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

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 [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* β1-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 B56δ 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-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, PP2A-B56δ 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 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 μm) 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 $20,000 \times g$ 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 rehydratation 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 NH_4HCO_3 solution for 10 min followed by a dehydratation 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 NH_4HCO_3, 5 mM $CaCl_2$, 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 NH_4HCO_3, 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 a-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 3000m/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''-nitrilotriethanol, 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-*bis*phosphatase 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			
Ppp2r1A	2AAA	Q76MZ3	PP2A 65 kDa regulatory subunit Aα or Aβ	2.24	−1.15	2	Cell cycle	Conflict (**25**)
Ccdc15	CCDC15	Q8C9M2	Coiled-coiled domain protein15	4.92	−1.55	1	Cell cycle	NEW
Ahctf1	ELYS	Q8CJF7	Protein ELYS	2.88	−1.32	1	Cell cycle	NEW
Rbbp7	RBBP7	Q60973	Histone-binding protein RBBP7	2.51	−1.02	1	Cell cycle	NEW
Serpina1b	A1AT2	P22599	**Alpha-1-antitrypsin 1-2**	2.68	−1.64	1	Coagulation	(**13, 14, 22, 25-27, 32**)
Serpinc1	ANT3	P32261	Antithrombin-III	2.06	−1.14	3	Coagulation	NEW
Anxa3	ANXA3	O35639	**Annexin A3**	1.9	1.28	1	Coagulation	(**15**)
Fgg	FIBG	Q8VCM7	**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	ANXA11	P97384	Annexin A11	1.9	1.28	1	Cytoskeleton	NEW
Anxa4	ANXA4	P97429	Annexin A4	3.91	−1.48	1	Cytoskeleton	Conflict (**20**)
Cdh23	CAD23	Q99PF4	Cadherin-23	1.83	−1.07	1	Cytoskeleton	NEW
Col6a2	CO6A2	Q02788	Collagen alpha-2 (VI) chain	−3.7	1.71	1	Cytoskeleton	NEW
Des	DESM	P31001	Desmin	3.51	−1.19	1	Cytoskeleton	NEW
Ezr	EZRI	P26040	Ezrin	−1.5	−1.01	1	Cytoskeleton	NEW
Krt18	K1C18	P05784	**Keratin, type I cytoskeletal 18**	3.69	−1.28	1	Cytoskeleton	(**19, 26, 31**)
Krt2	K22E	Q3TTY5	**Keratin, type II cytoskeletal 2 epidermal**	3.51	−1.19	1	Cytoskeleton	(**27**)
Krt77	K2C1B	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	RADI	P26043	**Radixin**	−1.93	−1.03	1	Cytoskeleton	(**19**)
Tuba1a	TBA1A	P68369	Tubulin alpha-1A chain	2.58	−1.02	3	Cytoskeleton	NEW
Tuba1a	TBA1A	P68369	Tubulin alpha-1A chain	−2.66	1.33	1	Cytoskeleton	
Tubb2a	TBB2A	Q7TMM9	**Tubulin beta 2A chain**	2.76	−1.03	2	Cytoskeleton	(**25, 33**)
Tubb2c	TBB2C	AAH05547	**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	
Calr	CALR	P14211	**Calreticulin**	2.41	−1.25	1	ER/protein folding	(**31**)
Dnajc3	DNAJC3	Q91YW3	**DnaJ homolog subfamily C member 3**	5.39	−1.25	1	ER/protein folding	(**21**)
Hsp90b1	ENPL	P08113	**Endoplasmin**	3.4	−1.24	1	ER/protein folding	(**21, 27**)
Erp44	ERP44	Q9D1Q6	Endoplasmic reticulum resident protein 44	−1.17	−1.41	1	ER/protein folding	
Hspa5	GRP78	P20029	**78-kDa Glucose-regulated protein**	3.46	−1.41	2	ER/protein folding	(**14, 22, 24, 26, 27, 31**)
Hspa8	HSP7C	P63017	**Heat-shock cognate 71-kDa protein**	4.08	−1.08	1	ER/protein folding	(**21, 23, 26, 27, 31**)
P4hb	PDIA1	P09103	**Protein disulfide-isomerase**	3.25	−1.63	5	ER/protein folding	(**19, 20, 26, 27, 31**)
Pdia3	PDIA3	P27773	**Protein disulfide-isomerase A3**	2.80	−1.32	4	ER/protein folding	(**17, 31**)
Txndc5	TXND5	Q91W90	Thioredoxin domain-containing protein 5	−1.17	−1.41	1	ER/protein folding	
Eno1	ENOA	P17182	**Alpha-enolase**	−2.11	1.23	2	Glucose metabolism	(**17, 26**)
Eno3	ENOB	P21550	Beta-enolase	−1.76	1.19	1	Glucose metabolism	NEW
Fbp1	FBP1	Q9QXD6	**Fructose-1,6-*bis*phosphatase 1**	−1.4	−1.46	2	Glucose metabolism	(**12, 13, 15, 17-20, 22, 26, 27, 31-33**)
Pgk1	PGK1	P09411	**Phosphoglycerate kinase 1**	−3.4	1.27	1	Glucose metabolism	(**26, 31**)
Pgm2	PGM2	Q7TSV4	Phosphoglucomutase-2	−2.95	−1.08	1	Glucose metabolism	NEW
Tpi1	TPIS	P17751	**Triosephosphate isomerase**	−2.05	−1.12	1	Glucose metabolism	(**21**)
Apoa1	APOA1	Q00623	**Apolipoprotein A-1**	15.57	−0.83	5	Lipids	(**22, 25, 31**)
Cpne1	CPNE1	Q8C166	Copine-1	1.86	−1.29	1	Lipids	NEW
Aspg	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 HCC/KO	KO/WT	Number of spots	Functional classes GO annotation	Reference
Got1	AATC	P05201	Aspartate aminotransferase, cytoplasmic	1.7	−1.14	1	Liver metabolism	Conflict (**20**)
Adk	ADK	P55264	**Adenosine kinase**	−2.38	−1.24	2	Liver metabolism	(**25**)
Apmap	APMAP	Q9D7N9	Adipocyte plasma-membrane associated protein	−3.44	1.77	1	Liver metabolism	NEW
Ass1	ASSY	P16460	**Argininosuccinate synthase**	−3.4	1.27	1	Liver metabolism	(**17, 20, 26, 28, 31**)
Bhmt	BHMT1	O35490	**Betaine homocysteine *S*-methyltransferase 1**	−3.4	1.27	1	Liver metabolism	**13, 14, 18, 20, 21, 26, 29, 30, 33**)
Cndp2	CNDP2	Q9D1A2	Cytosolic non-specific dipeptidase	2.35	1.18	1	Liver metabolism	NEW
Dpys	DPYS	Q9EQF5	Dihydropyrimidinase	−1.73	−1.1	1	Liver metabolism	NEW
Ces7	EST7	EDL11102	Carboxylesterase 7	2.94	−1.32	2	Liver metabolism	NEW
Aldh1l1	FTHFD	Q8R0Y6	**10-formyltetrahydrofolate dehydrogenase**	−2.98	1.13	2	Liver metabolism	(**15, 25, 27**)
Gnmt	GNMT	Q9QXF8	**Glycine *N*-methyl transferase**	−3.62	1.09	1	Liver metabolism	(**13-15, 22, 25, 26, 31**)
Gda	GUAD	Q9R111	Guanine deaminase	−3.09	1.9	1	Liver metabolism	NEW
Ephx2	HYES	P34914	**Epoxide hydrolase 2**	2.3	−1.27	1	Liver metabolism	(**31**)
Mdh1	MDHC	P14152	**Malate dehydrogenase, cytoplasmic**	−1.92	1.07	1	Liver metabolism	(**15, 18, 22, 25**)
Mat1a	METK1	Q91X83	**S-Adenosylmethionine synthase isoform type-1**	7.28	−1.1	1	Liver metabolism	(**12, 31**)
Mat1a	METK1	Q91X83	**S-Adenosylmethionine synthase isoform type-1**	−3.09	1.9	1	Liver metabolism	(**13, 14**)
P4ha2	P4HA2	Q60716	**Prolyl 4-hydroxylase subunit alpha 2**	2.88	−1.32	1	Liver metabolism	(**18**)
Pbld1	PBLD1	Q9DCG6	**Phenazine biosynthesis-like containing protein 1**	−2	1	1	Liver metabolism	(**26**)
Pah	PH4H	P16331	**Phenylalanine-4-hydroxylase**	3.59	1.15	1	Liver metabolism	(**15, 19**)
Pon3	PON3	Q62087	Serum paraoxonase/lactonase 3	4	1.05	1	Liver metabolism	NEW
Ctps1	PYRG1	P70698	CTP synthase 1	2.04	−1.06	1	Liver metabolism	NEW
Ahcy	SAHH	P50247	**Adenosyl homocysteinase**	−2.41	1.33	1	Liver metabolism	(**13, 22, 31**)
Uap1l1	UAP1L	Q3TW96	**UDP-*N*-acetylhexosamine pyrophosphorylase-like protein 1**	4.6	1.2	1	Liver metabolism	(**15**)
Acsm1	ACSM1	Q91VA0	Acyl-coenzyme A synthetase ACSM1, mitochondrial	−1.15	−1.35	1	Mitochondria	
Metap1d	AMP1D	Q9CPW9	Methionine aminopeptidase 1D, mitochondrial	1.43	−1.07	1	Mitochondria	NEW
Atp5pd	ATP5H	Q9DCX2	ATP synthase subunit d, mitochondrial	−1.1	−1.19	1	Mitochondria	NEW
Hspd1	CH60	P63038	**60-kDa Heat-shock protein, mitochondrial**	4.6	1.2	1	Mitochondria	(**23**)
Cps1	CPS1	Q8C196	**Carbamoyl-phosphate synthase, mitochondrial**	−2.36	1.71	12	Mitochondria	(**14, 17, 19, 22, 25, 26, 29, 31**)
Sdha	SDHA	Q8K2B3	Succinate dehydrogenase flavoprotein subunit, mitochondrial	−1.39	−1.36	3	Mitochondria	NEW
Hspa9	GRP75	P38647	**Stress-70 protein, mitochondrial**	8.54	−1.35	2	Mitochondria	(**22, 26**)
Hmgcs2	HMGCS2	P54869	**Hydroxymethylglutaryl-CoA synthase, mitochondrial**	1.7	−1.14	1	Mitochondria	(**19, 23, 29**)
Immt	IMMT	Q8CAQ8	Mitochondrial inner membrane protein	−1.5	−1.01	1	Mitochondria	NEW
Mccc1	MCCA	Q99MR8	**Methylcrotonoyl-CoA carboxylase subunit α, mitochondrial**	5.92	1.52	1	Mitochondria	(**21**)
Ndufs2	NDUS2	Q91WD5	NADH dehydrogenase iron-sulfur protein 2, mitochondrial	−3.44	1.77	1	Mitochondria	NEW
Ndufv2	NDUFV2	Q9D6J6	NADH dehydrogenase flavoprotein 2, mitochondrial	35.28	−1.3	1	Mitochondria	NEW
Oat	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			
Ogdh	ODO1	Q60597	2-oxoglutarate dehydrogenase, mitochondrial	−1.81	1.66	1	Mitochondria	
Dlst	ODO2	Q9D2G2	Dihydrolipoyllysine-residue succinyl-transferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.59	−1.17	1	Mitochondria	
Otc	OTC	P11725	**Ornithine carbamoyl transferase, mitochondrial**	−2.54	1.01	1	Mitochondria	**(22, 25, 31)**
Pc	PYC	Q05920	Pyruvate carboxylase, mitochondrial	−2.33	1.18	2	Mitochondria	NEW
Sardh	SARDH	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)**
Uqcrfs1	UQCRFS1	Q9CR68	Cytochrome B-C1 complex subunit Rieske, mitochondrial	−2	1	1	Mitochondria	NEW
Hnrnpk	HNRPK	P61979	**Heterogenous nuclear ribonucleoprotein K**	2.15	−1.28	3	mRNA process./translat.	**(15)**
Eif4b	IF4B	Q8BGD9	Eukaryotic translation initiation factor 4B	2.14	−1.37	1	mRNA process./translat.	NEW
Rplp0	RLA0	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	Q9JJA4	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	1	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
Ahsg	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
N/A	IGHA	P01878	Ig Alpha chain C region	2.02	−1.01	1	Not classified	NEW
Iigp1	IIGP1	Q9QZ85	Interferon-inducible GTPase 1	1.59	−1.17	1	Not classified	
Gaa	LYAG	P70699	**Lysosomal alpha-glucosidase**	2.5	−1.42	1	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	1	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	GDIB	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)**
Dhdh	DHDH	Q9DBB8	***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)**
Prdx2	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 HCC/KO	KO/WT	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	
Tg	THYG	O08710	Thyroglobulin	−5.74	1.17	1	Transport	NEW
Tf	TRFE	Q921I1	**Serotransferrin**	−1.5	−1.01	1	Transport	**(13, 32)**
Tf	TRFE	Q921I1	**Serotransferrin**	5.92	1.52	1	Transport	**(15, 22, 26, 31)**
Gc	VTDB	P21614	**Vitamin D-binding protein**	4.27	−1.08	1	Transport	**(22)**
Cops4	CSN4	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 secundary 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,
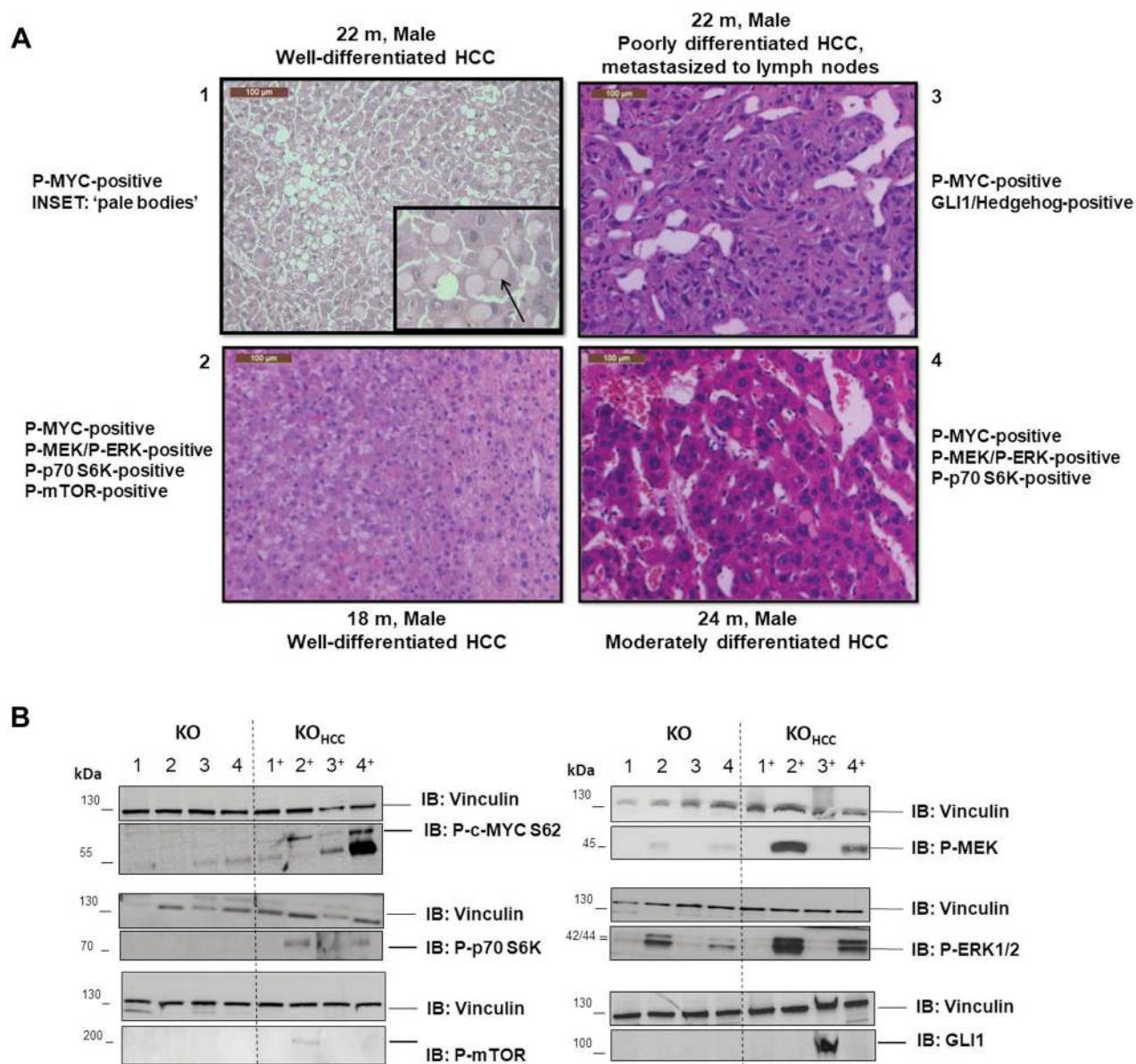
Figure 1. *Histopathology of murine protein phosphatase 2A regulatory subunit B56δ (Ppp2r5d) knockout (KO) hepatocellular carcinoma (HCC) samples used for proteomics analysis. A: Hematoxylin/eosin-stained sections of the analyzed HCC tissues (n=4) were evaluated by an experienced liver pathologist, and 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 μm. 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 endoplasmatic reticulum (RER), containing amorphous, granular material (Figure 6A), most likely representing fibrinogen deposits. Fibrinogen is a protein
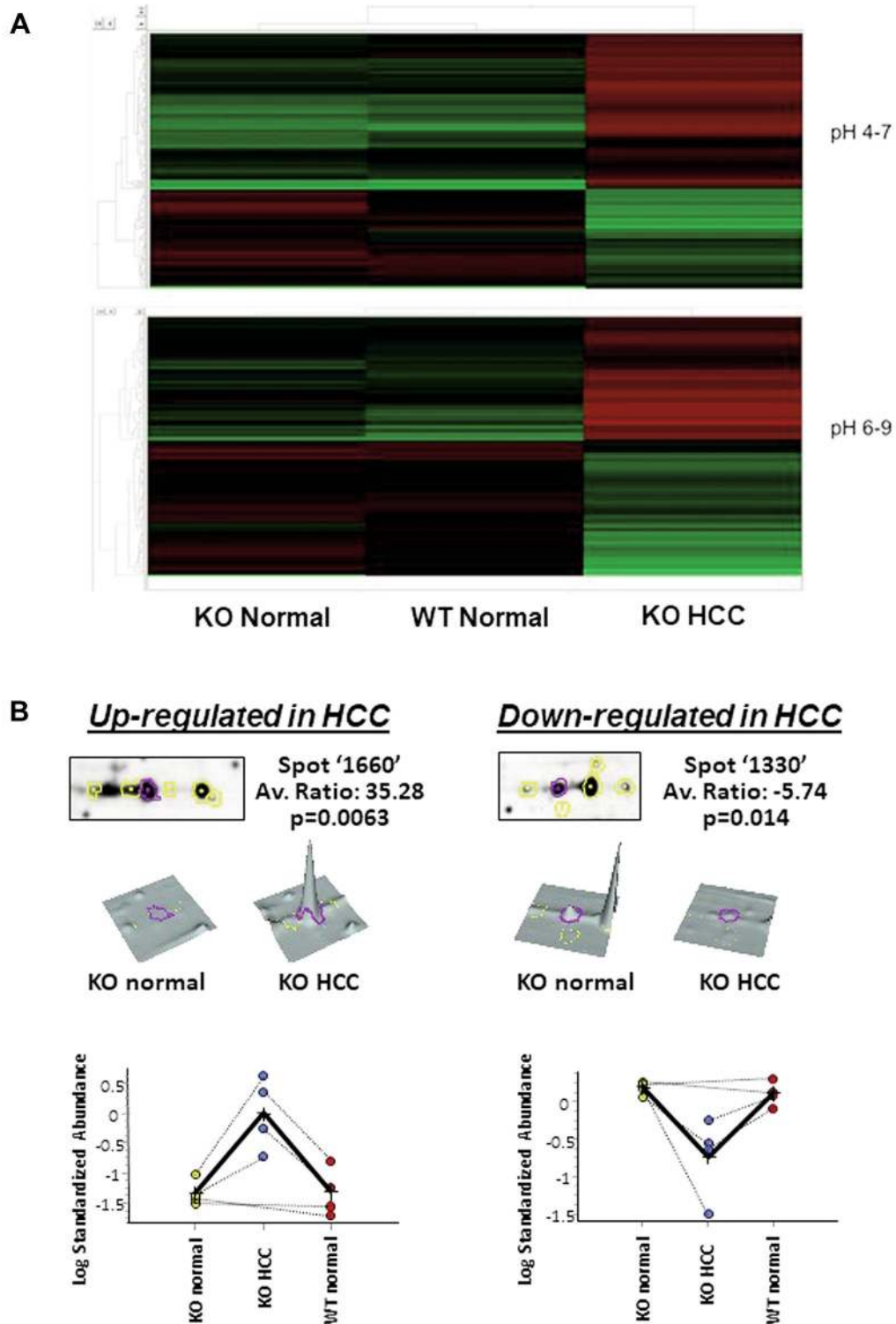
Figure 2. *Analysis of differentially regulated spots following two-dimensional differential gel electrophoresis (2D-DIGE) A: Cluster analysis of differentially regulated 'spots' between wild-type (WT), protein phosphatase 2A regulatory B56δ subunit (Ppp2r5d) knockout (KO) non-cancerous, and Ppp2r5d 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.*
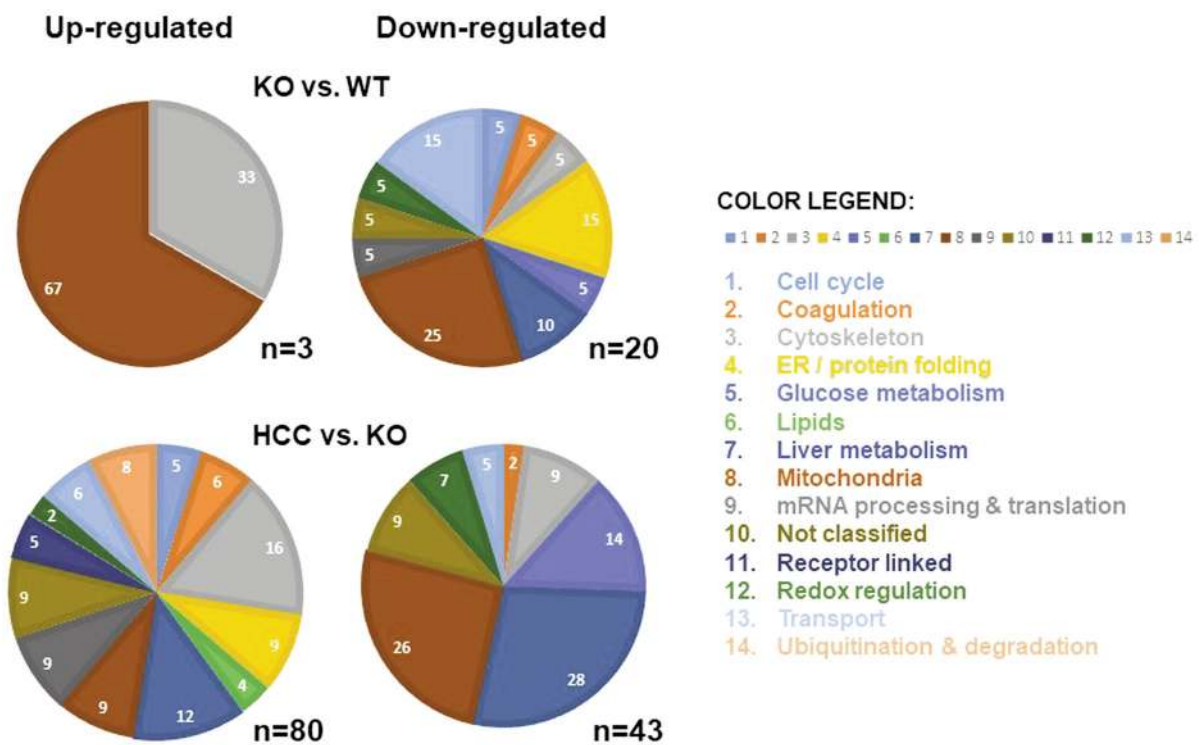
Figure 3. *Overview of differentially regulated proteins, grouped into 14 functional classes based on Gene Ontology (GO) annotation. n: Absolute number of up-regulated or down-regulated proteins found; the numbers within the diagrams indicate percentages. In the hepatocellular carcinoma (HCC) samples, 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 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
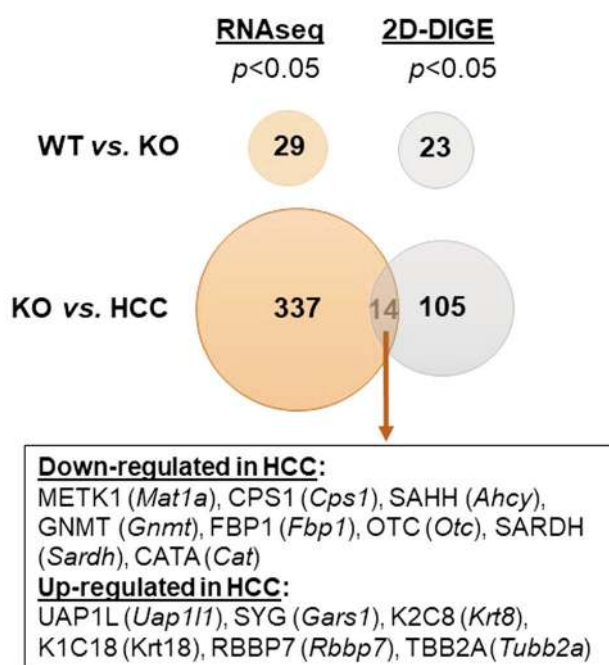
Figure 4. *Comparison between the number of differentially expressed mRNAs as determined by RNAseq (36) and as identified in the current study. While there was no overlap between both datasets in the livers from wild-type (WT) vs. non-cancerous knockout (KO) mice, 14 genes/proteins appeared to be commonly 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.*

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.*

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

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
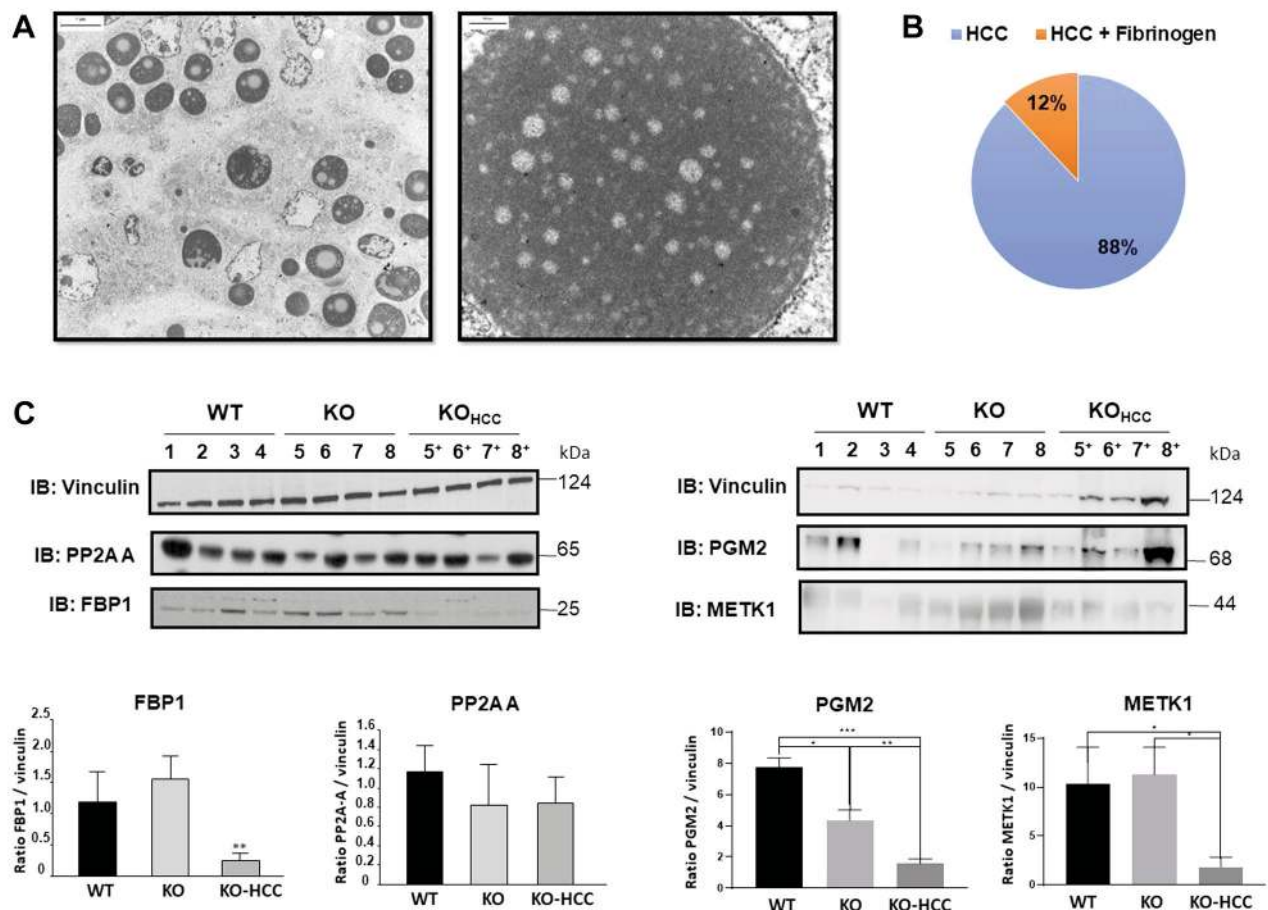
Figure 6. *Validation of two-dimensional differential gel electrophoresis (2D-DIGE) results. A: Additional analysis of 'pale body' structures by electron microscopy. B: Frequency of occurrence of fibrinogen deposits in hepatocellular carcinomas (HCCs) from protein phosphatase 2A (PP2A) regulatory B56δ 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).*

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* (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 (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); 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); 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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