Abstract. Background: Hepatocellular carcinoma (HCC) is the major type of primary liver cancer. Mice lacking the tumor-suppressive protein phosphatase 2A subunit B56δ (Ppp2r5d) spontaneously develop HCC, correlating with increased c-MYC oncogenicity. Materials and Methods: We used two-dimensional difference gel electrophoresis-coupled matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to identify differential proteomes of livers from wild-type, non-cancerous and HCC-affected B56δ knockout mice. Results: A total of 23 proteins were differentially expressed/regulated in liver between wild-type and non-cancerous knockout mice, and 119 between non-cancerous and HCC knockout mice (‘cancer proteins’). Overlap with our reported differential transcriptome data was poor. Overall, 56% of cancer proteins were reported before in HCC proteomics studies; 44% were novel. Gene Ontology analysis revealed cancer proteins mainly associated with liver metabolism (18%) and mitochondria (15%). Ingenuity Pathway Analysis identified ‘cancer’ and ‘gastrointestinal disease’ as top hits. Conclusion: We identified several proteins for further exploration as novel potential HCC biomarkers, and independently underscored the relevance of Ppp2r5d knockout mice as a valuable hepatocarcinogenesis model.

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, and the second leading cause of cancer-related death worldwide (1). Most patients with HCC are diagnosed at an advanced stage due to asymptomatic features during neoplastic progression, and hence their prognosis is grim. Curative therapies, including orthotopic liver transplantation, surgical resection and local destruction, can only be offered to early diagnosed patients, and is complicated by high recurrence rates. Advanced or non-resectable HCC is associated with a poor prognosis due to broad resistance to chemotherapeutic agents (2-5). Several risk factors for HCC development have been established, including viral hepatitis B and C infection, aflatoxin B1 exposure, excessive alcohol abuse and nonalcoholic fatty liver disease, all giving rise, to some extent, to (chronic) liver inflammation, cirrhosis or liver injury. Only in about 15% of cases does HCC arise in a context of normal liver (6).

In general, the molecular mechanisms resulting in HCC initiation and progression remain incompletely understood (7). In recent years, comparative genomic profiling, gene expression and exome mutational analysis studies in human HCC samples have enabled HCCs to be classified into two different molecular subgroups, termed ‘proliferation’ and ‘nonproliferation’ [reviewed in (8, 9)]. Tumors from the first class are highly heterogeneous and are associated with poor prognosis. They show increased protein kinase B (AKT)/mammalian target of rapamycin (mTOR), hepatocyte...
growth factor receptor (MET), transforming growth factor β (TGFβ), insulin-like growth factor (IGF), rat sarcoma virus oncogene (RAS)/mitogen-activated protein kinase (MAPK) or NOTCH signaling, high-level amplifications of chromosomes 11q13 [fibroblast growth factor 19 gene (FGF19)/cyclin D1 gene (CCND1)] or 6p21 [vascular endothelial growth factor A gene (VEGFA)], and frequent aberrations in chromosomes 1 or 8 [myelocyte maturation oncogene (MYC)]. Tumors from the second class are less aggressive, better differentiated and are associated with a better outcome. These tumors featured WNT signaling (mostly via β1-catenin gene (CTNNB1) mutations) or chromosome 7 gain [epidermal growth factor receptor gene (EGFR)].

Mechanistically, we identified lack of PP2A-B56δ-driven glycogen synthase kinase-3β (GSK-3β) dephosphorylation on Ser9 (resulting in constitutive GSK-3β inactivation) as the likely tumor-predisposing factor, which affected the GSK-3β substrate c-MYC in all HCC-bearing livers. Indeed, in the presence of a serendipitous oncogenic alteration affecting c-MYC Ser62 phosphorylation, lack of c-MYC Thr-58 phosphorylation by GSK-3β resulted in c-MYC stabilization and Ser62 hyperphosphorylation in all tumors (36), c-MYC is a well-established oncogenic driver in liver (42, 43), whose activation is essential for malignant conversion of dysplastic nodules into early HCC (44). Conversely, Ppp2r5d KO appears to act as a tumor suppressor in liver, keeping oncogenic activation of c-MYC in check (36).

HCCs from several c-MYC transgenic mouse models show interesting molecular overlaps with human HCCs of both the better and the poorer survival groups (45). In the present study, we subjected B56δ KO HCC samples, paired non-cancerous B56δ KO liver tissue, and wild-type (WT) livers to an unbiased quantitative proteomics approach using two-dimensional difference gel electrophoresis (2D-DIGE). With this approach, we aimed to provide further insights into the mechanisms of hepatocarcinogenesis in Ppp2r5d KO mice, as well as into the suitability of this model to mimic human HCC.

Materials and Methods

Sampling of biological material. All protocols involving mice were in compliance with the KEU Leuven University Animal Care and Usage Committee (ECD projects P034-2008 and P168-2013). The genetic background of the WT and Ppp2r5d KO mice was mixed (C57BL/6J: 87.5%; 12.5%) (F3) (36). All sampled mice were male and between 18 and 24 months old. Mice were anaesthetized with an intraperitoneal injection of pentobarbital (Nembutal) and transcardially perfused with ice-cold saline (NaCl 0.9% Baxter). Livers were entirely removed, or in the case of a macroscopically visible liver lesion, both the lesion and a part of the normal surrounding liver were kept separately. For each tissue, one part was snap-frozen in liquid nitrogen and kept at −80°C until further use.

Histopathology. Hematoxylin/eosin-stained sections (4 μm) of paraffin-embedded liver tissue were examined by a liver pathologist for the presence of pathological lesions. For the Ppp2r5d KO samples, the ‘healthy’, non-tumor state, as well as the HCC state, were additionally confirmed on a cryosection of the snap-frozen material that was eventually lyzed for 2D-DIGE use.

Electron microscopy. Small samples from the snap-frozen tissues were thawed and fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) at 4°C overnight. After 1 h post-fixation in 2% osmium tetroxide (in 0.1 M phosphate buffer, pH 7.2) at 4°C, the samples were dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections of 50-60 nm were cut,
stained with uranyl acetate and lead citrate, and examined at 50 kV using a Zeiss EM 900 electron microscope (Carl Zeiss AG, Oberkochen, Germany).

**Protein extraction and 2D-DIGE.** Frozen liver tissues were weighed, homogenized and sonicated on ice in lysis buffer [7 M urea, 2 M thiourea, 40 mM Tris, 1% dithiothreitol (DTT), 4% CHAPS] containing protease and phosphatase inhibitor cocktail (Roche). Samples were centrifuged for 15 min at 20,000 × g at 4°C. The supernatant was collected and desalted by dialysis (PlusOne Mini Dialysis Kit, GE Healthcare, Chicago, IL, USA), and the protein concentration was determined with the Bradford method. Fifty micrograms of protein was labeled with 200 pmol of amine reactive cyanine dyes Cy3 (samples of condition 1) or Cy5 (samples of condition 2), whereas 50 μg of an internal standard was labeled with 200 pmol of Cy2 (GE Healthcare). The internal standard consisted of a pool of equal amounts of all 12 samples. A total of 12 gels were run, each with three samples (condition 1 sample, condition 2 sample and internal standard). Thus, a WT liver sample (n=4) was compared with a healthy KO liver sample (n=4) (4 gels), as well as with a KO HCC sample (n=4) (4 gels); and a healthy KO liver sample (n=4) was pairwise compared with a KO HCC sample (n=4) (4 gels). IPG strips were rehydrated overnight in 450 μl rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% immobilized pH gradient (IPG) buffer with either 1% DTT for IPG strips pH 4-7 or 1.2% DeStreak (GE Healthcare) for IPG strips pH 6-9). The pooled labelled samples containing sample loading buffer were loaded into individual 24 cm IPG strips using anodic cup loading. Isoelectric focusing (first dimension) was carried out on an Ettan IPGphor II manifold (GE Healthcare). After isoelectric focusing, the IPG strips were equilibrated during two intervals of 15 min each in equilibration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer, 0.02% bromophenol blue), and either 1% DTT (pH strip 4-7) or 1.2% DeStreak (pH strip 6-9). Equilibrated strips were placed on top of a 12.5% sodium dodecyl sulfate polyacrylamide gel and separated on an Ettan DaltSix System (GE Healthcare). Gels were scanned on a Typhoon 9400 gel imager at 100 μm pixel size (GE Healthcare). Prior to analysis with DeCyder V 7.0 software (GE Healthcare), gel images were cropped using ImageQuant TL (GE Healthcare). Spot detection and matching was performed automatically using the “Batch Processor” module of DeCyder V 7.0 software followed by careful manual rematching of unmatched spots and wrongly matched spots. Proteins in spots were accepted as being differentially expressed when showing a statistically significant (p<0.05) increase or decrease when compared to the control in at least 75% of the spot maps (Supplementary Table: available at https://gbiomed.kuleuven.be/lambrecht-cgp).

**Immunoblotting.** Protein lysates were resolved on 4-12% 2,2'-bis(hydroxyethyl)-2,2'-nitroliothrianol, 2-bis(2-hydroxyethyl) amino-2-(hydroxyethyl)-1,3-propanediol, bis(2-hydroxyethyl)aminotris(hydroxyethyl)methane (BIS-TRIS) gels (BioRad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (GE Healthcare) by wet blotting. The membranes were blocked in 5% milk in tris(hydroxyethyl)aminomethane-buffered saline (TBS)/Tween buffer (0.1% Tween-20 in TBS) at 4°C overnight. After washing in TBS/Tween, incubation with the primary antibody was performed in TBS/Tween containing 5% bovine serum albumin. The following primary antibodies were used: polyclonal rabbit anti-B56 (dilution: 1/2,000); monoclonal mouse anti-PP2A A and PP2A C (clones C5.3D10 and F2.6A10, dilution: 1/3,000; kind gifts of Dr. Stephen Dillosworth, UK); monoclonal mouse anti-vinculin (dilution: 1/10,000; Sigma-Aldrich), polyclonal rabbit anti-fructose-1,6-bisphosphatase 1 (FBP1) (dilution: 1/1,000; Abcam, Cambridge, UK); mouse monoclonal anti-phosphoglycerate mutase 2 (PGM2) (sc-376718; dilution: 1/500; Santa Cruz Biotechnologies, La Jolla, CA, USA); mouse monoclonal anti-S-adenosylmethionine synthase 1 (METK1) (sc-166452; dilution 1/500; Santa Cruz Biotechnologies); polyclonal rabbit anti-phospho-MEK1/2 (S217/S221 (dilution 1/1,000; Cell Signaling Technology, Danvers, MA, USA); polyclonal rabbit anti-phospho-ERK1/2 T202/Y204 (dilution 1/1,000; Cell Signaling Technology, Danvers, MA, USA).
Table I. Representation of 129 differentially expressed proteins [green: up-regulated, p<0.05; red: down-regulated, p<0.05; grey: not dysregulated, p>0.05; bold: marker proteins confirmed in other proteomics studies of mouse, rat or human hepatocellular carcinoma, including references (last column)].

| Gene name | UniProtKB name | Accession number | Full protein name | Average ratio | Number of spots | Functional classes GO annotation | Reference |
|-----------|----------------|------------------|-------------------|---------------|----------------|---------------------------------|-----------|
| Ppp2r1A   | 2AAA           | Q76MZ3           | PP2A 65 kDa regulatory subunit Aα or Aβ | 2.24 – 1.15   | 2              | Cell cycle | Conflict (25) |
| Cdc5      | CDC5C          | Q8C9M2           | Coiled-coiled domain protein15 | 4.92 – 1.55   | 1              | Cell cycle | NEW |
| Ahcfl     | ELYS           | Q8CJF7           | Protein ELYS | 2.88 – 1.32   | 1              | Cell cycle | NEW |
| Rbp7p7    | RBBP7P         | Q60973           | Histone-binding protein RBBP7 | 2.51 – 1.02   | 1              | Cell cycle | NEW |
| Serpin1A  | A1AT2          | P22599           | Alpha-1-antitrypsin 1-2 | 2.68 – 1.64   | 1              | Coagulation | (13, 14, 22, 25-27, 32) |
| Serpin1C  | ANT3           | P32261           | Antithrombin-III | 2.06 – 1.14   | 3              | Coagulation | NEW |
| Anxa3     | ANXA3          | O35639           | Annexin A3 | 1.9 – 1.28    | 1              | Coagulation | (15) |
| Fgg       | FBG            | Q8VMC7           | Fibrinogen gamma chain | 7.28 – 1.1    | 1              | Coagulation | (13) |
| Tcp1      | TCPA           | P11983           | T-Complex protein 1 subunit alpha | -2.56 – 1.83  | 1              | Coagulation | (15) |
| Cct8      | TCPQ           | P42932           | T-Complex protein 1 subunit theta | 2.13 – 1.28   | 2              | Coagulation | NEW |
| Anxa11    | ANXAI1         | P97384           | Annexin A11 | 1.9 – 1.28    | 1              | Cytoskeleton | NEW |
| Anxa4     | ANXA4          | P97429           | Annexin A4 | 3.91 – 1.48   | 1              | Cytoskeleton | Conflict (20) |
| Cadh23    | CAD23          | Q99PF4           | Cadherin-23 | 1.83 – 1.07   | 1              | Cytoskeleton | NEW |
| Colba2    | COL6A2         | Q07288           | Collagen alpha-2 (VI) chain | -3.7 – 1.71   | 1              | Cytoskeleton | NEW |
| Des       | DESM           | P31001           | Desmin | 3.51 – 1.19   | 1              | Cytoskeleton | NEW |
| Ezh2      | EZRI           | P26040           | Ezrin | -1.5 – 1.01   | 1              | Cytoskeleton | (19, 26, 31) |
| Krt18     | K1C18          | P05784           | Keratin, type I cytoskeletal 18 | 3.69 – 1.28   | 1              | Cytoskeleton | (27) |
| Krt2      | K22E           | Q3T7T5           | Keratin, type II cytoskeletal 2 epidermal | 3.51 – 1.19 | 1 | Cytoskeleton | (27) |
| Krt77     | K21B           | Q6IFZ6           | Keratin, type II cytoskeletal 1b | 3.51 – 1.19 | 1 | Cytoskeleton | (27) |
| Krt8      | K2C8           | P11679           | Keratin type II, cytoskeletal 8 | 4.03 – 1.19 | 5 | Cytoskeleton | (15, 17, 19, 24) |
| Rdx       | RAD1           | P26043           | Radixin | -1.93 – 1.03 | 1 | Cytoskeleton | (19) |
| Tuba1a    | TBA1A          | P68369           | Tubulin alpha-1A chain | 2.58 – 1.02 | 3 | Cytoskeleton | NEW |
| Tuba1b    | TBA1B          | P68369           | Tubulin alpha-1A chain | -2.66 – 1.33 | 1 | Cytoskeleton | NEW |
| Tubb2a    | TBB2A          | QTMM9            | Tubulin beta 2A chain | 2.76 – 1.03 | 2 | Cytoskeleton | (25, 33) |
| Tubb2c    | TBB2C          | AAH5547          | Tubulin beta 2C chain | 2.76 – 1.03 | 2 | Cytoskeleton | (25, 26) |
| Tubb5     | TBB5           | P99024           | Tubulin beta-5 chain | 1.94 – 1.06 | 1 | Cytoskeleton | (25) |
| Vim       | VIME           | P20152           | Vimentin | 2.47 – 1.03 | 2 | Cytoskeleton | (17, 26, 27) |
| Wdr1      | WDR1           | O88342           | WD Repeat-containing protein 1 | 1.17 – 1.35 | 2 | Cytoskeleton | NEW |
| Calr      | CALR           | P14211           | Calreticulin | 2.41 – 1.25 | 1 | ER/protein folding | (31) |
| Dnaic3    | DNAJC3         | Q91YW3           | DnaJ homolog subfamily C member 3 | 5.39 – 1.25 | 1 | ER/protein folding | (21) |
| Hsp90b1   | ENPL           | P08113           | Endoplasm | 3.4 – 1.24 | 1 | ER/protein folding | (21, 27) |
| Erp4p     | ERP4P          | Q9JDQ6           | Endoplasmic reticulum resident protein 44 | -1.17 – 1.41 | 1 | ER/protein folding | (14, 22, 24, 26, 27, 31) |
| Hspa5     | GRP78          | P20029           | 78-kDa Glucose-regulated protein | 3.46 – 1.41 | 2 | ER/protein folding | (14, 22, 24, 26, 27, 31) |
| Hspa6     | HSP7C          | P63017           | Heat-shock cognate 71-kDa protein | 4.08 – 1.08 | 1 | ER/protein folding | (21, 23, 26, 27, 31) |
| P4hb      | PD1A           | P09103           | Protein disulfide-isomerase | 3.25 – 1.63 | 5 | ER/protein folding | (19, 20, 26, 27, 31) |
| Pdia3     | PDDA3          | P27773           | Protein disulfide-isomerase A3 | 2.80 – 1.32 | 4 | ER/protein folding | (17, 31) |
| Tnndc5    | TXNDC5         | Q91W90           | Thioredoxin domain-containing protein 5 | -1.17 – 1.41 | 1 | ER/protein folding | (17, 26) |
| Enol1     | ENOA           | P17182           | Alpha-enolase | -2.11 – 1.23 | 2 | Glucose metabolism | NEW |
| Enol3     | ENOB           | P21550           | Beta-enolase | -1.76 – 1.19 | 1 | Glucose metabolism | NEW |
| Fbp1      | FBPI           | Q9QXD6           | Fructose-1,6-bisphosphatase 1 | -1.4 – 1.46 | 2 | Glucose metabolism | (12, 13, 15, 17-20, 22, 26, 27, 31-33) |

Table I. Continued
| Gene name | UniProtKB name | Accession number | Full protein name | Average ratio | Number of spots | Functional classes GO annotation | Reference |
|-----------|----------------|------------------|-------------------|--------------|----------------|-----------------------------------|-----------|
| Got1      | AATC           | P05201           | Aspartate aminotransferase, cytoplasmic | 1.7          | 1              | Liver metabolism                  | Conflict (20) |
| Adk       | ADK            | P55264           | Adenosine kinase  | -2.38        | 2              | Liver metabolism                  | (25)      |
| Apmap     | APMAP          | Q9D7N9           | Adipocyte plasma-membrane associated protein | -3.44        | 1              | Liver metabolism                  | NEW       |
| Ass1      | ASSY           | P16460           | Argininosuccinate synthase | -3.4         | 1              | Liver metabolism                  | (17, 20, 26, 28, 31) |
| Bhmt      | BHMT1          | O35490           | Betaine homocysteine S-methyltransferase 1 | -3.4         | 1              | Liver metabolism                  | 13, 14, 18, 20, 21, 26, 29, 30, 33 |
Table I. Continued

| Gene name | UniProtKB name | Accession number | Full protein name | Average ratio | Functional classes | Reference |
|-----------|----------------|------------------|-------------------|---------------|--------------------|-----------|
|           |                |                  |                   | HCC/KO        | KO/WT              |           |
|           |                |                  |                   | Number of spots | annotation         |           |
|           |                |                  |                   |               |                    |           |
| Ogdh      | ODO1           | Q60597           | 2-oxoglutarate dehydrogenase, mitochondrial | -1.81         | 1.66               | 1 Mitochondria |
| Dlst      | ODO2           | Q9D2G2           | Dihydriopilysine-residue succinyl-transferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | 1.59          | -1.17              | 1 Mitochondria |
| Otc       | OTC            | P11725           | Ornithine carboxamyl transferase, mitochondrial | -2.54         | 1.01               | 1 Mitochondria (22, 25, 31) |
| Pc        | PYC            | Q05920           | Pyruvate carboxylase, mitochondrial | -2.33         | 1.18               | 2 Mitochondria NEW |
| Sarhd     | SARHD          | Q99LB7           | Sarcosine dehydrogenase, mitochondrial | -2.98         | 1.08               | 4 Mitochondria (19, 22, 25-27, 31) |
| Stoml2    | STML2          | Q99JB2           | Stomatin-like protein 2 | -3.40         | 1.27               | 1 Mitochondria NEW |
| Sucla2    | SUCB1          | Q9Z2I9           | Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial | 1.19          | -1.35              | 1 Mitochondria |
| Acat1     | THIL           | Q8QZT1           | Acetyl-CoA acetyltransferase, mitochondrial | -3.11         | 1.06               | 1 Mitochondria (13-15, 17) |
| Uqcrf5s1  | Q9CRFS1        | Q9CRC6           | Cytochrome B-C1 complex subunit | -2            | 1                  | 1 Mitochondria NEW |
| Hnrnpk    | HNRPK          | P61979           | Heterogenous nuclear ribonucleoprotein K | 2.15          | -1.28              | 3 mRNA process./translat. (15) |
| Ef4b      | E4FB           | Q8BG09           | Eukaryotic translation initiation factor 4B | 2.14          | -1.37              | 1 mRNA process./translat. NEW |
| Rplo0     | RPL0           | P14869           | 60S Acidic ribosomal protein P0 | 1.43          | -1.07              | 1 mRNA process./translat. (28) |
| Ruvbl2    | RUVB2          | Q9WTM5           | RuvB-like 2 | 7.28          | -1.1               | 1 mRNA process./translat. (16) |
| Hars1     | SYHC           | Q61035           | Histidyl-tRNA synthetase, cytoplasmic | 2.35          | 1.18               | 1 mRNA process./translat. NEW |
| Gars1     | SYG            | P41250           | Glycyl-tRNA synthetase | 2.43         | 1.09               | 1 mRNA process./translat. (15) |
| Wdr12     | WDR12          | Q9JJ4A           | Ribosome biogenesis protein WDR12 | 2.85          | -1.16              | 1 mRNA process./translat. NEW |
| Abhd14b   | ABHEB          | Q8VCR7           | α/β Hydrolase domain-containing protein 14B | -2.34         | -1.15              | Not classified (17, 25, 31) |
| Crtap     | CRTAP          | Q9CYD3           | Cartilage-associated protein | -3.09         | 1.9                | 1 Not classified NEW |
| Efhd2     | EFHD2          | Q9D8Y0           | EF-Hand domain-containing protein D2 | 2.35          | 1                  | 1 Not classified NEW |
| Ahsq      | FETUA          | P29699           | Alpha-2-HS-glycoprotein | 4.27          | -1.08              | 1 Not classified NEW |
| H2-K1     | HA1B           | P01901           | H-2 Class I histocompatibility antigen, K-B α chain | -3.09         | 1.9                | 1 Not classified NEW |
| Na/A      | IGHA           | P01878           | Ig Alpha chain C region | 2.02          | -1.01              | Not classified |
| Igg1      | IGG1           | Q9Q285           | Interferon-inducible GTPase 1 | 1.59          | -1.17              | Not classified |
| Gaa       | LGAY           | P07699           | Lysosomal alpha-glucosidase | 2.5           | 1.42               | Not classified (17) |
| Kat6a     | MYST3          | Q8BZ21           | Histone acetyltransferase KAT6a or MYST3 | 3            | -1.14              | 1 Not classified NEW |
| Pebp1     | PEBP1          | P70296           | Phosphatidylethanolamine-binding protein 1 | 1.51          | -1.15              | Not classified (25) |
| Rgn       | RGN            | Q64374           | Regucalcin | -5.14         | 1.03               | 3 Not classified (13, 15, 22, 25, 31, 32) |
| Rgn       | RGN            | Q64374           | Regucalcin | 8.4           | -1.18              | 1 Not classified (19) |
| Gdi1      | GDIA           | P50396           | Rab GDP dissociation inhibitor alpha | 2.09         | -1.2                | 2 Receptor linked NEW |
| Gdi2      | GDB            | Q61598           | Rab GDP dissociation inhibitor beta | 1.52          | -1.19               | 1 Receptor linked NEW |
| Atp6v1a   | VATA           | P50516           | V-Type proton ATPase catalytic subunit A | 5.52          | 1.22                | 1 Receptor linked NEW |
| Atp6v1b2  | VATB2          | P62814           | V-Type proton ATPase subunit B, brain isoform | 2.35          | 1.18                | 1 Receptor linked NEW |
| Cat       | CATA           | P24270           | Catalase | -2.19         | -1.07              | 2 Redox regulation (13-15, 20, 22, 32) |
| Ddhv      | DHDH           | Q9DBC8           | Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase | -1.4          | -1.33              | 1 Redox regulation (15) |
| Gpx1      | GPX1           | P11352           | Glutathione peroxidase 1 | -1.98         | -1.10              | 3 Redox regulation (15, 25, 31) |
| Grhpr     | GRHPR          | Q91Z53           | Glyoxylate reductase/hydroxy-pyruvate reductase | 1.7           | -1.14               | 1 Redox regulation Conflict (26) |
| Pdrx2     | PRDX2          | Q61171           | Peroxiredoxin 2 | 1.51          | -1.15              | 1 Redox regulation (24, 25) |

Table I. Continued
Table I. Continued

| Gene name | UniProtKB name | Accession number | Full protein name | Average ratio | Number of spots | Functional classes GO annotation | Reference |
|-----------|----------------|------------------|-------------------|---------------|----------------|----------------------------------|-----------|
| Alb       | ALBU           | P07724           | Serum albumin     | 4.94          | –1.32          | 22 | Transport | (14, 22, 26, 27, 31) |
| Ehd4      | EHD4           | Q9EQP2           | EH domain-containing protein 4 | –1.2          | –1.33          | 1 | Transport |          |
| Hpx       | HEMO           | Q91X72           | Hemopexin         | 2.93          | –1.15          | 5 | Transport | (25) |
| Selenbp1  | SELENBP1       | P17563           | Methanethiol oxidase/selenium-binding protein 1 | 3.60          | –1.06          | 2 | Transport | (19) |
| Snx6      | SNX6           | Q6P8X1           | Sorting nexin-6   | 1.59          | –1.17          | 1 | Transport |          |
| Tf        | THYG           | O08710           | Thyroglobulin     | –5.74         | 1.17           | 1 | Transport | (13, 32) |
| Tf        | TRFE           | Q92111           | Serotransferrin   | –1.5          | –1.01          | 1 | Transport | (15, 22, 26, 31) |
| Gc        | VTDB           | P21614           | Vitamin D-binding protein | 4.27          | –1.08          | 1 | Transport | (22) |
| Cops4     | CSON4          | O88544           | COP9 Signalosome complex subunit 4 | 4            | 1.05           | 1 | Ubiquitination/ degradation | NEW |
| Psmc4     | PRS6B          | P54775           | 26S Proteasome regulatory subunit 6B | 3.69          | –1.28          | 1 | Ubiquitination/ degradation | NEW |
| Psmc2     | PRS7           | P46471           | 26S Proteasome regulatory subunit 7 | 1.52          | –1.19          | 1 | Ubiquitination/ degradation | NEW |
| Psmd13    | PSD13          | Q9WVJ2           | 26S Proteasome non-ATPase regulatory subunit 13 | 1.50          | –1.06          | 1 | Ubiquitination/ degradation | NEW |
| Psmb4     | PSMB4          | P99026           | Proteasome subunit beta type 4 | 1.97          | 1 | 1 | Ubiquitination/ degradation | (31) |
| Uba1      | UBA1           | Q02053           | Ubiquitin-like modifier-activating enzyme 1 | 3.63          | –1.14          | 2 | Ubiquitination/ degradation | NEW |

KO: Knockout; WT: wild-type; HCC: hepatocellular carcinoma tissue.

1/1,000; Cell Signaling Technology); monoclonal mouse anti-phospho-c-MYC S62 (dilution 1/500; BioAcademia, Osaka, Japan), rabbit polyclonal anti-phospho-mTOR S2448 (dilution 1/500; Cell Signaling Technology); mouse monoclonal anti-phospho-p70 S6K T389 (dilution 1/1,000; Cell Signaling Technology); and rabbit polyclonal anti-GLI family zinc finger 1 (GLI1) (dilution 1/500; Santa Cruz Biotechnologies). Incubation with horseradish peroxidase-conjugated secondary antibodies (dilution 1/5,000; Cell Signaling Technology) was performed for 1h at 4˚C. To visualize the bands, the blots were developed on an ImageQuant LAS 4000 system (GE Healthcare) using densitometric quantifications were carried out with ImageJ software (Imagej.net/ImageJ). An unpaired two-tailed t-test was used for statistical analysis and p-values below 0.05 were considered significant.

Bioinformatics. The complete dataset of differentially expressed/regulated proteins was loaded into the Ingenuity Pathway Analysis (IPA) software (IPA version 8.7) and database (Ingenuity Systems, Redwood City, CA, USA) to find the most relevant canonical pathways, toxicity pathways or biological functions.

Results

Histopathological and molecular description of marine liver samples used for proteomics analysis. Four independent HCC samples from four randomly chosen Ppp2r5d KO mice exhibiting spontaneous hepatocarcinogenesis were collected and snap-frozen, together with a non-cancerous sample from the same livers (paired analysis). The pathological state of the frozen samples, side-by-side with a paraformaldehyde-fixed and hematoxylin/eosin-stained sample of the same tissues (Figure 1A), was confirmed by an experienced liver pathologist. Normal liver tissues from four age- and gender-matched WT mice (19m, 21m, 22m and 23m) were additionally collected as reference tissues. Although ranging from well- to poorly differentiated, all HCC samples showed increased c-MYC Ser62 phosphorylation, but differed in the activation of other oncogenic pathways, including MEK/ERK, mTOR/p70 S6K and Hedgehog signaling, as previously reported (36) (Figure 1B). One HCC sample showed ‘pale body’-like structures of unknown significance (Figure 1A).

Comparative 2D-DIGE analysis. We next generated protein-expression profiles of the protein lysates of these WT liver (n=4), KO HCC (n=4) and KO non-tumor tissues (n=4) using 2D-DIGE. For this purpose, an internal standard was created, in which 25 µg of each lysate (n=12) was mixed, and labelled with Cy2. Two WT, two KO tumor and two KO non-tumor tissue lysates were labelled with Cy3; the remaining two WT,
two KO tumor and two KO non-tumor lysates were labelled with Cy5. By normalizing the Cy3 or Cy5 intensities to the Cy2 control, it was possible to compensate for gel-to-gel variations. Direct comparison between Cy3 and Cy5 spot intensities allowed identification of differentially expressed or regulated proteins. All samples were first resolved on six gels with isoelectric focusing in pH range 4-7, and then re-run on another six gels with isoelectric focusing in pH range...
6-9. In pH range 4-7, 22 differentially expressed spots were detected between WT and non-cancerous KO liver samples (p<0.05), and 154 differentially expressed spots were identified between KO HCC and KO non-tumor tissue (p<0.05) (Figure 2A). In pH range 6-9, seven spots for differentially expressed proteins between WT and non-cancerous KO livers were found (p<0.05), while 58 were identified between KO HCC and KO non-tumor tissue (p<0.05) (Figure 2A). Representative examples of the analysis of spots that were up-regulated and down-regulated, respectively, in KO HCC are shown (Figure 2B).

MS-based protein identification. Subsequent MS analysis by MALDI TOF/TOF of the spots of differentially regulated proteins eventually resulted in the identification of 129 differentially expressed proteins between either or both conditions (Table I). Twenty proteins were down-regulated in non-tumor KO compared with WT livers, while only three proteins were up-regulated in non-tumor KO versus WT livers. Moreover, the fold-changes in expression between these samples were relatively small, ranging between 1.17- to 1.46-fold down-regulation, and between 1.66- to 1.71-fold up-regulation (Table I).

In contrast, many more proteins were found to be differentially expressed between KO HCC and KO non-tumor samples, with much higher absolute differences: 43 were down-regulated in the tumors (range=1.1- to 5.74-fold), while 80 proteins were up-regulated in the tumors (range=1.43- to 35.28-fold) (Table I). Interestingly, four proteins (tubulin alpha-1A chain, METK1, regucalcin and serotransferrin) showed apparently conflicting differential expression patterns in HCC versus non-tumor KO tissues, as they were found to be both up-regulated as well as down-regulated in the tumors (Table I). This might imply they may actually occur as different protein isoforms, for instance due to differing post-translational modifications or protein processing events. Hence, a total of 119 proteins showed different expression or regulation in normal KO liver versus HCC KO liver, and were denoted as ‘cancer proteins’. Based on Gene Ontology (GO) annotations, these cancer proteins can be grouped into 14 functional classes, covering a wide spectrum of biological functions (Table I and Figure 3). Notably, almost one-quarter of the cancer proteins (23%) belonged to ‘liver metabolism’ (n=22) or ‘glucose metabolism’ (n=6), and appeared mostly to be down-regulated in the tumors, consistent with a degree of hepatocyte de-differentiation and altered cancer cell metabolism. Another 18% of the cancer proteins were functionally associated with ‘mitochondria’ (mostly down-regulated in the tumors), and 15% with ‘cytoskeleton’ (mostly up-regulated in the tumors). Other functional classes were related to ‘cell cycle’, ‘endoplasmic reticulum/protein folding’, ‘lipid metabolism’, ‘mRNA processing/translation’, ‘transport’ and ‘protein ubiquitination/degradation (all predominantly up-regulated in the tumors) (Figure 3). Interestingly, 66 (56%) cancer proteins had previously been identified in other proteomic studies of HCC samples from patients, mice or rats (Table I, last column), while the remaining 55 (44%) others may represent potentially new HCC biomarkers (Table I, last column, ‘NEW’). Four proteins of the latter group had however been reported as differentially regulated in HCC in at least one other study, but in the opposite direction of what we found here, potentially testifying once more to the presence of differentially modified proteins rather than differential expression (Table I, last column, ‘conflict’).

Comparison between ‘cancer proteins’ and ‘cancer genes’. We compared the differentially expressed proteins between KO HCC and KO non-cancerous samples identified here with the differentially expressed genes previously determined by RNA-seq analysis in a similar set of Ppp2r5d KO mouse samples (36). Intriguingly, we found the overlap between both datasets was overall rather poor: of 29 differentially expressed genes between WT and KO samples (36), none overlapped with the 23 differentially regulated proteins identified here; and, of 351 differentially expressed genes between KO non-tumor and KO HCC samples (36), only 14 were also retrieved in the list of 119 differentially regulated proteins (Figure 4). For these 14 genes/proteins, the changes in expression were fully concordant at the mRNA and the protein level.

IPA-assisted pathway analysis of differentially regulated proteins. The cancer protein dataset was subsequently loaded into the IPA software and database (IPA; Ingenuity Systems) to identify links with the most relevant functional pathways or altered molecular networks. Importantly, the results confirm the diagnosis of HCC at the biochemical level, since ‘cancer’ and ‘gastrointestinal disease’ were the two top hits (Figure 5). Within the top altered pathways and molecular/cellular functions, several metabolic pathways were identified, including amino acid metabolism and the urea cycle, and methionine metabolism. Thus, this analysis indirectly confirms the quality and biological relevance of data obtained by the 2D-DIGE proteomics technique.

Validation of select differentially regulated proteins. One of the most up-regulated proteins in the HCCs was fibrinogen gamma (7.28-fold higher versus adjacent non-tumor tissue) (Table I). A closer inspection of the data revealed, however, that this up-regulation predominantly occurred in one of the four HCC samples analyzed, coinciding with the appearance of the ‘pale body’ structures identified during histopathological examination (Figure 1A). Additional electron microscopy examination of the ‘pale bodies’ showed rounded dilated cisternae of the rough endoplasmatic reticulum (RER), containing amorphous, granular material (Figure 6A), most likely representing fibrinogen deposits. Fibrinogen is a protein
Figure 2. Analysis of differentially regulated spots following two-dimensional differential gel electrophoresis (2D-DIGE) A: Cluster analysis of differentially regulated 'spots' between wild-type (WT), protein phosphatase 2A regulatory B56 δ subunit (Ppp2r5d) knockout (KO) non-cancerous, and Ppp2r5d KO hepatocellular carcinoma (HCC) liver tissue, grouped by pH range. Red: up-regulated; green: down-regulated. B: Examples of spot picking and analysis using DeCyder V 7.0 software, for typically up-regulated (left) and down-regulated (right) spots in the KO HCC samples. Proteins in spots were accepted as being differentially expressed when showing a statistically significant (p<0.05) increase or decrease when compared to the control in at least 75% of the spot maps.
synthesized exclusively by hepatocytes, and the intracellular deposition might reflect an impairment in its transport and/or secretion. Interestingly, such fibrinogen deposits have also been reported in human HCCs in 5-5.7% of cases, depending on the study (47, 48). In the B56δ KO mice, the overall incidence of pale body occurrence was 12% (3/25, n=25) (Figure 6B), matching well with the human HCC data.

Additional validation of the differential proteomics results was performed by western blot analysis of a select number of identified proteins for which antibodies were available. Consistent with the 2D-DIGE results (Table I), down-regulation of FBP1 was observed in all HCC samples (Figure 6C). Interestingly, this protein is also down-regulated in human HCC and was suggested to be one of five potential HCC biomarkers identified from a meta-analysis of several proteomic HCC profiling studies (49). In contrast, the slightly increased expression of the PP2A scaffolding A subunit (Table I) was not confirmed by immunoblotting (Figure 6C), suggesting it might rather represent a difference in post-translational modification of this protein. Supporting this hypothesis is another 2D-DIGE study which identified an opposite change in PP2A A subunit expression in HCC, i.e. down-regulation, neither confirming this by western blotting (25). Immunoblots further confirmed the down-regulation of phosphoglucomutase-2 (PGM2) (Figure 6C). For S-adenosyl methionine synthase 1 (METK1), immunoblots confirmed significant down-regulation (Figure 6C), in concordance with the mRNA expression data (Figure 4). However, the 2D-DIGE data additionally identified a spot of this protein significantly up-regulated in the HCCs (7.28-fold, p>0.05; Table I), suggestive of potential post-translational modification(s) of METK1 in the tumors.

Discussion

Genomic and mRNA analyses of human and mouse HCC have revealed significant overlaps between chromosomal aberrations and gene-expression signatures (50). However, because proteins rather than mRNA are the major effectors of cellular and tissue functions, complementary comparative analyses at the proteome level are equally important in gaining insights into the mechanisms of hepatocarcinogenesis.
and to discover novel prognostic or predictive markers for improved HCC (targeted) treatment. Here, we used 2D-DIGE to identify changes between the proteome of spontaneous HCCs in Ppp2r5d KO mice, their surrounding non-tumoral tissue and normal WT liver tissue. We identified 23 proteins differentially expressed or regulated between normal WT and KO livers, and 119 proteins differentially expressed or regulated between KO HCC and normal KO livers (the latter defined as ‘cancer proteins’). Intriguingly, the overlap with our previously published transcriptomics data, performed on mRNA isolated from similar tissue samples, was very poor, with only 14 genes/proteins appearing to be commonly regulated at the mRNA and protein levels in the non-cancerous KO livers vs. the KO HCCs. The identities of these ‘cancer’ genes/proteins are detailed in the box.

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Unbiased IPA analysis of the cancer proteins identified ‘gastrointestinal disease’ and ‘cancer’ as the top hits, independently confirming the diagnosis of our samples and hence, indirectly, the quality of the data obtained. In addition, within the cancer proteins, several established HCC biomarkers or differentially expressed proteins identified in former 2D-DIGE studies of human, rat or mouse HCC samples were confirmed (56%) (Table I, indicated in bold), further testifying to the data quality and suitability of the model.

When looking in more detail at the list of cancer proteins, an obvious finding is the overt decrease in ‘metabolism’-associated proteins in the Ppp2r5d KO HCCs, both in
proteins classified as belonging to ‘glucose metabolism’ as ‘liver metabolism’ – not only testifying to significant metabolic changes in the cancer cells, but likely also to a degree of de-differentiation of the hepatocytes. Down-regulation of Fbp1 (an enzyme promoting gluconeogenesis), seen at the mRNA level as well (Figure 4) and confirmed by immunoblotting (Figure 6B), suggests changes in glucose metabolism consistent with the Warburg effect (a change from oxidative phosphorylation to aerobic glycolysis) (51). Several enzymes involved in protein and amino acid degradation [e.g. phenylalanine-4-hydroxylase (PH4H), mitochondrial acetyl-CoA acetyltransferase (THIL), cytoplasmic malate dehydrogenase (MDHC)] were also reduced, while proteins involved in glutamine (a critical amino acid for proliferation of tumor cells) synthesis were up-regulated [e.g. ornithine aminotransferase (OAT)] as well as proteins involved in protein translation in general [e.g. eukaryotic translation initiation factor 4B (IF4B), 60S acidic ribosomal protein P0 (RLA0), histidyl-tRNA synthetase (SYHC), glycyl-tRNA synthetase (SYG), ribosome biogenesis protein WDR12 (WDR12)] and in protein maturation processing [e.g. methionine aminopeptidase 1D (AMP1D), protein disulfide-isomerase (PDIA1), protein disulfide-isomerase A3 (PDIA3)] (Table I). The urea cycle seemed significantly impaired in the HCCs [e.g. down-regulation of carbamoyl-phosphate synthase (CPS1) and

Figure 6. Validation of two-dimensional differential gel electrophoresis (2D-DIGE) results. A: Additional analysis of ‘pale body’ structures by electron microscopy. B: Frequency of occurrence of fibrinogen deposits in hepatocellular carcinomas (HCCs) from protein phosphatase 2A (PP2A) regulatory B56δ subunit (Ppp2r5d) knockout (KO) mice. Total number of hepatocellular carcinomas (HCCs) analyzed was 25. C: Validation of differentially expressed/regulated proteins by immunoblotting. FBP1 (fructose-1,6-bisphosphatase 1): 4.7-fold less expression in HCC compared to wild-type (WT) (p=0.015) and 6.2-fold less expression compared to non-tumor KO (p=0.002), PP2A scaffolding A subunit: no significant differences in expression between HCC, non-tumor KO or WT (p>0.05). Phosphoglucomutase-2 (PGM2): 5.5-fold less expression in HCC compared to WT (p=0.0001) and 3.3-fold less expression compared to non-tumor KO (p=0.0086). S-Adenosylmethionine synthase 1 (METK1): 5.7-fold less expression in HCC compared to WT (p=0.049) and 6.1-fold less expression compared to non-tumor KO (p=0.019).
ornithine carbamoyl transferase (OTC)] as was the liver
methylation cycle, with four essential enzymes in this
process, METK1, glycine N-methyl transferase (GNMT),
betaine-homocysteine-S-methyl transferase 1 (BHMT1) and
adenosyl homocysteinase (SAHH), all being down-regulated
(Table I). Down-regulation of METK1, validated by our
immunoblotting results (Figure 6B), and down-regulation of
SAHH and GNMT are likely caused by reduced gene
expression, as Mat1a, Ancy and Gnm1 mRNAs were also
found to be reduced in the ‘cancer genes’ list (Figure 4) (36).
Interestingly, however, we also found METK1 to be
significantly up-regulated in a different spot (Table I), likely
testifying from a different post-translationally modified form
or different isoform, being specifically increased in the
HCCs. Such explanation would also reconcile seemingly
contradicting data in literature, describing METK1 up-
regulation (12, 31), as well as down-regulation in HCC (13,
14). In any case, down-regulation of the methylation cycle
and, hence, chronic S-adenosyl-L-methionine deficiency,
have been shown to result in spontaneous HCC development
in Mat1a and Gnm1 KO mice (52, 53), which our data are
fully consistent with.

A similar explanation may hold true for tubulin alpha-1A
chain (TBA1A), serotransferrin (TRFE) and regucalcin
(RGN), which we and others (Table I) identified in inversely
altered spots, seemingly both up-regulated and down-
regulated in HCC. RGN is a senescence marker, suppresses
HCC growth in vitro (54, 55) and its expression is correlated
with improved survival of patients with HCC (54), suggesting
it is likely expressed less in the Ppp2r5d KO HCCs, while
the spot of up-regulated protein may represent a modified form.
In contrast, TBA1A is a cytoskeletal protein that was
likely up-regulated in the Ppp2r5d KO HCCs, as was the case
for at least three other members of the tubulin family that
were differentially regulated in our dataset (TBB2A, TBB2B,
TBB5; Table I); and TBB2A also at the mRNA level (Figure
4) alongside several other cytoskeleton proteins (different
cytokeratins, desmin and vimentin). Vimentin is known to be
abundantly expressed in human HCC and its overexpression
is significantly associated with HCC metastasis (56).
Likewise, expression levels of cytokeratin 8 (K2C8) have
been positively correlated with HCC metastatic ability (57),
and this up-regulation apparently occurs at the mRNA level
(Figure 4). Together with serum albumin and apolipoprotein
A1, serotransferrin was amongst the most highly up-
regulated proteins in the cancer protein list. We suggest that
the huge increase of these proteins in HCCs might actually
represent accumulation of extravascular plasma protein in the
tumor microenvironment, and not necessarily increased
protein synthesis by the tumor cells. Increased serotransferrin
levels in that environment might additionally induce
transferrin-mediated iron uptake, thereby satisfying the high
iron demand of the highly proliferating cancer cells. The spot
representing down-regulated serotransferrin may again
 correspond to an altered post-translationally modified form
or transferrin isoform.

Equally highly overexpressed in the HCCs was fibrinogen-
γ. Fibrinogen is a protein exclusively synthesized by
hepatocytes, and it was specifically found to be overexpressed
in one of four HCC samples analyzed, which at the
histological level featured ‘pale body’ structures (Figure 1A),
and at the ultrastructural level appeared as dilated cisternae of
the RER, filled with amorphous, granular material (Figure
6A). Since the RER is the site of protein synthesis, and
fibrinogen is a secreted protein, its retention in the RER likely
represents deficient translocation to the smooth ER or Golgi
apparatus, and eventually a defect in fibrinogen transport.
Interestingly, such pale bodies are observed in about 5-5.7%
of human HCCs (47, 48) and therefore represent another
clinical feature of Ppp2r5d (B56δ) KO HCC that we found in
12% of our analyzed mouse HCC samples.

The down-regulation of mitochondrial proteins, except
mitochondrial heat-shock proteins (HSPs), which were
significantly up-regulated, suggest a loss of function of this
organelle, with potentially increased stress. The up-regulation
of several molecular chaperones [mitochondrial stress-70
protein (GRP75), 78 kDa glucose-regulated protein (GRP78),
heat-shock cognate 71 kDa protein (HSP7C) and
mitochondrial 60 kDa heat-shock protein (CH60)] has been
reported in several HCC studies (Table I), and the
overexpression of these proteins may be interpreted as a
response to the stressful cancerous environment for
cytoprotective functions. Up-regulation of HSPs may also
contribute to the tumor cell adaptive response to altered
oxygen levels, as testified by altered expression of antioxidant
defense enzymes, such as catalase (CATA), glutathione
peroxidases (GPX1) and peroxiredoxins (PRDX2) (Table I).

The HCCs also showed an increased protein
ubiquitination/degradation signature, with upregulation of
proteins belonging to the ubiquitin-mediated degradation
machinery [e.g. 26S protease regulatory subunit 6B
(PSMB6B), 26S protease regulatory subunit 7 (PSMB7), 26S
proteasome non-ATPase regulatory subunit 13 (PSD13) and
proteasome subunit beta type 4 (PSMB4)], as well as of
proteins enzymatically regulating the poly-ubiquitination
process [e.g. ubiquitin-like modifier-activating enzyme 1
(UBA1)]. Increased ubiquitin immunopositivity has also
been observed in human HCC samples, and was predictive
for HCC re-occurrence (58).

Thus, as a first conclusion, our study confirmed many of
previously found protein HCC biomarkers. Such a successful
reproduction of previously reported results clearly underscores
the applicability of the approach. Moreover, it demonstrates the
suitability of spontaneous HCC development in Ppp2r5d KO
mice as a new valuable murine hepatocarcinogenesis model
that captures many characteristics of the human disease.
In addition, however, our study also identified many previously unreported molecules that might theoretically have significant roles in HCC tumorigenesis or progression and become novel HCC biomarkers (Table I, last column, ‘NEW’). For us, the most suitable candidates for further validation seem to be the three proteins categorized as ‘cell cycle’ proteins [coiled-coiled domain protein 15 (CCDC15), protein ELYS (ELYS) and histone-binding protein RBBP7 (RBBP7)], which were all significantly up-regulated in the HCCs (2.51- to 4.92-fold); the cytoskeletal protein desmin (3.51-fold up-regulated); phosphoglucomutase-2 (PGM2) (2.95-fold down-regulated), confirmed by immunoblotting; several liver metabolic enzymes [e.g. adipocyte plasma-membrane associated protein (APMAP) and guanine deaminase (GUAD), 3.44- and 3.09-fold down-regulated]; several mitochondrial enzymes, particularly NADH dehydrogenase flavoprotein 2 (35.28-fold up-regulated); elf4B and WDR12 (up-regulated 2.14- and 2.85-fold); receptor-linked proteins Rab GDP dissociation inhibitor alpha (GDIA), GDIB, V-type protein ATPase catalytic subunit A (VATA) and VATB2 (up-regulated 1.52- to 5.52-fold); thyroglobulin (5.74-fold down-regulated); and some ‘unclassified proteins’ (e.g. histone acetyltransferase MYST3, 3-fold up-regulated). Further validation and investigation of these proteins in human HCC samples may eventually result in the discovery of new molecular targets for therapy, or biomarkers for early detection or prognosis.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors’ Contributions

C.L. performed all mouse-related experiments, tissue isolation, protein extraction, MS sample preparation and overall data analysis; G.B.F. performed 2D gel electrophoresis and 2D-DIGE-related data analysis (spot picking; clustering); J.D.O. performed and analyzed histopathologic analysis of all liver tissues; R.D.V. performed electron microscopy; L.O and C.M. supervised 2D-DIGE analysis (spot picking; clustering); J.D.O. performed and analyzed data processing (Mascot search and protein identifications); VJ and EW designed and supervised the study, and wrote the article.

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