enhanced chemiluminescence (Perkin Elmer). Equal loading of the gels was assessed by Ponceau staining and hybridization with a β -actin specific antibody (Abcam).

2.4. Determination of SAM levels

SAM levels from Huh7, HepG2 and Hep3B cells were measured by high performance liquid chromatography as described by Perez-Mato et al. [34]. Cells were washed twice in DPBS and centrifuged at 1500 rpm at 4 °C and 5 min. Pellets were homogenized in 250 μ l of perchloric acid 0.4 M and centrifuged at 10,000 $\times$ g for 15 min at 4 °C. Supernatants (40 μ l) were loaded onto a Spherisorb ODS-2 column (particle size 5 μ , 25 $\times$ 0.46 cm; Waters) equilibrated with mobile phase A (10 mM ammonium formate, 4 mM heptanesulfonic acid, pH 4.0). Mobile phase B consisted of 50% HPLC grade acetonitrile (Merck). Elution was carried out with a 21-min linear gradient of acetonitrile (0–12.5%) at a constant flow rate of 1 ml/min, and the UV absorbance was monitored at 254 nm. Chromatograms were analyzed using UNICORN-^{TM} (GE Healthcare) software and verified using SAM standards of known concentrations.

2.5. 2D-DIGE, imaging, and mass spectrometry

Cells were washed three times with ice cold PBS. Subcellular fractionation was performed using the Qproteome Cell Compartment Kit from Qiagen. After acetone precipitation, protein samples were solubilized in 2-D DIGE sample buffer: 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris, buffered to pH 8. Protein concentration was determined using the Bradford's assay (Bio-Rad). Then 50 μ g protein were labelled with 400 pmol of CyDye DIGE Fluor minimal dyes (GE Healthcare) and incubated on ice in the dark for 30 min according to the manufacturer's instructions (Cy3, Cy5 for samples and Cy2 for internal control consisting of a mixture composed by equal amounts of protein from all samples). Paired samples were reverse-labelled in order to prevent potential dye labelling bias. The reaction was stopped by addition of 1 μ l of 10 mM lysine and incubated on ice for 10 min. Samples were cup-loaded onto IPG strips, 24 cm, pH 3–11NL (GE Healthcare), and subjected to isoelectric focusing (IEF) in IPGphor^{TM} IEF System (GE Healthcare) according to the manufacturer's recommendations. Upon IEF, strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue), containing

0.5% DTT for 15 min and thereafter in the same buffer with 4.5% iodoacetamide for 15 min. For the second dimension, strips were loaded on top of 12.5% polyacrylamide gels (21×24 cm) and run (1 W/gel) for 12–14 h until the bromophenol blue dye reached the gel bottom-end. Subsequently, 2-D gels were scanned using a Typhoon™ Trio Imager (GE Healthcare) at 100 µm resolution with $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5 respectively. The photomultiplier tube was set to ensure that the maximum pixel intensity was between 90,000 and 99,000 pixels. Image analysis was performed using DeCyder 6.5 software (GE Healthcare) as described in the user's manual. Three independent experiments were performed. Briefly, the differential in-gel analysis (DIA) module was used for spot detection, spot volume quantification and volume ratio normalization of different samples in the same gel. Then the Biological Variation Analysis (BVA) module was used to match protein spots among different gels and to identify protein spots that exhibit significant differences. Manual editing was performed in the BVA module to ensure that spots were correctly matched between different gels, and to get rid of streaks and speckles. Differentially expressed spots were considered for MS analysis when the fold change was larger than 1.2 and the *p*-value after *t*-test was below 0.05. Spots differentially represented were excised manually and gel specimens were processed with a MassPrep station (Waters) as described elsewhere [35]. In-gel tryptic digestion was performed with 12.5 ng/µl trypsin in 50 mM ammonium bicarbonate for 12 h at 37 °C. The resulting peptides were extracted with 5% formic acid (FA), 50% acetonitrile (ACN). Samples were then concentrated in a speed-vac before MS analysis. NanoLC-ESI-MS/MS analysis was performed as described previously [36]. Data processing was performed with MassLynx 4.0. Database searching was done with two independent search engines: Phenix 2.6 (GeneBio) and Mascot Server 2.2 (Matrix Science) against UniprotKB/Swiss-Prot Release 51.6 with 257964 entries (human taxonomy: 15720 entries). Parameters used in Mascot searches were: enzyme, trypsin; variable modifications, carbamidomethylation of cysteine and oxidation of methionine; maximum missed cleavages, 1; peptide mass tolerance settings/windows was 50 ppm; product mass tolerance, 0.1 Da. Probability *p* of random matches was set to the default value of 0.05.

2.6. Label free LC-MS/MS

Peptides were separated by reverse phase chromatography using a Dionex Ultimate 3000 MDLC pump fitted with a 75 µm ID column (Acclaim PepMap100 C18, 2 µm 100A), samples were first loaded for desalting and concentration into a 2 cm length 100 µm ID precolumn packed with the same chemistry as the separating column. The mobile phases were 100% water 0.1% formic acid (buffer A) and 100% Acetonitrile 0.1% formic acid (buffer B). Column was developed in a 90 min two step gradient from 5% B to 40% B in 75 min and 40% B to 70% B in 15 min. Column was equilibrated in 95% B for 5 min and 5% B for 20 min. During all process precolumn is in line with column and flow is maintained all along the gradient at 300 nl/min. Eluting peptides from the column were analyzed using an AB Sciex 5600 TripleTOF™ system. Information data acquisition was acquired upon a survey scan performed in a mass range from 350 *m/z* up to 1250 *m/z* in a scan time of 250 ms. Top 20 peaks were selected for fragmentation. Minimum accumulation time for MS/MS was set to 50 ms giving a total cycle time of 1250 ms. Product ions were scanned in a mass range from 100 *m/z* up to 1500 *m/z* and excluded for further fragmentation during 15 s.

After MS/MS analysis, data files were processed using ProteinPilot™ 4.0 software from AB Sciex which uses the algorithm Paragon™ for database search and Progroup™ for data grouping [37] and searched against IPI database. False discovery rate was performed using a non linear fitting method [38] and displayed results were those reporting a 1% Global false discovery rate or better. Data were analyzed using three technical replicas from each sample. Peaks list were generated in PeakView™ 1.1 Software from AbSciex, using the combined database search results generated in ProteinPilot™ 4.0 software. The peak list matrix generated, was exported to MarkerView™ 1.2.1 software for Principal Component Analysis (PCA). Sample comparison was performed using the first two components that explained a total of 75% of the variance between samples. Sample dispersion was measured using a *t*-test and proteins with extreme *t* values were chosen as candidates for validation.

2.7. Bioinformatic functional analysis

The biological knowledge extraction was carried out through the use of Ingenuity Pathway Analysis (Ingenuity Systems, version 11631407, www.ingenuity.com), which database includes manually curated and fully traceable data derived from literature sources. Access codes for differential proteins from both, 2D-DIGE and label free LC-MS/MS analyses were uploaded.

2.8. RNA isolation, RT-PCR and real-time PCR

Total RNA was extracted using TRI reagent (Sigma). RNA was treated (2 µg) with DNaseI (Invitrogen) prior to reverse transcription with M-MLV Reverse Transcriptase (Invitrogen) in the presence of RNaseOUT (Invitrogen). Primers for DDX3X amplification were: forward 3'-ACTGCAGCATTCTGTGTC and reverse 3'-TAAACCACGCAAGGACGAAC. Real-time PCR was performed with 1/20 of the PCR reaction using an iCycler (BioRad) and the iQ SYBR Green Supermix (BioRad). mRNA levels were normalized according to GAPDH quantitation in the same sample. To monitor the specificity, final PCR products were analyzed by melting curves and electrophoresis. The amount of transcripts was calculated and expressed as the relative difference to the control gene GAPDH (2^{-Ct} , where *Ct* represents the difference in threshold cycles between the target and control genes).

3. Results

3.1. Functional characterisation of Huh7 cells expressing MAT1A gene

To analyse the effects of increasing intracellular SAM concentration in human hepatoma cells, Huh7 cells were transiently transfected with the plasmid pMAT1 expressing *MAT1A* gene. Both, MATI/III and SAM accumulated in a time dependent manner with respect to control cells that were transfected with the same vector expressing EGFP (pEGFP), expressing basal levels of this enzyme. MAT and SAM levels increased 3.5 and 7.5 fold at 24 and 48 h post-transfection respectively (Fig. 1 panels A and B respectively). Interestingly, SAM increased in pMAT1A transfected cells to reach values close to those measured in normal hepatocytes [39], condition that elicit deleterious effects induced by excessive SAM accumulation [23]. As also evidenced in previous studies, re-expression of

MAT1A and normalisation of SAM concentration, induced apoptosis in human hepatoma cells [33].

3.2. 2D-DIGE analysis

To get a deep insight of the mechanisms mediating the response of Huh7 cells to an increase of SAM, a detailed proteome analysis was performed 48 h post-transfection. Cytosolic, microsomal and nuclear fractions were extracted to increase the proteome coverage and the extent of enrichment was assessed by monitoring the presence of selected marker proteins: MAT for the cytosolic, KDEL for the microsomal, and Histone H4 for the nuclear fractions (Supplemental Fig. 2). Cytosolic and microsomal sub proteomes were firstly studied by 2D-DIGE, leading to detection of 2983 ± 77 and 3159 ± 138 spots in the corresponding fractions. Among them, 29 cytosolic and 42 microsomal differential spots were assigned upon DeCyder analyses (Fig. 2), from which 13 and 19 unique proteins were identified by LC-MS/MS respectively (Table 1 and Supplemental Table 1). With respect to microsomal alterations, down-regulation of glutamate dehydrogenase, ATP synthase beta subunit and citrate synthase, revealed impairment of energy metabolism. Deregulation of 26S protease regulatory subunit 10B, that participates in the degradation of ubiquitinated proteins, and the 78 kDa glucose-regulated protein, involved in the assembly of multimeric complexes in the endoplasmic reticulum, suggest deficient protein degradation and ER stress. Reduced FUBP2 might be considered as an indicative of the interference of SAM in the transformed phenotype of Huh7 cells, as it activates the FUSE element of c-myc and induces its expression. Mitochondrial damage is highlighted by deregulation of prohibitin, Clp protease, Aralar1, cytochrome b-c1 and other metabolic enzymes. In the cytosolic fraction differential proteins indicate metabolic adaptation, impairment of proteasome activity and cytoskeletal alterations. The metabolic enzymes were 3'-phosphoadenosine 5'-phosphosulphate synthase 1 bifunctional, involved in sulphur metabolism, alpha and beta enolases that in addition to their participation in the glycolysis bind to myc promoter. Proteasome deregulation is highlighted by reduction of regulatory subunit 11 non ATPase of 26S proteasome, and 4 down-regulated proteins associates to cytoskeleton, plastin-3, protein 1 annexin types A8 and 6, cytosolic aminopeptidase and T2-TrpRS. Interestingly, the last one has been involved in angiogenesis and as a regulator of Erk, Akt and eNOS signalling pathways [40–43], in addition to its function in cytoskeleton remodelling.

3.3. Label free LC-MS/MS analysis

To dig deeper into the proteome alterations induced by *MAT1A* expression in Huh7 cells, the same samples mentioned above were studied using a label free LC-MS/MS approach. Assuming a FDR below 1%, 698 microsomal and 477 cytosolic proteins were identified, from which 38 and 58 respectively showed significant alterations (Table 2 and Supplemental Table 2). Most differential proteins are metabolic enzymes involved in energy (AK2), carbohydrate (MDH2), hyaluronic acid (ITI3), cholesterol (APOA1) and prostaglandin (PTGR2, PTGES3) metabolism, glycolytic enzymes (PKM2, HK1, ENO1) and others (FDH, ADH). The cluster of differential proteins highlight impairment of translation (RPL8, RPL35, RPLP1), transcription (PSIP1), elongation (EEF1A1, EEF1B2, EEF2), initiation (EIF4A1), splicing (PABPL, HNRNPA3), protein degradation (PSMC2, PSMC4, CACYBP, CAPNS1, SQSTM1), endocytosis (STON2, RAB7A, ANXA1, EPS15L1), cell cycle

(CDC42, SF1, SEPT7), proliferation (DNAJA2), cell signaling (YWHAE, YWHAZ), and transport processes (NACA, NAPA, HGS, SSR4, CANX, KPNB1, IPO5). IPO5 is an importin that has been involved in histone transport, including H3F3B and H2AFV, which are also altered in Huh7 cells upon *MAT1A* expression.

3.4. Data integration

Since no overlapping aside from ENO1 and HSPA5 was observed between differential proteins from 2D-DIGE and gel free analyses, we wondered if these proteins might be part of common cellular processes suggesting, therefore, the same functional alterations. Bearing that idea in mind data were analyzed conjointly with Ingenuity Pathway Analysis (version 11631407), a software providing network-type representations where the interactions among nodes (proteins) is based on experimental evidences. Functional networks could be constructed using proteins from 2D-DIGE and gel free experiments, supporting the functional complementarity of the information generated by these two approaches. Considering a statistical threshold scoring value of 8, two networks comfortably reached statistical significance, with score values of 53 and 44 (Fig. 3). The first one (Fig. 3A) included as nodes 53 of the differential proteins identified in the proteomic analyses, 12 from 2D-DIGE and 41 from label free analyses respectively. The second includes 19 proteins, 7 from DIGE and 12 from label free studies respectively. In addition to adaptor proteins such as 14-3-3 YWHAE and YWHAZ that play central roles in the regulation of many signaling pathways, NFkB arises as a central driver of the antiproliferative and proapoptotic effect of SAM in Huh7 cells. Although NFkB levels were not found to be modified in the proteomic analysis, hyperphosphorylation of p65 (Fig. 3C) support NFkB activation. Alterations of Ras homolog, as proposed by IPA analysis, and down-regulation of LAMTOR1, are of interest since it might suggest impairment of MAP kinases pathway. Ras deregulation was indicated by decreased phosphorylation of C-Raf at Ser338 (stimulatory site), which in turn is hyperphosphorylated at inhibitory C-Raf Ser259 (Fig. 3C). Inhibition of C-Raf is consistent with downstream dephosphorylation of Erk (Fig. 3C), adaptation suggested by IP analysis as the central core of network 2 (Fig. 3B), in addition to caspase changes, that were further validated by Western blot revealing activation of caspase 3 (Fig. 3C). We next wondered if PI3K/AKT pathway, involved in differentiation, cell proliferation and survival, is also deregulated, as proposed in network 2. Dephosphorylation of both, PI3K and downstream Akt in *MAT1A* expressing Huh7 cells (Fig. 3C) indicates the switch off of this survival pathway, which activation has been largely demonstrated in tumoral cells. Our attention was also captured on two RNA helicases connected to 14-3-3 YWHAZ, EIF4A1, and especially DDX3X as it has been implicated in the progression of liver disorders [44–46]. Down-regulation of DDX3X upon *MAT1A* expression was confirmed by Western blot (Fig. 3C).

3.5. Regulation of DDX3X by SAM in human hepatoma cells

In light of the abovementioned results and the relevance of DDX3X in the progression of HCV infection and HCC, we wanted to investigate whether this protein is a direct target of SAM and if there is a correlation between SAM and DDX3X levels. A time dependent down-regulation with maximum effect of 60% DDX3X decrease was induced after *MAT1A* expression (Fig. 4A), indicating a nice negative correlation with intracellular SAM. As

previous studies have shown DDX3X shuttling between cytosol and nucleus, and that this change of subcellular location may have functional implications [47–49], we next wanted to investigate DDX3X distribution upon *MAT1A* expression in Huh7 cells. In addition to DDX3X depletion, SAM accumulation induced DDX3X shuttling from the cytosol to the nuclear fraction (Fig. 4B). To further confirm the regulatory effect of SAM on DDX3X levels, a dose dependent experiment was performed, exposing Huh7 cells to increasing concentrations of SAM in the cell culture medium. Accumulation of SAM correlated with a decrease of DDX3X (Fig. 5A). It is worth to mention that intracellular SAM levels were in the concentration range of those found in Huh7 cells upon *MAT1A* expression and were close to normal values in hepatocytes [39], suggesting the physiological relevance of our results. Down-regulation of DDX3X by SAM involves a translation defect as suggested by the maintenance of the mRNA levels and the lack of DDX3X in the extracellular medium (Supplemental Fig. 4). Finally, time-dependent reduction of DDX3X in HepG2 and Hep3B cells upon incubation with SAM (Fig. 5B) indicates that the regulatory effect is not specific of a particular cell line and that it can be generalized to human hepatoma cells.

4. Discussion

The aim of this study was the identification of proteins mediating the response of human hepatoma cells to an increase of their basal SAM concentrations to further understand its proapoptotic and antiangiogenic activity in liver cancer cells and its putative beneficial effect on liver maladies, in particular HCC. Compromised SAM synthesis is a common trait of most liver disorders, resulting from liver MAT inactivation or by *MAT1A* silencing in advanced cirrhosis and HCC [9,25,50]. Expression of *MAT1A* in hepatoma cells lead to MATI/III and SAM accumulation, observation consistent with the kinetic properties of this MAT isoform. MATI/III, the form present in differentiated liver, is activated by methionine and ATP, the substrates of SAM synthetic reaction, and is not inhibited by SAM, as is the case of MATII [17] the predominant form in liver cancer cells. Therefore, the expression of MATI/III allows higher steady state SAM levels than MATII, which is central to preserve quiescent hepatocytes, preventing dedifferentiation and proliferation [51–53] and might be a mechanism to interfere the transformed phenotype of human hepatoma cells.

Increased SAM levels induced proteome alterations in human hepatoma cells suggesting mechanisms driving the modulation of essential cellular pathways such as cell proliferation, survival and apoptosis. Two distinct approaches were used, 2D-DIGE and LC-MS/MS, allowed identification of distinct but complementary protein sets that participate in the same cellular pathways, providing an integrated functional interpretation. Increased intracellular SAM in Huh7 cells switch-off signalling pathways typically activated in liver cancer cells, including ERK/MAPK and PI3K/AKT [54,55]. Moreover, inhibition of ERK/MAPK pathway has been correlated with apoptosis mediated by activation of caspase 3 and 7 [54]. Activation of caspase 3 has been also related with up-regulation of calpains [56–59] as we have also observed in our study. Inactivation of MAPK pathway is further supported by down regulation of upstream RAF activity, which also suggest uncoupling at the level of EGFR and impairment of cell cycle regulators YWHAE/YWHAZ [60]. Although no activation of caspase 7 was found in this study, these findings might explain the reduction of cell growth and increased apoptosis induced in Huh7 cells by expression of *MAT1A* gene.

Hyperphosphorylation of NFkB p65 that has been also found in HepG2 cells upon increasing intracellular SAM [61], and inactivation of PI3K/AKT pathway, which is typically switched on in tumoral cells [54,55] provide additional mechanisms supporting the anti proliferative effect of SAM in human hepatoma cells. The multifactorial effect of SAM on cell growth is illustrated by down-regulation of heat shock proteins (HSPs). It has been recently shown that HSPs are over expressed in different pathologies, including HCC [62] playing pivotal roles in cell differentiation, proliferation and tumorigenesis [63].

The up-regulation of SAM induced a dose and time dependent decrease of the RNA helicase DDX3X in Huh7 cells, a potential oncogene associated to HCC. DDX3X belongs to the family of DEAD-box helicases that shared a consensus sequence D-E-A-D and two recA domains with an RNA helicases activity that unfold RNA in an ATP hydrolysis dependent manner [64]. Recent studies demonstrated that this protein is central for normal replication of HCV and HIV [49,65] and consequently, its inhibition was proposed as an antiviral therapeutic strategy [66]. Although its participation in hepatocarcinogenesis is still unclear, Huang et al. [46] have reported DDX3X accumulation in HCC patients and its oncogenic capacity has been proposed [45]. We have shown here that DDX3X is a primary target of SAM, which has the ability to modulate the levels of this protein in a mechanism that may depend on protein degradation or protein synthesis by action of a specific miRNA. This observation provides a new mechanism to explain and support the hepatoprotective function of SAM in the context of severe liver diseases such as HCV and HCC, two of the major challenges of hepatology because of their incidence worldwide and the difficulties for the clinical management of the patients, especially in the case of HCC.

In addition to the decrease of DDX3X in human hepatoma cells in response to accumulation of SAM, a subcellular shuttling was also observed. Many studies have described a cytoplasmic location of DDX3X [47–49,67–69] although it has been suggested that the differentiation degree of a cell might be a factor regulating the organellar distribution of this protein, as DDX3X was found to be predominantly nuclear in primary epithelial cells, while it was cytoplasmic in epithelial cancer cells [45]. In parallel with these observations, we found that Huh7 cells display a mainly cytoplasmic location of DDX3X and that intracellular SAM accumulation induces its nuclear shuttling, further suggesting the capacity to interfere with the transformed phenotype of hepatoma cells. Nuclear transport of DDX3X is mediated by mechanisms dependent on exporting CRM1 [47–49] and TAP [67]. The biological functions of DDX3X in the different locations is still under investigation. In the nucleus it was formerly associated with splicing [70] and with the transport of viral proteins to the cytosol, as in VIH infection [49] and it may be also the case on HCV infection. DDX3X has been related with protein translation through its interaction with initiation factors eIF4e [71], eIF4a, eIF2alpha, PABP [67] and eIF3 [68] and its participation in the initiation of this process has been proposed [70,72,73]. In light of these data, it is tempting to suggest that an increase on intracellular SAM may lead to down regulation of protein translation by depleting DDX3X and translation initiation factors, including eIF3J and eIF4A. Participation of DDX3X in apoptosis has been also described, although the mechanism involved is still controversial. It has been suggested as a proapoptotic factor in HCV-derived HCC based on its capacity to activate caspases 6 and 9 [44]. In contrast, interference at the level of caspase 3 activation of complex GSK3, DDX3X, and c-IAP1

suggest the antiapoptotic capacity of DDX3X [74]. Although this apparent contradiction might be reconciled by assuming a dual effect of DDX3X, that would have a pro- or anti-apoptotic activity depending on the cell type and cell context, activation of caspase 3 in parallel to DDX3X depletion upon increased SAM content in human hepatoma cells, agree better with the second hypothesis.

In summary, our results show that expression of *MAT1A* in human hepatoma cells, leading to intracellular SAM concentration similar to quiescent hepatocytes, target cellular functions that are essential for the maintenance of the transformed phenotype. Subcellular proteomic analysis allowed the identification of target proteins mediating the effects of SAM on apoptosis, cell proliferation and survival. Interestingly, data are provided indicating that DDX3X is regulated by SAM in a dose and time dependent manner. Down-regulation of DDX3X and its shuttling to the cellular nucleus might be one of the central factors regulating the response of Huh7 cells to increased SAM.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2012.01.009.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AK2	Adenylate kinase 2
AKT	Protein kinase B
ANXA1	Annexin A1
APOA1	Apolipoprotein A1
CACYBP	Calcyclin-binding protein
CAPNS	Calpain small subunit 1
CANX	Calnexin
CDC42	Cell division control protein 42 homolog
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3 X-linked
DNAJA2	DnaJ (Hsp40) homolog subfamily A member 2
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
EEF2	Eukaryotic Elongation factor 2

EEF1B2	Eukaryotic translation elongation factor 1 beta 2
EGFR	Epidermal growth factor receptor
EIF4A1	Eukaryotic initiation factor 4A-I
EPS15L1	Epidermal growth factor receptor substrate 15-like 1
FUBP2	Far upstream element binding protein 2
ERK	Extracellular signal-regulated kinase
H2AFV	Histone H2A.V
H3F3B	Histone H3.3
HCC	Hepatocellular carcinoma
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HK1	Hexokinase 1
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3
HSPA5	Heat shock protein 5A
IPO5	Importin 5
ITIH3	Inhibitor heavy chain H3
KPNB1	Importin subunit beta-1
LAMTOR1	Late endosomal/lysosomal adaptor MAPK and MTOR activator 1
MAT1A	Methionine adenosyltransferase 1A
MAT2A	Methionine adenosyltransferase 2A
MAPK	Mitogen-activated protein kinase
MDH2	Malate dehydrogenase 2
NACA	Nascent-polypeptide-associated complex alpha polypeptide
NAPA	N-ethylmaleimide-sensitive factor attachment protein alpha
NFκB	Nuclear factor kappa B
PABPL1	Poly(A) binding protein cytoplasmic 1
PI3K	Phosphoinositide-3-kinase
PKM2	Pyruvate kinase isozyme M2
PSIP1	PC4 and SFRS1 interacting protein 1
PSMC2	26S protease regulatory subunit 7
PSMC4	26S protease regulatory subunit 6B
PTGES3	Prostaglandin E synthase 3
PTGR2	Prostaglandin reductase 2

RAB7A	Ras-related protein Rab-7A
RPL8	60S ribosomal protein L8
RPL35	60S ribosomal protein L35
RPLP1	60S acidic ribosomal protein
SAM	S-Adenosylmethionine
SEPT7	Septin 7
SF1	Splicing factor 1
SQSTM1	Sequestosome-1
SSR4	Translocon-associated protein subunit delta
STON2	Stonin 2
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide

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Fig. 1.

Effect of MAT1A overexpression in Huh7 cells. Western blotting and densitometric measurement for MATI/III protein estimation (A) and intracellular SAM levels (B) in pMAT1A and pEGFP transfected cells. Experiments were performed at different time intervals after transfection. MAT and SAM levels are expressed as % of control condition (100% SAM was 0.3286 ± 0.036 pmol/ μ g protein) at time 0 hours of transfection. Mean $\pm$ SEM from three different experiments is shown. Equal protein loading in SDS-PAGE gels was assessed using an antibody against β -ACTIN. Representative blot from three independent experiments is shown (A).

Fig. 2. Differential proteins in MAT1A expressing Huh7 cells as evidenced by DIGE analysis. Representative 2-D gels from cytosolic and microsomal proteomes of MAT1A expressing Huh7 cells are shown. Differential spots which were subsequently identified by nano-LC-ESI-MS/MS are indicated.

Fig. 3.

Functional clustering of differential proteins of MAT1A expressing Huh7 cells using Ingenuity. Data from both proteomic approaches was grouped according to Ingenuity interaction parameters. The best network with a score of 53 is represented in (A). Symbols representing the nodal proteins in nodes refer to their functional classification (red and green are up- and down-regulated, respectively) (see legend in Supplemental Fig. 3). Nodes are interconnected by specific links according to the type of interaction. A cluster of NFkB interacting proteins displayed differential expression patterns in MAT1A expressing Huh7 cells. Activation of NFkB was further confirmed by the increase of phospho-NFkB p65. The second top-rank network with a score of 44 is represented in (B). Ras homolog displayed differential expression patterns in MAT1A expressing Huh7 cells. Inhibition of Ras homolog was further suggested by the increase of the inhibitor molecule phospho-C-RAF Ser259 and the parallel decrease of activator molecule phospho-C-RAF Ser338 as evidenced

by Western blot analysis (C). ERK/MAPK and PI3-K/AKT impairment was suggested by the differential expression of some of their target proteins (B), as well as by their decreased phosphate content (C). In agreement, increased apoptosis in MAT1A expressing Huh7 cells was assessed by Western blot (C). Equal protein loading in SDS-PAGE gels was demonstrated using an antibody against β -ACTIN. Representative blot from three independent experiments is shown (C).

Fig. 4.

Downregulation of DDX3X in MAT1A expressing Huh7 cells. DDX3X levels were measured at different time intervals on MAT1A expressing Huh7 cells. Western blotting and their densitometric measurements (A), expressed as % of control at time 0. Mean $\pm$ SEM from three different experiments is shown. Subcellular localization of DDX3X protein was assessed by subcellular fractionation; a significant decrease in the cytosolic fraction and an increase in the nuclear fraction (B) were observed. Equal protein loading in SDS-PAGE gels was demonstrated using an antibody against β -ACTIN. Representative blot from three independent experiments is shown (B).

Fig. 5.

Effect of SAM on DDX3X levels on human hepatoma cell lines. DDX3X and SAM levels are expressed as % of control condition (100% SAM=0.3585±0.021 pmol/μg protein) (A). Mean ± SEM from three different experiments is shown. The sulfate-p-toluensulfonate salt of SAM was used. Western blot of DDX3X protein in Huh7 cells is shown in (A), whereas DDX3X content in HepG2 and Hep3B human hepatoma cell lines is shown in (B). A representative blot is shown from three independent experiments (A, B). Equal protein loading in SDS-PAGE gels was assessed using an antibody against β-ACTIN.

Table 1

Differential proteins identified from 2D-DIGE gel analysis. Spot number, International Protein Index, Protein Name, MW (molecular weight), pI (isoelectric point), Average ratio (MATIA expressing Huh7 vs. control cells) and associated t-test, peptide number and associated Phenyx Score are shown.

Spot	International protein Index	Protein name	Phenyx score	MW	pI	Av ratio	t-test	Peptides
<i>Microsomal proteins</i>								
1243	IP100926977.1	26S protease regulatory subunit 10B	34.6	44041	7.8	-1.9	0.018	5
1612	IP100008530.1	60S acidic ribosomal protein P0-like	25.3	34412	5.7	1.5	0.042	2
605	IP100003362.3	78 kDa glucose-regulated protein	167.0	70478	5.0	-1.9	0.0055	24
1832	IP100005161.3	Actin-related protein 2.3 complex subunit 2	70.0	34426	6.8	1.2	0.015	9
935	IP100440493.2	ATP synthase subunit alpha, mitochondrial	195.3	55209	8.5	-1.7	0.043	21
390	IP100215637.5	ATP-dependent RNA helicase DDX3X	111.5	73243	6.9	-1.4	0.024	2
939	IP100386271.4	Calcium-binding mitochondrial carrier protein Aralar1	134.1	75114	8.6	1.3	0.0031	14
1243	IP100025366.4	Citrate synthase, mitochondrial	32.8	49006	7.9	-1.9	0.018	5
1403	IP100013847.4	Cytochrome b-c1 complex subunit 1, mitochondrial	53.6	53297	5.9	1.3	0.011	8
1336	IP100937615.2	Elongation factor 1-gamma	51.8	50429	6.3	1.4	0.0001	5
525	IP100479786.5	Far upstream element-binding protein 2	32.1	72942	8.2	-1.5	0.039	5
1330	IP100027438.2	Flotillin-1	56.9	47554	7.08	1.2	0.0055	8
236	IP100016801.1	Glutamate dehydrogenase 1, mitochondrial	62.3	61397	8.1	-1.5	0.038	9
827	IP100024934.5	Methylmalonyl-CoA mutase, mitochondrial	63.0	83538	6.5	1.3	0.0072	8
2036	IP100017334.1	Prohibitin	31.9	29843	5.6	-2.7	0.00044	3
2313	IP100479306.1	Proteasome subunit beta type-5	27.7	28633	6.4	1.4	0.027	3
2098	IP100003870.1	Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial	43.9	30446	8.3	-3.0	0.0038	5
998	IP100220644.8	Pyruvate kinase isozymes M1	352.1	58470	8.0	-1.2	0.032	56
690	IP100022774.3	Transitional endoplasmic reticulum ATPase	649.2	89950	5.1	-1.2	0.021	102
<i>Cytosolic proteins</i>								
1049	IP100105598.3	26S proteasome non-ATPase regulatory subunit 11	69.4	47332	6.3	-1.3	0.036	8
1298	IP100012007.6	Adenosylhomocysteinase	25.6	48255	5.9	1.4	0.049	2
1049	IP100465248.5	Alpha-enolase	162.4	47037	7.6	-1.3	0.036	110
1319	IP100914849.1	Annexin A8-like protein 1	42.2	36880	5.6	-1.2	0.032	18
1049	IP100218474.6	Beta-enolase	153.3	46800	8.1	-1.3	0.036	69

Spot	International protein Index	Protein name	Phenyl score	MW	pI	Av ratio	t-test	Peptides
622	IP100011619.4	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 1	15.2	70833	6.5	-1.3	0.0073	2
898	IP100789806.2	Cytosol aminopeptidase	29.4	52771	6.5	-1.2	0.037	4
866	IP100177728.3	Cytosolic non-specific dipeptidase	52.7	52747	5.7	-1.3	0.030	6
1401	IP100022824.1	Endophilin-B1	116.8	41056	5.8	1.3	0.028	13
681	IP100216694.3	Plastin-3	96.0	70811	5.5	-1.4	0.039	12
2025	IP100029623.1	Proteasome subunit alpha type-6	37.6	27838	6.3	-1.3	0.033	5
2031	IP100301311.1	Protein SET	32.5	37688	4.2	-1.5	0.011	8
898	IP100295400.1	T2-TrpRS	61.2	53165	5.9	-1.2	0.037	7

Table 2

Identification of differential proteins from Label Free LC-MS/MS analysis. International Protein Index as the protein identifier, Protein Name, fold change of Huh7-MAT1A versus Huh7-GFP and associated t-test value, number of peptides with a 95% of coverage and Total ProtScore calculated by ProGroup™ algorithm for each protein is shown. More detailed information about each single identification can be found in Supplemental Table 2.

International Protein Index	Protein name	Fold change Huh7-MAT/Huh7-GFP	t-test	Peptides (95%)	Total ProtScore
<i>Downregulated cytosolic proteins</i>					
IP100000816.1	YWHAE 14-3-3 protein epsilon	0.50	0.00178	8	10.52
IP100219038.9	H3F3A:LOC440926;LOC644914;H3F3B Histone H3.3	0.57	0.00196	2	2
IP100797126.1	NACA nascent polypeptide-associated complex subunit alpha isoform a	0.43	0.002	2	2.02
IP100006176.3	HGS Isoform 1 of Hepatocyte growth factor-regulated tyrosine kinase substrate	0.60	0.00343	2	2.01
IP100218988.4	AK2 Isoform 2 of Adenylate kinase 2, mitochondrial	0.57	0.00428	3	4
IP100032406.1	DNAJA2 DnaJ homolog subfamily A member 2	0.41	0.00468	1	2
IP100013894.1	STIP1 Stress-induced-phosphoprotein 1	0.58	0.01391	7	7.14
IP100001639.2	KPNB1 Importin subunit beta-1	0.41	0.01438	2	4.85
IP100021435.3	PSMC2 26S protease regulatory subunit 7	0.32	0.01472	1	2
IP100167515.1	PTGR2 Isoform 1 of Prostaglandin reductase 2	0.38	0.0157	1	2
IP100797230.1	RPL8 32 kDa protein	0.15	0.02663	6	6.02
IP100924819.2	MANF mesencephalic astrocyte-derived neurotrophic factor	0.50	0.03391	1	1.33
IP100021263.3	YWHAZ 14-3-3 protein zeta/delta	0.61	0.03881	7	6.34
IP100455757.2	RPL35 Similar to 60S ribosomal protein L35	0.59	0.04035	2	2
IP100455423.4	LOC440577 similar to nucleophosmin 1	0.40	0.04048	7	6.1
IP100180675.4	TUBA1A Tubulin alpha-1A chain	0.64	0.04146	11	4.23
IP100412779.2	RPLP1 60S acidic ribosomal protein P1 isoform 2	0.61	0.0455	5	1.21
<i>Upregulated cytosolic proteins</i>					
IP100783862.2	BLVRB Flavin reductase	2.60	0.00122	5	1.21
IP100013933.2	DSP Isoform DPI of Desmoplakin	1.45	0.00178	6	5.62
IP100396485.3	EEF1A1 Elongation factor 1-alpha 1	2.02	0.00256	33	32.32
IP100010740.1	SFPQ Isoform Long of Splicing factor, proline- and glutamine-rich	1.57	0.00257	6	8
IP100477342.1	UFM1 Ubiquitin-fold modifier 1	3.45	0.00306	1	2
IP100913961.2	H2AFV 18 kDa protein	1.74	0.00417	5	5.55
IP100916847.1	OLA1 47 kDa protein	2.07	0.00508	6	7.64

International Protein Index	Protein name	Fold change Huh7-MAT/Huh7-GFP	t-test	Peptides (95%)	Total ProtScore
IP100893746.1	NHP2L1 NHP2 non-histone chromosome protein 2-like 1	1.99	0.00681	4	2.06
IP100008524.1	PABPC1 Isoform 1 of Polyadenylate-binding protein 1	1.76	0.0076	8	4.26
IP100514311.1	CTTNBP2NL CTTNBP2 N-terminal-like protein	1.68	0.00817	1	2
IP100910473.1	CDC42	2.44	0.00982	3	4
IP100012011.6	CFL1 Collin-1	2.30	0.01071	21	17.87
IP100006690.1	EPX Eosinophil peroxidase	2.20	0.0113	6	7.73
IP100386119.4	SF1 Isoform 5 of Splicing factor 1	1.76	0.01173	3	2.29
IP100941771.1	-24 kDa protein	2.30	0.01392	1	2
IP100025084.3	CAPNS1 Calpain small subunit 1	1.54	0.01976	3	2.1
IP100020042.2	PSMC4 Isoform 1 of 26S protease regulatory subunit 6B	1.84	0.02014	2	1.65
IP100759715.1	FH Isoform Cytoplasmic of Fumarate hydratase, mitochondrial	2.22	0.02138	1	2
IP100955848.1	SH3 domain binding glutamic acid-rich protein like 3, isoform CRA	2.37	0.02198	4	4.02
IP100790627.2	PCBP2 poly(rC)-binding protein 2 isoform e	1.44	0.02238	7	6.09
IP100180730.1	- Similar to elongation factor 1-alpha 1	4.28	0.02425	29	26
IP100953191.1	FAM114A2 cDNA FLJ56229	1.92	0.02513	1	1.17
IP100007074.5	YARS Tyrosyl-tRNA synthetase, cytoplasmic	1.29	0.03181	2	2
IP100941534.1	SEPT7 Isoform 1 of Septin-7	2.43	0.03181	3	3.26
IP100395627.3	CACYBP Isoform 1 of Calyculin-binding protein	4.38	0.03297	1	2
IP100873416.2	ITIH3 Putative uncharacterized protein ITIH3	5.34	0.03348	1	2
IP100939304.1	IPO5 Isoform 3 of Importin-5	1.98	0.0351	9	8.1
IP100465248.5	ENO1 Isoform alpha-enolase of Alpha-enolase	1.58	0.03631	13	12.01
IP100186290.6	EEF2 Elongation factor 2	1.67	0.0374	33	29.25
IP100746777.3	ADH5;ADH5P4 Alcohol dehydrogenase class-3	1.95	0.03957	6	6
IP100003923.1	UMPS Isoform 1 of Uridine 5'-monophosphate synthase	1.46	0.04036	3	2
IP100167490.1	TOLLIP protein	3.59	0.04056	1	1.19
IP100220667.3	HK1 Isoform 4 of Hexokinase-1	1.77	0.04067	1	2
IP100018783.1	ITPA Inosine triphosphate pyrophosphatase	1.43	0.04093	2	2.72
IP100028122.1	PSIP1 Isoform 1 of PC4 and SFRS1-interacting protein	1.85	0.04319	2	2.14
IP100178440.3	EEF1B2;SNORA41 Elongation factor 1-beta	1.43	0.04447	3	2
IP100479186.7	PKM2 Isoform M2 of Pyruvate kinase isozymes M1/M2	1.45	0.04472	13	14.84
IP100291006.2	MDH2 Malate dehydrogenase, mitochondrial	1.54	0.04572	7	4.09

International Protein Index	Protein name	Fold change Huh7-MAT/Huh7-GFP	t-test	Peptides (95%)	Total ProtScore
IP100005064.1	GPSM3 G-protein-signaling modulator 3	1.73	0.0463	1	2
IP100000874.1	PRDX1 Peroxiredoxin-1	1.65	0.04752	9	11.44
IP100003362.2	HSPA5 HSPA5 protein	1.16	0.0482	3	2.31
<i>Downregulated microsomal proteins</i>					
IP100179473.9	SQSTM1 Isoform 1 of Sequestosome-1	0.93	0.00025	13	7.61
IP100025491.1	EIF4A1 Eukaryotic initiation factor 4A-I	0.86	0.00036	15	13.95
IP100013296.3	RPS18;RPS18P9 40S ribosomal protein S18	0.80	0.0016	9	8.33
IP100016670.3	C11orf59 RhoA activator C11orf59	0.42	0.0021	1	2.37
IP100337705.1	STON2 Isoform 2 of Stonin-2	0.35	0.00358	1	2.01
IP100032881.2	MRPS23 28S ribosomal protein S23, mitochondrial	0.84	0.00449	3	2
IP100795263.1	DNAJC19 Putative uncharacterized protein DNAJC19	0.66	0.00586	1	1.06
IP100872818.2	C6orf136 hypothetical protein LOC221545 isoform 3	0.41	0.00646	1	1.27
IP100016342.1	RAB7A Ras-related protein Rab-7a	0.72	0.00815	6	9.95
IP100916517.1	HNRNPA2B1 Putative uncharacterized protein HNRNPA2B1	0.85	0.00886	46	39.12
IP100395646.2	MUTED TXNDC5 protein	0.85	0.00911	18	9.06
IP100290461.3	EIF3J Eukaryotic translation initiation factor 3 subunit J	0.88	0.00942	6	10.2
IP100455134.1	HNRNPA3 Isoform 2 of Heterogeneous nuclear ribonucleoprotein A3	0.86	0.00967	21	22.05
IP100793322.1	DUT 24 kDa protein	0.61	0.00995	1	1.58
IP100793201.1	AIMP1 isoform b precursor	0.52	0.01473	2	2.01
IP100011770.1	NDUFA4 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	0.48	0.01637	2	3.55
IP100019385.3	SSR4 Translocon-associated protein subunit delta precursor	0.71	0.01652	3	0.84
IP100910575.1	PTGES3 cDNA FLJ59888, moderately similar to Prostaglandin E synthase 3	0.66	0.01843	1	2.25
IP100021841.1	APOA1 Apolipoprotein A-I	0.85	0.01882	11	14.79
IP100002349.2	NUFIP2 Nuclear fragile X mental retardation-interacting protein 2	0.79	0.02024	6	6.49
IP100941747.1	CANX Calnexin	0.82	0.02254	20	15.45
IP100927496.1	ACAA1 Putative uncharacterized protein ACAA1	0.84	0.02257	1	2.34
IP100013297.1	PDAP1 28 kDa heat- and acid-stable phosphoprotein	0.88	0.02389	4	5.61
IP100017448.1	LOC100291837;RPS21 40S ribosomal protein S21	0.84	0.02414	6	4.14
IP100294536.2	STRAP cDNA FLJ51909	0.83	0.02417	2	2.98
IP100412579.6	RPL10A 60S ribosomal protein L10a	0.79	0.02527	2	3.74
IP100221093.7	RPS17 40S ribosomal protein S17	0.72	0.02551	5	7.06

International Protein Index	Protein name	Fold change Huh7-MAT/Huh7-GFP	t-test	Peptides (95%)	Total ProtScore
IP100956475.1	SEBOX cDNA FLJ51266, highly similar to Vitronectin	0.81	0.02597	4	2.63
IP100009253.2	NAPA Alpha-soluble NSF attachment protein	0.81	0.02715	3	5.8
IP100045396.2	CALU Calumenin isoform 4	0.84	0.02733	68	34.69
IP100218918.5	ANXA1 Annexin A1	0.77	0.02926	8	10.08
IP100942431.1	EPS15L1 Epidermal growth factor receptor substrate 15-like 1	0.71	0.0293	1	1.07
IP100783625.2	SERPINF5 Isoform 1 of Serpin B5	0.79	0.02947	4	2.34
<i>Upregulated microsomal proteins</i>					
IP100646794.1	CFDP1 Isoform 2 of Craniofacial development protein 1	1.51	0.01488	1	1.05
IP100902909.1	highly similar to NUCLEOSOME ASSEMBLY PROTEIN 1-LIKE 1	1.86	0.01722	1	1.85
IP100017592.1	LETM1 Isoform 1	1.29	0.01793	5	7.84
IP100796366.2	MYL6 cDNA FLJ56329, highly similar to Myosin light polypeptide 6	1.14	0.01902	7	7.8
IP100021266.1	RPL23A;SNORD4A 60S ribosomal protein L23a	1.30	0.02857	10	11.71