

Differential Proteomic Analysis of Hepatocellular Carcinomas from *Ppp2r5d* Knockout Mice and Normal (Knockout) Livers

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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

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growth factor receptor (MET), transforming growth factor β (TGF β), 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 [myelocytomatosis oncogene (*MYC*)]. Tumors from the second class are less aggressive, better differentiated and are associated with a better outcome. They feature activated WNT signaling (mostly *via* β 1-catenin gene (*CTNNB1*) mutations) or chromosome 7 gain [epidermal growth factor receptor gene (*EGFR*)]. The most prevalent mutations in HCC pathogenesis include those in the telomerase reverse transcriptase gene (*TERT*) promoter, tumor protein p53 gene (*TP53*), *CTNNB1*, axin1 gene (*AXIN1*), and AT-rich interaction domain 1A and 2 genes (*ARID1A* and *ARID2*) (10, 11). Up to 10 different mutational signatures associated with HCC have been identified in various studies [reviewed in (8)]. Additional proteomics studies have further contributed to HCC classification and biomarker discovery (12-33). Disappointingly, however, these insights have not yet resulted in improved, molecular-targeted therapies.

To help unraveling the molecular mechanisms underlying hepatocarcinogenesis in different pre-clinical settings *in vivo*, rodent HCC models remain valuable tools. This is especially true for models in which the human pathology is well mimicked, and considering that available human HCC tissues can be limiting. In addition to chemically or virally induced liver cancer models, several genetically engineered transgenic, knockout (KO) or knockin (KI) mouse models of HCC have been described, each featuring its own specific tumor initiation/progression, tumor microenvironment and mechanistic characteristics [reviewed in (34, 35)]. These models have significantly contributed to identifying molecular signatures relevant to HCC *via* cross-species comparative and integrative analyses with human data. Moreover, they have facilitated the discovery of potential biomarkers for diagnostic or prognostic purposes.

Recently, we described significantly increased spontaneous HCC development, arising within a largely normal liver context (36), in *Ppp2r5d* KO mice. These mice lack expression of the regulatory B56 δ subunit of protein phosphatase 2A (PP2A) (37), a large family of predominantly tumor-suppressive Ser/Thr protein phosphatases (38, 39) regulating cell signaling processes in many organs and tissues (40). Tumor-suppressive PP2A holoenzymes negatively control a large variety of oncogenic pathways, relevant for HCC, including phosphatidylinositol 3-kinase (PI3K)/AKT, mTOR/p70 ribosomal S6 Kinase (p70 S6K), MAP-extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) and WNT signaling (41).

Mechanistically, we identified lack of PP2A-B56 δ -driven glycogen synthase kinase-3 β (GSK-3 β) dephosphorylation on Ser9 (resulting in constitutive GSK-3 β inactivation) as the likely tumor-predisposing factor, which affected the GSK-3 β 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-3 β 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, PP2A-B56 δ 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 B56 δ KO HCC samples, paired non-cancerous B56 δ 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 KU 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/6;129 - 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 fixed in 4% paraformaldehyde at 4°C overnight, washed the next day and stored in 70% ethanol until paraffin embedding; another part was snap-frozen in liquid nitrogen and kept at -80°C until further use.

Histopathology. Hematoxylin/eosin-stained sections (4 μ m) of paraffin-embedded liver 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 lysed 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>).

Spot digestion and protein identification by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF analysis. A pick list was generated and exported into Spot Picker V1.20 software operating the Ettan Spot Picker (GE Healthcare). Spots were picked in MilliQ water, transferred to 100 µl of fixation solution (50% methanol, 5% acetic acid and 45% MilliQ) and rinsed three times with MilliQ water and three times with acetonitrile (ACN) (Sigma-Aldrich, St. Louis, MO, USA). The gel pieces were hydrated in a 100 mM NH₄HCO₃ solution for 10 min followed by a dehydration step in 100% ACN for 10 min with vigorous vortexing. This step was repeated twice prior to dehydrating the gel pieces in a SpeedVac (Thermo Fisher Scientific, Bremen, Germany). The gel pieces were rehydrated in digestion buffer [50 mM

NH₄HCO₃, 5 mM CaCl₂, 5 ng/µl of sequencing grade modified trypsin (Promega, Madison, WI, USA)] on ice for 45 min, followed by overnight incubation at 37°C. The resulting peptides were extracted out of the gel plugs in four steps: once with 50 mM NH₄HCO₃, twice with 50% ACN/5% formic acid, and once with 95% ACN/5% formic acid (30 min each). Supernatants were dried in a SpeedVac. Upon concentrating and desalting the tryptic fragments using ZipTip C-18 (Millipore, Milford, MA, USA), the samples were mixed in a 1:1 ratio with α-cyano-4-hydroxycinnamic acid matrix (saturated solution in 50% ACN/2.5% trifluoroacetic acid in HPLC water), spotted onto the MALDI target plate, and allowed to air dry. MS/MS analyses were performed on a 4800 MALDI TOF/TOF (Applied Biosystems, Waltham, MA, USA). Measurements were taken in the positive ion mode between 900 and 3000 *m/z*. Sequences were automatically acquired by scanning first in MS mode and selecting the 15 most intense ions for MS/MS using an exclusion list of peaks arising from tryptic autodigestion. Air was used as the collision gas, whereas the collision energy was adapted automatically. Data interpretation was carried out using the GPS Explorer software and database searching was carried out using MASCOT. MS/MS searches were conducted with the following settings: Swiss-Prot-TrEMBL as databases, taxonomy *Mus musculus*, MS/MS tolerance for precursor ions was set on 0.2 Da and for fragment ions 0.4 Da, methionine oxidation as variable modification, and carbamidomethylation of cysteine as fixed modification. Only peptides with MASCOT scores above the identity threshold and probability-based MOWSE scores greater than the given cut-off value for MS/MS fragmentation data were retained and taken as significant (*p*<0.05). Using these parameters, 118 picked spots could be linked to at least one reliable protein identification (Supplementary Table: available at <https://gbiomed.kuleuven.be/lambrecht-cgp>). For proteins confirmed to be present within different spots, either in horizontal spot ‘trains’ or dispersed over the whole gel map (32 in total), an average relative abundance value was calculated, keeping up-regulated values separate from down-regulated ones (when applicable). These average values were used for eventual representation in Table I.

Immunoblotting. Protein lysates were resolved on 4-12% 2,2-bis(hydroxymethyl)-2,2',2''-nitrioltriethanol, 2-bis(2-hydroxyethyl) amino-2-(hydroxymethyl)-1,3-propanediol, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)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(hydroxymethyl)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-B56δ (46) (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 Dilworth, 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

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
				HCC/KO	KO/WT			
<i>Ppp2r1A</i>	2AAA	Q76MZ3	PP2A 65 kDa regulatory subunit A α or A β	2.24	-1.15	2	Cell cycle	Conflict (25)
<i>Ccdc15</i>	CCDC15	Q8C9M2	Coiled-coiled domain protein15	4.92	-1.55	1	Cell cycle	NEW
<i>Ahctf1</i>	ELYS	Q8CJF7	Protein ELYS	2.88	-1.32	1	Cell cycle	NEW
<i>Rbbp7</i>	RBBP7	Q60973	Histone-binding protein RBBP7	2.51	-1.02	1	Cell cycle	NEW
<i>Serpina1b</i>	A1AT2	P22599	Alpha-1-antitrypsin 1-2	2.68	-1.64	1	Coagulation	(13, 14, 22, 25-27, 32)
<i>Serpinc1</i>	ANT3	P32261	Antithrombin-III	2.06	-1.14	3	Coagulation	NEW
<i>Anxa3</i>	ANXA3	O35639	Annexin A3	1.9	1.28	1	Coagulation	(15)
<i>Fgg</i>	FIBG	Q8VCM7	Fibrinogen gamma chain	7.28	-1.1	1	Coagulation	(13)
<i>Tcp1</i>	TCPA	P11983	T-Complex protein 1 subunit alpha	-2.56	-1.83	1	Coagulation	(15)
<i>Cct8</i>	TCPQ	P42932	T-Complex protein 1 subunit theta	2.13	-1.28	2	Coagulation	NEW
<i>Anxa11</i>	ANXA11	P97384	Annexin A11	1.9	1.28	1	Cytoskeleton	NEW
<i>Anxa4</i>	ANXA4	P97429	Annexin A4	3.91	-1.48	1	Cytoskeleton	Conflict (20)
<i>Cdh23</i>	CAD23	Q99PF4	Cadherin-23	1.83	-1.07	1	Cytoskeleton	NEW
<i>Col6a2</i>	CO6A2	Q02788	Collagen alpha-2 (VI) chain	-3.7	1.71	1	Cytoskeleton	NEW
<i>Des</i>	DESM	P31001	Desmin	3.51	-1.19	1	Cytoskeleton	NEW
<i>Ezr</i>	EZRI	P26040	Ezrin	-1.5	-1.01	1	Cytoskeleton	NEW
<i>Krt18</i>	K1C18	P05784	Keratin, type I cytoskeletal 18	3.69	-1.28	1	Cytoskeleton	(19, 26, 31)
<i>Krt2</i>	K22E	Q3TTY5	Keratin, type II cytoskeletal 2 epidermal	3.51	-1.19	1	Cytoskeleton	(27)
<i>Krt77</i>	K2C1B	Q6IFZ6	Keratin, type II cytoskeletal 1b	3.51	-1.19	1	Cytoskeleton	(27)
<i>Krt8</i>	K2C8	P11679	Keratin type II, cytoskeletal 8	4.03	-1.19	5	Cytoskeleton	(15, 17, 19, 24)
<i>Rdx</i>	RADI	P26043	Radixin	-1.93	-1.03	1	Cytoskeleton	(19)
<i>Tuba1a</i>	TBA1A	P68369	Tubulin alpha-1A chain	2.58	-1.02	3	Cytoskeleton	NEW
<i>Tuba1a</i>	TBA1A	P68369	Tubulin alpha-1A chain	-2.66	1.33	1	Cytoskeleton	
<i>Tubb2a</i>	TBB2A	Q7TMM9	Tubulin beta 2A chain	2.76	-1.03	2	Cytoskeleton	(25, 33)
<i>Tubb2c</i>	TBB2C	AAH05547	Tubulin beta 2C chain	2.76	-1.03	2	Cytoskeleton	(25, 26)
<i>Tubb5</i>	TBB5	P99024	Tubulin beta-5 chain	1.94	-1.06	1	Cytoskeleton	(25)
<i>Vim</i>	VIME	P20152	Vimentin	2.47	-1.03	2	Cytoskeleton	(17, 26, 27)
<i>Wdr1</i>	WDR1	O88342	WD Repeat-containing protein 1	1.17	-1.35	2	Cytoskeleton	
<i>Calr</i>	CALR	P14211	Calreticulin	2.41	-1.25	1	ER/protein folding	(31)
<i>Dnajc3</i>	DNAJC3	Q91YW3	DnaJ homolog subfamily C member 3	5.39	-1.25	1	ER/protein folding	(21)
<i>Hsp90b1</i>	ENPL	P08113	Endoplasmic	3.4	-1.24	1	ER/protein folding	(21, 27)
<i>Erp44</i>	ERP44	Q9D1Q6	Endoplasmic reticulum resident protein 44	-1.17	-1.41	1	ER/protein folding	
<i>Hspa5</i>	GRP78	P20029	78-kDa Glucose-regulated protein	3.46	-1.41	2	ER/protein folding	(14, 22, 24, 26, 27, 31)
<i>Hspa8</i>	HSP7C	P63017	Heat-shock cognate 71-kDa protein	4.08	-1.08	1	ER/protein folding	(21, 23, 26, 27, 31)
<i>P4hb</i>	PDIA1	P09103	Protein disulfide-isomerase	3.25	-1.63	5	ER/protein folding	(19, 20, 26, 27, 31)
<i>Pdia3</i>	PDIA3	P27773	Protein disulfide-isomerase A3	2.80	-1.32	4	ER/protein folding	(17, 31)
<i>Txndc5</i>	TXND5	Q91W90	Thioredoxin domain-containing protein 5	-1.17	-1.41	1	ER/protein folding	
<i>Eno1</i>	ENOA	P17182	Alpha-enolase	-2.11	1.23	2	Glucose metabolism	(17, 26)
<i>Eno3</i>	ENOB	P21550	Beta-enolase	-1.76	1.19	1	Glucose metabolism	NEW
<i>Fbp1</i>	FBP1	Q9QXD6	Fructose-1,6-bisphosphatase 1	-1.4	-1.46	2	Glucose metabolism	(12, 13, 15, 17-20, 22, 26, 27, 31-33)
<i>Pgk1</i>	PGK1	P09411	Phosphoglycerate kinase 1	-3.4	1.27	1	Glucose metabolism	(26, 31)
<i>Pgm2</i>	PGM2	Q7TSV4	Phosphoglucomutase-2	-2.95	-1.08	1	Glucose metabolism	NEW
<i>Tpi1</i>	TPIS	P17751	Triosephosphate isomerase	-2.05	-1.12	1	Glucose metabolism	(21)
<i>Apoa1</i>	APOA1	Q00623	Apolipoprotein A-1	15.57	-0.83	5	Lipids	(22, 25, 31)
<i>Cpne1</i>	CPNE1	Q8C166	Copine-1	1.86	-1.29	1	Lipids	NEW
<i>Aspg</i>	LPP60	A0JNU3	60-kDa Lysophospholipase	2.5	-1.42	1	Lipids	(21)

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
				HCC/KO	KO/WT			
<i>Got1</i>	AATC	P05201	Aspartate aminotransferase, cytoplasmic	1.7	-1.14	1	Liver metabolism	Conflict (20)
<i>Adk</i>	ADK	P55264	Adenosine kinase	-2.38	-1.24	2	Liver metabolism	(25)
<i>Apmmap</i>	APMAP	Q9D7N9	Adipocyte plasma-membrane associated protein	-3.44	1.77	1	Liver metabolism	NEW
<i>Ass1</i>	ASSY	P16460	Argininosuccinate synthase	-3.4	1.27	1	Liver metabolism	(17, 20, 26, 28, 31)
<i>Bhmt</i>	BHMT1	O35490	Betaine homocysteine S-methyltransferase 1	-3.4	1.27	1	Liver metabolism	13, 14, 18, 20, 21, 26, 29, 30, 33)
<i>Cndp2</i>	CNDP2	Q9D1A2	Cytosolic non-specific dipeptidase	2.35	1.18	1	Liver metabolism	NEW
<i>Dpys</i>	DPYS	Q9EQF5	Dihydropyrimidinase	-1.73	-1.1	1	Liver metabolism	NEW
<i>Ces7</i>	EST7	EDL11102	Carboxylesterase 7	2.94	-1.32	2	Liver metabolism	NEW
<i>Aldh1l1</i>	FTHFD	Q8R0Y6	10-formyltetrahydrofolate dehydrogenase	-2.98	1.13	2	Liver metabolism	(15, 25, 27)
<i>Gnmt</i>	GNMT	Q9QXF8	Glycine N-methyl transferase	-3.62	1.09	1	Liver metabolism	(13-15, 22, 25, 26, 31)
<i>Gda</i>	GUAD	Q9R111	Guanine deaminase	-3.09	1.9	1	Liver metabolism	NEW
<i>Ephx2</i>	HYES	P34914	Epoxide hydrolase 2	2.3	-1.27	1	Liver metabolism	(31)
<i>Mdh1</i>	MDHC	P14152	Malate dehydrogenase, cytoplasmic	-1.92	1.07	1	Liver metabolism	(15, 18, 22, 25)
<i>Mat1a</i>	METK1	Q91X83	S-Adenosylmethionine synthase isoform type-1	7.28	-1.1	1	Liver metabolism	(12, 31)
<i>Mat1a</i>	METK1	Q91X83	S-Adenosylmethionine synthase isoform type-1	-3.09	1.9	1	Liver metabolism	(13, 14)
<i>P4ha2</i>	P4HA2	Q60716	Prolyl 4-hydroxylase subunit alpha 2	2.88	-1.32	1	Liver metabolism	(18)
<i>Pbld1</i>	PBLD1	Q9DCG6	Phenazine biosynthesis-like containing protein 1	-2	1	1	Liver metabolism	(26)
<i>Pah</i>	PH4H	P16331	Phenylalanine-4-hydroxylase	3.59	1.15	1	Liver metabolism	(15, 19)
<i>Pon3</i>	PON3	Q62087	Serum paraoxonase/lactonase 3	4	1.05	1	Liver metabolism	NEW
<i>Ctps1</i>	PYRG1	P70698	CTP synthase 1	2.04	-1.06	1	Liver metabolism	NEW
<i>Ahcy</i>	SAHH	P50247	Adenosyl homocysteinase	-2.41	1.33	1	Liver metabolism	(13, 22, 31)
<i>Uap1l1</i>	UAP1L	Q3TW96	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	4.6	1.2	1	Liver metabolism	(15)
<i>Acsml</i>	ACSM1	Q91VA0	Acyl-coenzyme A synthetase ACSM1, mitochondrial	-1.15	-1.35	1	Mitochondria	
<i>Metap1d</i>	AMP1D	Q9CPW9	Methionine aminopeptidase 1D, mitochondrial	1.43	-1.07	1	Mitochondria	NEW
<i>Atp5pd</i>	ATP5H	Q9DCX2	ATP synthase subunit d, mitochondrial	-1.1	-1.19	1	Mitochondria	NEW
<i>Hspd1</i>	CH60	P63038	60-kDa Heat-shock protein, mitochondrial	4.6	1.2	1	Mitochondria	(23)
<i>Cps1</i>	CPS1	Q8C196	Carbamoyl-phosphate synthase, mitochondrial	-2.36	1.71	12	Mitochondria	(14, 17, 19, 22, 25, 26, 29, 31)
<i>Sdha</i>	SDHA	Q8K2B3	Succinate dehydrogenase flavoprotein subunit, mitochondrial	-1.39	-1.36	3	Mitochondria	NEW
<i>Hspa9</i>	GRP75	P38647	Stress-70 protein, mitochondrial	8.54	-1.35	2	Mitochondria	(22, 26)
<i>Hmgcs2</i>	HMGCS2	P54869	Hydroxymethylglutaryl-CoA synthase, mitochondrial	1.7	-1.14	1	Mitochondria	(19, 23, 29)
<i>Immt</i>	IMMT	Q8CAQ8	Mitochondrial inner membrane protein	-1.5	-1.01	1	Mitochondria	NEW
<i>Mccc1</i>	MCCA	Q99MR8	Methylcrotonoyl-CoA carboxylase subunit α, mitochondrial	5.92	1.52	1	Mitochondria	(21)
<i>Ndufs2</i>	NDUS2	Q91WD5	NADH dehydrogenase iron-sulfur protein 2, mitochondrial	-3.44	1.77	1	Mitochondria	NEW
<i>Ndufv2</i>	NDUFV2	Q9D6J6	NADH dehydrogenase flavoprotein 2, mitochondrial	35.28	-1.3	1	Mitochondria	NEW
<i>Oat</i>	OAT	P29758	Ornithine aminotransferase, mitochondrial	1.52	-1.19	1	Mitochondria	(15, 19, 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
				HCC/KO	KO/WT			
<i>Ogdh</i>	ODO1	Q60597	2-oxoglutarate dehydrogenase, mitochondrial	-1.81	1.66	1	Mitochondria	
<i>Dlst</i>	ODO2	Q9D2G2	Dihydrolipoylysine-residue succinyl-transferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.59	-1.17	1	Mitochondria	
<i>Otc</i>	OTC	P11725	Ornithine carbamoyl transferase, mitochondrial	-2.54	1.01	1	Mitochondria	(22, 25, 31)
<i>Pc</i>	PYC	Q05920	Pyruvate carboxylase, mitochondrial	-2.33	1.18	2	Mitochondria	NEW
<i>Sardh</i>	SARDH	Q99LB7	Sarcosine dehydrogenase, mitochondrial	-2.98	1.08	4	Mitochondria	(19, 22, 25-27, 31)
<i>Stoml2</i>	STML2	Q99JB2	Stomatin-like protein 2	-3.40	1.27	1	Mitochondria	NEW
<i>Sucla2</i>	SUCB1	Q9Z2I9	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	1.19	-1.35	1	Mitochondria	
<i>Acat1</i>	THIL	Q8QZT1	Acetyl-CoA acetyltransferase, mitochondrial	-3.11	1.06	1	Mitochondria	(13-15, 17)
<i>Uqcrrf1</i>	UQCRFS1	Q9CR68	Cytochrome B-C1 complex subunit Rieske, mitochondrial	-2	1	1	Mitochondria	NEW
<i>Hnrnpk</i>	HNRPK	P61979	Heterogenous nuclear ribonucleoprotein K	2.15	-1.28	3	mRNA process./translat.	(15)
<i>Eif4b</i>	IF4B	Q8BGD9	Eukaryotic translation initiation factor 4B	2.14	-1.37	1	mRNA process./translat.	NEW
<i>Rplp0</i>	RLA0	P14869	60S Acidic ribosomal protein P0	1.43	-1.07	1	mRNA process./translat.	(28)
<i>Ruvbl2</i>	RUVB2	Q9WTM5	RuvB-like 2	7.28	-1.1	1	mRNA process./translat.	(16)
<i>Hars1</i>	SYHC	Q61035	Histidyl-tRNA synthetase, cytoplasmic	2.35	1.18	1	mRNA process./translat.	NEW
<i>Gars1</i>	SYG	P41250	Glycyl-tRNA synthetase	2.43	1.09	1	mRNA process./translat.	(15)
<i>Wdr12</i>	WDR12	Q9JJA4	Ribosome biogenesis protein WDR12	2.85	-1.16	1	mRNA process./translat.	NEW
<i>Abhd14b</i>	ABHEB	Q8VCR7	α/β Hydrolase domain-containing protein 14B	-2.34	-1.15	1	Not classified	(17, 25, 31)
<i>Crtap</i>	CRTAP	Q9CYD3	Cartilage-associated protein	-3.09	1.9	1	Not classified	NEW
<i>Efh2</i>	EFHD2	Q9D8Y0	EF-Hand domain-containing protein D2	2.35	-1	1	Not classified	NEW
<i>Ahsg</i>	FETUA	P29699	Alpha-2-HS-glycoprotein	4.27	-1.08	1	Not classified	NEW
<i>H2-K1</i>	HA1B	P01901	H-2 Class I histocompatibility antigen, K-B α chain	-3.09	1.9	1	Not classified	NEW
N/A	IGHA	P01878	Ig Alpha chain C region	2.02	-1.01	1	Not classified	NEW
<i>Iigp1</i>	IIGP1	Q9QZ85	Interferon-inducible GTPase 1	1.59	-1.17	1	Not classified	
<i>Gaa</i>	LYAG	P70699	Lysosomal alpha-glucosidase	2.5	-1.42	1	Not classified	(17)
<i>Kat6a</i>	MYST3	Q8BZ21	Histone acetyltransferase KAT6A or MYST3	3	-1.14	1	Not classified	NEW
<i>Pebp1</i>	PEBP1	P70296	Phosphatidylethanolamine-binding protein 1	1.51	-1.15	1	Not classified	(25)
<i>Rgn</i>	RGN	Q64374	Regucalcin	-5.14	1.03	3	Not classified	(13, 15, 22, 25, 31, 32)
<i>Rgn</i>	RGN	Q64374	Regucalcin	8.4	-1.18	1	Not classified	(19)
<i>Gdi1</i>	GDIA	P50396	Rab GDP dissociation inhibitor alpha	2.09	-1.2	2	Receptor linked	NEW
<i>Gdi2</i>	GDIB	Q61598	Rab GDP dissociation inhibitor beta	1.52	-1.19	1	Receptor linked	NEW
<i>Atp6v1a</i>	VATA	P50516	V-Type proton ATPase catalytic subunit A	5.52	1.22	1	Receptor linked	NEW
<i>Atp6v1b2</i>	VATB2	P62814	V-Type proton ATPase subunit B, brain isoform	2.35	1.18	1	Receptor linked	NEW
<i>Cat</i>	CATA	P24270	Catalase	-2.19	-1.07	2	Redox regulation	(13-15, 20, 22, 32)
<i>Dhdh</i>	DHDH	Q9DBB8	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	-1.4	-1.33	1	Redox regulation	(15)
<i>Gpx1</i>	GPX1	P11352	Glutathione peroxidase 1	-1.98	-1.10	3	Redox regulation	(15, 25, 31)
<i>Grhpr</i>	GRHPR	Q91Z53	Glyoxylate reductase/hydroxy-pyruvate reductase	1.7	-1.14	1	Redox regulation	Conflict (26)
<i>Prdx2</i>	PRDX2	Q61171	Peroxioredoxin 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
				HCC/KO	KO/WT			
<i>Alb</i>	ALBU	P07724	Serum albumin	4.94	-1.32	22	Transport	(14, 22, 26, 27, 31)
<i>Ehd4</i>	EHD4	Q9EQP2	EH domain-containing protein 4	-1.2	-1.33	1	Transport	
<i>Hpx</i>	HEMO	Q91X72	Hemopexin	2.93	-1.15	5	Transport	(25)
<i>Selenbp1</i>	SELENBP1	P17563	Methanethiol oxidase/selenium-binding protein 1	3.60	-1.06	2	Transport	(19)
<i>Snx6</i>	SNX6	Q6P8X1	Sorting nexin-6	1.59	-1.17	1	Transport	
<i>Tg</i>	THYG	O08710	Thyroglobulin	-5.74	1.17	1	Transport	NEW
<i>Tf</i>	TRFE	Q921I1	Serotransferrin	-1.5	-1.01	1	Transport	(13, 32)
<i>Tf</i>	TRFE	Q921I1	Serotransferrin	5.92	1.52	1	Transport	(15, 22, 26, 31)
<i>Gc</i>	VTDB	P21614	Vitamin D-binding protein	4.27	-1.08	1	Transport	(22)
<i>Cops4</i>	CSN4	O88544	COP9 Signalosome complex subunit 4	4	1.05	1	Ubiquitination/degradation	NEW
<i>Psmc4</i>	PRS6B	P54775	26S Proteasome regulatory subunit 6B	3.69	-1.28	1	Ubiquitination/degradation	NEW
<i>Psmc2</i>	PRS7	P46471	26S Proteasome regulatory subunit 7	1.52	-1.19	1	Ubiquitination/degradation	NEW
<i>Psmc13</i>	PSD13	Q9WVJ2	26S Proteasome non-ATPase regulatory subunit 13	1.50	-1.06	1	Ubiquitination/degradation	NEW
<i>Psmb4</i>	PSMB4	P99026	Proteasome subunit beta type 4	1.97	1	1	Ubiquitination/degradation	(31)
<i>Uba1</i>	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-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 Western Bright ECL detection kit (Advansta, San Jose, CA, USA). All 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 murine 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,

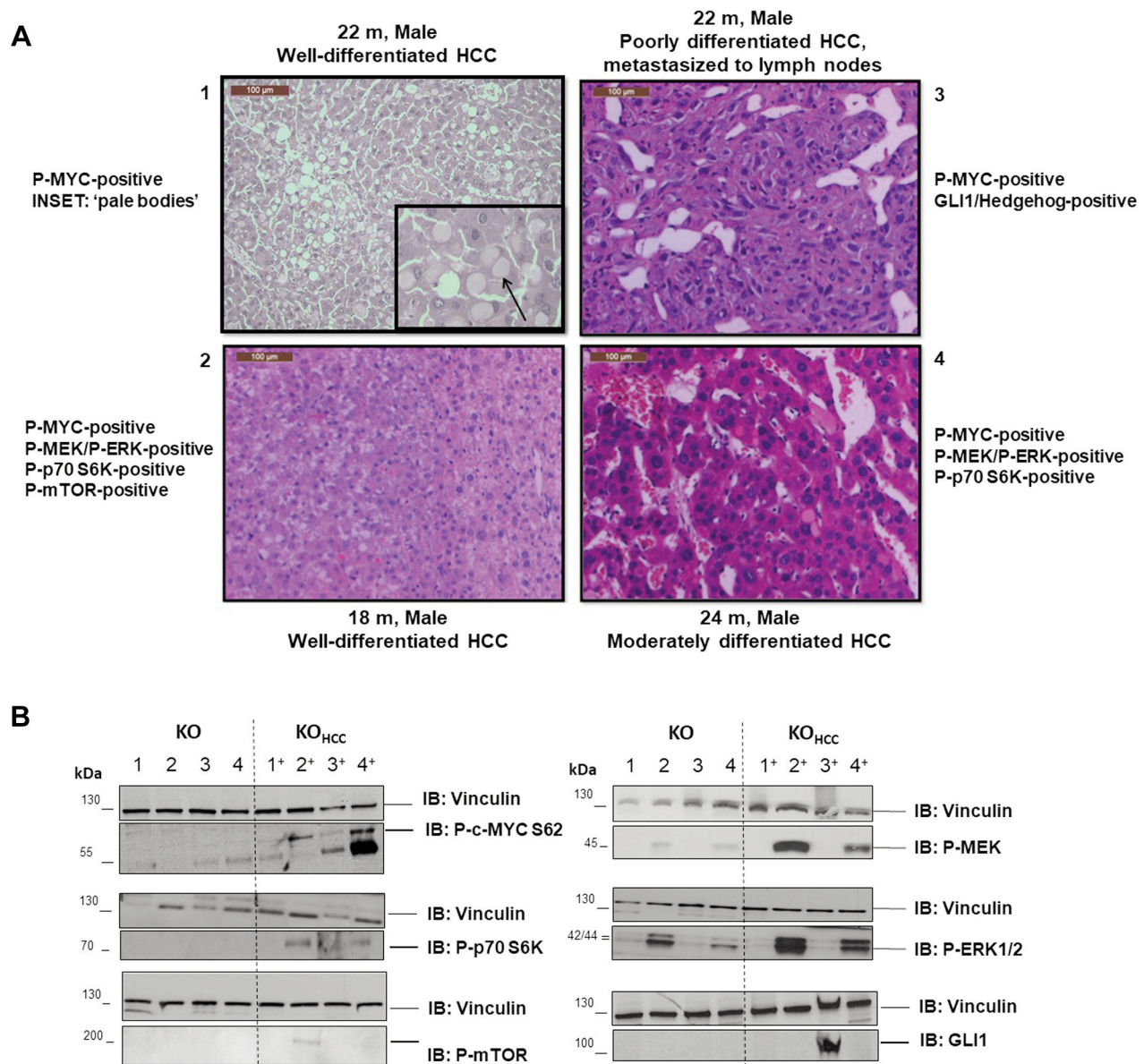


Figure 1. Histopathology of murine protein phosphatase 2A regulatory subunit B56 δ (Ppp2r5d) knockout (KO) hepatocellular carcinoma (HCC) samples used for proteomics analysis. A: Hematoxylin/eosin-stained sections of the analyzed HCC tissues (n=4) were evaluated by an experienced liver pathologist, and diagnosed as well-differentiated (samples 1 and 2), moderately differentiated (sample 4) and poorly differentiated (sample 3). The latter tumor had been metastasized to the lymph nodes and was positive for expression of the GLI family zinc finger 1 (GLI1) Hedgehog signaling transcription factor. Sample 1 showed 'pale bodies' (arrow in inset) of unknown significance. All scale bars: 100 μ m. B: Immunoblotting analysis with indicated antibodies of the upper HCC samples and adjacent non-cancerous liver samples of the same mice (paired analysis) showed sporadic activation of MAP-extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70 S6K) and Hedgehog signaling, while c-MYC S62 phosphorylation was up-regulated in all HCC samples analyzed.

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 RNAseq 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 endoplasmic reticulum (RER), containing amorphous, granular material (Figure 6A), most likely representing fibrinogen deposits. Fibrinogen is a protein

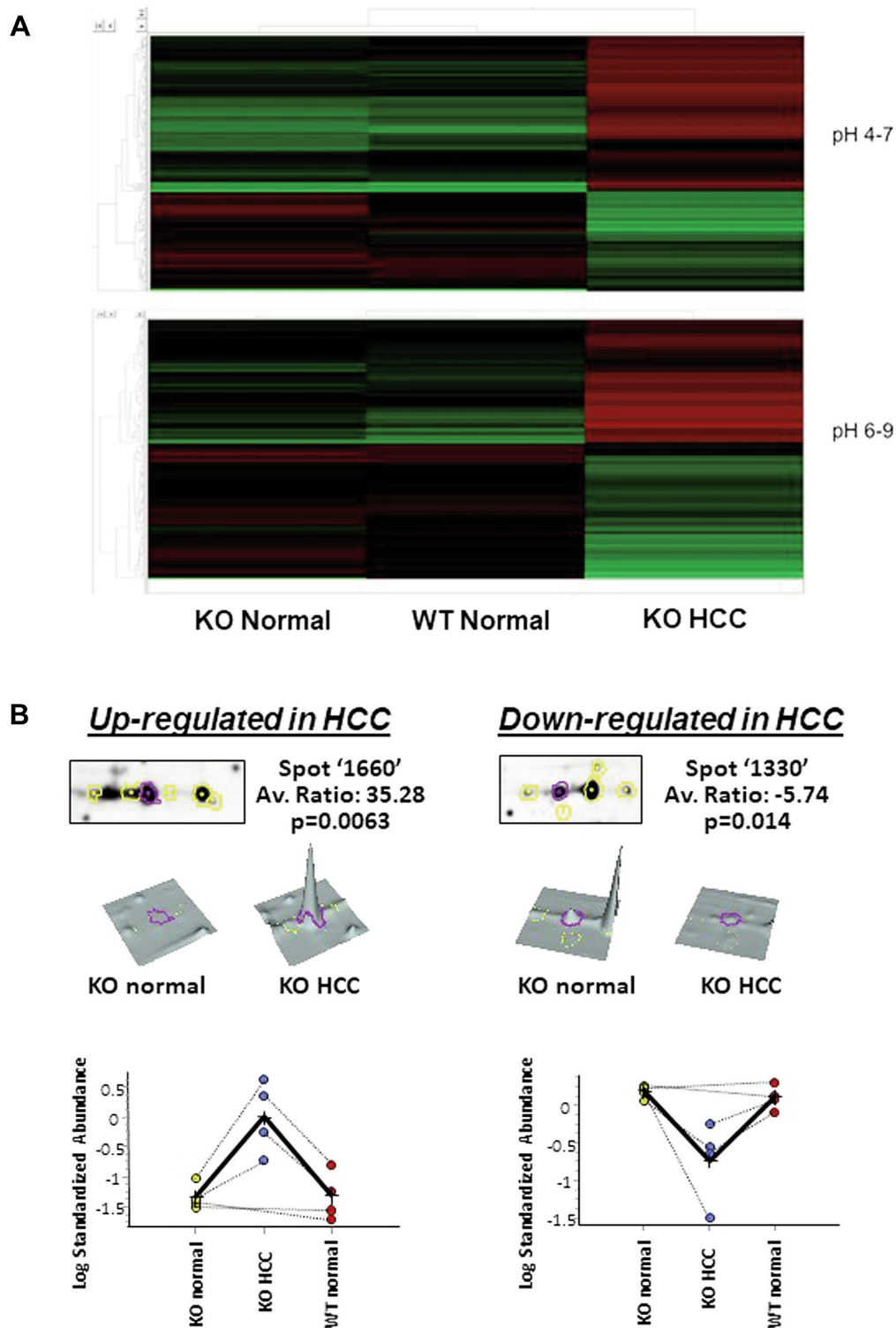


Figure 2. Analysis of differentially regulated spots following two-dimensional differential gel electrophoresis (2D-DIGE) A: Cluster analysis of differentially regulated 'spots' between wild-type (WT), protein phosphatase 2A regulatory B56 δ subunit (Ppp2r5d) knockout (KO) non-cancerous, and Ppp2r5d 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.

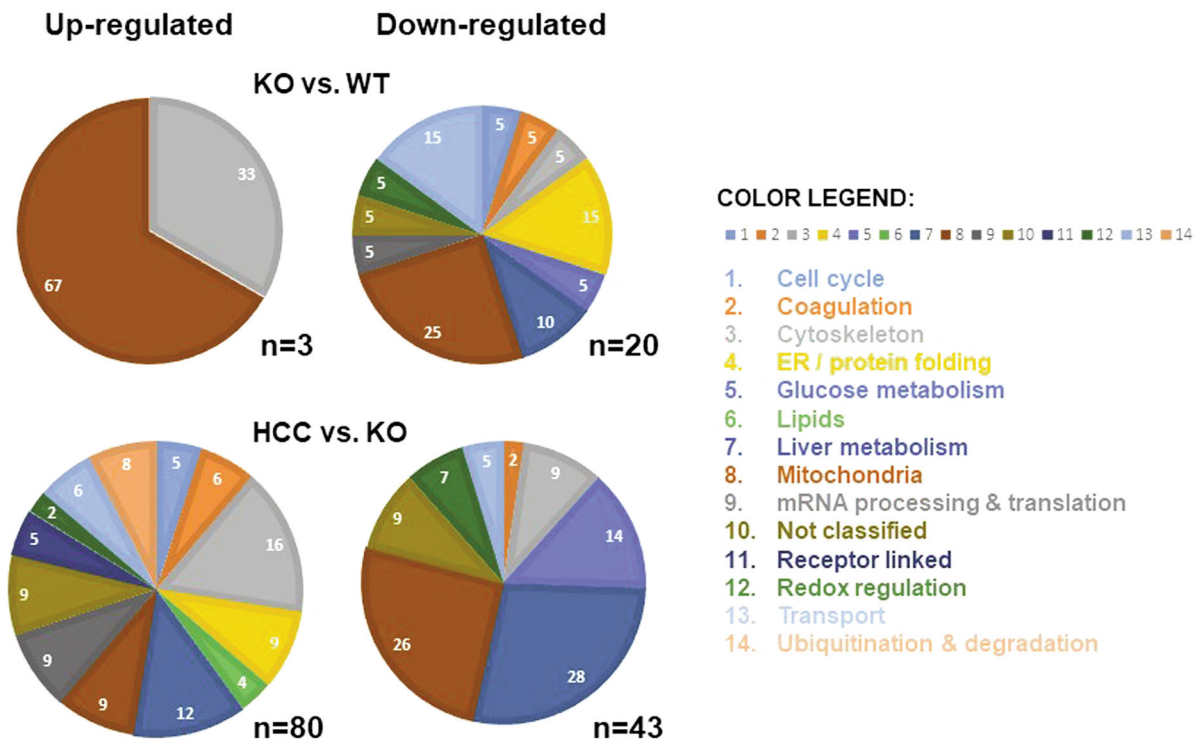


Figure 3. Overview of differentially regulated proteins, grouped into 14 functional classes based on Gene Ontology (GO) annotation. n: Absolute number of up-regulated or down-regulated proteins found; the numbers within the diagrams indicate percentages. In the hepatocellular carcinoma (HCC) samples, the most frequently altered proteins belonged to liver metabolism (28% of those down-regulated in HCC, and 12% of those up-regulated in HCC), mitochondria (26% of those down-regulated in HCC, and 9% of those up-regulated in HCC), glucose metabolism (14% of those down-regulated in HCC) and cytoskeleton (16% of those up-regulated in HCC, and 9% of those down-regulated in HCC).

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 B56d 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

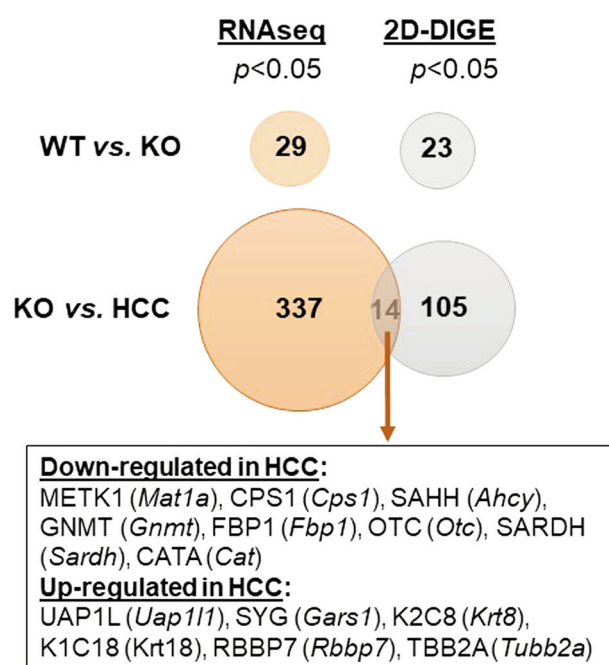


Figure 4. Comparison between the number of differentially expressed mRNAs as determined by RNAseq (36) and as identified in the current study. While there was no overlap between both datasets in the livers from wild-type (WT) vs. non-cancerous knockout (KO) mice, 14 genes/proteins appeared to be commonly 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.

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 proteins/mRNAs commonly found differentially expressed between KO HCC and non-tumor KO tissue, and thus, only 12% of the cancer proteins concordantly regulated at the mRNA level. It is known that proteins with very high or very low pI as well as hydrophobic proteins are in general more difficult to detect by 2D-DIGE, and that there is some bias in favor of cytoplasmic and soluble proteins (18). Moreover, 2D-DIGE is definitely biased towards the identification of medium to highly abundant proteins and does not provide a comprehensive nor representative view of

IPA Summary of Analysis

Top networks		score:
1	Small molecule biochemistry, cancer, reproductive system disease	55
2	Immunological disease, hematological disease, inflammatory disease	46
3	Cellular compromise, organismal injury, abnormalities lipid metabolism	23
4	Lipid metabolism, molecular transport, small molecule biochemistry	23
5	Cell death, tumor morphology, cell-to-cell signaling and interaction	2
Top bio functions		number of molecules
Diseases and disorders		
1	Cancer	38 (1.7E-09 - 3.0E-02)
2	Gastrointestinal disease	22 (1.7E-09 - 3.0E-02)
3	Hematological disease	22 (2.5E-09 - 2.5E-02)
4	Immunological disease	23 (5.3E-08 - 2.6E-02)
5	Inflammatory disease	30 (6.7E-08 - 2.6E-02)
Molecular and cellular functions		
1	Small molecule biochemistry	31 (5.8E-08 - 3.3E-02)
2	Amino acid metabolism	11 (1.9E-06 - 2.5E-02)
3	Cellular assembly and organization	15 (1.7E-05 - 2.9E-02)
4	Cell morphology	8 (5.2E-05 - 3.2E-02)
5	Post-translational modification	13 (5.2E-05 - 2.9E-02)
Physiological system development and function		
1	Hair and skin development and function	4 (1.7E-05 - 2.1E-02)
2	Organismal development	9 (1.7E-05 - 3.1E-02)
3	Hepatic system development and function	2 (5.2E-05 - 3.0E-02)
4	Endocrine system development and function	2 (1.0E-04 - 2.5E-02)
5	Tissue development	11 (7.2E-04 - 3.3E-02)
Top canonical pathways		p-Value:
1	Acute phase response signaling	7.70E-05
2	14-3-3 Mediated signaling	1.14E-04
3	Urea cycle and metabolism of amino groups	1.23E-04
4	Breast cancer regulation by stathmin 1	1.22E-03
5	Methionine metabolism	1.49E-03

Figure 5. In silico pathway analysis of 'cancer proteins'. Ingenuity Pathway Analysis (IPA) of 119 proteins differentially regulated between knockout (KO) hepatocellular carcinoma (HCC) and non-cancerous KO livers, with indication of Top networks, bio-functions and canonical pathways.

the tissue proteome. On the other hand, a change in mRNA level does not always reflect a corresponding change in protein level, especially for proteins with a low turnover. Finally, an obvious difference in protein expression as identified by 2D-DIGE may actually also represent expression of an altered isoform, post-translationally modified or processed form of the protein, which cannot be detected by RNAseq analysis. This all illustrates the complementarity of transcriptomic and proteomic analyses.

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

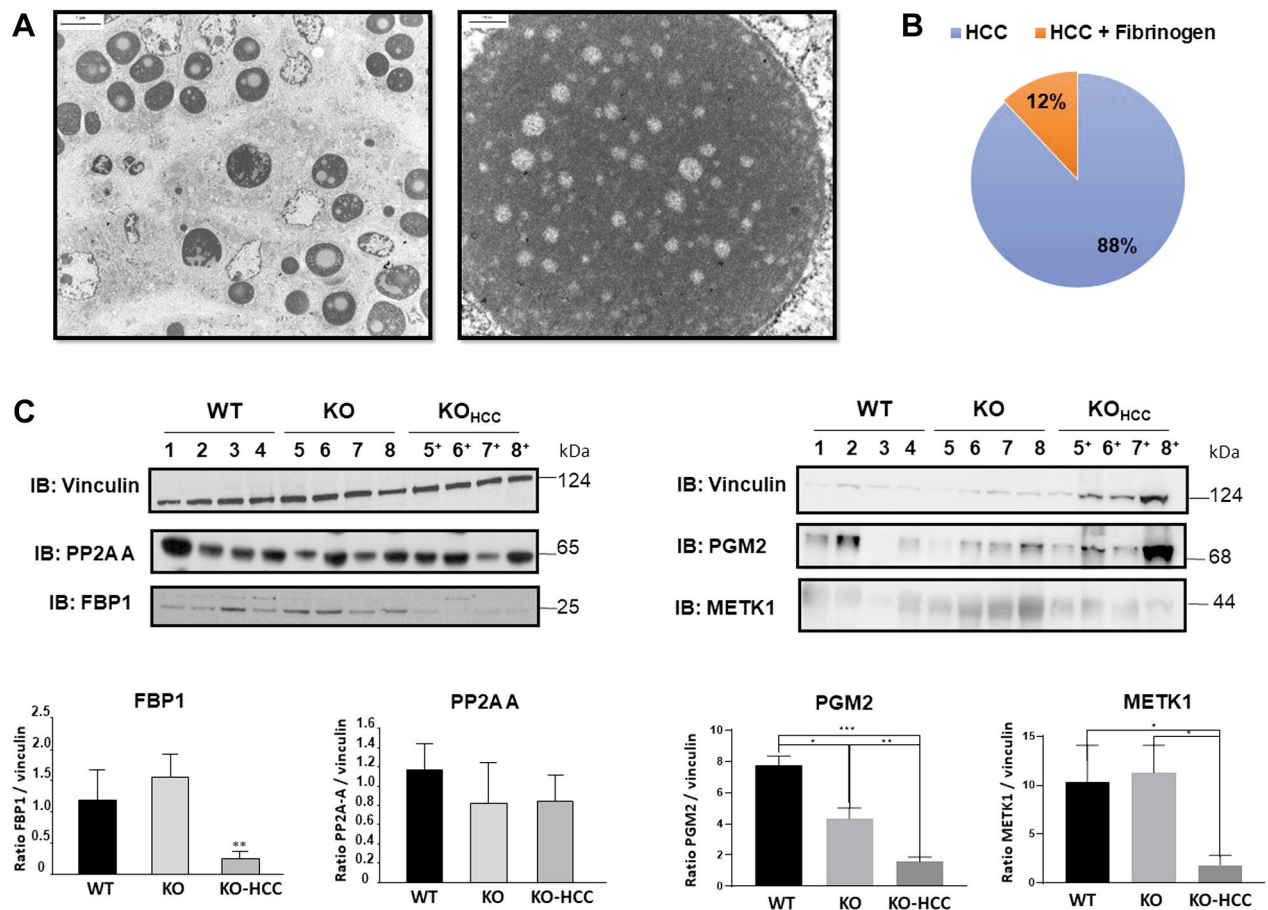


Figure 6. Validation of two-dimensional differential gel electrophoresis (2D-DIGE) results. A: Additional analysis of 'pale body' structures by electron microscopy. B: Frequency of occurrence of fibrinogen deposits in hepatocellular carcinomas (HCCs) from protein phosphatase 2A (PP2A) regulatory B56 δ 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 ($p=0.019$).

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

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*, *Ahcy* and *Gnmt* 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 *Gnmt* 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* (B56 δ) 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 (PRS6B), 26S protease regulatory subunit 7 (PRS7), 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); phosphoglucosyltransferase-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); eIF4B 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); protein degradation-linked proteins COP9 signalosome complex subunit 4 (CSN4), UBA1 and PRS6B (4-, 3.63- and 3.69-fold up-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 immunoblot validation experiments; L.L. performed histopathologic analysis of all liver tissues; R.D.V. performed electron microscopy; R.D. analyzed data by IPA software; L.O. and C.M. supervised 2D-DIGE analysis; E.W. performed MS analysis and data processing (Mascot search and protein identifications); VJ and EW designed and supervised the study, and wrote the article.

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