universität wien

DISSERTATION

Titel

An experimental and bioinformatic tissue proteomics-strategy applied to human hepatocellular carcinoma focussing on functional data interpretation

Verfasser

Mag. Hannes Zwickl

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer. nat.)

Wien, 2010

Studienkennzahl lt. Studienblatt:	A 091441
Dissertationsgebiet lt. Studienblatt:	Genetik-Mikrobiologie
Betreuerin / Betreuer:	Ao. Prof. Dr. Christopher Gerner

„Durch Kenntnis der Stärken und Schwächen des Feindes ist der Angriff deiner Armee wie der Schlag eines Mühlsteines gegen ein Ei.“

Sūnzǐ (chinesischer General, Militärstratege und Philosoph, um 500 v. Chr., „Über die Kriegskunst“)

„Suche das Einfache und mißtraue ihm.“

Alfred North Whitehead (Logiker und Philosoph, 1861-1947)

Content

Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gelelectrophoresis
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
BLAST	Basic local alignment Search tool of NCBI
Dp	Distinct peptides
EHCO	Encyclopedia of hepatocellular carcinoma goes online
ER	Endoplasmatic reticulum
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IEF	Isoelectric focussing
IPG	Immobilized pH gradient
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ORF	Open reading frame
REL	Relative expression level
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAGE	Serial analysis of gene expression
SQL	Structured query language
Tp	Total peptides

Figures

Tables

1 Summary

The main challenge of tissue proteomics arises from the intrinsic complexity of samples which impedes reliable data interpretation. Tissues are composed of different cell types and a specific extracellular matrix. Although tumors are supposed to arise from a single cell type (e.g. HCC from hepatocytes or liver stem cells), their composition is additionally influenced by e.g. the infiltration of immune cells, by changes in the abundance of cells constituting also non-tumorous tissues and by epithelial-mesenchymal transition of cells due to an increase of developmental plasticity and loss of differentiation state usually accompanying tumor progression as well as the extracellular matrix as crucial constituent of the tumor microenvironment.

The aim of this work was to determine functional differences of non-tumorous liver and HCC tissue via proteomics methods (shotgun, mass spectrometry, 2D-PAGE). For data interpretation, the focus was laid on basic cell biological and metabolic alterations resulting in the loss or gain of functions rather than those occuring at the single gene (protein) level. Proteins were functionally clustered based on several databases (e.g. KEGG, PubMed) as well as biochemical and toxicological literature. Moreover, functional proteome alterations were correlated to physiologically, histologically, and cytologically observable alterations. In order to enable the comparison of immunohistochemically (from www.proteinatlas.org) and tissue proteomics-derived protein expression, a method for the elaboration of histologic data was introduced. In addition, proteome data were compared to those of "Encyclopedia of Hepatocellular Carcinoma genes Online" (EHCO), a database compiling eight gene set collections including PubMed, SAGE, microarray, and proteomics data (see citation in 1.1.2).

HCC tissues generally exhibit reduced secretion performance and concomitantly enhanced cytoplasmic protein synthesis (see paper "A novel technique to specifically analyze the secretome of cells and tissues" [1]) although liver-specifically synthesized plasma proteins were found to be enriched in HCC compared to non-tumorous liver at the tissue proteome level. Hence, the quantitative and qualitative aspects of the tumor secretome are discussed as well as functional groups with influence on this phenotype including protein synthesis, import of proteins into the ER as prerequisite for secretion as well as chaperones and protein degradation. Furthermore, alterations of enzyme expression involved in glycolysis (Warburg effect), glycogen metabolism and fatty acid metabolism (altered composition of lipid composition of tumors) were determined. In additon, some liver-specific functions concerning detoxification in a broader sense are covered (urea cycle, phase I- and II-system). Moreover, the involvement of e.g. c-Myc and Ras in tumor development or maintenance is indicated, the first based on the coordinated expression levels of five independent proteins, the second by being highly upregulated in HCC tissue. In addition, some other tumor-relevant proteins such as anti-apoptotic and drug resistance-mediating sorcin, the supposedly locally enriched cardiotrophin-1, and HRPAP20, which enhances the growth and survival of hormone-responsive tumor cells but has not been associated with primary liver cancer thus far, were found to be pronouncedly more abundant in HCC.

2 Zusammenfassung

Die Interpretation von pathophysiologischen Veränderungen des Gewebsproteoms stellt aufgrund der Vielzahl von Einflussgrößen eine besondere Herausforderung dar. Gewebe bestehen aus verschiedenen Zelltypen, deren quantitative Zusammensetzung und qualitativer Funktionszustand sich unter pathophysiologischen Bedingungen drastisch verändert. So spiegeln sich beispielsweise die Infiltration von Immunzellen und die gesteigerte Durchblutung des Gewebes bei Entzündung, die epitheliale-mesenchymale Transition von transformierten Hepatozyten oder die Transdifferenzierung von Stellatzellen der Leber in Myofibroblasten im Zuge der Leberkanzerogenese im Proteomprofil des hepatozellulären Karzinoms wider.

Das Ziel dieser Arbeit war, die Unterschiede der Proteomprofile von Leber und hepatozellulärem Karzinom auf funktioneller Ebene zu charakterisieren. Ein Hauptaugenmerk wurde auf Veränderungen basaler zellbiologischer (z.B. Proteinsynthese, -faltung, -degradation) und metabolischer Funktionen (Glykolyse, Fettsäuremetabolismus) sowie auf leberspezifische Funktionen (Fremdstoffmetabolismus, Harnstoffzyklus) gelegt. Die funktionelle Kategorisierung wurde anhand verschiedener Datenbanken (z.B. KEGG, PubMed) sowie biochemischer und toxikologischer Literatur erarbeitet. Soweit möglich, wurden funktionelle Proteomveränderungen mit physiologisch, histologisch und cytologisch beobachtbaren Veränderungen korreliert (z.B. Erbdefekte, dominant-negative Zelllinien, usw.) um die biologische Wirkung von gesteigerter oder verminderter Expression von Proteinen bewerten zu können. Um Proteomdaten mit immunohistochemischen Expressionsdaten („Human Protein Atlas“, www.proteinatlas.org) vergleichen zu können, wurde ein Verfahren etabliert, das es ermöglicht die unabhängigen histologischen Datensätze „Intensität“ und „Quantität“ miteinander zu verknüpfen. Weiters wurden die Proteomdaten mit jenen der „Encyclopedia of Hepatocellular Carcinoma genes Online“ (EHCO)-Datenbank korreliert. Diese umfasst u.a. SAGE-, Micorarray- und Proteomics-Daten.

Im Vergleich zu normalem Lebergewebe zeigt das hepatozelluläre Karzinom charakterischerweise eine veringerte Sekretionsleistung bei gleichzeitig gesteigerter Synthese von zytoplasmatischen Proteinen (“A novel technique to specifically analyze the secretome of cells and tissues” [1]). Allerdings wurden im hepatozellulären Karzinom auf Gewebsproteomebene vermehrt leberspezifisch sekretierte Plasmaproteine detektiert. Daher werden mögliche physiologische und zellbiologische Einflussgrößen auf die Proteinsynthese und die quantitative und qualitative Zusammensetzung des Tumorsekretoms im Kontext des funktionell charakterisierten Tumorproteoms diskutiert. Weiters werden die tumor-relevanten Funktionen Glykolyse (Warburg-Effekt) und Fettsäuremetabolismus sowie Detoxifikation (Harnstoffzyklus, Phase I- und Phase II- Enzyme; Resistenz gegen Chemotherapeutika) behandelt und Proteine, die tumor-charakteristische Eigenschaften vermitteln, in Bezug auf vorhandene Literatur diskutiert.

3 Introduction

3.1 The proteome

3.1.1 General remarks

The term proteome, coined in 1996 by M.R. Wilkins, indicates the set of proteins expressed by a genome or tissue [2]. It already implicates the ‘dichotomy’ of the theoretical (i.e. primary structure of proteins derived from DNA-sequences of all 'Open Reading Frames' (ORFs)) and the physiologically relevant proteome in anology to the difference between 'genome' and 'functional genome'.

Sequencing of the human genome not only pushed open the door to comprehensively gather the theoretical proteome but put it also center stage for phylogenetic considerations. Decoding of the human genome revealed that it comprises a constant but astonishingly modest number of protein-coding genes [3] which has actually been revised downwards in recent estimates and is now assumed to comprise about 22.000 ORFs [4, 5]. It is commonly accepted that sheer gene counts scarcely correlate with the complexity of organisms. Thus, the nematode *Caenorhabditis elegans* has a similar and the fruit fly *Drosophila melanogaster* about half the number of genes as humans [6]. The interactome, i.e. the total number of protein interactions, is now suggested the more reliable parameter to reflect the complexity of biological systems. Indeed, based on presently available interaction data, *Stumpf et al.* recently estimated the human, *C. elegans*, and *D. melanogaster* interactomes to contain about 650.000, 250.000, and 100.000 protein interactions, respectively, which seemingly approximate at least “our perception of the relative complexity" (*Stumpf et al.*, [7]) of those species better than gene numbers [7]. In addition, genome-sequencing uncovered many proteins of yet uncharacterized or pleiotropic functions constituting a significant section of the theoretical proteome [8, 9] and offering the opportunity to targetly focus on their integration into the physiological context as constituents of a functional proteome.

In contrast to the intrinsically static species-specific collection of genes as well as the theoretical proteome and protein interaction network derived thereof, gene expression is highly dynamic and temporally, spatially, and contextually regulated. In fact, only minor moieties of genes are expressed and thus just sections of the total proteome and interactome are realized under distinct physiological conditions. This implicates the emanation of numerously distinct functional proteomes and transitional states from a single theoretical proteome in the course of e.g. development, differentiation, regeneration, and cancer development. Indeed, the expression of the physiologically appropriate proteomes is decisive for development, maintenance, and homeostasis of (multi-cellular) organisms. Therefore, gene expression is tightly regulated at many levels in the course of information flow from DNA to RNA to proteins and beyond in order to provide systemic fidelity, robustness, and adaptability.

The regulation of protein abundance and diversity concerns the accessibility of promoter elements for transcription factors by assembly, regulation, and remodeling of chromatin (reviewed in [10-12]), mRNA maturation including ‘capping’ [13] and alternative splicing (reviewed in [14, 15]), transport of mature mRNAs from the nucleus into the cytoplasm (reviewed in [16], see also [13]), mRNA stability by deadenylases, miRNAs [17, 18], and the exosome (reviewed in [19, 20], translation initiation (reviewed in [21, 22]), and protein degradation [23].

Moreover, many proteins require co- or post-translational, transient or constitutive modifications to exert their functions, to switch between active and inactive states, and to bind to interaction partners and, hence, to become integral parts of the physiologically relevant dynamic interactome. Frequent modifications include e.g. ubiquitinylation, sumoylation, neddylation, acetylation, phosphorylation, methylation, glycosylation, and prenylation. Ubiquitinylation is the common cellular mechanism dedicating proteins to proteasomal degradation. However, ubiquitinylation is also involved in DNA repair, transcription regulation including gene silencing (e.g. ubiquitinylation of histone H2A in yeast), regulation of transcription factor turnover and estrogen receptor degradation, the control of stability and activity of the tumor suppressor p53 (TP53), signal transduction (e.g. by modulating the transcripton of NFκB-dependent genes), and endocytosis (reviewed in [24] and citations therein). Phosphorylation plays a pivotal role in signal transduction. TP53, for instance, is a nodal point for many signaling pathways integrating a broad variety of stress signals such as DNA damage, hypoxia, and dNTP depletion. However, the cellular outcomes (cell-cycle arrest, DNA repair, apoptosis, senescence) are as diverse as the signals triggering the response and are cell type-, tissue-, and stimulus-dependent indicating the activation of different gene sets. In addition to stability- and activity-providing modifications (see above), sequence-specific phosphorylation of p53 affords promoter selectivity in response to genotoxic stress leading to the expression of p53AIP1 which exerts pro-apoptotic activity and eventually triggers programmed cell death (reviewed in [25]). A decisive aspect of the biological tumor-suppressing activity of p53 is its translocation from the cytoplasm into the nucleus. Interference with the contextually proper nuclear location is indeed a common pathogenetic mechanism of tumor-inducing viruses such as adenovirus and hepatitis B virus ([26, 27], for the latter see below).

The subcellular localization of proteins is a crucial determinant of their function and has substantial implications for proteome analyses. It is either co-translationally (e.g. ER-resident, secreted, integral membrane proteins), post-translationally (e.g. mitochondrial proteins, nuclear proteins like histone proteins, RNA- and DNA-polymerases), or signal-dependently (e.g. nuclear localization of p53 (see above), reversible association of vesicle traffic-regulatory Rab GTPases with lipid membranes [28]) established. An impressive example for the correlation of biological function and subcellular location is cytochrome c which participates in the mitochondrial electron-transport chain under normal conditions but is released from mitochondria into the cytoplasm upon apoptotic stimuli and constitutes an integral component of the apoptosome there (reviewed in [29]). However, cytoplasmic cytochrome c does not necessarily lead to programmed cell death. The outcome, apoptosis vs. survival, rather depends on the cell biological context since autophagy as well as elevated levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and inhibitors of apoptosis proteins (IAPs) can effectively counteract the induction of apoptosis [30]. Indeed, increased expression of GAPDH and IAPs has been found in various cancers and is supposed to be involved in mediating resistance against apoptosis, one of the hallmarks of cancer [31-35].

3.1.2 The challenge

The interpretation of proteome data derived from 2D-PAGE and shot-gun experiments is based on the comparative acquisition of differential gene expression patterns at the protein level (proteome profiling). The latter reflect e.g. characteristics of cell types (differentiation states), drug effects (pharmacoproteomics), disease-mediated alterations (normal vs. tumorous tissues), and physiological states (inflammation, activation, proliferation) [36-38]. Proteomics proves to be almost universally applicable, complements and extends insights from alternative investigative approaches (e.g. genomics, transcriptomics, etc.), and, hence, contributes to a more comprehensive understanding of (patho-) physiological processes and mechanisms.

The biological and molecular heterogeneity of hepatocellular carcinoma tissues is a major challenge for extracting common features of statistical and medical relevance (biomarkers, treatment options, etc.). Serious efforts are made to globally assess the transcriptome and proteome of liver and tumor tissues in order to derive significant tissue-type specific differences. Although being promising approaches, they have methodically intrinsic limitations which might be generally assigned to the difficulty to distinguish the effect of different influencing variables on gene expression from actual cancer-relevant alterations and, hence, to define filter and selection criteria for the elaboration of large datasets.

Variables with substantial impact on the molecular status of investigated samples are interindividual differences, different tumor etiologies, and the "ontogenesis" of liver cancer including e.g. the stage of cancer progression and the grade of dedifferentiation. It is, therefore, evident that the results of transcriptome and proteome studies strongly depend on and reflect the preselection of samples for comparative analysis which already presumes the relevance of the applied criteria. In addition, the availability and, hence, investigation of solid tumors is restricted to resection or transplantation-derived and, hence, advanced-stage samples. Nevertheless, the major goal is the identification of reliable sets of prognostic, predictive, and pharmacodynamic biomarkers as basis for decisions concerning the questions if a given treatment is advantageous for a patient, if a patient is responsive to a given drug, and which dose of a given drug should be applied, respectively.

However, considering the heterogeneity and complexity of biological systems which e.g. manifest in the multitude of ways to gain common tumor traits (e.g. unrestricted proliferation, resistance against apoptosis), it is obviously improbable to find common biomarkers fitting to or reflecting the features of e.g. all hepatocellular carcinomas. Indeed, expression levels of proteins in histologic slices of normal liver tissues are commonly in a narrow (tissue-specific) range whereas those of tumorous tissues vary pronouncedly (www.proteinatlas.org). However, the probabilities of a given protein to be less, equally, or more abundant with respect to its expression level in normal liver tissue can be calculated and deliver ancillary infomations for the interpretation and evaluation of tissue proteomics data (see below).

3.1.3 The strategy

The main intention of this work is to integrate proteome data of tissues of hepatocellular carcinoma and adjacent non-tumorous liver and in order to determine "real" functional shifts and to correlate those with anatomically, physiologically, histologically and cytologically observable alterations of liver vs. tumor tissue as well as with published data gathered via proteomics and alternative/complementary methodical approaches. Taken together, this strategy might allow a broader and more fundamental, since integrative, interpretation of alterations concerning gene expression and functionality of liver and HCC tissue although the intrinsic complexity of the biological network rising from the multitude of interdependencies and interconnections of system components are far from being understood. Nevertheless, there are some adjuvant data available which can be consulted to assess the impact of reduced/increased abundance of a given protein on the molecular process it accounts for (e.g. dominant-negative cell lines, genetic disorders ascribed to a certain gene, knock-out models). A further level of complexity results from the fact that liver and HCC tissue comprise several and most possibly different cell types and that the proteome of transformed hepatocyte-like cells of HCC tumor tissue itself might converge to that of cellular components of non-tumorous liver tissue due to epithelial-mesenchymal transition. For that reason and in order to reduce complexity the main focus was laid on determining the functionality of the tissue/organ as a whole rather than to assigning it to certain cell types. It has to be stated that this work naturally represents just one of "many roads that lead to Rome". The shotgun proteomics approach for data acquisition is intrinsically holistic and has to be understood as hypotheses-delivering one. It is, therefore, well applicable to provide the basis for questions worth to be dealt with in detail. Although this method enables the detection of usually 1000- 1500 proteins in one experiment, this fraction represents just a section of the actually expressed functional proteome. However, it can be concluded that many biological functions are indispensable for the survival of cells in general although to a different extent and in dependency of the physiolgical contexts (e.g. glycolysis as primary energy-providing metabolic pathway for cells of avascular and hence oxygen-deprived tissues like cartilage or as pathway providing intermediates for biosynthesis by hepatocytes). Hence, the respective enzyme machinery has to be complete to fulfill its function. This assumption allows to fill gaps of not detected or detectable proteins and is also useful to conclude or hypothesize the involvement of e.g. signaling pathways involved in tumor progression and maintenance from proteins which are more easily detectable than the low-abundant, membrane-associated, or nuclearly localized signaling components themselves (see below).

Principal approaches for the interpretation of tissue proteomics data:

1. The analytical approach is based on the dissection of the proteomes of tissue-constituting and pathologically relevant cell types (e.g. cells of the immune system, blood cells, fibroblasts) in order to determine their contribution to the tissue proteome. This is achieved by the isolation of the distinct cell types, their expansion *in vitro*, and the subsequent determination of the cell type-specific proteomes. This approach has successfully applied by Slany and Haudek *et al.* who gathered the proteome profiles of primary human hepatocytes and of the hepatoma-derived cell lines HepG2 and Hep3B [36]. They could demonstrate that the latter represent

two completely different manifestations of liver cancer cells. E.g. HepG2 expressed more liver-specific proteins than Hep3B whereas the latter was suggested to having undergone epithelial-mesenchymal transition and, hence, to be originally derived from a more aggressive tumor than HepG2. The understanding of the spectrum of molecular alterations realized by cells constituting HCC tumors substantially facilitates the interpretation of tissue proteomes. In addition, it potentially helps to identify markers for the proper classification of tumors extending and supplementing the panel of criteria applied thus far (e.g. tumor-stage, etiology) which might significantly contribute to a more comprehensive insight into tumor biology. The cell-based analytical strategy, however, has intrinsic limitations due to excluding the histological and molecular composition of the tumor stroma. The specific tumor microenvironment comprising e.g. the distinct composition of the extracellular matrix and soluble factors mediating autocrine stimulation or paracrine crosstalk between different cell types is supposed to have decisive impact on gene expression of not only transformed cells. Furthermore, gene expression and the microenvironment are influenced by physical parameters such as blood supply, material exchange processes, and pressure regimen (e.g. hydrostatic or colloid osmotic pressure). Liberating cells from their systemic context and, hence, deprivation of physical or molecular stimuli provided by the microenvironment (e.g. due to the enrichment of growth and survival factors) possibly lead to unpredictable changes in gene expression and phenotype.

2. The data-driven approach correlates patterns to certain tumor traits (e.g those reviewed in [32]), thereby requiring no assumptions and being unbiased. A decisive aspect concerning the management of large datasets commonly gained by proteomics experiments is a reliable database featuring data quality-ensuring functions and faciltating multi-sample comparisons at the protein and even the functional level as it was introduced at the Medical University Vienna (lab of Prof. Gerner) in 2009 by Wimmer *et al*. (CPL/MUW database) [39].
3. The knowledge-based approach uses data from literature concerning distinct traits. Alterations of the gene expression should be reproducibly present in the respective sample as starting point to consequently discover new molecular players and to reveal their interrelations and/or correlated expression.

Principle considerations, assumptions, and procedures

1. The interpretation of proteomics data applied in this study is primarily based on the knowledge-based approach. The aim was to determine and to functionally characterize alterations of the proteomes of hepatocellular carcinoma and adjacent non-tumorous tissue. The applied data interpretation strategy is based on the construction of functional clusters (e.g. ribosomal proteins, glycolysis, respiratory chain, fatty acid metabolism, etc.) and is different from cluster analyses applied in order to extract gene-expression signatures (clusters), referring to patterns shared by or unique for (sub)groups of investigated samples [40]. Whereas the principal entity of the latter is the expression level of a given gene, the functional cluster comprises the expression level of all (at the best) proteins (genes) involved in a pathway. The output of the functional cluster-based data interpretation is the loss or gain

of function rather than the up- or downregulation of individual proteins. Supposed that cancerogenesis is in part the result of the gain or loss of function irrespective of its actual biological/biochemical realization (e.g. involvement of different signaling pathways in HCC, defects of several enzymes involved in glycogen metabolism cause glycogen storage disorders manifesting in common symptoms like hepatomegaly, hypoglycemia, lipidemia) and hence the up- or downregulation of individual proteins, this approach might explain the contradictory or inconsistent data concerning the involvement of individual proteins in cancers (e.g. putative biomarkers) [41].

2. The proteome of HCC and non-tumorous tissue of one patient was analyzed by mass spectrometry. The efficiency of protein detection of both samples was comparable (similar overall total peptide numbers).
3. For the semi-quantitative assessment of the abundance of a given protein the total peptide numbers of the respective proteins were taken. The preparation of protein samples for mass spectrometric analysis includes the digestion with site-specific proteases (e.g. trypsin, chymotrypsin) delivering peptides of defined amino acid compositions and, hence, predictable peptide masses. The latter are determined by the respective protein species and the protease used for digestion. The mass spectrometrical identification of proteins basically relies on the computational matching of actually determined and database-compiled theoretically calculated peptide masses of proteins. The proteins are characterized by distinct peptides assignable to the respective protein species. The number of distinct peptides (Dp) of a given protein and the percentage of amino acids identified (coverage (%)) are a measure for the reliability of protein identification. Although the number of distinct peptides reflects the abundance of protein species of the sample, the number of total peptides (Tp) is more convenient for quantification from theoretical considerations. The maximum number of Dp of a given protein is constant for a given protein species in contrast to Tp. This pertains particularly for small and/or highly abundant proteins. The correlation of Dp and Tp and their relevance for protein quantification is exemplified in figure 1. In addition, the mass spectrometrical identification of Dps (and Tps of the corresponding peptide) is influenced by covalent modifications (e.g. phosphorylation, glycosylation, acetylation) and requires algorithms incorporating the mass variance due to the attached functional groups.
4. Proteins were assigned to functional clusters comprising highly homologous proteins which deliver similar sets of peptides after tryptic digest and are therefore prone to be falsely listed as distinct proteins. Peptides of those proteins were checked for specificity (BLAST, Basic Local Alignment Search Tool of NCBI). Proteins which were not uniquely identified were grouped (e.g. P…../Q…..) and the highest number of total peptides was taken to quantitatively represent the group.

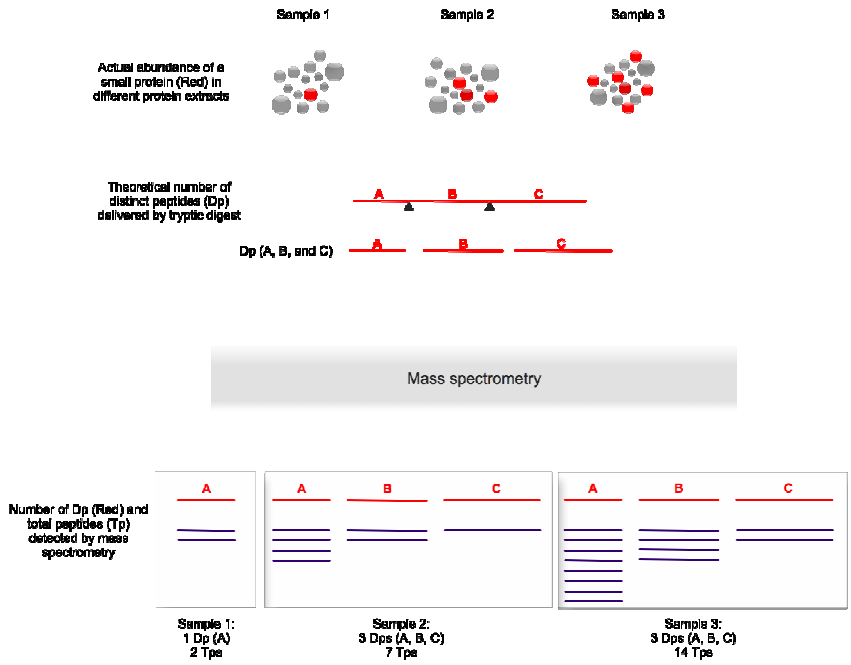


Figure 1: The number of total and distinct peptides (Tp and Dp) as measure for the abundance of proteins identified via mass spectrometry

5. Functional clustering was refined using the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes, see appendix), biochemical and toxicological literature, UNIPROT database, Pubmed. For clustering and, in particular, for chapter 3.3 and 5.9 the books “Biochemie und Pathobiochemie” (Löffler, Petrides, and Heinrich, 8th edition, 2006), “Taschenatlas der Biochemie” (Koolman and Röhm, 3rd edition, 2003), “Taschenatlas der Physiologie” (Silbernagl and Despopoulos, 6th edition, 2003), “Taschenatlas der Pathophysiologie” (Silbernagl and Lang, 2nd edition, 2005), “Lehrbuch Anatomie” (Lippert, 3rd edition, 1993), “Biochemistry” (Berg, Tymoczko, and Stryer, 5th edition, 2006), “Casarett & Doull's Toxicology: The Basic Science of Poisons”, “Histologie” (Lüllmann-Rauch, 2003), “Immunobiology” (Janeway *et al*, 6th edition, 2005), “Allgemeine und spezielle Pharmakologie und Toxikologie” (Aktories *et al*, 9th edition, 2005), and “Molecular biology of the cell” (Alberts *et al*., 5th edition, 2007) were consulted.
6. Shotgun results were compared to immunohistochemical data from “Human Protein Atlas (www.proteinatlas.org) and data derived from “Encyclopedia of Hepatocellular Carcinoma genes Online” (EHCO) which was introduced in 2007 and compiles eight gene set collections, ranging across PubMed, SAGE, microarray, and proteomics data [41]. Intriguingly, the authors state that 77% of the 2,906 genes covered are included just once and that their relevance for HCC is not clear. A rich source for the evaluation of protein abundances in tissues is the antibody-based immunohistochemical staining of histological slices. “Human Protein Atlas” (www.proteinatlas.org) which delivers expression data of more than 5000 proteins was chosen to compare and correlate shotgun results with immunohistochemically obtained protein abundance data. However, in this database protein expression data are represented by two independent datasets, “intensity” and “quantity”. The first corresponds to the expression level *per se* whereas the second gives the percentage of cells expressing the respective protein.

Since both equally influence the amount of proteins extracted from homogenized tissue samples prior to mass spectrometry, “intensity” and “quantity” had to be correlated in order to make immunohistochemical and tissue proteomics data comparable. For that, a new method was introduced. Immunohistochemically derived protein expression data are represented either as probabilities for up- or downregulation or equal expression compared to liver (see appendix) or statistically processed using SPSS and depicted as boxplots for proteins discussed in more detail. The three data sets are compiled and their compliance is given in the appendix.

7. For the determination of the biological impact of up- or downregulation of a given protein, data concerning dominant-negative cell lines, hereditary diseases, knock-out mice from PubMed and Uniprot were gathered.

3.2 Hepatocellular carcinoma (HCC)

3.2.1 Why investigating HCC- the epidemiologist's and clinician's point of view

The general term "liver cancer" comprises histologically distinct tumors like cholangiocarcinoma, hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma, epitheloid haemangioendothelioma, and hepatocellular carcinoma, the latter making up 83% of all cases. Hepatocellular carcinoma (HCC, hepatoma) is a global health problem in terms of incidence and lethality with about 1 million new cases yearly and median survival rates of <25 months after resection or <6 months with symptomatic treatment, respectively [42]. Several coinciding parameters contribute to the fatality and the difficulty to manage this malignancy. Most patients are diagnosed not before having developed advanced-stage tumors [43] partially due to the lack of biomarkers for early detection. In addition, malignant hepatocytes commonly develop against the background of permanent inflammation (chronic hepatitis), tissue remodeling processes (fibrosis), and the loss of liver parenchyme (cirrhosis) [44-46]. Both, late detection and hepatic dysfunction, drastically limit the options for curative treatments like tumor resection and liver transplantation or predispose to early tumor recurrence after surgical intervention (recurrence rate up to 50% at 2 years) [47-50]. Moreover, HCC proves to be highly resistant to conventional chemotherapeutics and hormonal therapies diminishing the efficacy of adjuvant and palliative treatments [51, 52]. Today's treatment options and their benefit to patients are summarized by Thomas and Zhu (Table 1, obtained from [50]).

Treatment Option	Comments	References
Liver transplantation	Historically low survival rates (20%-36%) recent improvement (61.1%; 1996-2001), likely related to adoption of Milan criteria at US transplantation centers Currently HCC represents 20+% of liver transplantations performed annually in the United States	[53, 54]
Surgical resection	Historical 5-year survival rates 30%-40% Recent series indicates 5-year progression-free survival as high as 48%; majority of patients develop recurrence or second primary tumors Resection in cirrhotic patients carries high morbidity and mortality	[54-58]
TACE (transarterial embolization/ chemoembolization)	Multiple trials show objective tumor responses and slowed tumor progression but questionable survival benefit compared to supportive care; greatest benefit seen in patients with preserved liver function, absence of vascular invasion, and smallest tumors Modest survival benefit demonstrated for repeated TACE (82% 1-year survival) vs. supportive care (63%) in patients with preserved liver function, performance status 0, and small tumor burden; improvement in 1-year survival from 32% in controls (supportive care) to 57% for TACE shown in randomized study of 279 primarily HBV-positive patients with tumors < 7 cm	[59-62]
Intra-arterial iodine-131–lipiodol administration	Efficacy demonstrated in unresectable patients, those with portal vein thrombus, and as adjuvant therapy in resected patients	[63-65]
Percutaneous treatments (ethanol injection, radiofrequency ablation)	Percutanous ethanol injection well tolerated, high response rate in small (< 3 cm) solitary tumors; no randomized trial comparing resection to percutaneous treatments; recurrence rates similar to those for postresection	[66-69]
Hormonal therapy	Antiestrogen therapy with tamoxifen studied in several trials, mixed results across studies, but generally considered ineffective Octreotide (somatostatin analogue) showed 13-month median survival vs. 4-month in untreated patients in a small randomized study; results not reproduced	[70-74] [75]
Chemotherapy	Adjuvant: No randomized trials showing benefit of neoadjuvant or adjuvant systemic therapy in HCC; single trial showed decrease in new tumors in patients receiving oral synthetic retinoid for 12 months after resection/ ablation; results not reproduced Palliative: Regimens that included doxorubicin, cisplatin, fluorouracil, interferon, epirubicin, or taxol, as single agents or in combination, have	[52, 76-79] [52]

	not shown any survival benefit (response rate 0%-25%); a few isolated major responses allowed patients to undergo partial hepatectomy; no published results from any randomized trial of systemic chemotherapy	

Table 1:Treatment options for HCC

3.2.2 Hepatocarcinogenesis

3.2.2.1 General remarks

Hepatocellular carcinomas (Figure 2) exhibit many phenotypic traits subsumed as “hallmarks of cancer” referring to e.g. independency from growth signals, reduced apoptosis, and unlimited proliferation potential [32]. In addition, metabolic alterations and the involvement of the immune system are also considered as characteristics of tumor cells and crucial determinants for tumor formation, respectively [80, 81].

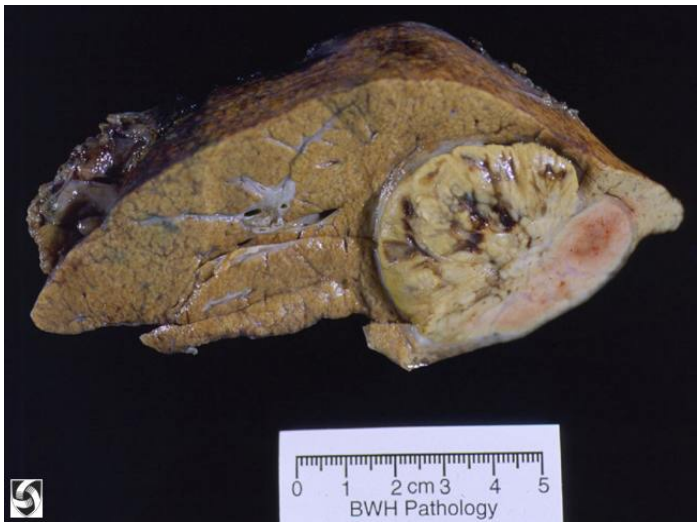


Figure 2: Liver tumor (Permission to use this image granted by the Brigham and Women's Hospital, Department of Radiology)

However, apart from those common traits, the HCC-characteristic clinical aspects and sequence/timeframe of cell biological events occuring in hepatocarcinogenesis (Figure 3, modified from [82]), individual hepatocellular carcinomas are highly heterogenous concerning the underlying etiologies, the molecular alterations and differentiation status of transformed cells, the proliferative and metastatic potential, the histological appearance and their integration in the systemic context (e.g. vascularization). Nevertheless, some pathophysiological mechanisms are suggested to be commonly involved in hepatocarcinogenesis (Figure 4).

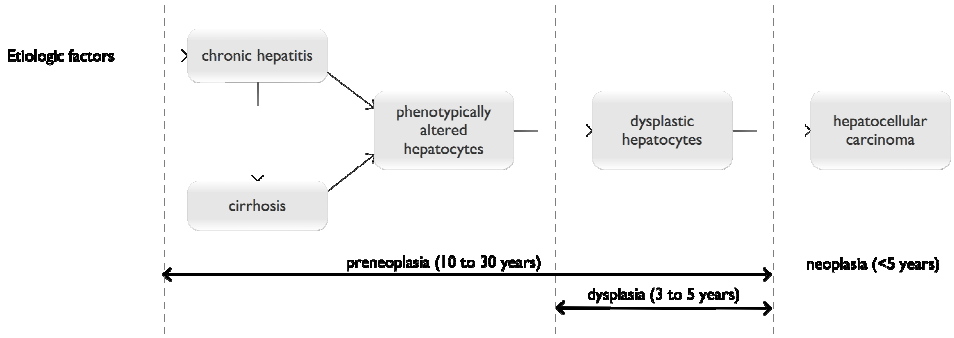


Figure 3: Timeframe for the development of HCC

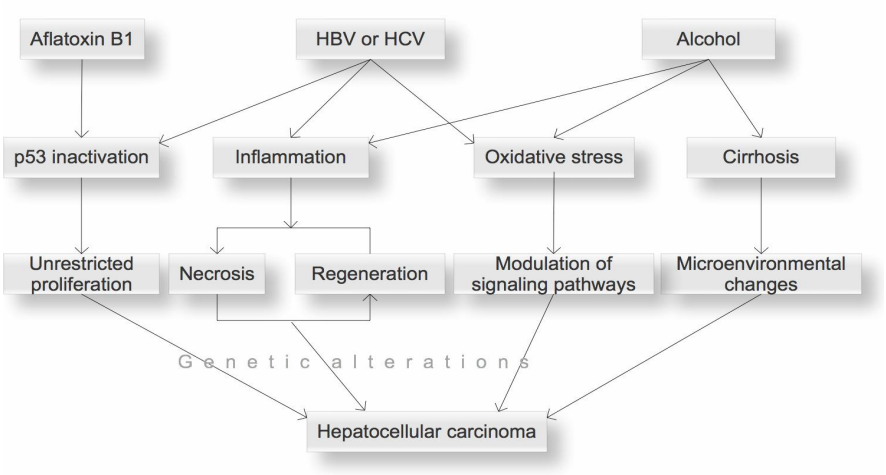


Figure 4: Pathophysiological mechanisms involved in hepatocarcinogenesis (modified from [83])

3.2.2.2 Etiologies

3.2.2.2.1 Hepatotropic viruses and their role in heaptocarcinogenesis

3.2.2.2.1.1 Hepatitis B virus...

####### 3.2.2.2.1.1.1 ...from the epidemiologist's point of view

Hepatitis B virus (HBV) infection is one of the major risk factors for the development of HCC. About 30-50% of HBV-related deaths can be attributed to HCC [83, 84]. However, just a minor proportion of HBV-positive individuals develop cirrhosis and of those only 5% end up with HCC

(www.who.int/csr/disease/hepatitis/). The majority of acute HBV infections delivers alternative clinical outcomes and is overcome by up to 95% of individuals (Figure 5). However, there are about 350 million people infected chronically and about 75% of the world's population lives in areas with high infection levels (www.who.int/csr/disease/hepatitis/) (Figure 6).

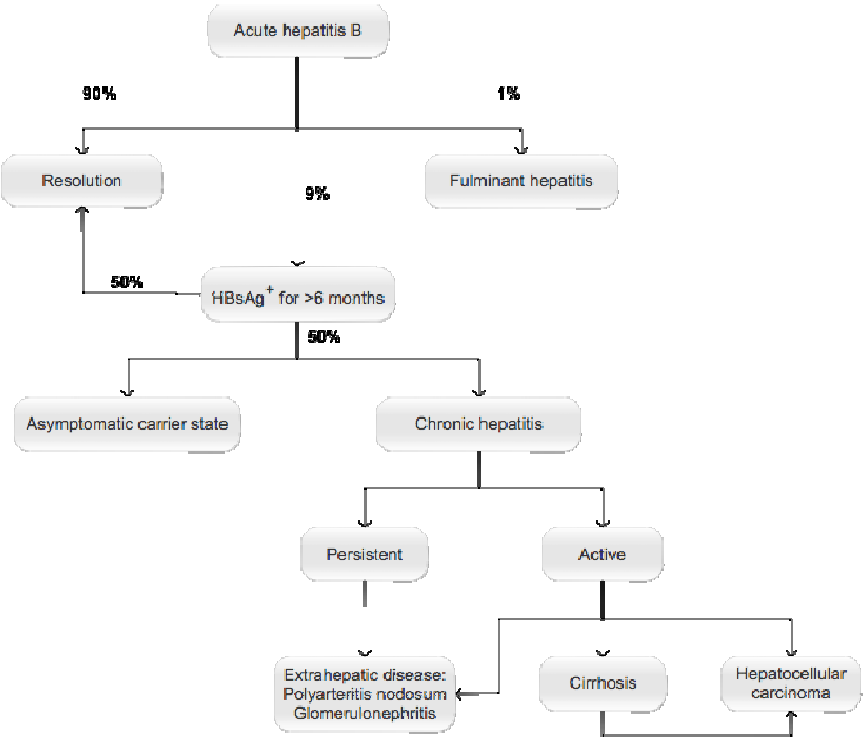


Figure 5: Clinical outcomes of acute HBV infections (modified from [85])

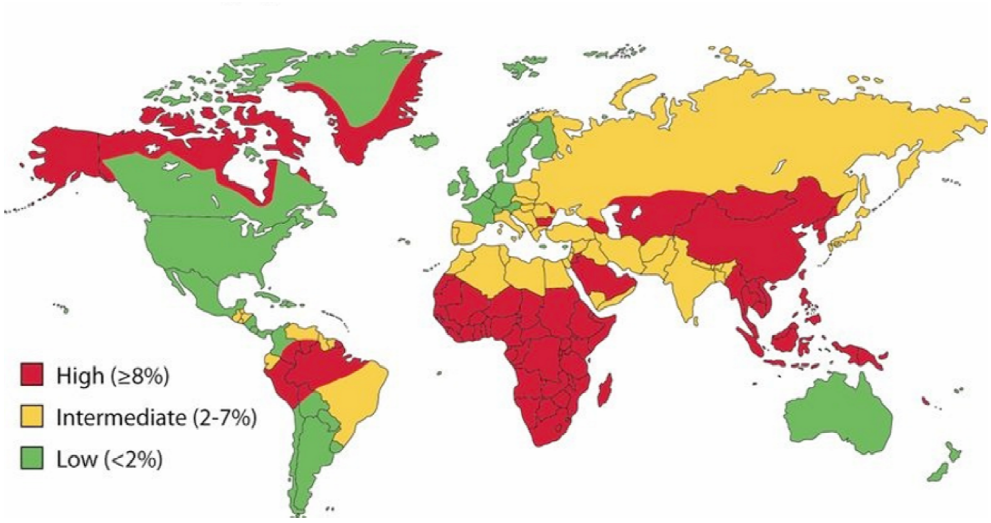


Figure 6: Geographic distribution of chronic HBV infections (adapted from CDC[1])

[1] Centers for Disease Control and Prevention

3.2.2.2.1.1.2 ...from the virologist's point of view

HBV is a small partially double-stranded DNA virus of the hepadnaviridae family [86]. The viral genome encodes proteins involved in replication (reverse transcriptase/DNA polymerase (pol), kinase), structural proteins (the capsid proteins HBc and HBe (core), envelope proteins L, M, S), and HBx, which fosters viral replication and transcription. The morphology of an infectious HBV particle is depicted in figure 7.

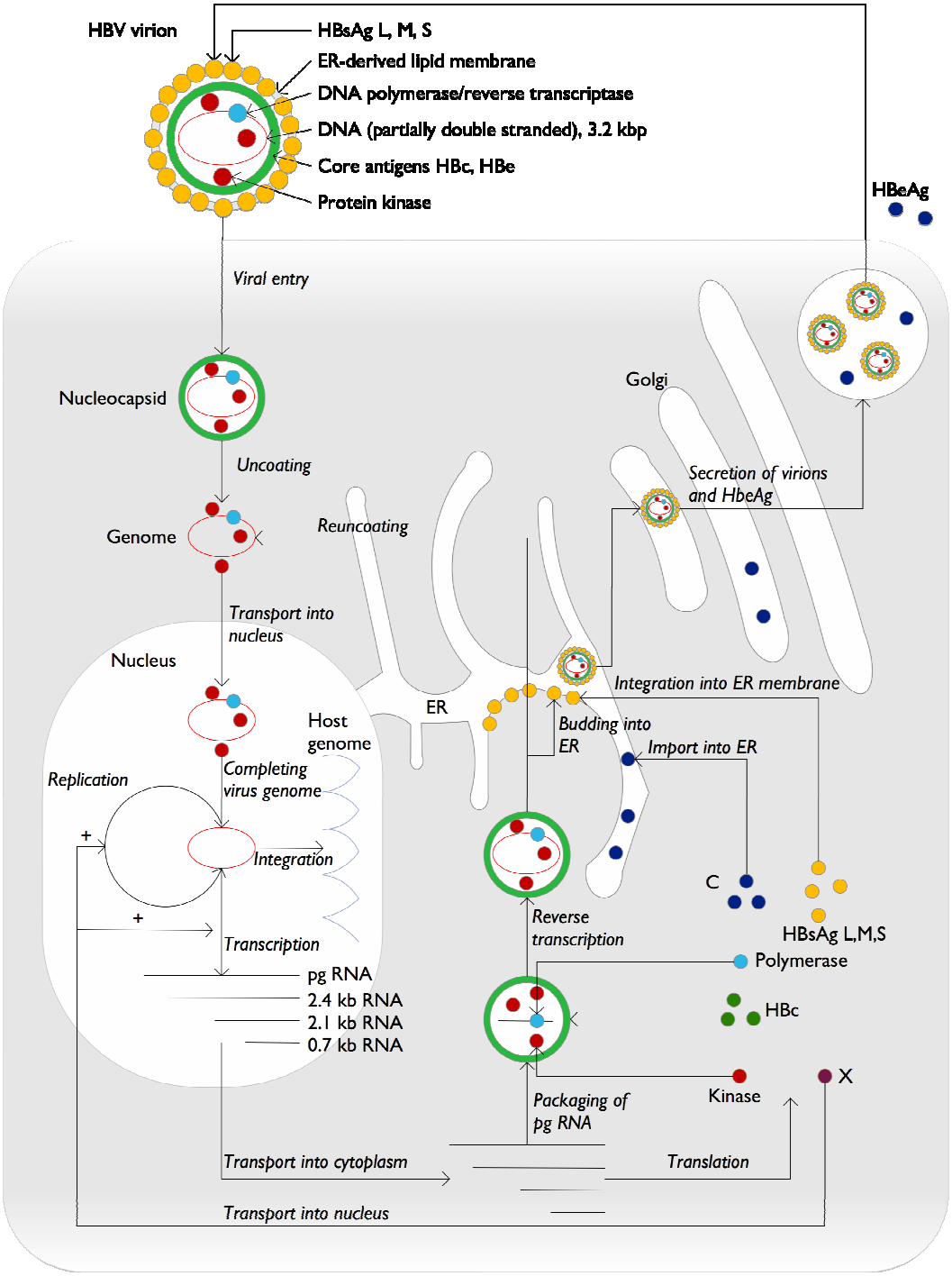


Figure 7: Morphology and life cycle of HBV

The 'life cycle" of HBV (Figure 7): Infection is initiated by the binding of an HBV particle to a receptor at the cell surface of hepatocytes (possibly transferrin receptor, asialoglycoprotein receptor, liver endonexin). Then, the nucleocapsid enters the cell via a yet unkown mechanism and uncoats near the nucleus delivering the viral genome. The covalently bound HBV polymerase mediates its transport into the nucleus and completes subsequently the viral genome yielding a covantly closed circular (ccc) supercoiled DNA which serves as transcription template for viral RNAs and semiconservative replication. After poly-adenylation, the four primary transcripts (pgRNA (pregenomic RNA, 3.5 kb), 2.4 kb, 2.1 kb, 0.7 kb) are transferred to the cytoplasm where translation of viral proteins takes place. The pregenomic RNA, DNA polymerase, and kinase are packaged into newly formed nucleocapsids for the conversion of viral RNA into genomic DNA. The latter process involves reverse transcription of RNA to DNA, DNA-dependent DNA polymerase activity, and nuclease activity for degradation of the original RNA template, all of which are accomplished by the multifunctional viral DNA polymerase. The new viral nucleocapsids can follow two alternative pathways leading either to the amplification of viral DNA or to the release of HBV virions. For amplification, nucleocapsids are reuncoated for the release of viral DNA which follows the way described for primary infection (see above). The virion assembly pathway comprises the integration of the envelope proteins (L, M, S) into the ER membrane and the budding of nucleocapsids into the ER gaining the native viral envelope. Viral particles are released via the cellular secretion pathway. Informations concerning the viral 'life cycle' were gathered from www.who.int/csr/disease/hepatitis/ (see also citations therein).

3.2.2.2.1.1.3 … and hepatocarcinogenesis

Hepatitis B virus DNA is capable of integrating into the host genome and is indeed commonly detected in cells isolated from HBV-associated HCCs. However, the impact of this mechanism on hepatocarcinogenesis is not clear yet. Integration is invariably associated with deletions and mediates secondary genomic rearrangements like translocations, inversions, deletions and possibly amplifications [87, 88]. However, contradictory results concerning the site-specificity of integration and, hence, the targeted activation of distinct proto-oncogenes have been reported [87, 89]. Although no preference for distinct pathways has been identified, cancer-relevant genes such as telomerase reverse transcriptase (TERT), platelet-derived-growth-factor receptor-β (PDGFRβ), mitogen activated protein kinase 1 (MAPK1), tumor suppressor genes, and genes for apoptosis control have been found to be affected disproportionately [90].

HBx transactivates the expression of genes involved in cell cycle control and proliferation (e.g. Ras, Raf, MAPK, c-fos, c-jun, c-myc, EGF) [91-95] and can bind to and inactivate the tumor suppressor and master regulator of the DNA damage-checkpoint control, p53, which further fosters the accumulation of mutations [96]. In addition, there are indications for the X-mediated modulation of Ca-signaling and the consequent activation of Ca-dependent kinases (e.g. Pyk2) as well as the activation of protein kinase C (PKC), both pathways possibly leading to the activation of e.g. NFκB [97, 98].

The envelope protein L has proved to be hepatotoxic and sufficient for inducing hepatocarcinogenesis [99, 100]. It is hypothesized that ER stress is at the basis of the observed carcinogenic effect. The overexpression and, hence, intracellular accumulation of viral glycoproteins induces ER stress which

in turn predispose cells to transformation [101]. The endoplasmic reticulum (ER) of hepatocytes is crucial for the assembly of HBV particles by providing the lipid membrane with integral viral proteins L, M, and S for the constitution of the viral envelope (Figure 7). Direct interaction of provirus particles with the ER [102] or the overloading of the cellular secretion apparatus induces ER stress and lead eventually to oxidative stress which can consequently stimulate growth- and survival signaling pathways, cause mutations due to free radicals, and activate stellate cells [103, 104].
Persistent rounds of hepatocyte necrosis, inflammation, and regeneration due to chronic active infections are supposed to contribute decisively to carcinogenesis (Figure 4) [103, 105]. Apart from inefficient virus particle clearance by the host immune system, mutations of HBV might lead to their retention within hepatocytes eventually leading to cellular damage and liver disease [106].

3.2.2.2.1.2 Hepatitis C virus...

3.2.2.2.1.2.1 ...from the epidemiologist's point of view

Hepatitis C virus (HCV) infection is a considerable risk factor for the development of chronic hepatitis, liver cirrhosis, and HCC. Unless HBV, the majority of acute HCV-infections (60-80%) end up in a chronic disease state. There is currently no preventive vaccine available and today's standard therapy has only low efficacy. It is consequently expected that the number of HCV-infected individuals will further increase over the next 20 years and with that the incidences for liver cirrhosis and HCC [107-109]. The geographical distribution of the prevalence of HCV infection is depicted in figure 8. HCV-infection increases the risk to develop HCC 17-fold [110].

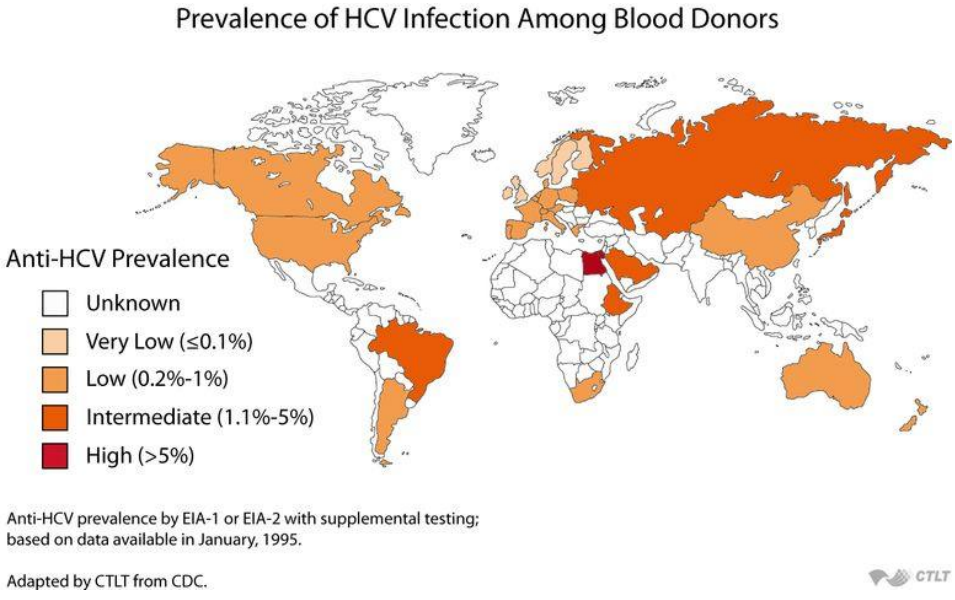


Figure 8: Geographical distribution of the prevalence of HCV infection (from CTLT[2])

[2] Center for Transforming Learning and Teaching

3.2.2.2.1.2.2 ...from the virologist's point of view

HCV is a member of the flaviviridae family. Its positive-strand RNA genome encodes the core protein and two envelope proteins (E1 and E2) as well as a number of non-structural proteins comprising p7 ion channel, the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins, and an RNA-dependent RNA polymerase (NS5B) [109].

The 'life cycle' of HCV: HCV exists in various forms in the systemic circulation. It can be associated with low-density lipoprotein (LDL) and very-low-density-lipoprotein (VLDL) as well as bound to antibodies or circulate as free virion [111]. Several receptors have been proposed to mediate viral binding to the host cell surface including CD81 [112], the LDL receptor (LDLR) [113], SR-BI [114], and claudin-1 [115]. There are indications that they act cooperatively to allow viral attachment and subsequent entry into the host cell. HCV is internalized via clathrin-mediated endocytosis [116] and passes subsequently through endosomal compartments with decreasing pH (Figure 9) [117, 118].

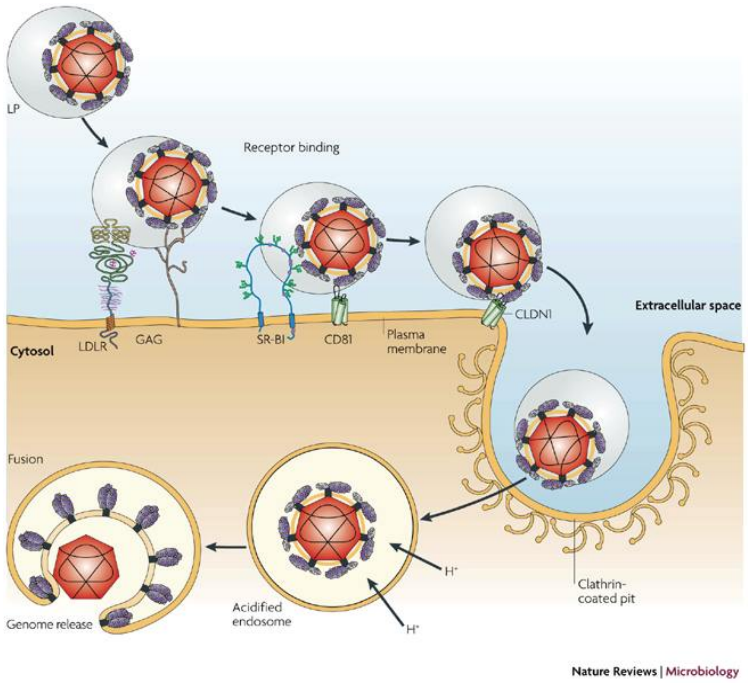


Figure 9: Mechanism of HCV cell entry (from [109])

The low pH triggers the fusion of the viral envelope and the endosomal membrane resulting in the release of the viral capsid into the cytoplasm. After uncoating, the 9.6-kb positive RNA genome is translated in an IRES-dependent manner and delivers a (potential) polyprotein which is co- and posttranslationally processed. The expression and processing of viral proteins strongly depends on the cellular machinery including e.g. translation initiation factors or ER signal peptidase, respectively. Amplification of viral genomic RNA occurs through the viral replication complex which is functionally associated with the host's endoplasmic reticulum. At those sites, the shape and structure of the endoplasmic reticulum is drastically altered [119]. Several host factors contribute to productive replication. It has been shown that HCV replication is stimulated by saturated fatty acids and

decreased by polyunsaturated fatty acids or by inhibition of fatty acid synthesis indicating the importance of lipid metabolism [109]. Moreover, cellular cyclophilin B, a peptidyl-prolyl cis-trans isomerase, fosters replication by stimulating the RNA binding activity of NS5B. Recently, it has been shown that FKBP8 (FK506-binding protein 8) and Hsp90 regulate viral replication by forming a complex with NS5B [120].

The late steps of HCV infection- packaging, assembly, particle release- is still obscure. Presumably, viral release occurs similar to that of HBV by budding into the ER and exiting through the secretory pathway. An overview of the infectious cycle is depicted in figure 10.

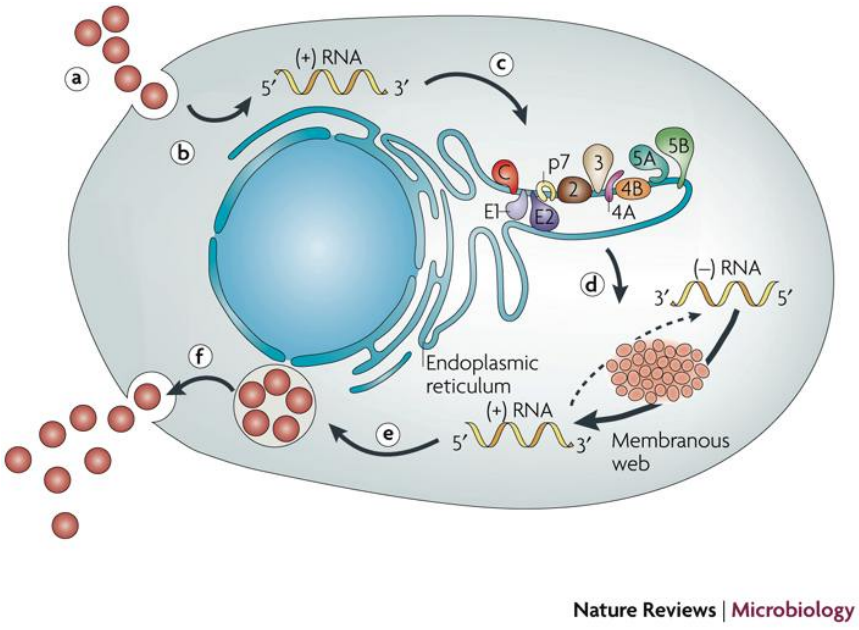


Figure 10: Lifecycle of HCV (from [109])

3.2.2.2.1.2.3 … and hepatocarcinogenesis

Mechanisms and proteins contributing to HCV-induced metabolic alterations and hepatocarcinogenesis: HCV infection is a major cause for liver injury such as steatosis, fibrosis, cirrhosis, and hepatocarcinogenesis. The impact of viral proteins on the latter, however, is still unclear considering the generally long latency of HCC in the face of a persistent infection (figure 3). However, epidemiologic studies proved differences in terms of hepatitis progression and tumor promotion caused by HCV and HBV infections concerning the incidence of and the predictive parameters for HCC development, the proportion of cirrhosis among individuals suffering from HBV- and HCV-mediated HCC (50% vs. 70%), and the three-year-survival rates [121, 122]. At least three HCV proteins feature oncogenic potential by modulating cancer-relevant signaling pathways (see below). Accordingly, the carcinogenesis rate due to HCV infection has been shown to be significantly higher than due to HBV [122]. In addition, liver fibrosis is suggested a major risk factor and predictor for HCV- rather than for HBV- associated hepatocarcinogenesis [122]. This might indicate that the substantial tissue alterations occurring in consequence of liver injury play distinct roles in hepatocarcinogenesis with respect to the underlying viral etiologies albeit they are indisputably pivotal for HCC development

in general (Figure 4). Therefore, it is appropriate to regard HCC as the product of a complex interplay between viral and host factors, both contributing to a microenvironment susceptible for cancer progression.

HCV vs. host immune system- persistent infection and collateral damage of the liver: Unlike HBV, HCV does not integrate into the host genome. Instead, persistent infection is established by interfering with and modulating the antiviral immune defense, in particular many parts of the adaptive immune system.

HCV inhibits the trafficking machinery of cells and therewith prevents the release of cytokines (e.g. interferon-β) and the presentation of MHC class I-molecules loaded with viral proteins, both of which triggering the host immune response. Interferon-β is secreted by virus-infected cells and plays a crucial role for the antiviral defense. Binding to its receptor on the surface of cells enhances the synthesis of proteasomal and MHC class I proteins for antigen-presentation to T-cells as well as that of miRNAs inhibiting the translation of viral RNA [123, 124]. At least two viral proteins, NS4A/B precursor and NS5A, are involved in reducing the rate of ER to Golgi transport and, hence, attenuate cellular secretion performance [125]. Mechanistically, NS5A annexes TBC1D20, a Rab-GTPase activating protein for Rab1, to the viral-replication complex and prevents its normal cellular function in ER-Golgi transport [126, 127].

HCV abrogates efficiently viral detection by infected hepatocytes and hinders consequently the onset of defense mechanisms such as the transcriptional activation of IFNs. In general, sensing of viral invaders is achieved by pathogen-recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) like double-stranded RNA [128]. In hepatocytes, RIG-I activation by viral RNA triggers its binding to IPS-1 (IFN promotor stimulator 1) resulting eventually in transcription of IFN [129]. IPS-1 localizes to the mitochondrial membrane which has proved to be critical in this context. The NS3-4A protease cleaves IPS-1 which leads to the release from the mitochondrial membrane and thereby prevents RIG-I signaling [128, 129]. Futhermore, NS3-4A targets TRIF (Toll-interleukin-1 receptor domain-containing adaptor inducing IFN) which is involved in signaling via TLR3, an additional sensor of viral infection. Hence, NS3-4A contributes decisively to the impairment of the host's first-line defense against viral invasion [130].

Apart from interfering with antiviral mechanisms of hepatocytes, HCV induces alterations of the immune response at the systemic level as well. Plasmacytoid dendritic cells, a subtype of dendritic cells occurring in blood and peripheral lymphoid organs, are capable to sense efficiently viral invasion and respond to it with the secretion of type I interferons (IFN-α, IFN-β). The HCV core protein, released into the systemic circulation, is implicated in the reduction of the number and the attenuation of the activatability of these cells and by means of that exerts a general immunosuppressive effect [131, 132].

It is supposed that HCV affects globally the virus-induced cytokine expression profile which is crucial for the onset of an appropriate immune response including the activation and expansion of T-cells as hub of the adaptive immune system [133-135].

Interference with host cell-mediated antiviral defense mechanisms: IFN is a pivotal trigger for the onset of the cellular antiviral defense. It acts via the Jak-STAT pathway and eventually leads to the transcriptional activation of IFN-regulated genes such as PKR (protein kinase R), Mx proteins, and

RNase L. HCV replication strongly depends on the host cell endoplasmic reticulum which is consequently overloaded with viral proteins. Host cells react to that with the induction of the ER stress response which manifests in the upregulation of proteins for resolving this potentially harmful condition. One of those proteins is PP2A, a phosphatase involved in cell-cycle regulation and signaling and an effective inhibitor of the Jak-STAT pathway [136-139].

PKR shuts down protein synthesis and, hence, inhibits translation of cellular and, most notably, viral RNA. The HCV proteins, E2 and NS5A, are known to prevent PKR activation [139, 140].

The contribution of HCV proteins- C, NS3, and NS5A- to hepatocarcinogenesis is comprehensively treated by Kasprzak and Adamek [141]. Those proteins affect many cell biological functions by modulating a broad variety of signaling pathways.

The *core protein (C)* is the structural component constituting the HCV-nucleocapsid in which viral genomic RNA is encapsidated. Intracellularly, it is found on ER membranes, in membranous webs, on the surface of lipid droplets and in the nucleus [109]. The association with lipid droplets fosters the folding of C, might play a role in viral replication/virion morphogenesis, and is suspected to alter cellular lipid metabolism and hence to contribute to liver steatosis [109, 142, 143].

Moreover, there are indications that it contributes to the establishment of persistent HCV infection and to transformation of hepatocytes and hence development of HCC. Several reports suggest that the core protein interferes with TNFα signaling. TNFα is a proinflammatory cytokine secreted by activated macrophages and T-cells and plays a pivotal role for the eventual clearance of viral infected cells by inducing FAS-mediated apoptosis [103]. The core protein prevents TNFα/FAS-mediated apoptosis by interacting with the cytoplasmic domains of TNFRI, lymphotoxin B receptor, and gC1q receptor [144-147]. Furthermore, it modulates signaling upon proinflammatory stimuli by targeting transcription factors involved in immune response and proliferation such as NFκB and AP-1. Ectopic expression of the core protein has been shown to inhibit NFκB activation by preventing degradation of its inhibitor IκB whereas AP-1 is constitutively activated via JNK and MAPKK [148]. Moreover, core protein-induced activation of ERK, JNK, and MAPK enables growth-factor (EGF, TGF-α) independent proliferation, a hallmark of cancer [149, 150]. However, there are indications that NFκB is rather upregulated and activated as has been shown in HCV core protein-transfected liver cells and in hepatocytes from chronically infected patients [151]. NFκB is frequently upregulated and activated in tumor tissue suggesting its role in hepatocarcinogenesis [152]. Indeed, core protein-expressing transgenic mice show increased HCC rates [153].

NS3 is a non-structural multifunctional viral protein. It has protease, helicase, and NTPase activity [154]. In addition, it can translocate to the nucleus and bind to histones thereby interfering with PKA-mediated histone phosphorylation [155]. However, NS3 mainly resides in the cytoplasm and the ER of infected hepatocytes [156, 157]. Cytoplasmic NS3 prevents translocation of PKA into the nucleus and hence histone phosphorylation and might interfere with other PKA-mediated functions as well [155].

NS3 expression is sufficient to induce cell transformation *in vitro* [158, 159]. It is suggested that this effect is partially mediated by the protease activity which possibly degrades regulatory proteins [141]. In addition, NS3 can induce the activation of MAPK and JNK, foster DNA-binding activity of AP-1, and enhance c-jun expression [160, 161]. The NS3 protein binds to and affects the activity to p53 leading to the inhibition of p21 transcription, enhanced proliferation, and to the inhibition of apoptosis [162,

163]. NS3 secreted by infected hepatocytes can activate stellate cells which manifests in the augmented secretion of chemokines and TGF-β. The latter is a pro-fibrogenic factor which fosters collagen deposition by stellate cells eventually leading to liver fibrosis [164, 165].

NS5A is a component of the viral replicase and implicated in HCV-induced carcinogenesis. It serves as a transcription activator for many genes. A disproportionately high number contain NFκB-binding sites in their promotors suggesting a crucial role of NS5A-induced NFκB activation [166]. The latter is suggested to prevent apoptosis and hence to contribute to hepatocarcinogenesis. The anti-apoptotic effect of NS5A is mediated by the inhibition of caspase-3 activation as well as poly(ADP-ribose) polymerase cleavage in TNFα-treated cells [167]. NS5A transcriptionally represses p21 in a p53-dependent manner [168, 169].

3.2.2.2.2 Dietary carcinogens and tumor promotors

3.2.2.2.2.1 Alcohol

Chronic alcohol intake induces fibrosis, cirrhosis, and increases the risk for hepatocarcinogenesis. Several mechanisms are supposed to contribute decisively to alcohol-induced pathophysiological alterations including the activation of cells of the innate immune system and the steady elevation of oxidative stress.

Chronic alcohol consumption entails the activation of monocytes which in turn produce pro-inflammatory cytokines like TNF and IL-8 as well as decreased levels of the antiinflammatory IL-10 [170]. Moreover, alcohol sensitzes Kupffer cells to endotoxins delivered by the portal vein which causes the activation of LPS-responsive signaling pathways (TLR-4) and the consequent upregulation of proinflammatory cytokines and chemokines (TNFα, IL-1β, IL-6) [171, 172]. This effect might be further enhanced by the increased amount of endotoxins getting into the circulation due to the alcohol-induced damage of the mucosa of the gastrointestinal tract [173]. In this context, the effect of chronic inflammation is further exacerbated by the increased sensitivity of hepatocytes to TNFα-mediated cytotoxicity [172].

3.2.2.2.2.2 Aflatoxin B1

Ingestion of aflatoxin B1-contaminated food increases the risk for the development of HCC. Aflatoxin B1 is a mycotoxin produced by *Aspergillus flavus* and *parasiticus* which infect crop particularly in warm and humid climates. Aflatoxin B1 is metabolized to aflatoxin M1 by hepatocytes. The latter is a highly reactive epoxide which forms DNA-adducts that might eventually lead to mutations. Indeed, the mutagenic activity seems to be the primary mechanism for the initiation of hepatocarcinogenesis since exposure does not correlate with cirrhosis incidence in contrast to other HCC etiologies [83]. Aflatoxin-induced development of HCC is frequently associated with a specific mutation of p53, whereas the ras gene seems to be not affected by aflatoxin [174]. The co-occurence of aflatoxin and HBV increases the risk for liver cancer 4-10-fold compared to the carcinogenic effect of the single agents.

3.2.2.3 Common molecular themes in hepatocarcinogenesis- TP53

The signaling pathways involved in HCC-tumor development, progression, and invasion have been comprehensively discussed by Zender et al. [175]. However, the tumor suppressor p53 occupies a central role by being functionally compromized in most human cancers and is indeed a common feature of hepatocellular carcinoma irrespective of the causative factor.

The failure to accomplish its tasks contributes to the manifestation of well-defined tumor characteristics [32]. However, there are distinct mechanisms leading to its malfunction by interfering with the regulatory circuit controlled by p53 (Table 2, from [176]).

Mechanism of inactivating p53	Typical tumors	Effect of inactivation
Amino-acid-changing mutation in the DNA-binding domain	Colon, breast, lung, bladder, brain, pancreas, stomach, oesophagus and many others	Prevents p53 from binding to specific DNA sequences and activating the adjacent genes
Deletion of the carboxyterminal domain	Occasional tumors at many different sites	Prevents the formation of tetramers of p53
Viral infection	Cervix, liver, lymphomas	Products of viral oncogenes bind to and inactivate p53 in the cell, in some cases stimulating p53 degradation
Deletion of the p19ARF gene	Breast, brain, lung and others, expecially when p53 is not mutated	Failure to inhibit MDM2 and keep p53 degradation under control
Mislocalization of p53 to the cytoplasm, outside of the nucleus	Breast, neuroblastomas	Lack of p53 function (p53 functions only in the nucleus)
Genomic multiplication of the MDM2 gene	Sarcomas, brain	Extra MDM2 stimulates the degradation of p53

Table 2: Mechanisms involved in the inactivation of p53

The transcription factor p53 occupies center stage in an extended molecular network connecting pathways for cell-cycle control, apoptosis, DNA repair, and blood-vessel formation. It is activated upon cellular stress and damage induced by DNA double-strand breaks (e.g due to ionizing radiation), aberrant growth signals, as well as chemotherapeutics, phosphatase inhibitors, and UV radiation [176]. Although three distinct pathways are involved in sensing and signaling of those potentially harmful cellular conditions, all of them bring about the stabilization and hence an increase in concentration of the otherwise short-lived and latent p53.

The abundance of p53 is principally regulated by the rate of its degradation. Under normal conditions p53 exists as heterodimer with MDM2, an E3 ubiquitin-protein ligase, which tags it for ubiquitin-mediated proteolysis. Moreover, p53 serves as a transcriptional activator for MDM2 which in turn affects half-life of p53. This feedback loop allows the maintenance of a constantly low base level of p53 and provides concomittantly a rapid mechanism to increase its concentration and hence to adapt to cellular stress by factors interfering with MDM2-mediated degradation. This is the principal avenue pursued by pathways leading to p53 stabilization described below.

However, additional regulatory levels are implemented controlling transcriptional activity of p53. An important role for p53 function play conformational changes triggered by the attachment or removal of functional groups like phosphate, acetyl, glycosyl, ubiquitin, methyl, NEDD (an ubiquitin-like protein), or sumo [177-180]. The carboxyterminus of p53 normally covers its DNA-binding domain inhibiting its transcription factor activity. Acetylation and phosphorylation at the carboxyterminus are supposed to

interfere with this folding and hence release the block of DNA-binding as basis for the observed increase of transcriptional activity. Although a consensus sequence in the promotor region of p53 target genes exists, unique combinations of posttranslational modifications of p53 might provide the basis for the temporal and contextual finetuning of p53-mediated gene expression as proposed by Kruse and Gu [180].

Crucial components of the pathway triggered by DNA double-strand breaks are the checkpoint proteins ATM (ataxia telangiectasia mutated), DNA-dependent kinase, Chk1, and Chk2 (Checkpoint kinases 1 and 2) [181]. All of these kinases, once activated by DNA damage, are capable to phosphorylate p53 in the proximity to its MDM2-binding region, thereby preventing interaction with MDM2 and hence ubiquitin-mediated proteolysis. However, there are indications that an alternative mechanism for p53 stabilization might exist in this context [177-179, 182].

Aberrant expression of oncogenes like Ras and Myc activate p53 via $p14^{ARF}$, a product of the Ras-responsive INK4a/ARF tumor supppressor locus [183, 184]. Those oncogenes increase the concentration of $p14^{ARF}$ by stimulating its transcription or by stabilizing the protein. Two cooperatively acting or alternative mechanisms are involved to protect p53 from MDM2-dependent degradation. $P14^{ARF}$ directly binds to and inactivates MDM2. In addition, $P14^{ARF}$ mediates the sequestration of MDM2 in the nucleolus, a subcompartment of the nucleus dedicated to ribosomal biosynthesis, and hence spatially prevents its interaction with p53 which remains outside of the nucleolus. However, $p16^{INK4a}$, an antagonist of cyclin D-dependent kinases, has to be expressed concomittantly for effective cell cycle arrest due to oncogenic Ras [185].

The third pathway is triggered by chemotherapeutics, phosphatase inhibitors, and UV light and does not depend on the afore-mentioned factors for p53 activation (ATM, Chk2, $p19^{ARF}$). In this context, the kinases ATR (ataxia telangiectasia related) and casein kinase II are considered to be the crucial signaling components [186].

The main function of activated p53 is the transcriptional activation of target genes which is prevented or compromized by all mutations known to occur in p53 [187, 188]. The first effect of p53 expression is the cell-cycle arrest. One of the target genes upregulated by p53 is $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs) which, together with cyclins, drive cell cycle progression. Inhibition of CDKs leads to a block at the G1-to-S and G2-M checkpoints [176]. 14-3-3σ, another p53-dependent protein, participates in the maintenance of the G2 arrest by sequestering the cyclin B1- CDK1 complex in the cytoplasm [189, 190]. The impact of 14-3-3σ on the anti-proliferative effect of p53 is underlined by the observation that its inhibition is sufficient to induce indefinite proliferation in epithelial cell culture [191].

Another effect of p53 expression is the transcriptional activation of apoptosis-inducing proteins, such as Bax [192], NOXA [193], and P53AIP1 [194] or PIDD, a 'death signal'-receptor [195]. An alternative mechanism of apoptosis-induction might be the p53-mediated stimulation of mitochondria to produce ROS (reactive oxygen species).

P53 is also involved in preserving genetic stability by inducing gene expression of components for DNA-repair (e.g. nucleotide-excision repair, recombination, chromosome segregation) [196, 197]. Moreover, it prevents the expression of genes triggering vascularization and inhibits angiogenesis [198, 199], a characteristic feature of tumor suppressors [200].

3.3 The liver

3.3.1 General remarks

The liver is a prime example for complexity at many organization levels. Its enormous metabolic capacity is sustained by a variety of different cell types, spatially organized to small hexagonal functional units which, in their collectivity, constitute the largest gland and the most versatile organ of the human body. The liver is anatomically and functionally associated with the small intestinum. It serves as a filter for blood leaving the digestive tract via the portal vein before entering the systemic circulation. Hence, most absorbed compounds like nutrients (carbohydrates, amino acids, lipids), vitamins, ions, drugs or toxicants pass through the liver where they are metabolized, stored and/or released into the blood, (de)toxified and/or excreted into bile. Thus, the liver is crucial for the maintenance of metabolic homeostasis of the body and protection against potentially harmful substances.

The high demands and its key position in metabolism require mechanisms to compensate for damage or loss of tissue. Indeed, the liver features an outstanding potential for regeneration which implies the ability of cells to dedifferentiate, proliferate, and redifferentiate in a temporally and spatially highly coordinated manner to recover full functionality. However, exuberant and/or permanent damage of liver tissue overloads or compromizes the fidelity of the regeneration process consequently leading to the loss of liver parenchyme (cirrhosis) or disproportioning of liver cell types by deposition of connective tissue (fibrosis). In addition, chronic or repetitive liver injury inducing persistent rounds of necrosis and regeneration associated with the obligatory activation of physiological mechanisms involved in defense and repair (inflammation, oxidative stress) lay also the (micro-) environmental basis for the initiation and/or progression of primary liver cancer and are, indeed, considered as common molecular prerequisites for hepatocarcinogenesis almost irrespective of its etiology (see figure 4).

3.3.2 Microstructure of the liver and hepatocytes and how they account for liver physiology and function

3.3.2.1 Liver parenchyme- hepatocytes

Hepatocytes constitute about 70% of the overall liver volume and partially contain two nuclei or are tetraploid (age-dependently). The pronounced polarization of these epithelial cells and their arrangement in plates (Muralium simplex-architecture) provide the structural basis (concerning surface-volume ratio) for directed and regulated exchange processes. For that, two distinct compartments are in close contact with and are spatially separated by hepatocytes, the liver sinusoids as specialized part of the vascular system and the bile canaliculi derived from the apical cell surfaces of adjacent hepatocytes of the muralium simplex. The sinusoids are capillaries with an inner width of

up to 15 μm and are composed of extensively fenestrated endothelial cells lacking a basal membrane. This discontinous endothel (10-50 pores/ μm^2) enables free access of blood components apart from blood cells and chylomikrones to the perisinusoidal space (space of Disse), the space between the sinusoidal lining cells and the microvilli-containing basal membrane of hepatocytes (extended surface area). The sinusoids receive oxygen-deprived, nutrient-enriched blood from the capillary bed of the gastrointestinal tract via the portal vein (Vena portae) as well as oxygenated blood via the liver artery (Arteria hepatica propria). On its way towards the central vein of the lobuli the blood serum enters the space of Disse, comes into contact with the hepatocytic cell surface where exchange processes occur and consequently its composition is drastically altered due to the metabolic and synthetic performance of hepatocytes. The blood is eventually released again into the systemic circulation via the Venae hepaticae.

3.3.3 Other cell types of the liver

3.3.3.1 The sinusoidal lining cells

The sinusoids are built from fenestrated sinusoidal endothelial cells. They constitute sieve plates with 10-50 pores/ μm^2.

3.3.3.2 Endothelial cells

The pore size of endothelial cells is regulatable by serotonin. They synthesize v.Willebrand factor and contain heparin-releasable lipase, a clearing factor for lipoproteins which are consequently clathrin-dependently endocytosed.

3.3.3.3 Kupffer cells

Kupffer cells are immigrated yolk sac or bone marrow macrophages. They are phagocytotic cells crucial for the clearance of immune complexes (via Fc-receptors, Crb-receptors), protease-antiprotease complexes (e.g. thrombin-antithrombin), old erythrocytes, endotoxin or bacteria and lysosomal enzymes derived from peripheral tissue degradation. In addition, they serve as antigen-presenting cells and participate in inflammation by producing e.g. IL-1 and IL-6.

3.3.3.4 Stellate cells

Stellate cells contribute to normal liver function constituting approximately 1/3 of non-parenchymal cells or 15% of the total number of resident cells of the normal liver and mediate fibrosis following liver injury. They are located in the subendothelial space, are typically spindle-shaped with prominent dendritic processes extending beneath endothelial cells and wrapping around sinusoids. These projections bear numerous spines making contact to hepatocytes on the lumenal side facilitating intercellular transport of soluble mediators and cytokines. Moreover, direct connections to nerve

endings might be important for neurally mediated vasoregulation. Stellate cells are the primary storage of vitamin A (histologically visible droplets).

Following liver injury, they are activated and consequently transdifferentiate into myofibroblasts, a process mainly triggered by Kupffer cells and cytokines (e.g. IL-1, TGF-β). Protein synthesis and secretion performance is increased reflected by the enlargement of the rough endoplasmic reticulum and Golgi apparatus. Indeed, they synthesize collagen type I and III, dermatan- and heparan-sulfate and matrixproteins (fibronectin, laminin) as well as PDGF receptor (platelet-derived growth factor receptor) and α-smooth muscle actin.

3.3.4 Metabolic and synthetic performance of hepatocytes and their role in the systemic/physiological context

3.3.4.1 Carbohydrate homeostasis

The liver plays a crucial role in glucose homeostasis by equilibrating resorption/postresorption phase-derived fluctuations of glucose in blood plasma. An elevation of plasma levels of energy-rich compounds leads to an increased secretion of insulin and reduced synthesis of glucagon by β-cells and α–cells of the pancreatic Langerhans' island, respectively. The increased insulin-glucagon ratio consequently forces their uptake and storage by hepatocytes. Glucose as well as fructose and galactose after being converted into glucose are stored as glycogen. In the postresorption phase the insulin-glucagon ratio is inverted triggering the mobilization of the glycogen depot and the release of free glucose into the systemic circulation. In the case of complete glycogen deprivation due to a prolonged starvation period, the liver performs gluconeogenesis from lactate and glycerine (products of glycolysis (Cori cycle) and lipolysis) as well as from glucogenic amino acids in order to ensure the glucose supply for erythrocytes, the central nervous system, and the Medulla renalis. Cortisol is the main signaling molecule which induces an increase of the activity of enzymes involved in gluconeogenesis and amino acid metabolism and a decrease of those involved in glycolysis.

3.3.4.2 Lipid metabolism

A main function of the liver in the resorption phase is the biosynthesis of triacyl glycerines, phosphoglycerides, and sphingolipids from dietary carbohydrates and lipids (degradation of LDL- and HDL-lipoproteins) as well as the biosynthesis and secretion of VLDL-lipoproteins. Depending on the availability of cholesterin, it also synthesizes and thereof provides cholesterin for the organism. In the postresorption phase, energy for hepatocytes is gained by fatty acid oxidation. Excess fatty acids are metabolized to keton bodies in order to cover the energy requirements of extrahepatic tissues.

3.3.4.3 Amino acid metabolism

Amino acids from nutriental proteins (resorption phase) as well as those released by protein degradation in extrahepatic tissues (postresorption phase) are taken up via a variety of amino acid

transporters of the cell membrane of hepatocytes. Glucogenic amino acids are used for gluconeogenesis whereas ketogenic amino acids serve as anaplerotic substrates for the citrate cycle following deamination. Ammonium is detoxicated in the urea cycle by conversion to urea, a speciality of hepatocytes, and consequently renally excreted. In the course of this process bicarbonate ions are needed which is a key metabolite for the regulation of the pH of blood. Indeed, the urea cycle is reduced in the case of acidosis. Under those conditions ammonium is fixed by the conversion of glutamate to glutamine by glutamine synthetase in order to avoid toxic effets.

3.3.4.4 Protein metabolism

Hepatocytes synthesize and release plasma proteins like coagulation factors, protease inhibitors (e.g. α1-antitrypsin, α-2-makroglobulin), transport proteins (e.g. transferrin, albumin) into sinusoids as well as prohormones (angiotensinogen, kininogen) and acute phase proteins. The latter increase 2- 1000-fold 6-48 h after infection upon stimulation via IL-1 and IL-6 released by macrophages, endothelial cells, and fibroblasts and in the presence of glucocorticoids. Moreover, hepatocytes participate in the lysosome-mediated degradation of proteins, especially glycoproteins which are bound and internalized via the asialoglycoprotein receptor.

3.3.4.5 Storage of vitamins and trace elements

Hepatocytes contain significant amounts of water-soluble vitamins (e.g. thiamin, folic acid, vitamin B12, biotin). In addition, the liver hydroxylates vitamin D to the biologically active form 1,25-Dihydroxycholecalciferol and converts thyroxin (T_4) to triiodthyronin. The liver stores significant amounts of the trace elements iron and copper.

3.3.4.6 Biotransformation- the liver as excretion organ

The bile canaliculi comprise the second compartment of the liver. They unify to higher-order bile ducti and eventually end up in the ductus coledochus which discharge into the small intestine. The primary or liver bile is further modified by cholangiocytes in the course of its passage.

Most xenobiotics, to which organisms are exposed, are lipophilic fostering absorption through epithelia and thereby hindering or retarding their elimination. Therefore, biotransformation processes for conversion to hydrophilic metabolites or derivatives are required to accelerate the excretion of potentially harmful substances. The intensity and duration of drug effects (pharmacodynamics) are largely determined by these processes. Hepatocytes express a variety of enzymes (constitutively or inducibly by xenobiotics) involved in biotransformation which characteristically feature broad substrate specificity by e.g. metabolizing endogenous substrates as well. They are divided into phase I and phase II enzymes reflecting the common two-stage biotransformation process. Phase I reactions expose or introduce functional groups (hydroxyl-, thiol-, carboxyl-groups) that largely constitute the molecular targets for phase II reactions (e.g. glucuronidation, acetylation) mediating the hydrophilic substance properties for biliary or urinary excretion.

4 Methods: Proteome analysis of liver and HCC tissues

4.1 Tissue homogenization and cell lysis prior to proteome analysis

For shotgun analysis, HCC and adjacent liver tissue samples (kept at -196°C) were thawed on ice, minced into small pieces, passed through a metal sieve of 80 µm mesh size, then through a 40 µm filter (Millipore), and were eventually homogenized using a tissue-grinder. All steps were performed at 4°C and in the presence of protease inhibitors. Cells of the tissue homogenate were lysed in hypotonic lysis buffer (10mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100) supplemented with protease inhibitors and pressed through a 26 g syringe to induce cell lysis. The cytoplasmic fraction was separated from nuclei by centrifugation and precipitated by the addition of ethanol. Afterward, all protein samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.5% SDS, 100 mM DDT) [36, 201], the protein mixture was pre-fractionated by PAGE and further processed for mass spectrometry analyses (see below).

Metabolic labeling of tissue slices: Freshly resected human liver and HCC tissues were cut in slices of about 200 mm thickness, rinsed in Hank's buffered saline solution (HBSS) and incubated in serum-free, methionine and cysteine-free William's E medium (ICN) in the presence of [35S]-labelled methionine and cysteine (Trans35- Slabel, Biomedica, MP Biomedicals) for 6 h at 377C. Supernatants were collected, filtered through a 0.22 mm filter (Millex-GP, Millipore, Billerica, MA, USA) to get rid of cell debris, soluble proteins were precipitated by the addition of ethanol. For the isolation of cytoplasmic proteins, all buffers were supplemented with protease inhibitors: PMSF (1 mm), aprotinin, leupeptin,and pepstatin A (each at 1 mg/mL). Cells were lysed in lysis buffer (10mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10mM NaCl, 3mM MgCl2, 0.5% Triton X-100). The cytoplasmic fraction was separated from the nuclei by centrifugation and ethanol precipitated. Protein samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4%CHAPS, 0.5%SDS, 100mM DTT) and subjected to 2D-PAGE.

4.2 Separation of complex protein mixtures

4.2.1 2D polyacrylamid gel electrophoresis

Proteins were loaded by passive rehydration of IPG strips pH 5–8, 17 cm (BioRad, Hercules, CA) at room temperature. IEF was performed in a stepwise fashion (1 h 0– 500 V linear; 5 h 500 V; 5 h 500–3500 V linear; 12 h 3500 V). After IEF, the strips were equilibrated with 100 mM DTT and 2,5 % iodacetamide according to the instructions of the manufacturer (BioRad). For SDS-PAGE using the Protean II xi electrophoresis system (BioRad), the IPG strips were placed on top of 1,5 mm 12 % polyacrylamide slab gels and overlaid with 0,5 % low-melting agarose. The gels were stained with a 400 nM solution of Ruthenium II tris (bathophenanthroline disulfonate) (RuBPS) as described [202]. Fluorography scanning was performed with the FluorImager 595 (GE Healthcare, Fairfield, CT) at a

resolution of 100 µm. After scanning, the radioactively labeled gels were dried using the slab gel dryer SE110 (Hoefer, San Francisco CA, USA). After exposure to phosphor screens (Molecular Dynamics), the screens were scanned using the Phosphorimager SI (Molecular Dynamics) at a resolution of 100 µm as previously described [1]. All 2-D gel data were independently reproduced for at least four times. Alternatively, gels were silver-stained, protein spots cut out of the gel and tryptically digested. After the trypitc digest of proteins the resulting peptides were mass spectrometrically analyzed and MS/MS data interpreted using the Spectrum Mill MS Proteomics Workbench software (see figure 11).

Figure 11: Workflow for protein identification of 2D-PAGE- separated samples

4.2.2 PAGE for subsequent shotgun analysis

Cytoplasmic protein fractions were loaded on 12 % polyacrylamid gels, electrophoresis was performed until complete separation of a pre-stained molecular marker (Dual Color, Biorad, Hercules, CA) was visible. Gels were fixed with 50 % methanol/10 % acetic acid and subsequently silver stained as described below. Gel lanes were cut out of the gel and digested with trypsin as described below.

4.3 Selection and preparation of proteins for mass spectrometry

4.3.1 MS-compatible silver staining procedure

2D gels were fixed with 50 % methanol, washed and sensitized with 0,02 % $Na_2S_2O_3$. The gels were stained with 0,1 % $AgNO_3$ ice cold for 20 minutes, rinsed with bi-distillated water and subsequently developed with 3 % Na_2CO_3/0,05 % formaldehyde as previously described [203].

4.3.2 Tryptic digest

Protein spots were cut out of the gel, the gel-pieces were destained with 15 mM $K_3Fe(CN)_6$/50 mM $Na_2S_2O_3$ and intensively washed with 50 % methanol/10 % acetic acid. The pH was adjusted with 50 mM NH_4HCO_3, and proteins were reduced with 10 mM DTT/50 mM NH_4HCO_3 for 30 minutes at 56°C and alkylated with 50 mM iodacetamide/50 mM NH_4HCO_3 20 minutes in the dark. Afterwards the gel-pieces were treated with acetonitril and dried in a speedvac. Between each step, the tubes were shaken 5-10 minutes (Eppendorf Thermomixer comfort). Dry gel-spots were treated with trypsin 0,1 mg/ml (Trypsin sequencing grade, Roche Diagnostics, Germany)/50 mM NH_4HCO_3, in a ratio of 1:8 for 20 minutes on ice, afterwards covered with 50 mM NH_4HCO_3 and were subsequently incubated over night at 37°C. The digested peptides were eluted by adding 50 mM NH_4HCO_3, the supernatant was transferred into silicon-coated tubes, and this procedure was repeated two times with 5 % formic acid/50 % acetonitril. Between each elution step the gel-spots were ultrasonicated for 10 minutes. Finally the peptide solution was concentrated in a speedvac to an appropriate volume.

4.4 Mass Spectrometry analysis

For the identification of 2D spots, peptides were loaded on a Zorbax 300SB-C8 (5 µm, 0,3 mm, 5 mm) column and separated by nanoflow LC (1100 Series LC system, Agilent, Palo Alto, CA) with a Zorbax 300SB-C18 (5 µm, 75 mm, 150 mm) column at a flow rate of 250 nl/min using a gradient from 0,2 % formic acid and 3 % acetonitrile (ACN) to 0,2 % formic acid and 45 % ACN over 12 minutes. In case of shotgun analysis, peptides were separated by nano-flow LC (1100 Series LC system, Agilent, Palo Alto, CA) using the HPLC-Chip technology (Agilent) equipped with a 40 nl Zorbax 300SB-C18 trapping column and a 75 µm x 150 mm Zorbax 300SB-C18 separation column at a flow rate of 400 nl/min, using a gradient from 0,2 % formic acid and 3 % ACN to 0,2 % formic acid and 50 % ACN over 60 minutes. Peptide identification was accomplished by MS/MS fragmentation analysis with an iontrap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MS/MS data, including peaklist-generation and search engine, were interpreted by the Spectrum Mill MS Proteomics Workbench software (Version A.03.02, Agilent) allowing for two missed cleavages and searched against the SwissProt Database for human proteins (Version 20061207 containing 15.265 entries) allowing for precursor mass deviation of 1,5 Da, a product mass tolerance of 0,7 Da and a minimum matched peak intensity (%SPI) of 70 %. Due to previous chemical modification, carbamidomethylation of cysteines was set as fixed modification. No other modifications were

considered here. The listed peptides were identified with the indicated scores. The scores were essentially calculated from sequence tag lengths, but also consider mass deviations. To assess the reliability of the peptide scores, we performed searches against the corresponding reverse database. 5,9 % positive hits were found with peptides scoring >9,0, while 0,21 % positive hits were found with peptides scoring >13,0. Consequently, we set the threshold for protein identification to at least one peptide scoring higher than 13,0.

To eliminate redundancy in the case of peptides matching to multiple members of a protein family, the following procedure was applied and realized with a self-made algorithm. All proteins containing the peptides in question were proposed. Each protein proposal listed all matching peptides provided with a number indicating how many proteins contained the identical peptide. A peptide found in three different proteins got the protein-count three, while a peptide occurring only in a specific isoform got the protein-count one. Of the proposed proteins, the candidates containing at least one peptide with a protein-count one were positively selected. If no peptide with a protein-count one was identified, the protein entry containing the largest number of different peptides was positively selected. If several proteins were proposed containing the same number of identical peptides, the first entry of the isoforms in question was chosen (one before two, A before B etc.). Proteins not fulfilling these criteria were negatively selected. As a result of the employed strategy, the reliability of each protein identification can be individually scrutinized based on the identification data contained in the html-files.

4.5 Data preparation and correlation of histological data with shotgun results

The „Human Protein Atlas" (www.proteinatlas.org) provides protein expression data derived from antibody-based proteomics. It comprises about 5 million images of immunohistochemically stained normal (e.g. liver) and tumorous tissues (e.g. liver cancer) representing more than 5000 human proteins [204]. Protein expression levels are delivered by means of two independent parameters, the staining intensity (negative, weak, moderate or strong) and the fraction of stained cells (rare, <25%, 25-75% or >75%) (Figure 12). Both parameters equally contribute to the abundance of proteins reflected by the number of total proteins (Tp) delivered by mass spectrometry. In order to achieve comparability of immunohistochemical and shotgun data in terms of up- or downregulation with respect to normal tissue, intensity and quantity data have to be combined and compared to the combined data of normal liver tissue.

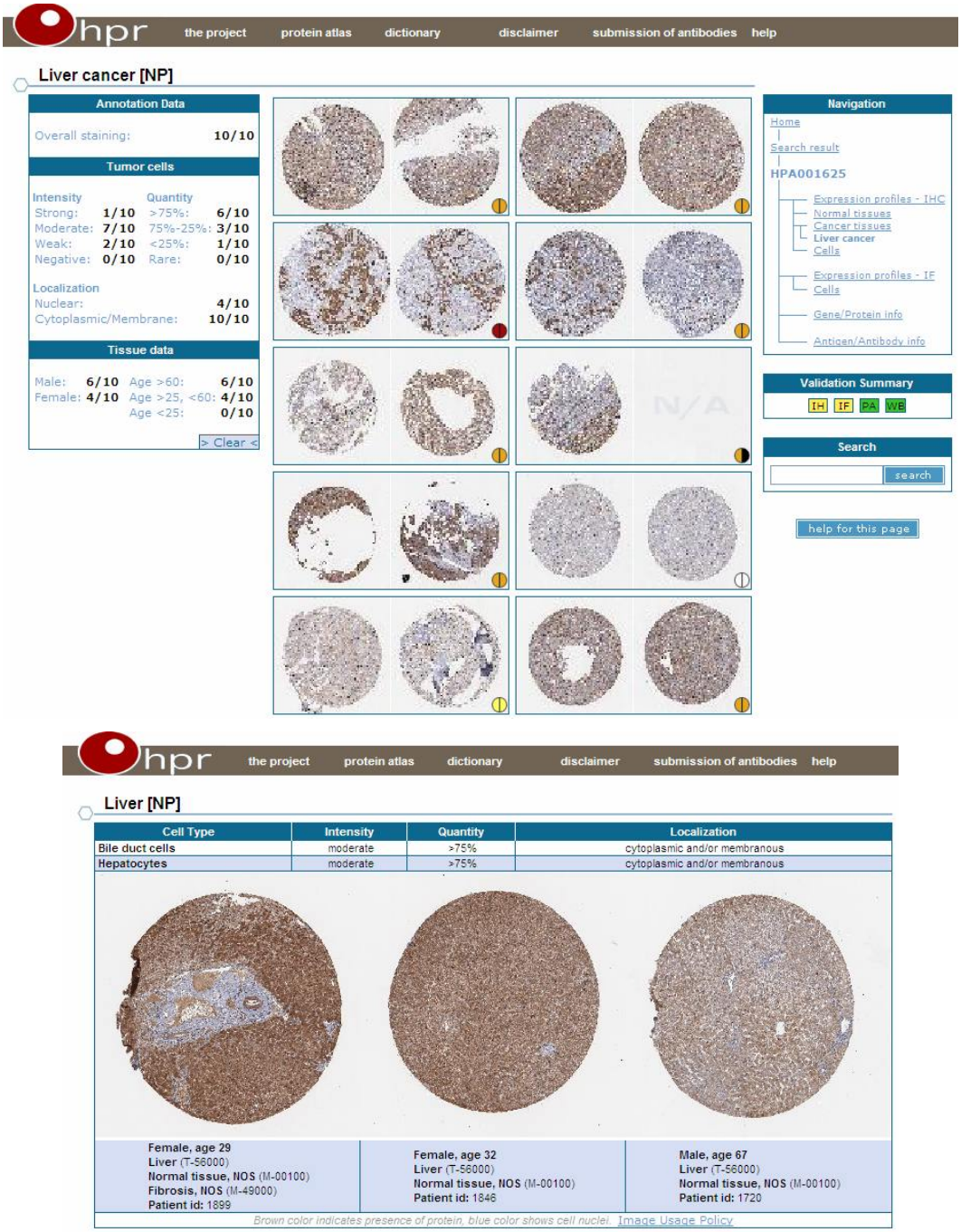


Figure 12: Representation of immunohistochemically derived protein expression data for liver cancer (above) and liver (below) in "Human Protein Atlas" (www.proteinatlas.org); example "Purine nucleoside phosphorylase" (P00491)

The following approach was applied and is exemplified via data for purine nucleoside phosphorylase (Figure 12):

1. Assignment of weighted values to intensity and quantity levels of protein expression (table 3).

Intensity	Quantity	Weighted value
Strong	>75%	1
Moderate	75- 25%	0,6
Weak	<25%	0,2
Negative	Rare	0

Table 3: Assignment of common weighted values to intensity and quantity levels

2. Multiplicative correlation of the weighted values according to table 4. The resulting values correspond to relative expression levels (REL).

		Intensity		
		Strong	**Moderate**	**Weak**
Quantity	**>75%**	1	0,6	0,2
	75- 25%	0,6	0,36	0,12
	<25%	0,2	0,12	0,04

Table 4: Matrix for the multiplicative correlation of weighted values

3. Calculating the number of liver cancer samples belonging to the respective RELs. Two combinatorial border cases can be defined assuming a) the highest and b) the lowest possible inter-sample variability with respect to expression levels. Intensity and quantity data of purine nucleoside phosphorylase are summarized in table 5 (derived from figure 12), the combinatorial elaboration of the border cases is depicted in table 6. The results expressed as the number of samples of distinct RELs are depicted in table 7.

Intensity	Quantity
Strong: 1	>75%: 6
Moderate: 7	75- 25%: 3
Weak: 2	<25%: 1
Negative: 0	Rare: 0

Table 5: Number of samples corresponding to the respective intensity and quantity levels

Intensity	Quantity
Strong: 1	>75%: 6
Moderate: 7	75- 25%: 3
Weak: 2	<25%: 1
Negative/ Rare: 0	

Intensity	Quantity
Strong: 1	<25%: 1
Moderate: 7	75- 25%: 3
Weak: 2	>75%: 6
Negative/ Rare: 0	

Table 6: Combinations for case a (left) and case b (right)

		Intensity		
		Strong	Moderate	Weak
Quantity	>75%	1	5/4	2
	75- 25%		2/3	1
	<25%	1		1

REL	Case a	Case b
1	1	0
0,6	5	4
0,36	2	3
0,2	0	3
0,12	1	0
0,04	1	0
0	0	

Table 7: Number of samples corresponding to distinct RELs for case a and b in the matrix (above) and tabularized (below)

4. The expression level of the protein in liver is: Moderate intensity and quantity of >75% (figure 12, below), hence, corresponds to the relative expression level (REL) of 0.6 which represents the treshold expression level for equal, up- and down-regulation of liver cancer. Since the total number of samples is 10, the result for case a is: 10% of the investigated HCC samples showed higher, 50% equal, and 40% lower expression levels compared to liver tissue. For case b: 40% equal and 50% lower expression levels (Table 8).

up	equal	down
10- 0%	50- 40%	40- 60%

Table 8: Percentage of liver cancer samples with higher, equal, or less abundance of purine nucleoside phosphorylase compared to liver tissue

All proteins (936) identified by mass spectrometry were searched for protein expression data in “Human Protein Atlas”. Expression data for proteins represented in the data base (471 proteins) were elaborated as described above and compared with shotgun data (see list 2 in appendix). The data coincided with respect to up- and downregulation and equal expression levels to 83 % (392 proteins). For proteins covered in more detail, data for case a and b were combined and elaborated via SPSS and depicted as Boxplots. The blue lines and numbers given for each protein denotes the REL of normal liver.

5 Results: Functional characterization of HCC vs. liver proteomes

5.1 Global comparison of the proteome profiles of non-tumorous liver and HCC tissues

Proteins identified in non-tumorous liver and HCC tissue (548 distinct proteins) were divided in groups according to their relative abundance (Figure 13). The latter was calculated as the ratio of total peptide (Tp) numbers of the respective proteins. Proteins detected in both tissues showed a normal distribution with 343 proteins featuring a difference less than factor 2. They are suggested to exert functions indispensable for the survival of cells in general. Remarkably, 231 and 146 from overall 936 different proteins were exclusively detected in liver and HCC tissue, respectively, reflecting a high level of adaptation to the respective tissue challenges (metabolism in liver vs. proliferation in HCC). Comparison of 2D-gels from liver and HCC cytoplasmic protein samples confirmed the diversification of protein expression patterns and the loss of liver-specific expression performance in HCC (Figure 14, note the spot pattern particularly at the basic part of the gels typically enriched with liver-specific proteins).

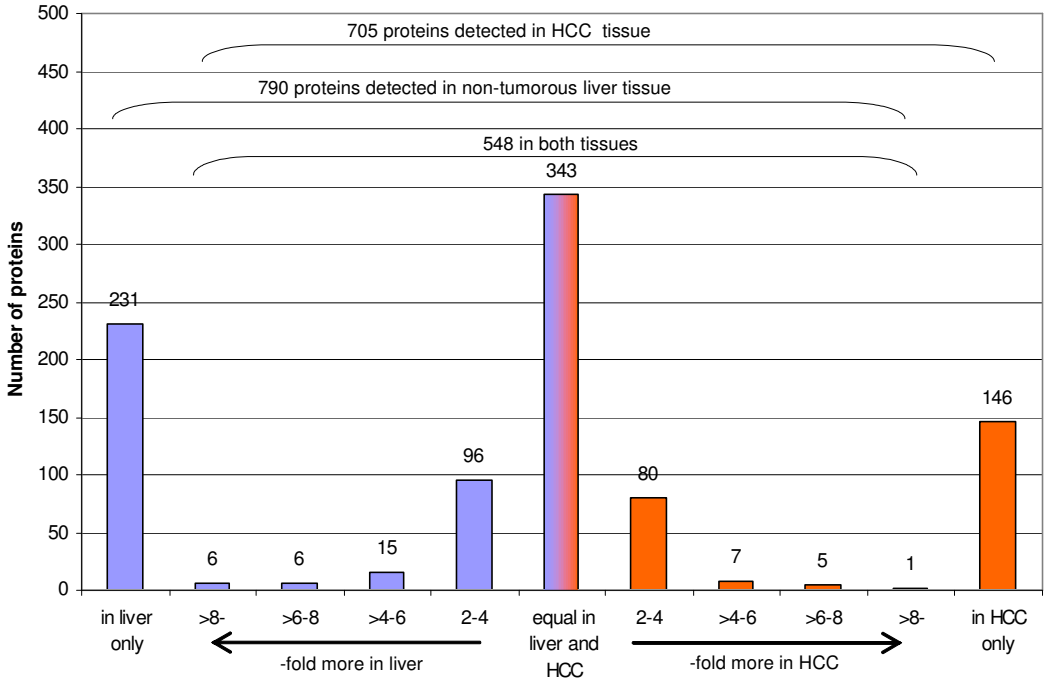


Figure 13: Overview of the differential protein expression of non-tumorous liver and HCC tissue

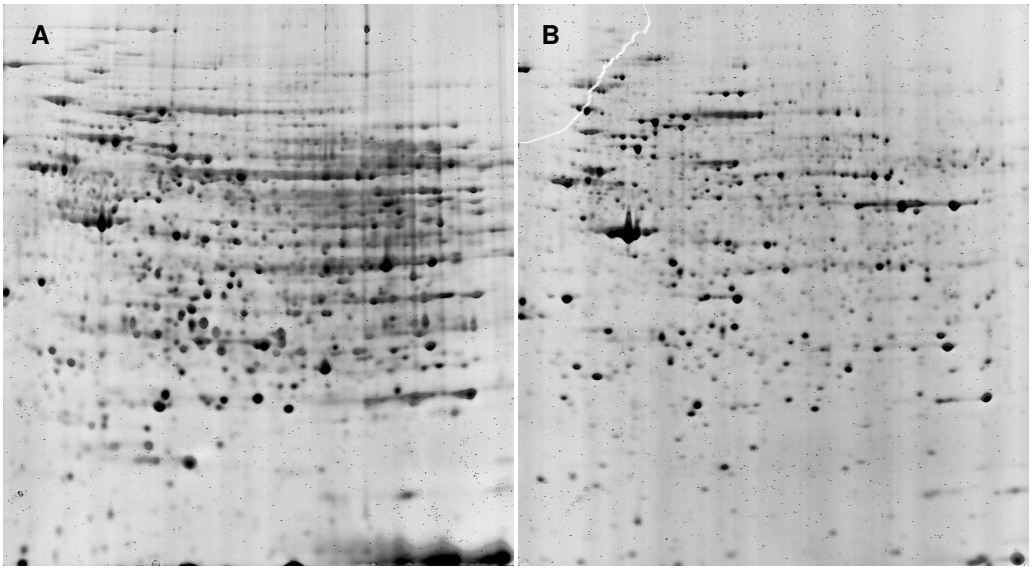


Figure 14: Expression patterns of cytoplasmic proteins of non-tumorous liver (A) and HCC tissue (B) separated by 2D-PAGE

In this study, the systemic and functional aspects were emphasized by accounting the loss or gain of groups of functionally associated proteins rather than distinct proteins. Thereof important determinants for cellular or tissue functionality were gathered with respect to enzymes involved in e.g. glycolysis and fatty acid metabolism as metabolic parameters or proteins mediating protein synthesis, protein folding as cellular functionality parameters. This approach might compensate for sensitivity limits of the method and reveals gaps (all enzymes of a pathway should be there for functionality) possibly worth to be challenged.

The schematic representation of gene expression summarizes fundamental processes cells depend on in general (Figure 15). Basic cellular functions have a major impact on metabolic functionality in the broader sense. Therefore, a focus was laid on protein synthesis of cytoplasmic proteins (including those that are translocated into mitochondria and peroxisomes) and organelle-resident and secreted proteins. Another focus was laid on proteins mediating processes which influence the abundance and/or functionality of newly synthesized proteins (e.g. protein folding, protein degradation). The compartmentalization of certain metabolic functions implicates that they are considerably influenced by not only the expression and abundance of certain enzymes involved in the respective parts of metabolism but also by their proper location (e.g. machinery for co-translational import of proteins into the ER as well as consequent processes for protein maturation) and transport to their final destination. The latter is accomplished via vesicular traffic which strongly depends on the cytoskeleton (note the marked downregulation of cytoskeletal proteins indicated in figure 16, chapter 5.2) and on proteins for vesicle targeting and fusion. In addition, processes involved in "organelle distribution" of proteins are also crucial for secretion performance and, hence, the tumor microenvironment and/or the abundance of putative biomarkers in the serum of patients suffering from HCC.

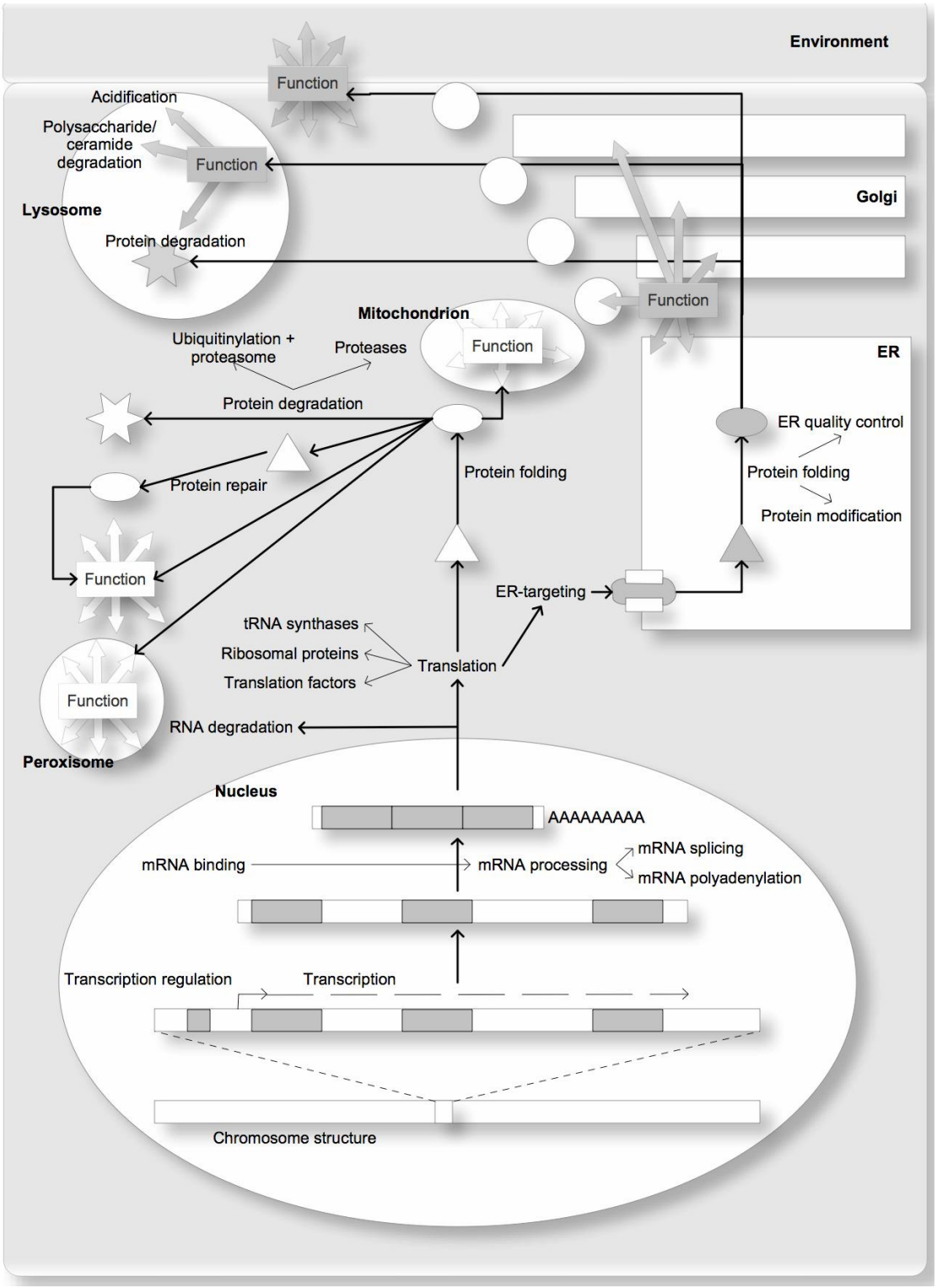
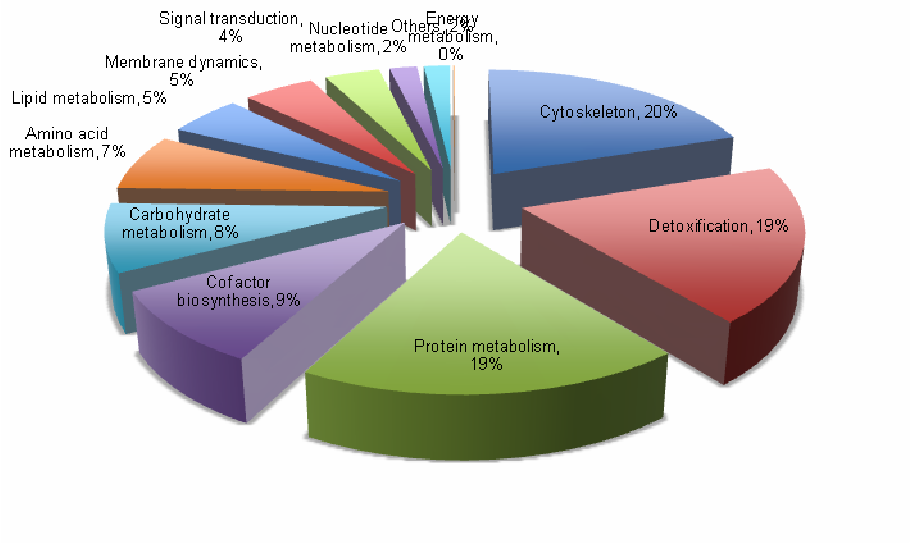


Figure 15: Outline of gene expression and cellular processes with impact on results and their interpretation gathered via proteomics

5.2 Functions mediated by cytoplasmic proteins of liver vs. HCC- a global view

Figure 16 depicts the functions associated with overall 427 cytoplasmic proteins detected in liver (A) and HCC tissue (B). Percentage values correspond to the sum of total peptides of proteins assigned to the respective functional group related to the overall number of total peptides derived from cytoplasmic proteins of non-tumorous liver tissue. Thus, values are directly comparable and allow the global assessment of "loss of function" and "gain of function" of HCC compared to non-tumorous liver tissue. Details with special emphasis on differential protein expression or on proteins being significantly up- or downregulated in terms of total peptide numbers in one of the two tissues are discussed in the context of the respective functional groups. The abundance of cytoplasmic proteins was markedly reduced in HCC in contrast to that of mitochondrial proteins (figure 16 and figure 32 in chapter 5.7.1).

A



B

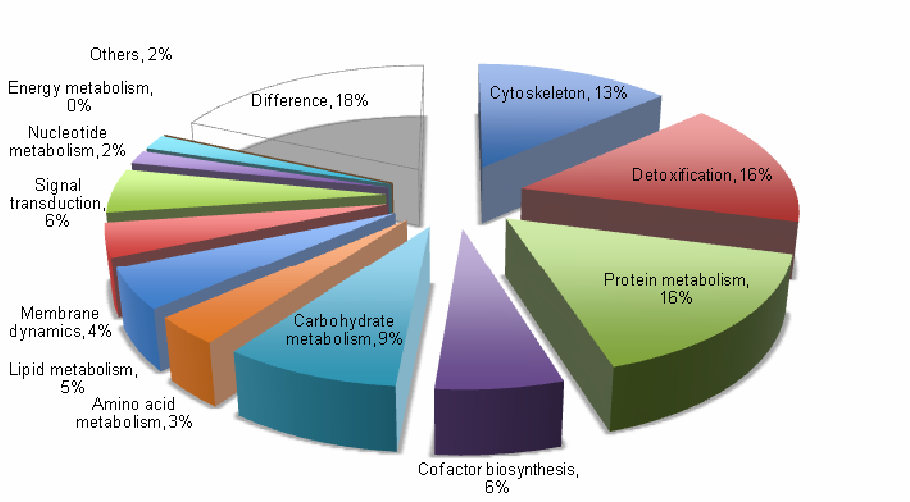


Figure 16: Comparison of cytoplasmic protein-mediated functions of liver (A) and HCC tissue (B)

Apart from components of the cytoskeleton, cytoplasmic enzymes involved in amino acid metabolism constituted the most pronouncedly affected functional group as it was observed for mitochondrial enzymes (figure 16 and figure 32 in chapter 5.7.1). This is exemplified by enzymes for serine biosynthesis (D-3-phosphoglycerate dehydrogenase (**O43175**), phosphoserine aminotransferase (**Q9Y617**)) and phenylalanine degradation (Homogentisate 1,2-dioxygenase (**Q93099**), phenylalanine-4-hydroxylase (**P00439**) (Table 9). Immunohistochemial data available for the serine biosynthetic enzymes were in accordance to shotgun results. Shotgun results are depicted in figure 17, immunohistochemical and EHCO data for those proteins are summarized in figure 18.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
O43175	D-3-phosphoglycerate dehydrogenase	8	**13**	19.9	0	**0**	0	In liver only
Q9Y617	Phosphoserine aminotransferase	6	**8**	18.9	0	**0**	0	
Q93099	Homogentisate 1,2-dioxygenase	5	**6**	17.3	2	**2**	8.3	0.3
P00439	Phenylalanine-4-hydroxylase	3	**5**	8.8	0	**0**	0	In liver only

Table 9: Cytoplasmic proteins involved in amino acid metabolism identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

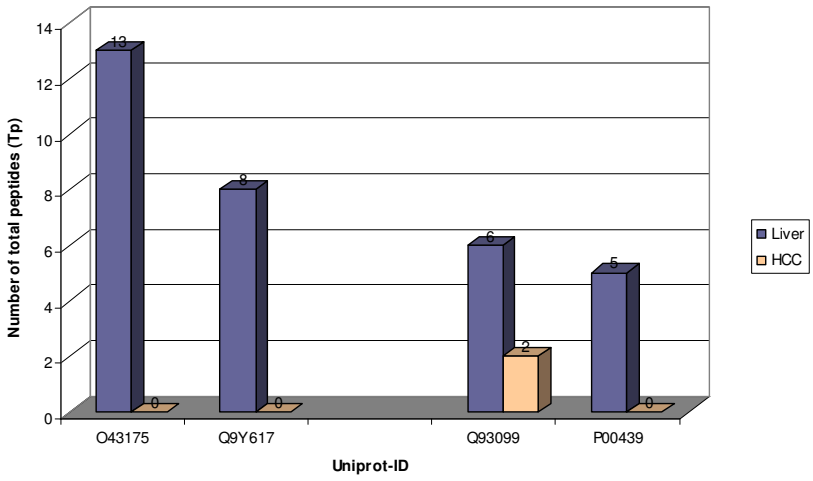
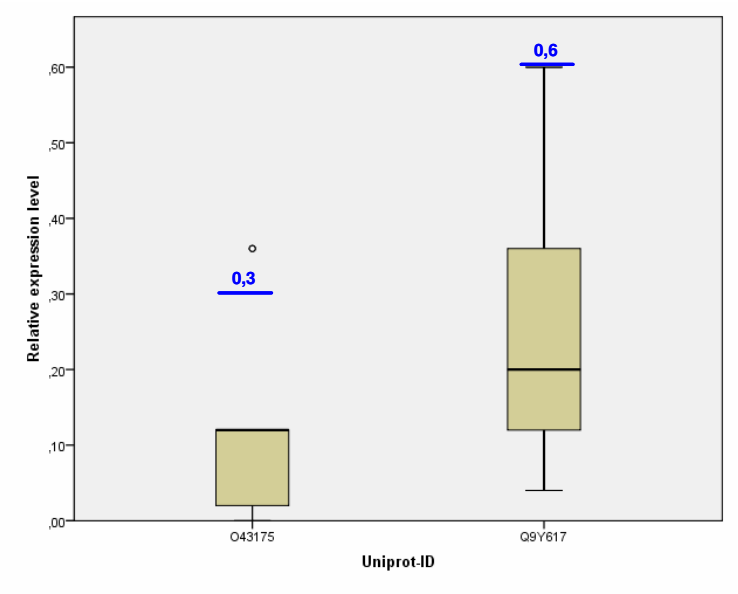


Figure 17: Abundance of cytoplasmic proteins involved in serine biosynthesis and phenylalanine degradation determined by mass spectrometry



Uniprot-ID	up	down
O43175	2	0
Q9Y617	No results	

Q93099	0	2
P00439	0	3

Figure 18: Immunohistochemical (left) and EHCO data (right) concerning the relative abundance of cytoplasmic proteins involved in amino acid metabolism in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.3 The tumor secretome

A characteristic feature of HCC tissue is the reduced synthesis and release of secreted proteins and the concomitantly increased expression of cytoplasmic proteins compared to adjacent non-tumorous liver tissue as revealed by 2D-PAGE of metabolically labeled proteins (method described in [1]). Figure 19 exemplifies the differential expression of the cytoplasmic and secretory protein fraction, respectively, associated with liver and HCC tissue slices. Some molecular features of HCC with implications for this phenotype are discussed in more detail below.

However, secreted proteins were pronouncedly more abundant in HCC compared to liver tissue as determined by mass spectrometrical analysis of non-labeled (cold) proteins extracted from the respective tissues. In particular, levels of plasma proteins synthesized and secreted by hepatocytes (e.g. albumin (**P02768**), transthyretin (**P02766**) and transferrin (**P02787**)) as well as plasma retinol-binding protein (**P02753**) which delivers liver retinol to peripheral tissues and is bound to transthyretin in plasma (Uniprot) was increased consistently about 2.5-fold compared to liver (see protein list in appendix). However, inconsistent data are delivered by EHCO and protein atlas (data not shown) possibly reflecting fundamental method-derived differences of sample preparation and data acquisition which manifest in particular in the non-cell-associated secretory and/or plasma protein fraction. This obvious contradiction, reduced expression and secretion vs. elevated levels of secreted proteins associated with the tumorous tissue, might be explicable by the massive histological and anatomical alterations known to occur in the course of hepatocarcinogenesis. The liver parenchyme is structurally optimized for efficient material exchange in contrast to tumorous tissue. In addition, haemodynamic parameters determining organ or tumor vascularity and perfusion are characteristically altered in HCC tissue. “Blood Flow” (BF (ml/100 g/min)), “Blood Volume” (BV (ml/100 g)) are increased and, correspondingly, the “Mean Transit Time” (MTT (sec)) of blood is reduced in advanced HCC [205]. Furthermore, BV correlates with the staging of astroglial brain tumors [206]. Increased blood volume might account for the elevated levels of plasma proteins detected in HCC. This is in accordance with the elevated abundance of hemoglobins found to be associated with the HCC tissue (data not shown). The “Permeability-Surface Area Product” (PS (ml/100g/min)) describes the “leakiness” of a capillary wall. Increased PS is a common feature of tumor blood vessels [205, 206] due to e.g. the effect of proangiogenic VEGF which is also a potent permeability factor [207]. Neoangiogenic vessels constitute higher permeability to macromolecules due to large endothelial cell gaps, incomplete basement membrane, and the lack of smooth muscle [206, 208]. Other permeability-influencing factors are the luminal surface area (see liver parenchyme as optimized structure) and interstitial, hydrostatic, and osmotic pressure across the endothelium [206]. In the face of an increased abundance of albumin which largely mediates colloid osmotic pressure of plasma, altered pressure ratios might be an essential parameter determining the protein composition of HCC tumors and, hence, the microenvironment of cancer cells. In addition, HCC tumors are embedded in a distinct physiological context. In fact, HCC progression is associated with an increasing arterial blood supply in contrast to the liver parenchyme which is largely perfused via the portal vein [209]. The microenvironmental aspect is also crucial concerning local concentrations of tumor-promoting, maintaining and protective proteins (and substances) possibly far exceeding that expectable from gene expression data. This putative local enrichment of otherwise low abundant proteins (e.g. in

plasma) is one of the most important advantages of tissue proteomics with respect to the quest for biomarkers.

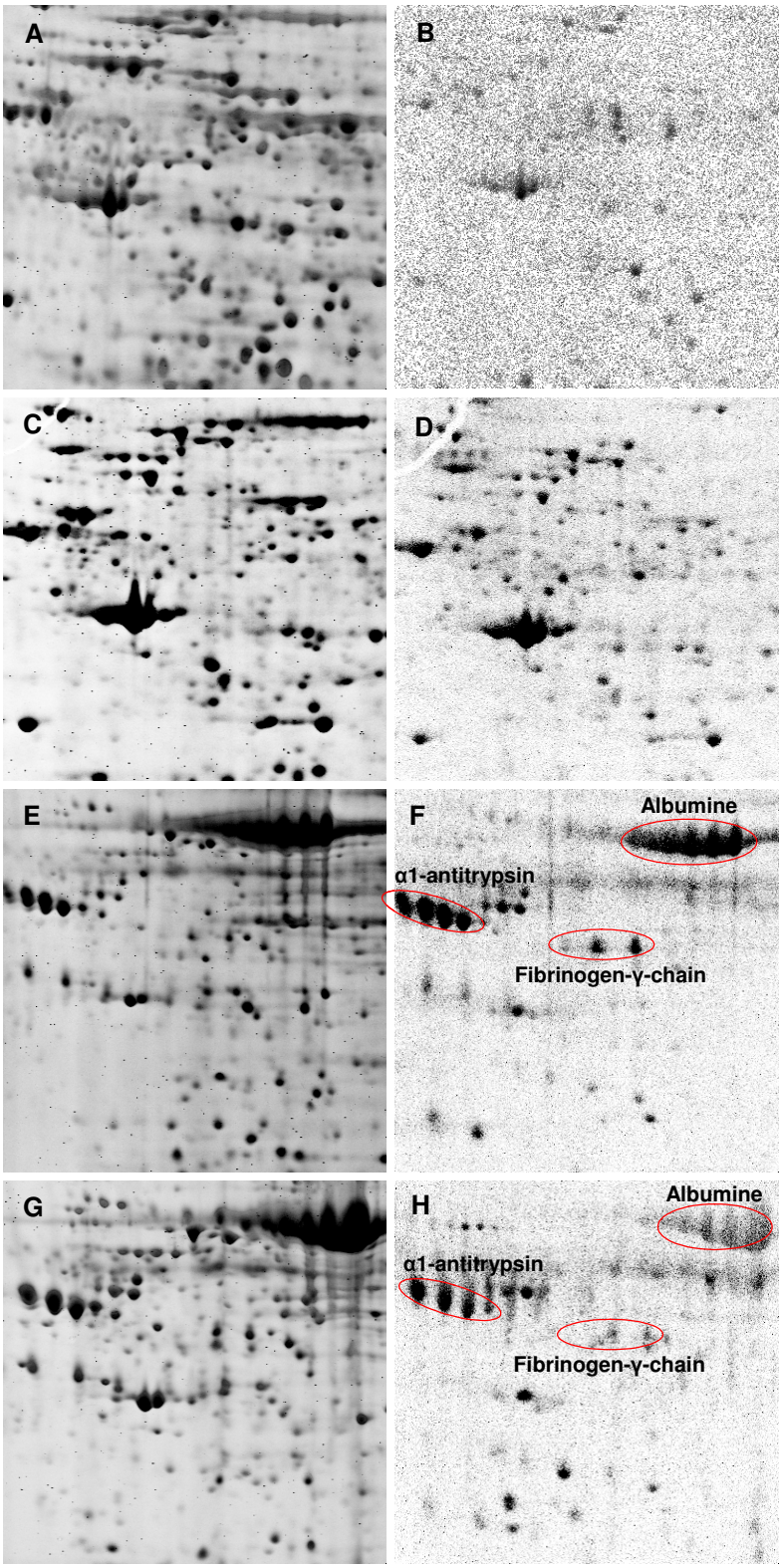


Figure 19: Sections of 2D-gels of cytoplasmic proteins and secreted proteins isolated from radioactively labeled liver and HCC tissues. A,B: Cytoplasmic proteins of liver, C,D cytoplasmic proteins of HCC tissue; E,F: Secreted proteins of liver; G-H: Secreted proteins of HCC tissue (A,C,E,G: Proteins fluorescently labeled; B,D,F,H: corresponding autoradiographs)

5.3.1 Proteases and protease inhibitors

Interestingly, proteases and protease inhibitors were differentially abundant in HCC compared to non-tumorous liver tissue.

The coagulation cascade is regulated by the molecular counter-players prothrombin (**P00734**) and anti-thrombin-III (AT-III, **P01008**). Thrombin exerts functions in blood homeostasis, inflammation, and wound healing. Its proteolytical targets are fibrinogen and the factors V, VII, VIII, and XIII leading to the formation of fibrin and the activation of the coagulation cascade, respectively. AT-III inhibits thrombin as well as several coagulation factors especially in the presence of heparin. Prothrombin and AT-III were differentially expressed in liver vs. HCC tissue. Notably, prothrombin was detected only in liver whereas AT-III was found only in HCC suggesting a modulation of the blood coagulation system in the tumor tissue. In addition, fibrinogen chains, which constitute the proteinous matrix of blood clots after being cleaved by thrombin, were less abundant in HCC compared to non-tumorous tissue. Alpha-2-antiplasmin (**P08697**) is an inhibitor of the proteases plasmin, trypsin, and chymotrypsin. Alpha-2-antiplasmin deficiency is associated with a hereditary disease leading to hemorrhagic diathesis, the predisposition for the leakage of blood from vessels. Plasmin cleaves fibrin, fibronectin, thrombospondin and other components of the extracellular matrix suggesting its role in tissue remodeling and tumor invasion. Alpha-2-antiplasmin was not detected in HCC in contrast to liver tissue. Alpha-1-antitrypsin (**P01009**) is an inhibitor of serine proteases. Its primary target is elastase with moderate affinity for plasmin and thrombin. Moreover, it inhibits trypsin, chymotrypsin and plasminogen activator. Alpha-1-antitrypsin was highly abundant in liver tissue but was not detected in HCC. Alpha-2-macroglobulin (**P01023**) is a potent inhibitor of all four classes of proteinases. Again, it was solely detected in liver. Taken together, inhibitors of proteinases involved in blood coagulation, mediating blood vessel leakage and the turnover of the extracellular matrix were consistently lacked or less abundant in HCC tissue suggesting tumor-associated alterations with respect to e.g. the structure and function of blood vessels.

5.3.2 Clusterin

The abundance of clusterin (**P10909**) was pronouncedly increased in HCC tissue (see protein list in appendix). Clusterin is a heterodimeric glycoprotein involved in apoptosis, cell cycle regulation, complement regulation and many other processes decisive for carcinogenesis. Its expression is upregulated in many human cancers like prostate, breast, lung, bladder, colon as well as in a majority of HCC cancers whereas it is downregulated in some others ([210] and citations therein). Interestingly, expression data obtained from EHCO and “Protein Atlas” delivered contradictory results and indicate tendential downregulation of clusterin, respectively (data not shown). One of the many biological implications of clusterin is its complement-modulatory effect. It is noteworthy, that it is possibly involved in preventing complement-mediated cell lysis via the classical pathway even in the increased presence of immunoglobulins and the concomitant low abundance of the C1 inhibitor protein in HCC tissue compared to the non-tumorous liver tissue.

5.3.3 Concluding remarks concerning the secreted protein fraction determined via shotgun-based tissue proteomics

Many other secreted/extracellular proteins were found to be differentially abundant in liver and HCC tissue (e.g. cardiotrophin-1, hepatoma-derived growth factor described in chapter 5.10 and serum paraoxonase in chapter 5.9) reflecting the tissue-specific microenvironment. However, it is noteworthy, that the latter is determined by several parameters. Apart from the differential expression of non-tumorous and tumorous tissues, it is potentially influenced e.g. by haemodynamic parameters, by pressure ratios of plasma and the tumorous interstitial fluid, by the unique composition of the extracellular matrix possibly providing the physical capability to retain and, hence, locally enrich distinct proteins even at normal or low expression levels exerted by tumor cells (e.g. cardiotrophin-1), by recruitment and local enrichment of plasma proteins potentially serving as a protective shield against oxidative stress or chemotherapeutics (e.g. albumin). All of those parameters might cooperatively act to lay the systemic basis for the development and maintenance of tumors. By all means, inconsistencies of data in literature especially concerning the expression and biological relevance of secreted proteins for carcinogenesis in general and discrepancies of results obtained by tissue proteomics and protein expression studies in particular might be partially ascribed to the different hierarchical levels treated (cell vs. tissue). However, the combination of 2D-PAGE of metabolically labeled protein fractions and shotgun-based tissue proteomics enables to bridge the gap between gene expression and the tumor-specific microenvironment, respectively.

5.4 Protein synthesis, folding, and degradation

5.4.1 Ribosomal proteins

Comparison of the abundance of ribosomal proteins constituting the 40S and 60S ribosomal subunits revealed a distinct tissue-specific pattern. Of overall 25 different 40S ribosomal proteins, two proteins were detected only in HCC and five proteins were exlusively found in liver or were significantly downregulated in HCC (Table 10, figure 20). Proteins of the 60S ribosomal subunit showed even higher diversification than those of the small subunit. Overall 32 identified 60S ribosomal proteins included 10 proteins which were significantly more abundant or solely detected in HCC and 11 were found only in liver or considerably downregulated in HCC tissue (Table 11, figure 20). In addition, translation initiation factors and elongation factors were also differentially expressed in liver and HCC tissue (data not shown). The differential expression of ribosomal proteins was also underlined by immunohistochemical and EHCO data (figure 21).

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P62266	40S ribosomal protein S23	0	**0**	0	2	**2**	15,4	In HCC only
P61247	40S ribosomal protein S3a	0	**0**	0	1	**1**	6,4	
P62269	40S ribosomal protein S18	9	**16**	43,4	12	**27**	53,9	1,7
P62753	40S ribosomal protein S6	2	**2**	6,4	3	**3**	18,1	1,5
P25398	40S ribosomal protein S12	3	**5**	22,7	2	**6**	15,9	1,2
P62701	40S ribosomal protein S4, X isoform	3	**4**	13,3	2	**4**	4,6	1,0
P46782	40S ribosomal protein S5	2	**5**	14,2	3	**5**	18,6	1,0
P62249	40S ribosomal protein S16	6	**7**	30,8	6	**7**	41,1	1,0
P08708	40S ribosomal protein S17	6	**8**	51,1	5	**8**	34,8	1,0
P23396	40S ribosomal protein S3	1	**1**	3,7	1	**1**	5,3	1,0
P62277	40S ribosomal protein S13	6	**13**	28,5	6	**12**	32,5	0,9
P62081	40S ribosomal protein S7	8	**10**	27,3	6	**9**	22,7	0,9
P62851	40S ribosomal protein S25	3	**5**	24	2	**4**	16	0,8
P62847	40S ribosomal protein S24	3	**5**	29,3	2	**4**	20,3	0,8
P39019	40S ribosomal protein S19	8	**11**	36,6	5	**8**	27,6	0,7
P60866	40S ribosomal protein S20	2	**8**	19,3	3	**5**	25,2	0,6
P62854	40S ribosomal protein S26	2	**5**	20,9	2	**3**	20,9	0,6
P62244	40S ribosomal protein S15a	5	**5**	38,5	2	**3**	16,9	0,6
P62841	40S ribosomal protein S15	3	**5**	22,1	2	**3**	22,1	0,6
P62263	40S ribosomal protein S14	7	**12**	41,7	6	**7**	41,7	0,6
P46783	40S ribosomal protein S10	8	**11**	48,5	4	**5**	27,3	0,5
P62241	40S ribosomal protein S8	4	**6**	24,5	2	**2**	12	0,3
P62280	40S ribosomal protein S11	2	**2**	19,6	0	**0**	0	In liver only
P08865	40S ribosomal protein SA	4	**4**	23,7	0	**0**	0	
P15880	40S ribosomal protein S2	2	**3**	8,5	0	**0**	0	

Table 10: Components of the 40S ribosomal subunit identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)
P46779	60S ribosomal protein L28	0	**0**	0	2	**3**	17,5
P62899	60S ribosomal protein L31	0	**0**	0	3	**3**	18,4
P83731	60S ribosomal protein L24	0	**0**	0	3	**4**	19,1
P40429	60S ribosomal protein L13a	0	**0**	0	1	**1**	5,4
P49207	60S ribosomal protein L34	0	**0**	0	1	**1**	6,8
P61353	60S ribosomal protein L27	0	**0**	0	1	**1**	6,6
P62917	60S ribosomal protein L8	1	**1**	4,3	2	**4**	10,5
P05386	60S acidic ribosomal protein P1	1	**1**	14	2	**4**	14
P62750	60S ribosomal protein L23a	2	**2**	8,3	3	**4**	21,8
P46776	60S ribosomal protein L27a	1	**1**	14,9	2	**2**	22,3
P35268	60S ribosomal protein L22	5	**7**	39,8	5	**8**	39,8
P50914	60S ribosomal protein L14	4	**5**	21,1	2	**5**	10,8
P30050	60S ribosomal protein L12	6	**6**	47,3	5	**6**	43
P47914	60S ribosomal protein L29	1	**1**	9,4	1	**1**	9,4
P62829	60S ribosomal protein L23	2	**4**	27,1	3	**4**	34,3
P18124	60S ribosomal protein L7	2	**4**	10,1	4	**4**	21
P05388	60S acidic ribosomal protein P0 (L10E)	4	**7**	16,1	3	**6**	11
P62913	60S ribosomal protein L11	6	**6**	23	5	**5**	21,9
P05387	60S acidic ribosomal protein P2	4	**10**	41,7	4	**7**	31,3
P62910	60S ribosomal protein L32	2	**3**	17	1	**2**	9,6
Q07020	60S ribosomal protein L18	4	**5**	20,2	3	**3**	19,7
P62888	60S ribosomal protein L30	4	**6**	51,3	3	**3**	34,8
P32969	60S ribosomal protein L9	1	**2**	5,7	1	**1**	7,3
P46777	60S ribosomal protein L5	3	**3**	12,5	1	**1**	5,7
P62424	60S ribosomal protein L7a	3	**6**	15	1	**2**	4,9
Q02878	60S ribosomal protein L6	5	**5**	24,3	1	**1**	5,2
P26373	60S ribosomal protein L13	3	**5**	13,7	1	**1**	6,2
P39023	60S ribosomal protein L3	1	**2**	5,5	0	**0**	0
P18621	60S ribosomal protein L17 (Human)	2	**2**	15,8	0	**0**	0
P36578	60S ribosomal protein L4 (L1)	5	**5**	16,4	0	**0**	0
P18077	60S ribosomal protein L35a	1	**1**	9,1	0	**0**	0
P42766	60S ribosomal protein L35	3	**3**	26,8	0	**0**	0

Table 11: Components of the 60S ribosomal subunit identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) are quoted.

Diversification with respect to the expression and modification (e.g. methylation) of ribosomal proteins as mechanisms to regulate, in a broader sense, the availability of components of the translation machinery and, hence, translation efficiency of all (or distinct subgroups) of mRNAs seems to be rather the rule than an exception (see references in this chapter). With respect to modification it has to be stated that low amounts of a given peptide and its highly abundant but modified counterpart could not be dissolved by the applied algorithm for protein identification via mass spectrometry. Consequently "low abundance" of proteins in terms of total peptide numbers might mask the existence of high amounts of peptides which are methylated/ glycosylated and vice versa. Therefore, the applied methodical approach impacts the result and has to be taken into account especially with respect to comparability of data concerning differential expression of ribosomal proteins (or proteins in general) gathered via e.g. RNA-based methods (e.g. microarray, RT-PCR).

Nevertheless, the structure of ribosomes and/or the abundance of their components is influenced by a variety of parameters like cell type and developmental stage [211] as well as physiological conditions

like starvation or hypothermia [212, 213]. Studies in yeast confirmed the impact of distinct ribosomal proteins on the translation machinery as a whole (including the differential requirement of translation factors). On the one hand they demonstrated that some ribosomal proteins were dispensable although with influence on translation efficiency and on the other hand that alternative ways of compensating for the loss of distinct ribosomal proteins might exist [214-216]. These results and others concerning the putative mechanistic base led to the postulation of the "ribosome filter hypothesis" by Mauro and Edelmann [217]. In short, they postulate a regulatory function of ribosomal proteins apart from their known function with respect to translation of certain subpopulations of mRNAs (based on the presence of cis-sequences to which ribosomal proteins can bind) which can thereby either be enhanced or inhibited (by sequestration). Nevertheless, this issue is far from being commonly accepted and subject of discussions up to date [218]. A systematic investigation of this question might have considerable impact on cancer biology and possibly targeted (local) cancer treatment (ribosomal proteins as drug targets, e.g. cycloheximide) taking into account that ribosomal proteins are reported to be differentially expressed in tumor vs. non-tumorous tissues in liver/HCC and many others [219-224].

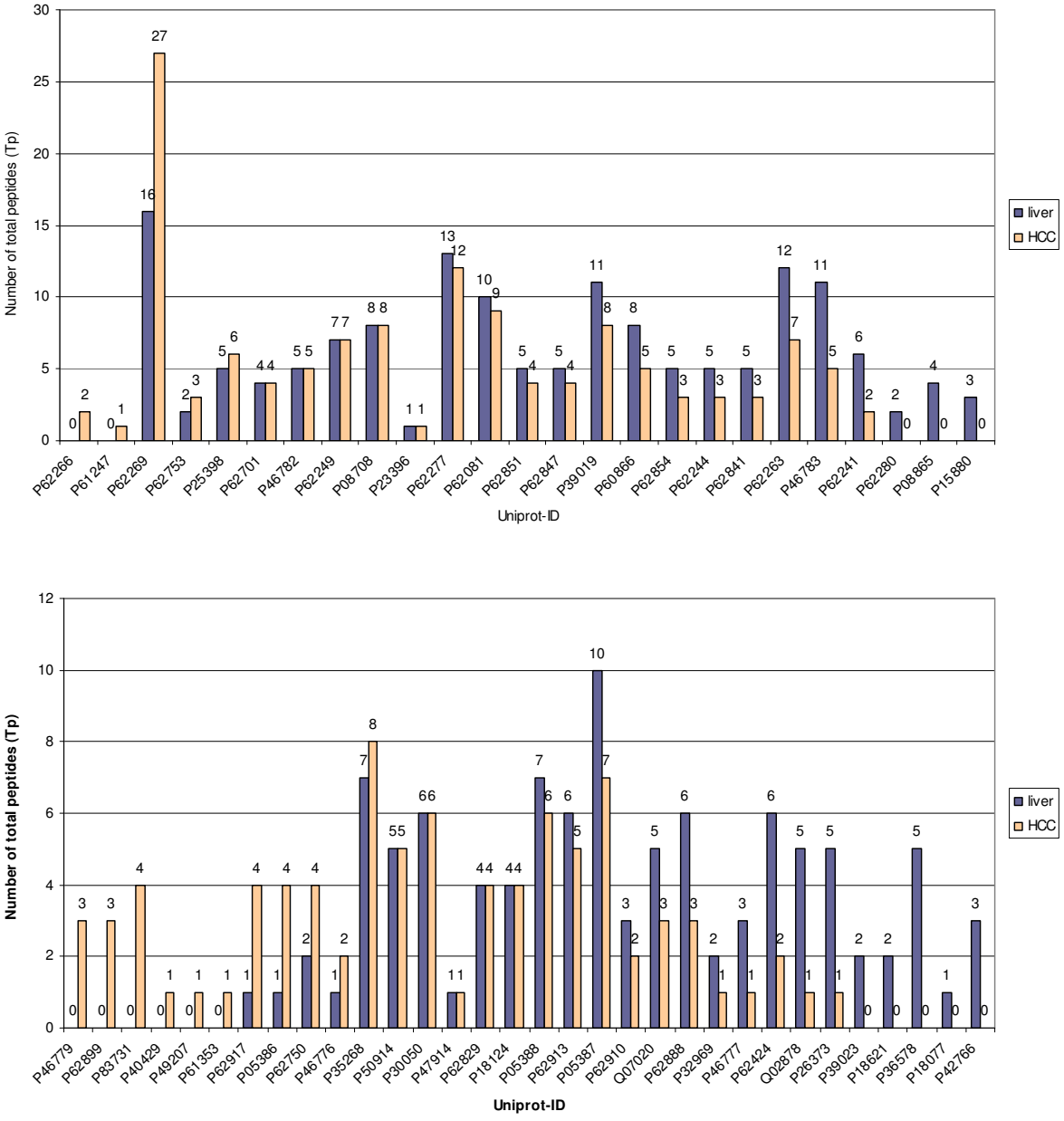
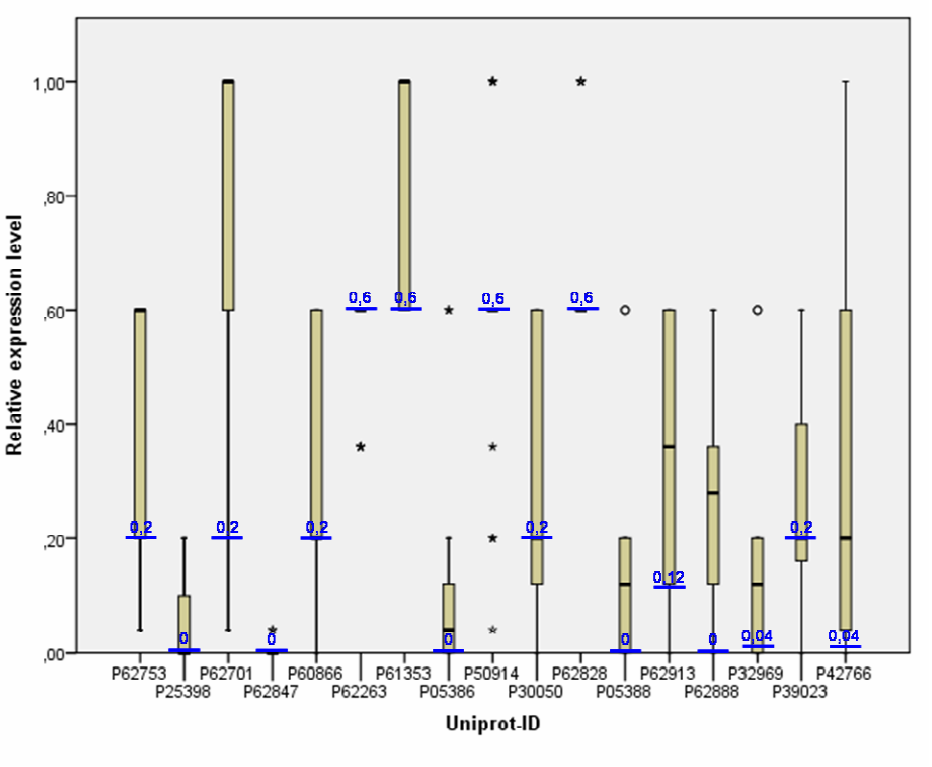


Figure 20: Abundance of 40S (above) and 60S ribosomal proteins (below) determined via mass spectrometry



Uniprot-ID	up	down
P62269	1	0
P62753	1	0
P62701	1	0
P62847	3	0
P60866	2	1
P62241	2	0
P08865	4	0
P15880	1	1
P61353	1	0
P62917	2	0
P05386	3	0
P30050	1	0
P18124	2	0
P05388	3	0
P05387	2	0
P62888	4	0
P39023	1	0
P42766	2	1

Figure 21: Immunohistochemical (left) and EHCO data (right) concerning the relative abundance of ribosomal proteins in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

In conclusion

1. Ribosomal proteins are differentially expressed in HCC and liver.
2. The possible biological implications are far from being understood although the requirement/usage of diverse sets of ribosomal proteins seems to be a common phenomenon.
3. Differential expression of translation factors has to be regarded in the context of those of ribosomal proteins (e.g. EF-2- independent translation mechanism).

5.4.2 Proteins involved in protein folding

5.4.2.1 Cytoplasmic chaperones

Cytoplasmic chaperones identified via mass spectrometry are listed in table 12. Shotgun data, immunohistochemical and EHCO data are compiled in figure 22. Hsp27 (**P04792**) was markedly upregulated in HCC. This is in accordance with reports concerning the obviously decisive implications of this multi-faceted protein on the expression of cancer-related phenotypes and resistance against anti-cancer treatments. Therefore, considerable efforts were undertaken to elucidate the mechanism of action of Hsp 27 and the signaling pathways it is involved in or depends on. Hsp27 was related to multidrug resistance (e.g. against vincristine) in hepatocellular, gastric carcinoma and breast cancer cell lines [225-227] and was suggested a factor influencing radiosensitivity of bladder cancer [228]. Moreover, overexpression of Hsp27 correlated with poor prognosis for patients suffering from prostate cancer [229]. The mechanisms of action and signaling pathways in which it is integrated comprises a putative effect of Hsp27 on apoptosis in metastatic HCC cells via modulation of the NF-kB pathway activation [230], the correlation of high amounts of non-phosphorylated Hsp27 to a high extracellular signal-regulated kinase activity which is known to provide a crucial proliferation signal in hepatocarcinogenesis [231] and the involvement in PKCβ-mediated HCC cell motility and invasion with Hsp27 as phosphorylation target [232]. The latter seems to be crucial for promoting cancer phenotype and is additionally regulated by PKCδ and p38 MAPK [232]. Hsp27 was one of 3 chaperones (among Hsp70 and Grp78, and ER-resident chaperone (**P11021**), see table 13 and figure 24 in chapter 5.4.3) which were significantly upregulated in HCC based on a proteomics approach [233]. Indeed, Hsp70 and Grp78 (see below) were also upregulated in the investigated HCC sample compared to liver tissue although to a lesser extent than Hsp27. Upregulation of these proteins was confirmed by immunohistochemical data (Figure 22). However, Hsp70 isoforms (Heat shock 70 kDa protein 1/2/6; **P08107**/**P54652**/**P17066**) are highly homologous and therefore could not be uniquely identified via mass spectrometry. Hence, histologically derived expression data of all three isoforms are represented in figure 22. Furthermore, two different antibodies were used to detect Grp78 delivering contradictory results. Expression data of both are included in figure 22 (P11021-a and P11021-b for antibodies CAB005221 and CAB019420, respectively; see www.proteinatlas.org).

Hsp27 is also increased in an HCV-infected cell line and interacts with HCV proteins playing a putative role in virus maintenance [234] and is, furthermore, involved in progesterone signaling which is underlined by the fact that the expression of Hsp27 gene is upregulated in response to progesterone [235]. Of special importance with respect to (early) diagnosis of HCC is that Hsp27 might be a useful biomarker since it was found to be enriched in the serum proteome of HCC patients [236]. By the way, it would be interesting to know the mechanism of delivery of a normally cytoplasmic protein into the patient's serum. It might be interpreted as a consequence of simple cell decay by necrosis or possibly provide an indication for alternative ways for protein release occuring in solid tumors like HCC (see chapter 5.3).

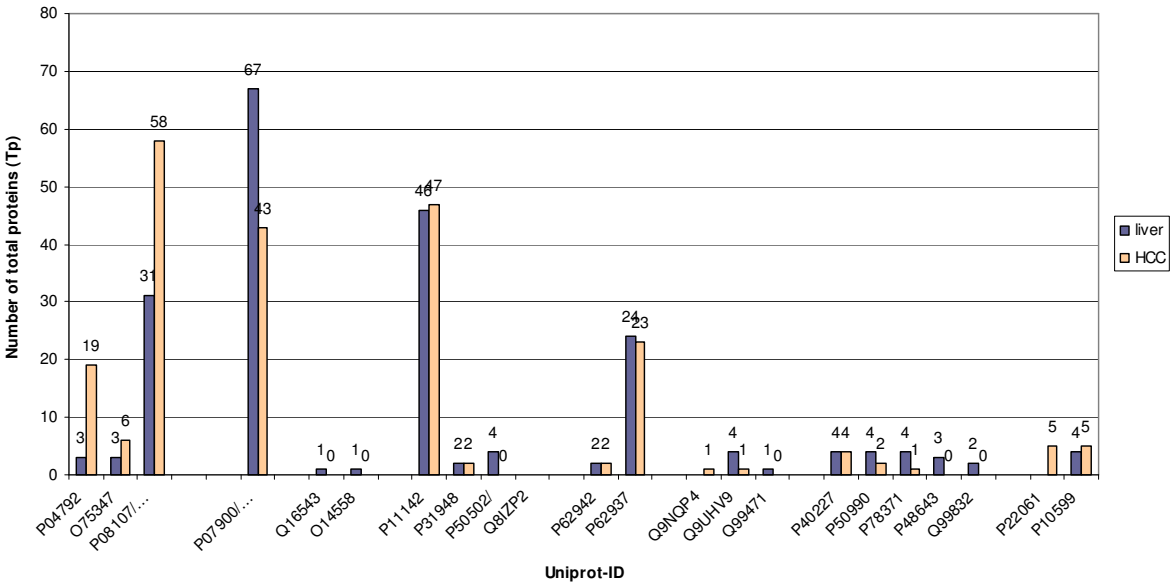
Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/Liver)
P04792	Heat shock 27 kDa protein	2	**3**	12,7	12	**19**	60,5	6,3
O75347	Tubulin-specific chaperone A	3	**3**	26,9	4	**6**	26,9	2,0
P08107/ P54652/ P17066	Heat shock 70 kDa protein 1/2/6	15	**31**	30,4	17	**58**	28,4	1,9
P07900/ P08238	Heat shock protein HSP 90-alpha/-beta	19	**67**	29,8	10	**43**	17	0,6
Q16543	Hsp90 co-chaperone Cdc37	1	**1**	3,7	0	**0**	0	In liver only
O14558	Heat-shock protein beta-6	1	**1**	8,1	0	**0**	0	
P11142	Heat shock cognate 71 kDa protein	23	**46**	36,4	22	**47**	36,2	1,0
P31948	Stress-induced-phosphoprotein 1 (STI1)	1	**2**	2,4	1	**2**	2,9	1,0
P50502/ Q8IZP2	Hsc70-interacting protein (Hip)/ Protein FAM10A4	1	**4**	3,8	0	**0**	0	In liver only
P62942	FK506-binding protein 1A(Immunophilin FKBP12)	2	**2**	13	2	**2**	13	1,0
P62937	Peptidyl-prolyl cis-trans isomerase A	13	**24**	67,9	13	**23**	67,9	1,0
Q9NQP4	Prefoldin subunit 4			0	1	**1**	9	In HCC only
Q9UHV9	Prefoldin subunit 2	3	**4**	29,9	1	**1**	7,8	0,3
Q99471	Prefoldin subunit 5	1	**1**	11,7	0	**0**	0	In liver only
P40227	T-complex protein 1 subunit zeta (TCP-1-zeta)	2	**4**	7,2	2	**4**	4,3	1,0
P50990	T-complex protein 1 subunit theta (TCP-1-theta)	2	**4**	4,9	1	**2**	2,7	0,5
P78371	T-complex protein 1 subunit beta (TCP-1-beta)	3	**4**	8,4	1	**1**	3,7	0,3
P48643	T-complex protein 1 subunit epsilon (TCP-1-epsilon)	2	**3**	6,1	0	**0**	0	In liver only
Q99832	T-complex protein 1 subunit eta (TCP-1-eta)	1	**2**	4,6	0	**0**	0	
P22061	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	0	**0**	0	4	**5**	27,3	In HCC only
P10599	Thioredoxin (ATL-derived factor) (ADF)	3	**4**	23,8	2	**5**	12,4	1,3

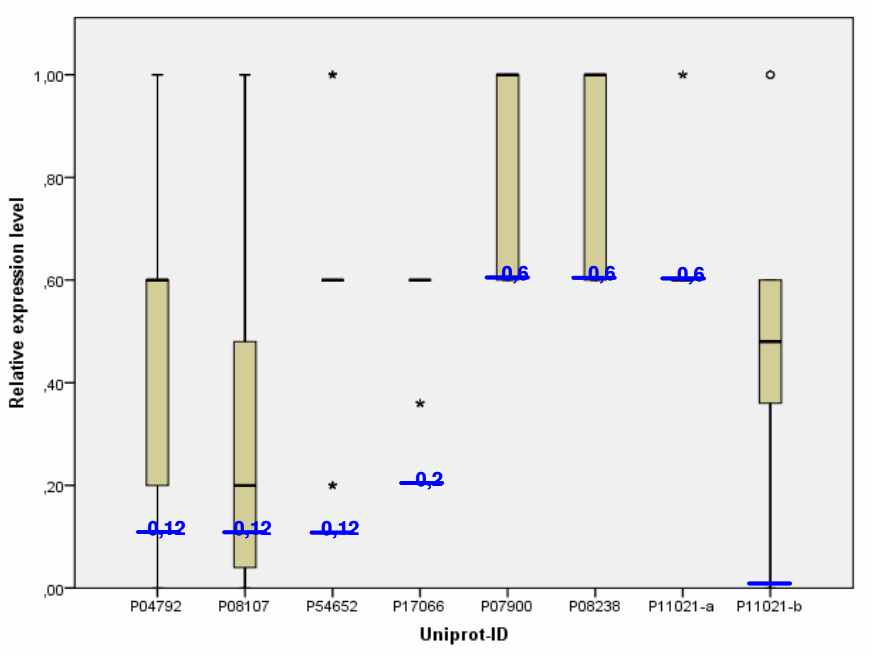
Table 12: Cytoplasmic chaperones identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Two cytoplasmic peptidyl-prolyl cis-trans isomerases (**P62942**, **P62937**) were identified. They show no differences in the expression levels of HCC compared to liver indicating that the catalysis of the otherwise slow cis-trans isomerization of proline in nascent proteins is not affected in HCC. Irrespective of their putative role as target for the anti-cancer drug rapamycin they seem to prepare proline for phosphorylation for conformation-specific proline-directed kinases and phosphatases (reviewed in [237]).

Prefoldin subunits and TCP1 subunits (see table 12) are involved in the folding of nascent actin and tubulin monomers to their native conformation (reviewed in [238]). Both show significant downregulation in HCC as well as differential expression of subunits comprising the functional complex. Interestingly the TCP1-chaperonin cofactor A which supports folding of those cytoskeleton proteins is one of the significantly upregulated proteins in HCC. Nevertheless, there might be a

correlation with the finding that actin and tubulin are also pronouncedly downregulated in HCC compared to liver (data not shown).





Uniprot-ID	up	down
P04792	4	1
P08107	2	1
P54652	No results	
P17066	No results	
P07900	3	0
P08238	No results	
P11021	3	1

Figure 22: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of cytoplasmic chaperones in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

One methyltransferase, Protein-beta-aspartate methyltransferase or PIMT, involved in protein repair was exclusively detected but most probably just strongly upregulated in HCC considering its postulated ubiquitous expression [239]. PIMT catalyzes the repair of proteins with isoaspartyl residues which enrich during aging of proteins. Aged or irreparably non-functional proteins are delivered to the ubiquitin-proteasome machinery for degradation and recycling of amino acids. Upregulation of this

protein might be induced in the case of insufficiency of this machinery. Indeed there is an indirect indication for deprivation of activated ubiquitin (loss of ubiquitin-activating enzyme in HCC) as a prerequisite for the consequent steps of the degradation process (see protein degradation). It might be assumed that an increased proportion of proteins found in HCC are altered and concomittantly might cause increased cytosolic stress by the enrichment of misfolded proteins (proteinous inclusion bodies?) which in turn would induce the expression of stress-response proteins (see chaperones mentioned above).

5.4.3 Proteins for co-translational import of proteins into ER, quality-control proteins, and ER-resident folding proteins

Proteins for protein import into the ER, proteins involved in quality control and folding identified via mass spectrometry are listed in table 13. Shotgun data are depicted in figure 24, immunohistochemical and EHCO data available for those proteisn are compiled in figure 23 and figure 25.

Translation into ER		**Dp**	**Tp**	**Cov. (%)**	**Dp**	**Tp**	**Cov. (%)**	**Ratio of Tp (HCC/Liver)**
P51571	Translocon-associated protein delta subunit precursor (TRAP-delta)	3	**5**	24,9	1	**1**	11	0,2
Q9P2E9	Ribosome-binding protein 1 (Ribosome receptor protein)	18	**35**	16,9	6	**7**	5,9	0,2
Glycosylation/reglycosylation								
O75356	Ectonucleoside triphosphate diphosphohydrolase 5 precursor, (NTPDase 5)	9	**10**	27,3	3	**4**	10,7	0,4
P04844	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit	1	**1**	1,9	0	**0**	0	
Q9NYU2	UDP-glucose:glycoprotein glucosyltransferase 1 precursor	1	**1**	1,1	0	**0**	0	
Q14165	Malectin	1	**1**	4,1	0	**0**	0	In liver only
Quality control								
P27824	Calnexin precursor	0	**0**	0	1	**2**	3	In HCC only
O60613	15 kDa selenoprotein precursor	1	**1**	9,9	1	**3**	9,9	3,0
P27797	Calreticulin precursor (CRP55)	8	**10**	33,3	12	**17**	37,2	1,7
Chaperones								
Q9UBS4	DnaJ homolog subfamily B member 11 precursor (ErJ3)	1	**1**	3,1	2	**2**	7,8	2,0
P11021	78 kDa glucose-regulated protein precursor (GRP 78)	32	**52**	46,5	43	**92**	56,7	1,8
Q9Y4L1	150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia up-regulated 1)	8	**14**	13,3	11	**18**	18,3	1,3
P14625	Endoplasmin precursor (94 kDa glucose-regulated protein) (GRP94)	38	**102**	52,8	32	**110**	45	1,1
Protein disulfide-isomerases								
P07237	Protein disulfide-isomerase precursor (PDI) (Prolyl 4-hydroxylase beta subunit)	27	**50**	51,8	29	**69**	60,6	1,4
P13667	Protein disulfide-isomerase A4 precursor (ERp72)	20	**23**	38,8	17	**29**	28,2	1,3
P30101	Protein disulfide-isomerase A3 precursor (ERp57)	23	**33**	50,5	24	**41**	43,8	1,2
Q15084	Protein disulfide-isomerase A6 precursor (Protein disulfide isomerase P5)	12	**22**	39,3	12	**25**	37,7	1,1

Thioredoxindomain containing proteins								
P30040	Endoplasmic reticulum protein ERp29 precursor (ERp31) (ERp28)	6	**6**	19,9	9	**10**	38,7	1,7
Q8NBS9	Thioredoxin domain-containing protein 5 (Endoplasmic reticulum protein ERp46)	5	**8**	14,1	5	**8**	12,5	1,0
O95881	Thioredoxin domain-containing protein 12 (Endoplasmic reticulum protein ERp19)	3	**3**	22,1	3	**3**	22,1	1,0
Q9BS26	Thioredoxin domain-containing protein 4 precursor (Endoplasmic reticulum resident protein ERp44)	3	**3**	11,6	2	**3**	6,4	1,0

Peptidyl-prolyl cis-trans isomerases								
P23284	Peptidyl-prolyl cis-trans isomerase B precursor	16	**24**	69,2	20	**37**	63	1,5
P26885	FK506-binding protein 2 precursor	6	**7**	47,9	6	**8**	33,8	1,1

Table 13: ER chaperones and folding proteins identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

The capability to perform co-translational transport of proteins through the ER membrane might be strongly compromized although not completely eliminated in HCC. Two proteins involved in this process were identified, TRAP-delta (**P51571**) which is a component of the translocon complex of the ER membrane and ribosome-binding protein 1 (p180, **Q9P2E9**) which mediates the interaction of the ribosome with the ER membrane. Expression of both was reduced and that to the same extent with a ratio of HCC/ liver of 0,2. Benyamini et al. recently demonstrated that p180 is necessary and sufficient for the secretory phenotype in mammalian cells by extending rough ER and Golgi as well as the secretion performance as a whole although it seems to be not the master regulator for enhancement of protein secretion since protein synthesis remained unaffected by overexpression of p180 [240, 241]. Reduced abundance of p180 is in accordance to EHCO data (data not shown). However, immunohistochemical evaluation revealed a pronounced tendency for upregulation (see histologic slices at www.proteinatlas.org and figure 23; note that normal liver as well as liver cancer tissue have RELs of 1).

Glycosylation of proteins in the ER might also be reduced in HCC. Four proteins involved in this process were identified and all of them were either significantly less abundant or not detected in HCC tissue in accordance to immunohistochemical and EHCO data (Figure 23). Apart from enzymes involved in primary glycosylation and the functionally yet un-characterized protein malectin, NTPDase (O75356) is assumed to promote reglycosylation reactions in the course of protein folding and quality control in the ER (http://www.uniprot.org/uniprot/**O75356**). Although the identified proteins represent just a small section of the whole spectrum of proteins participating in this process, there is a noticable "trend" towards reduction of glycosylation capability in HCC.

Interestingly, proteins mediating the calnexin-calreticulin cycle, calnexin (p90, **P27824**), calreticulin (Erp60, **P27797**), and Erp57 (**P30101**), a protein-disulfide isomerase, were found to be upregulated in HCC. Calnexin was detected only in HCC, calreticulin was moderately and Erp57 slightly upregulated in HCC, the latter two with ratios of total peptides found in HCC/liver of 1,7 and 1,2, respectively. The

elevated abundance of these proteins is underlined by histological and EHCO data (Figure 23). The primary function of this triade is the quality control of newly synthesized glycoproteins. An additional function of calnexin and calreticulin might be the regulation of Ca-homeostasis (reviewed in [242]). The biological meaning or significance of this finding against the background of a putative reduction of proteins translocated into the ER as well as diminished glycosylation in the ER might be worth to be considered in detail. Nevertheless, it might be suggested either as a reaction of HCC cells to enhanced ER-stress by accumulation of non- and falsely glycosylated proteins (irrespective of the reduced protein import into the ER) or reflect changes of Ca homeostasis in HCC compared to liver (or both). The first suggestion presumes that glycosylation deficiency comprises a bottleneck for proteins on their track through the secretory pathway but not the reduced protein import rate. Whereas it is evident that both parameters together influence the secretion performance as a whole, just the first might have impact on the expression of other ER-resident proteins responding to stress derived from accumulation of misfolded or insufficiently glycosylated proteins.

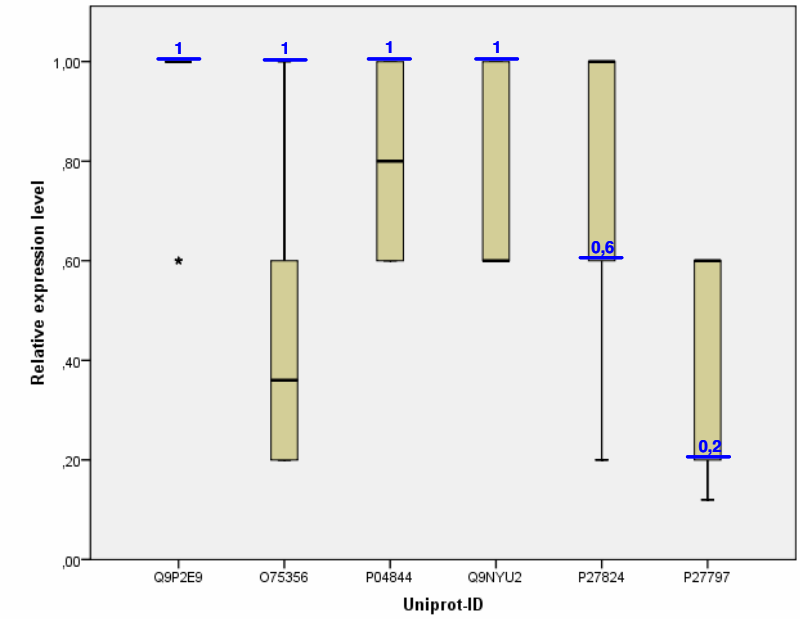


Figure 23: Immunohistochemical data concerning the abundance of proteins involved in the co-translational ER transport, glycosylation, and quality control in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

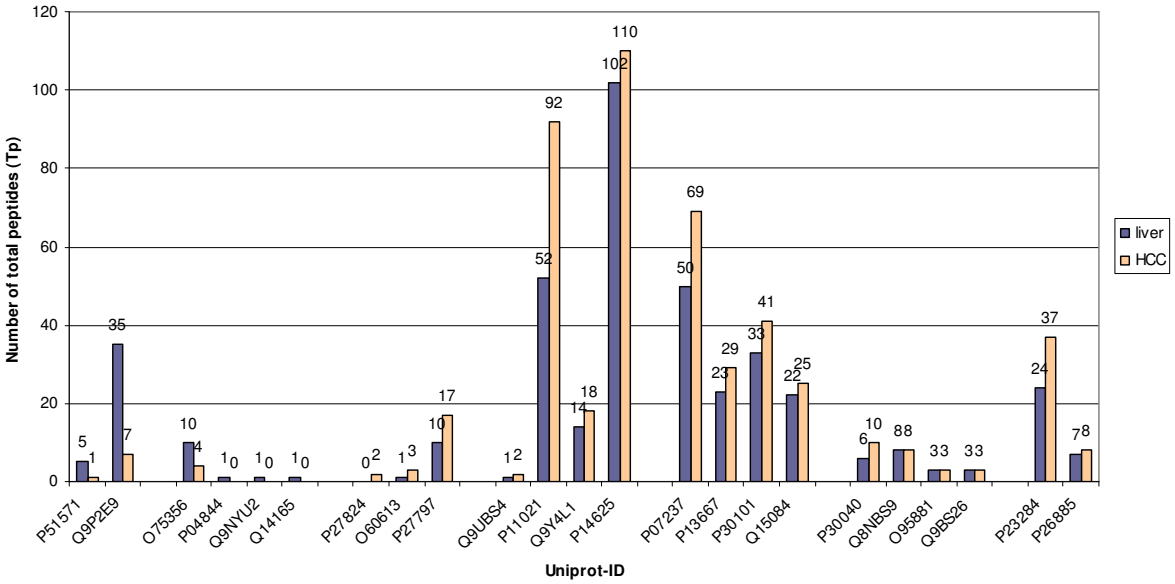
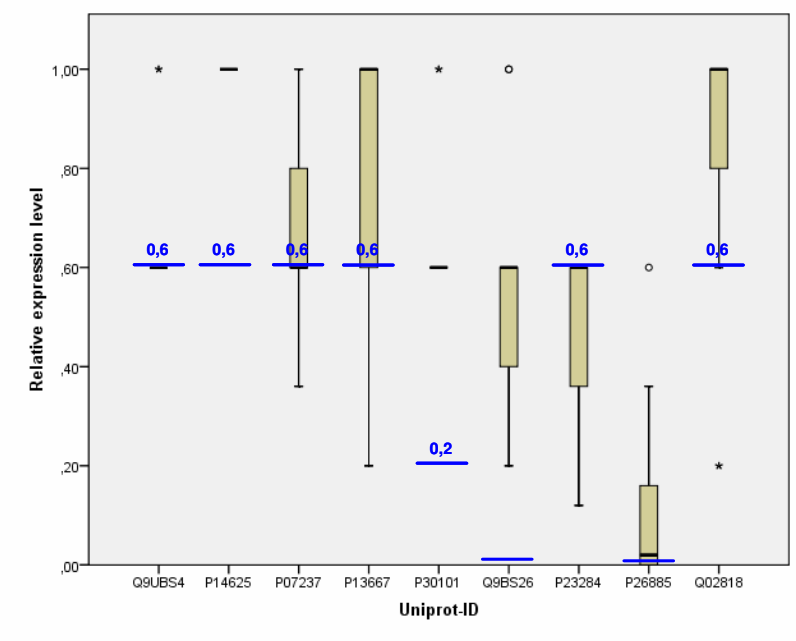


Figure 24: Abundance of proteins mediating protein import into ER, quality-control proteins, and ER-resident folding proteins



Uniprot-ID	up	down
Q9UBS4	No results	
P11021-a	3	1
P14625	1	3
P07237	0	2
P13667	No results	
P30101	3	0
Q15084	0	1
Q8NBS9	1	0
Q9BS26	2	0
P23284	3	1
P26885	No results	
P55327	2	0
Q02818	1	2

Figure 25: Immunohistochemical (left) and EHCO data (right) concerning the relative abundance of ER chaperones and disulfide isomerases in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

None of the identified chaperones, proteins with protein disulfide-isomerase and peptidyl-prolyl cis-trans isomerase activity was downregulated in HCC vs. liver. Among the proteins classified as chaperones, ErJ3 (**Q9UBS4**) showed the most pronounced upregulation in HCC, followed by Grp78 (**P11021**), Orp150 (**Q9Y4L1**), and endoplasmin (**P14625**), the latter with an expression level almost

equal to that detected in liver tissue. Four different members of the protein-disulfide isomerase family were identified (**P07237**, **P13667**, **P30101**, **Q15084**), all of which were slightly to moderately more abundant in HCC whereas the thioredoxin domain-containing proteins (**Q8NBS9**, **O95881**, **Q9BS26**) which are functionally (catalytically) equivalent to the disulfide isomerases were equally expressed in both tissues except for ERp29 (**P30040**) which proved to be moderately overexpressed in HCC (ratio of HCC/liver of 1,7). Focusing on the enzyme-mediated functions instead of individual proteins, the chaperone group features an average ratio of HCC/liver (based on total peptide numbers) of 1,55, disulfide-isomerase activity-mediating enzymes (protein-disulfide isomerases and thioredoxin domain-containing proteins) an average ratio of 1,2, and peptidyl-proly cis-trans isomerases (**P23284**, **P26885**) a ratio of 1,3. The upregulation of proteins involved in protein folding is less pronounced as expectable (see above). Nevertheless, it is evident that the effects of reduced protein import and reduced glycosylation implicating a possible accumulation of not correctly folded and/or glycosylated proteins are opposed. Thereof it might be assumed that the findings (most of the ER proteins slightly to moderately overexpressed irrespective of their individual functions/targets) reflect a tissue-specific shift of the balance between protein import and export into and out of the ER, respectively.

In conclusion these results indicate

1. the differential expression of chaperones in accordance to published data,
2. that co-translational import into and modification of proteins in the ER might be compromized. As a possible consequence, proteins for protein folding in the ER are upregulated although to a different extent with respect to their catalytic functions.

5.4.4 The ubiquitin-proteasome system

Ubiquitin (Ub) is an important player in a broad variety of cell-physiological processes including protein degradation, cell division, signal transduction, vesicle trafficking, and protein quality control (reviewed in [243]). The mechanism is based on the regulated cycling of free and protein-bound ubiquitin. For targeting of proteins to proteasome-mediated degradation highly specific ubiquitin ligases conjugate Ub which constitute the degradation signal or "degron" for the 26S proteasome. A prerequisite for that is the activation of Ub by the energy-dependent attachment to the ubiquitin-activating enzyme E1 which subsequently transfers monomeric Ub to members of a second class of enzymes involved in this process, the ubiquitin-conjugating enzymes E2. By means of the substrate specificity-providing E3 ligases Ub is transferred from E2 to their substrates in order to constitute poly-Ub degrons. Ubiquitinylation can also be reversed by deubiquitination enzymes delivering the substrate and ubiquitin which is again available for activation (see figure 26; reviewed in [244]).

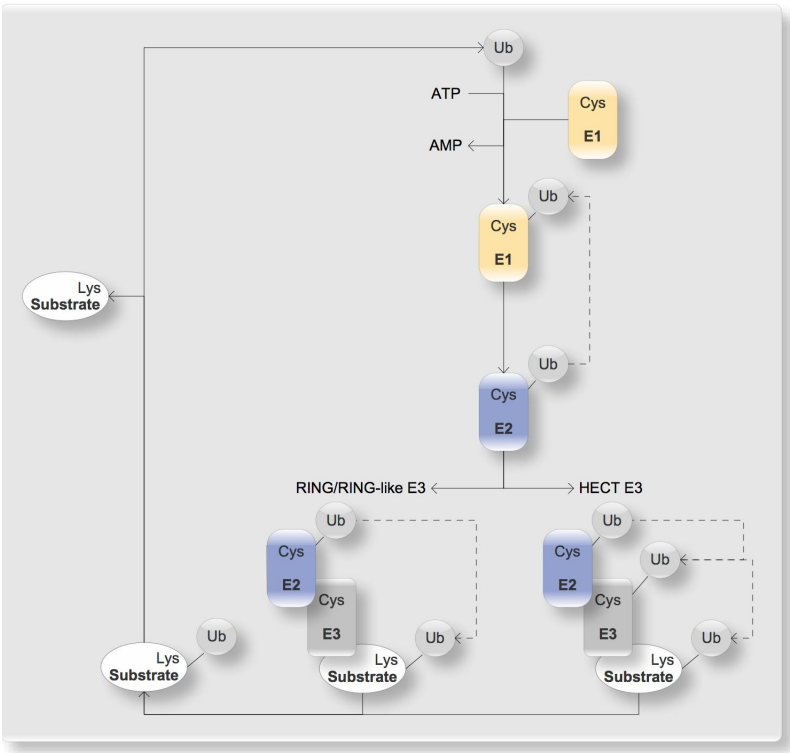


Figure 26: The ubiquitin conjugation machinery (slightly modified from [244])

The kind of linkage of Ub with respect to the involvement of one of its seven lysines contributes to the determination of the fate of the target proteins. E.g. linkage through Lys^{48} provides proteasome targeting whereas linkage via Lys^{63} is involved in DNA damage tolerance, endocytosis, and ribosomal protein synthesis [245]. In addition, Ub signal-specificity is achieved by selective Ub conjugation and deubiquitination.

The possible implications of disturbed ubiquitination for tumor biology are manifold. Of special importance might be a possibly altered growth factor abundance at the cell surface and thereof responsivity to signals [246]. This effect relies on the alteration of the preferentially used endocytosis

mechanism which massively influences the fate of the receptor inside the cell. E.g. association of the TGF-beta receptor with the Smad7-Smurf2 E3 ligase determines its association with caveolae and subsequent rapid degradation [247]. Otherwise it is internalized via clathrin-coated pits enabling further signaling from an endosomal compartment [248]. Moreover, it has been shown that signals initiated at the plasma membrane and at internal compartments are integrated differently by cells. Whereas membrane-located TrkA supports NGF (nerve growth factor)-mediated cell survival the internalized receptor induces differentiation [249]. In addition, it is suggested that mono-ubiquitination of EGFR forces its uptake via clathrin-independent endocytosis in the presence of the normally clathrin-coated pit-localized proteins Eps15, Eps15R, and epsin [250, 251]. A further example is the strong impact of ubiquitination on Notch (regulator of cell development and cell fate [252]) signaling and its modulation and termination. Whereas Delta and Serrate (type I membrane proteins and ligands of Notch) need to be ubiquitination-dependently internalized for activity it has been shown that ubiquitination is also necessary to terminate signaling. E.g. Uba1 was implicated as possible tumor suppressor since failure in ubiquitylation leads to exuberant Notch signaling and overgrowth in mutant Drosophila [253].

Proteins involved in ubiqutinylation and detected via mass spectrometry are listed in table 14.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P61956	Small ubiquitin-related modifier 2 precursor (SUMO-2)	1	**6**	12,6	1	**9**	12,6	1,5
P61081	NEDD8-conjugating enzyme Ubc12	2	**2**	10,9	2	**4**	9,3	2,0
P22314	Ubiquitin-activating enzyme E1 (A1S9 protein)	12	**13**	16,9	0	**0**	0	In liver only
P60604	Ubiquitin-conjugating enzyme E2 G2	0	**0**	0	1	**1**	14,5	In HCC only
P62256	Ubiquitin-conjugating enzyme E2 H	0	**0**	0	1	**1**	8,2	
P62837	Ubiquitin-conjugating enzyme E2 D2	0	**0**	0	2	**4**	23,8	
P61088	Ubiquitin-conjugating enzyme E2 N	6	**8**	48,7	6	**6**	42,1	0,8
P68036	Ubiquitin-conjugating enzyme E2	5	**7**	48,7	4	**4**	31,2	0,6
Q13404	Ubiquitin-conjugating enzyme E2 variant	5	**14**	19	4	**7**	16,3	0,5
Q15819	Ubiquitin-conjugating enzyme E2 variant 2	6	**15**	29	0	**0**	0	In liver only
O14933	Ubiquitin-conjugating enzyme E2 L6	1	**1**	17,1	0	**0**	0	
P15374	Ubiquitin carboxyl-terminal hydrolase isozyme L3	0	**0**	0	1	**3**	8,7	In HCC only
P54578	Ubiquitin carboxyl-terminal hydrolase 14	1	**1**	4,9	0	**0**	0	In liver only

Table 14: Proteins involved in ubiquitinylation identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Interestingly, the ubiquitin-activating enzyme E1 was not detected in HCC whereas it was significantly abundant (in terms of Tp) in liver. Taking into account the crucial role in basic cell physiology this finding might be worth to be considered in detail with respect to consequences for tumor growth and maintenance. Indeed, it has been shown that inhibition of E1 in the presence of wild-type p53 leads to apoptosis of tumor cells [254]. Since this mechanism relies on functional p53, it might be supposed that this tumor suppressor is functionally inactive in the investigated sample. To my knowledge this is the first report concerning a pronounced downregulation of the ubiquitinylation capacity in HCC cells.

Furthermore, this finding underlines that the efficiency of tumor drugs to affect tumor growth by inhibition of E1 has to be regarded in the context of e.g. the expression of functional p53. Indeed, UBE1, an E1-like enzyme, causes suppression of lung cancer growth by targeting cyclin D1 [255] underlining the importance of E1 for cell cycle progression and, hence, tumor growth. In addition, altered ubiquitinylation capacity might affect protein degradation even in the face of the more or less unaltered expression of proteasome components (table 15, figure 28).

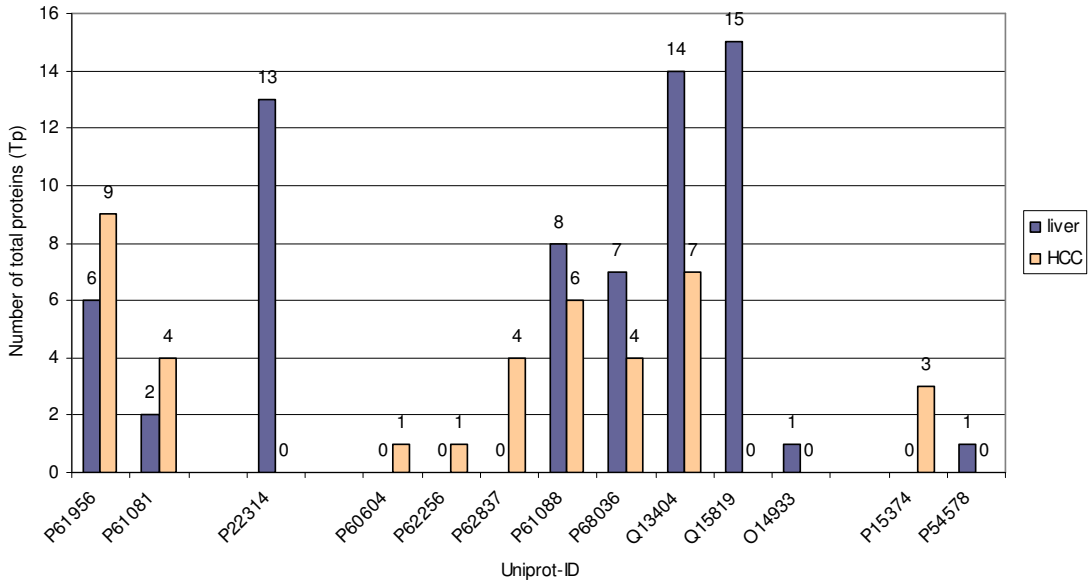


Figure 27: Abundance of proteins involved in ubiquitinylation in non-tumorous liver and HCC tissues determined by mass spectrometry

In general, a pronounced diversification concerning the expression of different ubiquitin-conjugating enzymes was observed (Figure 27). Ubiquitin-protein ligase G2 (**P60604**), H (**P62256**) and D2 (**P62837**) were detected only in HCC tissue. G2 is associated with ER-associated degradation (ERAD) and ubiquitinylates Cyp3A4 which is involved in the metabolism of more than 50% of clinically prescribed drugs [256]. Ubiquitin-protein ligase H capable to ubiquitinylate histone H2A and D2 mediates the selective degradation of short-lived and abnormal proteins and functions in the E6/E6-AP-induced ubiquitination of p53.

E2 L3 mediates the selective degradation of short-lived and abnormal proteins and functions in the E6/E6-AP-induced ubiquitination of p53/TP53. E2 variant 1 (**Q13404**) has no ubiquitin ligase activity on its own. The UBE2V1/UBE2N heterodimer (**Q13404**, **P61088**) catalyzes the synthesis of non-canonical poly-ubiquitin chains that are linked through Lys-63. This type of poly-ubiquitinylation is involved in NF-κ-B mediated signaling and contributes to the control of cell cycle and differentiation.

Ubiquitin carboxyl-terminal hydrolase isozyme L3 (L3, **P15374**), a deubiquitinylating enzyme, was detected solely in HCC tissue. It is involved in the processing of ubiquitin precursors and of ubiquitinated proteins by hydrolyzing a peptide bond at the C-terminal glycine of ubiquitin or NEDD8. The upregulation of L3 at the mRNA level was detected in breast cancer tissue and was suggested a

biomarker for tumor recurrence and bad prognosis [257]. In addition, its activity was consistently increased in cervical carcinoma biopsies compared to adjacent non-tumorous tissue [258].

Proteasome activator complex subunits		**Dp**	**Tp**	**Cov. (%)**	**Dp**	**Tp**	**Cov. (%)**	**Ratio of Tp (HCC/liver)**
Q9UL46	Proteasome activator complex subunit 2	6	**8**	39,3	8	**13**	51	1,6
Q06323	Proteasome activator complex subunit 1	6	**8**	36,1	8	**11**	42,2	1,4
26S protease regulatory subunits								
P43686	26S protease regulatory subunit 6B	0	**0**	0	1	**1**	3,3	In HCC only
P62195	26S protease regulatory subunit 8	0	**0**	0	1	**1**	3,9	
P62191	26S protease regulatory subunit 4	1	**1**	3,4	1	**1**	3,4	1,0
P62333	26S protease regulatory subunit S10B	2	**4**	7,7	0	**0**	0	In liver only
26S proteasome non-ATPase regulatory subunits								
P48556	26S proteasome non-ATPase regulatory subunit 8			0	1	**1**	5,8	In HCC only
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	2	**2**	8,8	1	**2**	4	1,0
O00231	26S proteasome non-ATPase regulatory subunit 11	2	**2**	5,9	1	**1**	3,1	0,5
O00232	26S proteasome non-ATPase regulatory subunit 12	1	**1**	2,6	0	**0**	0	In liver only
Proteasome alpha subunits								
Q8TAA3	Proteasome subunit alpha type 7-like	0	**0**	0	2	**3**	9,8	In HCC only
P25786	Proteasome subunit alpha type 1	2	**2**	8,7	4	**5**	14,8	2,5
O14818	Proteasome subunit alpha type 7	2	**3**	12,1	5	**6**	23,4	2,0
P25789	Proteasome subunit alpha type 4	1	**2**	3,8	2	**2**	9,2	1,0
P60900	Proteasome subunit alpha type 6	4	**5**	13	4	**4**	16,3	0,8
P25787	Proteasome subunit alpha type 2	4	**7**	23,9	4	**4**	20,1	0,6
P25788	Proteasome subunit alpha type 3	1	**3**	5,5	1	**1**	3,5	0,3
P28066	Proteasome subunit alpha type 5	3	**3**	21,6	1	**1**	5	0,3
Proteasome beta subunits								
P28072	Proteasome subunit beta type	1	**1**	4,6	3	**3**	13,4	3,0
P49720	Proteasome subunit beta type 3	5	**7**	30,2	5	**8**	28,3	1,1
P28070	Proteasome subunit beta type 4	1	**2**	8,3	2	**2**	11,7	1,0
P49721	Proteasome subunit beta type 2	6	**8**	34,8	6	**7**	39,3	0,9
P28065	Proteasome subunit beta type 9	3	**4**	12,3	3	**3**	12,8	0,8
P20618	Proteasome subunit beta type 1	6	**8**	31,1	5	**5**	36,9	0,6
P28074	Proteasome subunit beta type 5	1	**1**	6,3	0	**0**	0	In liver only
P28062	Proteasome subunit beta type 8	2	**3**	8,3	0	**0**	0	
P40306	Proteasome subunit beta type 10	1	**1**	7,3	0	**0**	0	

Table 15: Proteasome components identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

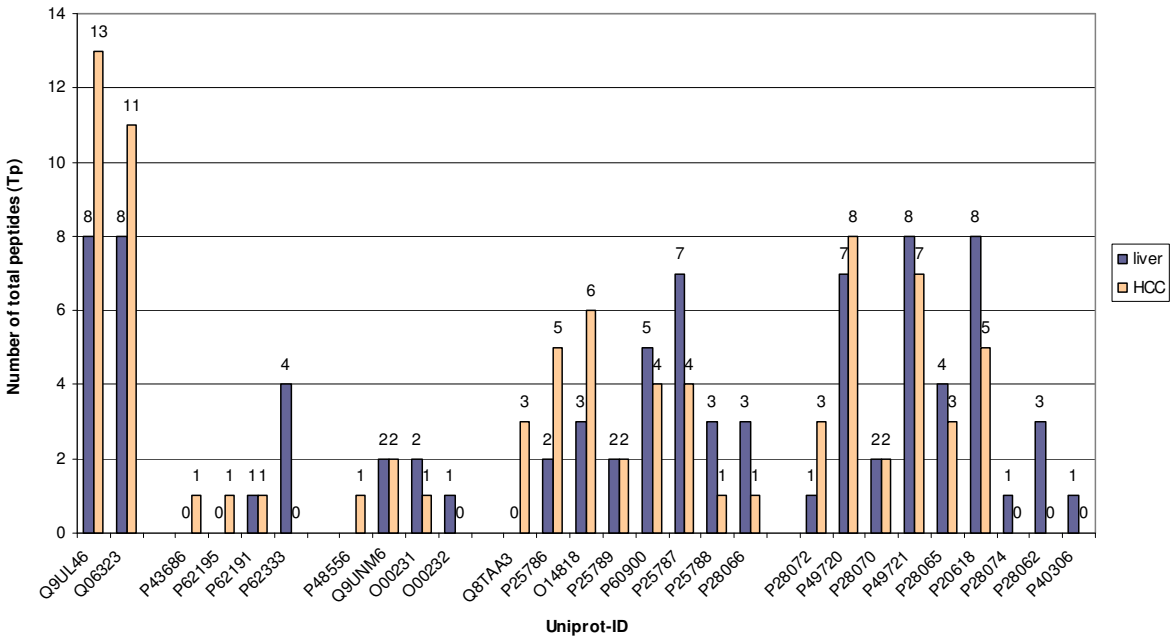


Figure 28: Abundance of proteasomal components in non-tumorous liver and HCC tissues determined by mass spectrometry

5.5 Glycolysis and glycogen metabolism

Many tumors gain energy via aerobic glycolysis, known as Warburg effect [259]. The latter is characterized by the increased uptake of glucose and the concomitant reduction of oxidative phosphorylation even in the presence of oxygen. Hence, pyruvate is predominantly reduced to lactate in order to regenerate reduction equivalents (NAD^+). Rapidly growing cells take up nutrients at high rates and use them for energy acquisition and for biosyntheses. The pyruvate kinase isoform M2 has recently gained center stage concerning the balance of catabolic and anabolic processes of tumor cells [260, 261]. In addition, the overexpression of glycolytic genes is considered a common feature of cancer cells [262]. However, hepatocytes primarily use intermediates of glycolysis for biosyntheses whereas its contribution for energy acquisition is negligible. Furthermore, the liver stores glucose as glycogen and releases it in response to insulin in order to keep blood glucose levels constant. Hence, the assumption of overexrression of glycolytic enzymes is discussed in the face of data acquired via shotgun and correlated to immunohistochemical and EHCO data. Enzymes involved in glycolysis and glycogen metabolism are depicted in figure 29.

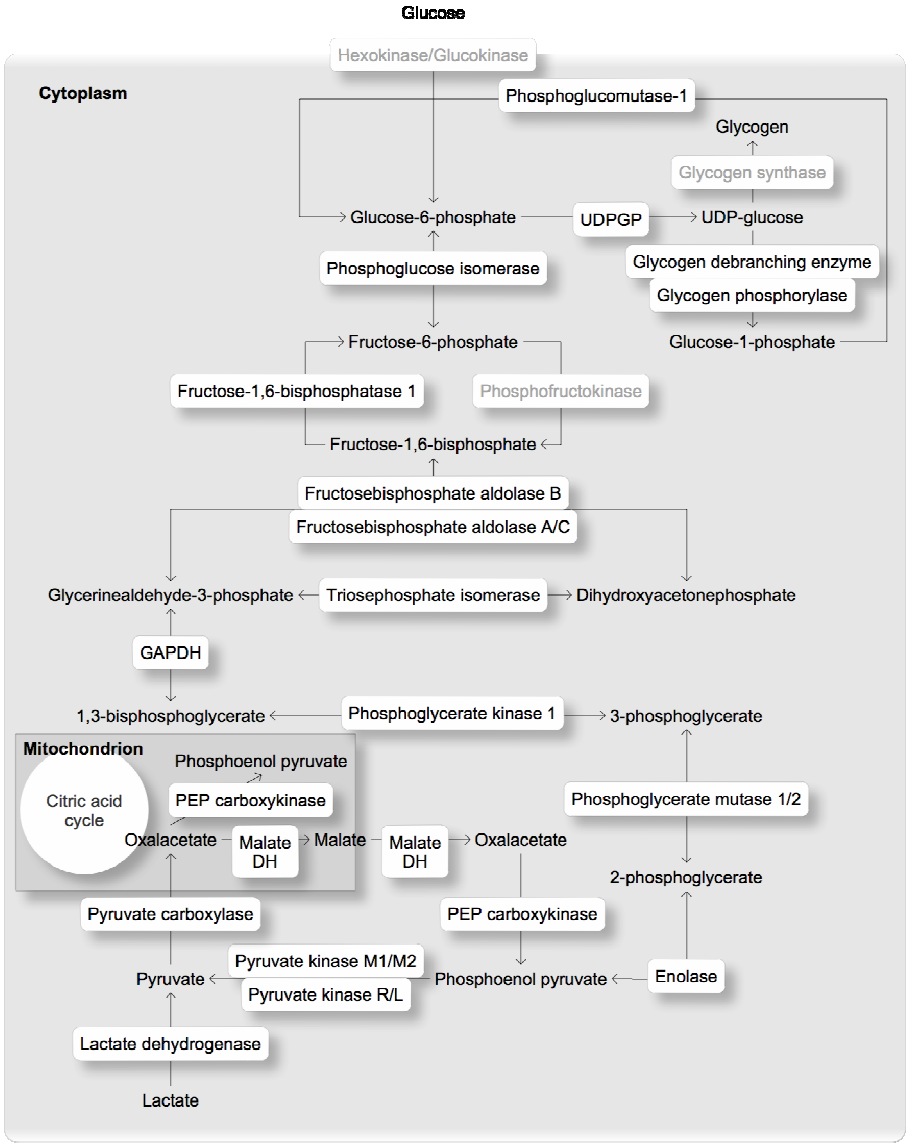


Figure 29: Proteins involved in glucose metabolism identified via mass spectrometry

Shotgun data of proteins involved in glucose metabolism (glycolysis, gluconeogenesis, and glycogen metabolism) are listed in table 16. Mass spectrometrical, immunohistochemical, and EHCO data are compiled in figure 31. Neither hexokinase (3 isoforms) nor glucokinase (~hexokinase IV) which catalyze the first step in the utilization of glucose (phosphorylation) in glycolysis were detected. Interestingly, immunohistochemical data state unaltered expression of liver cancer compared to normal liver tissue (data not shown).

The second step in glycolysis is catalyzed by glucose-6-phosphate isomerase (**P06744**) which was strongly downregulated in accordance to histologically derived and EHCO data. Phosphofructokinase (liver-form) which phosphorylates fructose-6-phosphate to fructose-1,6-bisphosphate was not detected, neither in HCC nor in non-tumorous liver tissue. Interestingly, data form “Human Protein Atlas” state downregulation or even loss of expression by tendency for that protein.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P06744	Glucose-6-phosphate isomerase	3	7	8,2	3	3	7,5	0,4
P09467	Fructose-1,6-bisphosphatase 1	15	27	53,3	9	25	36,7	0,9
P05062	Fructose-bisphosphate aldolase B	15	29	41,2	13	25	38,7	0,9
P04075	Fructose-bisphosphate aldolase A	1	2	5,5	3	5	12,9	2,5
P09972	Fructose-bisphosphate aldolase C	1	2	4,4	1	3	4,4	1,5
P60174	Triosephosphate isomerase	14	34	54,2	20	57	79,5	1,7
P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	14	23	50,4	13	30	53,4	1,3
P00558	Phosphoglycerate kinase 1	14	17	51,1	8	18	34,3	1,1
P18669	Phosphoglycerate mutase 1	6	12	40,6	8	19	47,6	1,6
P06733	Alpha-enolase	15	47	54,6	25	73	68	1,6
P30613	Pyruvate kinase isozymes R/L	0	0	0	2	2	6,1	In HCC only
P14618	Pyruvate kinase isozymes M1/M2	2	3	5,3	0	0	0	In liver only
P07195	L-lactate dehydrogenase B chain	2	2	7,5	0	0	0	In liver only
P00338	L-lactate dehydrogenase A chain	9	20	33,1	3	3	10,8	0,2
P11498	Pyruvate carboxylase, mitochondrial	36	64	43,2	20	32	25,2	0,5
Q16822	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	30	71	46,1	35	150	50,5	2,1
P40926	Malate dehydrogenase, mitochondrial	18	26	61,8	31	74	71,3	2,8
P40925	Malate dehydrogenase, cytoplasmic	4	5	12,9	5	6	19,2	1,2
P35558	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	6	12	11,9	4	12	6,4	1,0
P35573	Glycogen debranching enzyme	2	2	2,5	0	0	0	In liver only
P06737	Glycogen phosphorylase, liver form	16	37	23,6	0	0	0	
Q16851	UTP--glucose-1-phosphate uridylyltransferase 2	13	57	37,6	11	26	30,9	0,5
P36871	Phosphoglucomutase-1	24	37	55,3	11	14	29,9	0,4

Table 16: Proteins participating glucose metabolism identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Three tissue-specifically expressed or at least categorized isoforms of fructosebisphosphate aldolase were identified. Blasting of peptide sequences (NCBI Blast) confirmed the presence of aldolase B, liver-form (**P05062**), aldolase A (**P04075**), and aldolase C (**P09972**). Indeed, all peptides of aldolase A and C were specific for the respective isoform. Interestingly, EHCO data indicate a strong tendency for downregulation of aldolase B and upregulation of aldolase A in liver cancer. The latter is in accordance with shotgun and immunohistochemical data. Indeed, aldolase A was described as the predominant isoform in primary liver cancer and was suggested the more reliable serum marker than α-fetoprotein [263] which was not detected in the investigated sample (see also the enhanced expression of α-fetoprotein by the hepatoma-derived cell line Hep3B compared to primary human hepatocytes and in contrast to HepG2 [36]).Triosephosphate isomerase (**P60174**) was found to be moderately more abundant in HCC tissue. However, immunohistochemical evaluation revealed equal or downregulated expression rather than upregulation as suggested by EHCO data. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; **P04406**) was slightly more abundant in accordance to the immunohistochemically based estimation of about 73% of liver cancer samples showing unaltered expression (see proteinlist in appendix). However, this is in contrast to EHCO data with 6 citations (of 8 datasets) stating upregulation of GAPDH. Phosphoglycerate mutase 1 (**P18669**) and α-enolase (**P06733**) were slightly upregulated in HCC whereas immunohistochemical and EHCO data indicate tendential downregulation of enolase. It is noteworthy that all enzymes described for the conversion of 3-carbon glycolytic intermediates described thus far were slightly to moderately more abundant in HCC. In addition, pyruvate kinase isozymes R/L (**P30613**) and M1/M2 (**P14618**) were differentially expressed by being solely detected in HCC and liver, respectively. However, EHCO data state liver cancer-related upregulation for both isoforms which is at least confirmed for isoform M1/M2 by immunohistochemistry (data not shown, see www.proteinatlas.org).

Interestingly, mitochondrial PEP carboxykinase (PEP: phosphoenolpyruvate; **Q16822**) and malate dehydrogenase (**P40926**) were significantly upregulated in HCC tissue in contrast to their catalytically equivalent cytoplasmic counterparts (**P35558**, **P40925**). They are involved in the conversion of oxalacetate, a metabolite of the citrate cycle, in mitochondria. However, citrate synthase (**O75390**) as well as transaminase A (**P17174**) were significantly downregulated, possibly reflecting a metabolic preference towards formation of PEP and malate instead of citrate and aspartate (Figure 30).

Lactate dehydrogenase dehydrogenase chains A (**P00338**) and B (**P07195**) were detected solely in liver or pronouncedly less abundant in HCC, respectively. Lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate and converts pyruvate to lactate in conditions of short oxygen supply. In addition, lactat dehydrogenase catalyzes the reverse reaction in the course of the Cori cycle in liver. The latter is crucial for gluconeogenesis from muscle-derived lactate by hepatocytes. Notably, increased lactate is characteristic for the Warburg effect.

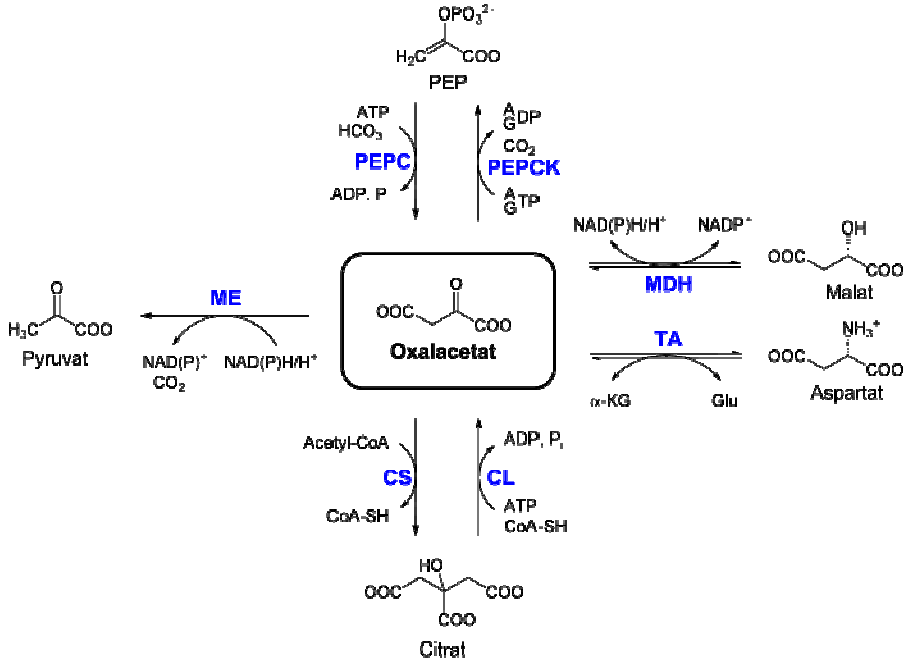
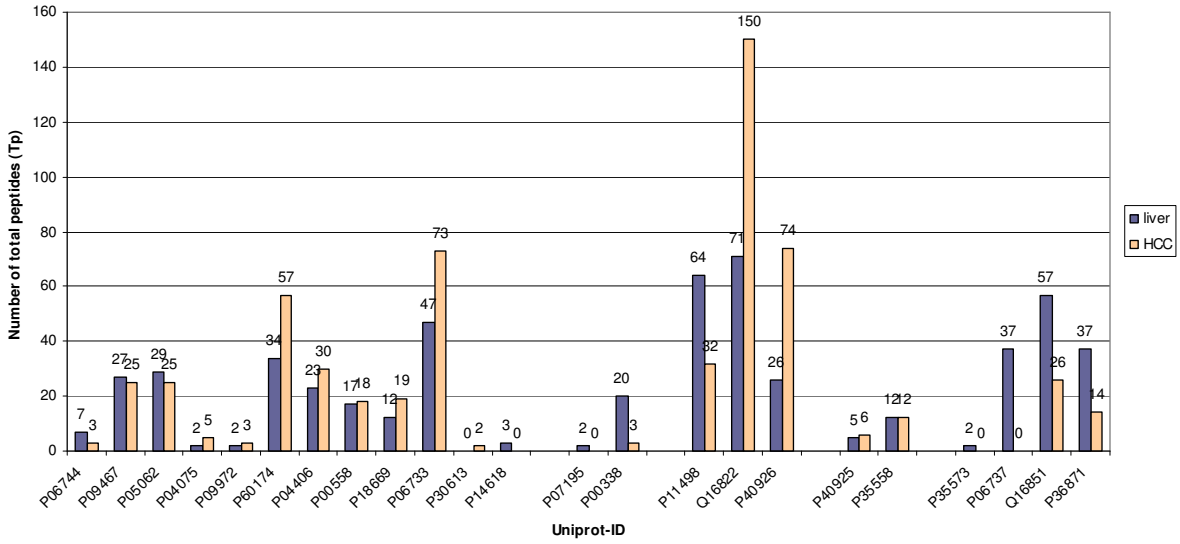


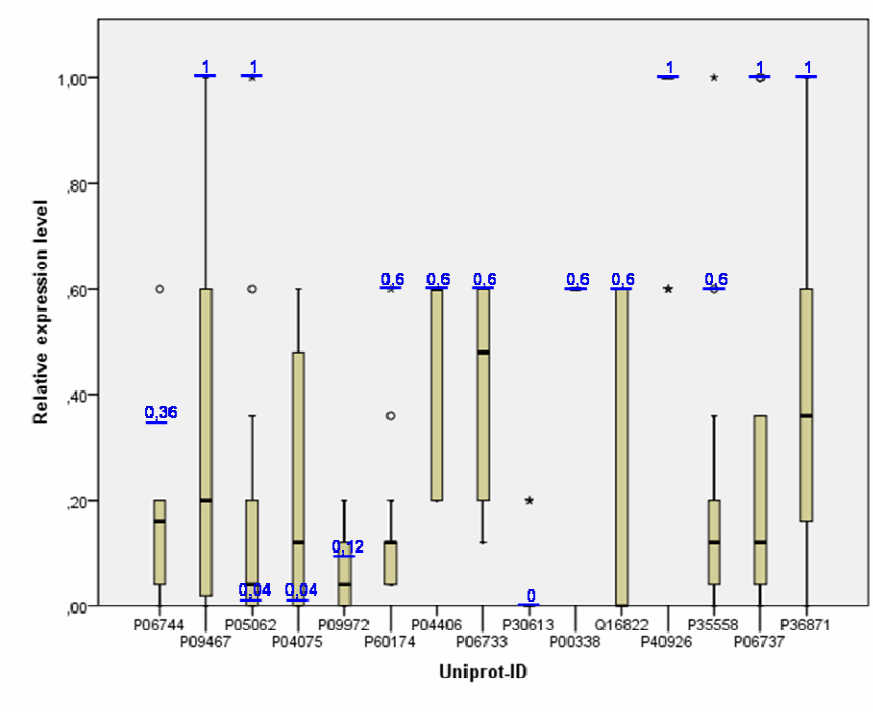
Figure 30: Metabolism of oxalacetate (Abbreviations: PEPC Phosphoenolpyruvate carboxylase, PEPCK Phosphoenolpyruvate carboxykinase, MDH Malate) (derived from Wikipedia)

Four enzymes involved in glycogen metabolism were detected (**P35573**, **P06737**, **Q16851**, **P36871**), all of which were significantly downregulated in HCC suggesting the impairment of this crucial liver-specific pathway in liver cancer. For glycogen phosphorylase (**P06737**) and phosphoglucomutase-1 (**P36871**) immunohistochemical expression data were available and confirmed the shotgun results.

In conclusion:

1. Glucose-6-phosphate isomerase was found to be less abundant in HCC tissue according to immunohistochemical and EHCO data. In addition, protein expression data for hexokinase/glucokinase and phosphofructokinase derived from “Human Protein Atlas” indicate the downregulation of both proteins in liver tumors.
2. Aldolase isoform A normally repressed in adult liver was more abundant in HCC. This result was supported by immunohistochemical and EHCO data.
3. Interestingly, the expression of enzymes for the conversion of 3-carbon glycolytic intermediates inclusive 2-phosphoglycerate were consistently upregulated in HCC. In addition, shotgun data indicate the differential expression of pyruvate kinase isoforms.
4. Mitochondrial enzymes involved in the conversion of oxalacetate were differentially expressed in HCC and normal liver. Based on these data, a preference for the formation of phosphoenolpyruvate and malate might be supposed.
5. Surprisingly, lactate dehydrogenase was found to be less abundant in HCC (see Warburg effect)
6. Enzymes involved in glycogen metabolism were less abundant in HCC indicating the impairment of this liver-specific pathway





Uniprot-ID	up	down
P06744	No results	
P09467	0	4
P05062	0	6
P04075	3	0
P09972	No results	
P60174	1	0
P04406	4	0
P18669	No results	
P06733	0	2
P30613	1	0
P14618	2	0
P07195	0	1
P00338	1	2
P11498	0	1
Q16822	0	4
P40926	1	0
P40925	1	0
P35558	0	6
P35573	1	1
P06737	0	1
Q16851	0	2
P36871	0	1

Figure 31: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of glycolytic enzymes in non-tumorous liver and liver cancer/HCC tissues The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.6 Comparison of functions mediated by mitochondria and the peroxisomes

5.6.1 Mitochondrial functions of HCC and liver- a global view

Mitochondrial enzymes mediate liver-specific and common metabolic features. A part of the urea cycle takes place in mitochondria as well as fatty acid metabolism, the citrate cycle, and oxidative phosporylation. In terms of total peptides of mitochondrial proteins detected in liver and HCC tissue, the difference was negligible (2% difference) (Figure 32). However, a pronounced reduction of proteins invoved in amino acid metabolism and an increase in enzymes involved in energy metabolism and fatty acid metabolism was observed. Examples of amino acid-degrading enzymes found to be downregulated in HCC were glutamate dehydrogenase 1/2 (P00367/P49448 with Tp of 68/16 in liver/HCC), glycine dehydrogenase (P23378 with Tp of 8 detected exclusively in liver), and aspartate aminotransferase (P00505 with Tp of 41/14 in liver/HCC)). These data were consistent with immunohistochemical and EHCO data (data not shown).

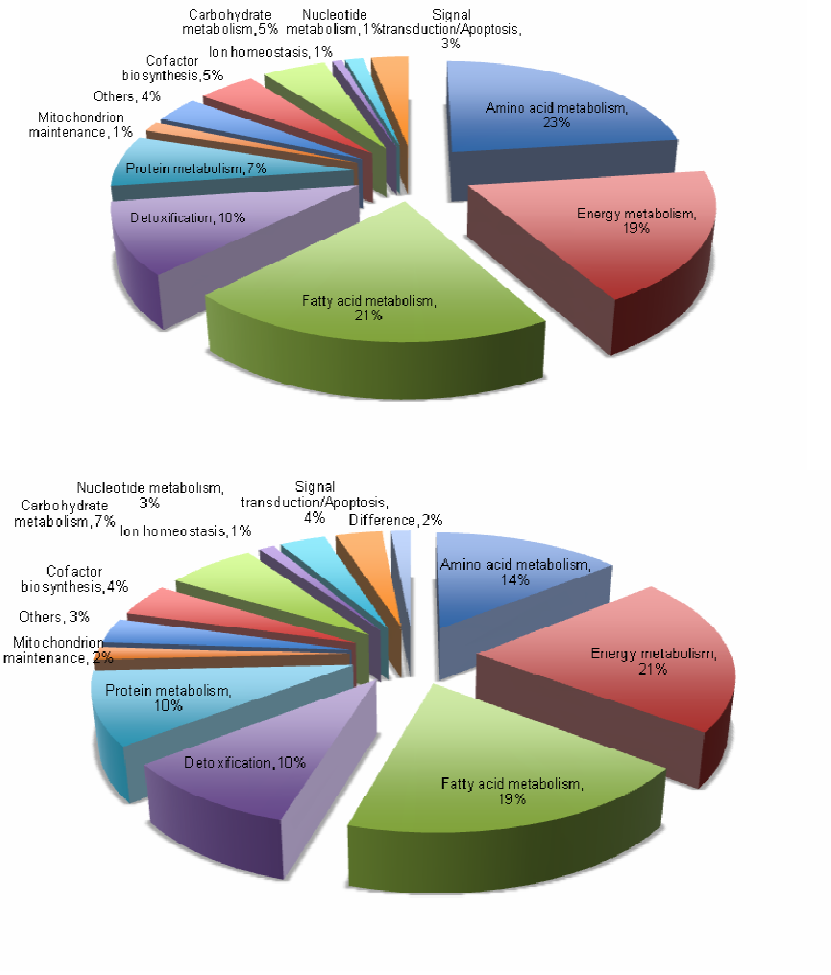


Figure 32: Functional comparison of mitochondrial proteins of liver (above) and HCC tissue (below). Percentage given for each functional group correspond to total peptide numbers of the respective group related to the total peptide number of proteins of non-tumorous liver tissue.

5.6.2 The urea cycle

The metabolic conversion of ammonia to urea is a specific function of hepatocytes. Its physiological impact is underlined by “urea cycle disorders”, classified as “inborn errors of metabolism”, resulting from the genetic deficiency of enzymes involved in the urea cycle. Moreover, liver failure leads to hyperammonaemia and eventually manifests in “Coma hepaticum”. All enzymes, located in mitochondria and the cytoplasm, were identified by shotgun and mass spectrometry (Table 17).

Uniprot-ID	Description	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp HCC/liver
P31327	Carbamoyl-phosphate synthase	87	**257**	62,2	76	**150**	54,5	0,6
P00480	Ornithine carbamoyltransferase	13	**24**	54	12	**15**	49,2	0,6
P00966	Argininosuccinate synthase	12	**19**	37,4	4	**5**	18,4	0,3
P04424	Argininosuccinate lyase	5	**7**	18,8	6	**9**	21,1	1,3
P05089	Arginase-1 (liver-type)	19	**31**	57,8	14	**16**	50	0,5

Table 17: Proteins of the urea cycle identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

There is a remarkable trend towards lower expression levels compared to non-tumorous liver, indicating a reduced capability of tumorous cells to detoxify ammonia. The data are in accordance with immunohistochemical and protein expression data obtained from “Protein Atlas” and EHCO, respectively. Figure 34 depicts the expression levels of the respective proteins of HCC relative to non-tumorous liver tissue. Immunohistochemical data were elaborated as described above. Expression levels of all urea cycle enzymes are comparably high in terms of intensity and quantity (exemplified in figure 33) resulting in a REL of 1. Figure 34 depicts shotgun data, relative values for REL derived from immunohistochemical data, and compiles the numbers of citations with respect to up- and downregulation from EHCO.

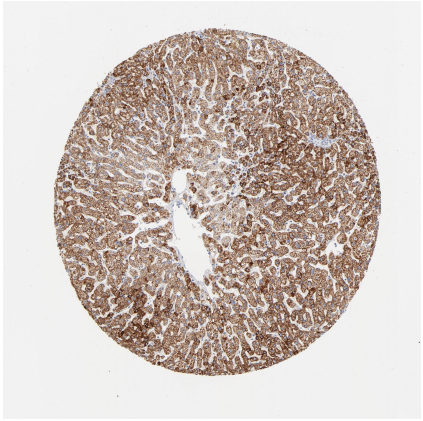
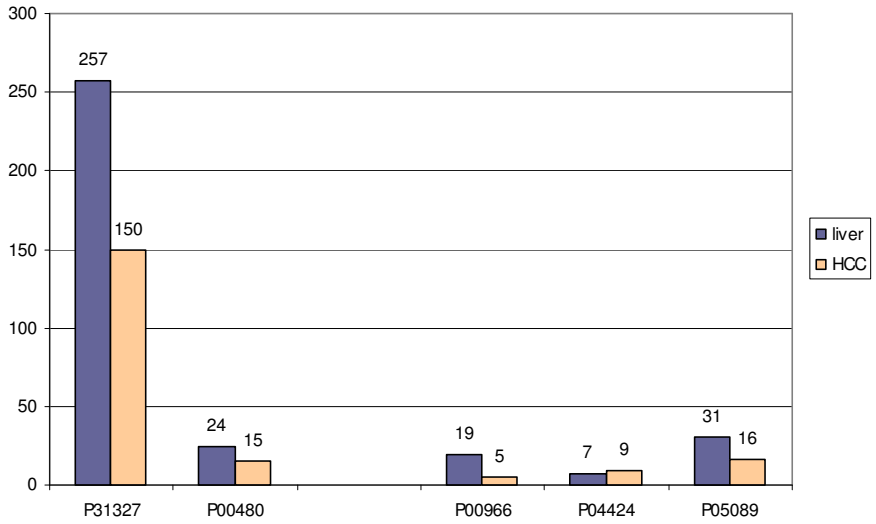
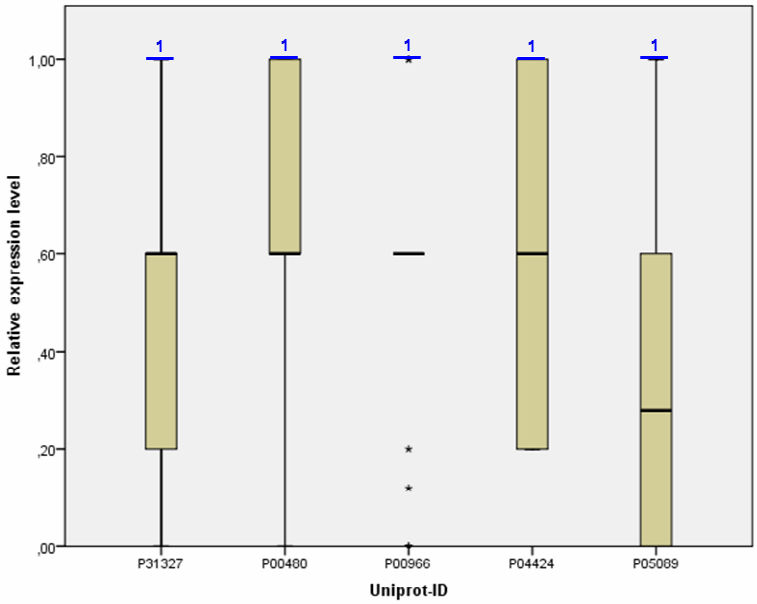


Figure 33: Immunohistochemical detection of carbamoyl-phosphate synthase (P31327) in liver indicating strong expression (intensity: strong) of more than 75% of hepatocytes (quantity: >75%) (from www.proteinatlas.org).





Uniprot-ID	up	down
P31327	1	4
P00480	0	2
P00966	0	3
P04424	No results	
P05089	0	6

Figure 34: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of enzymes of the urea cycle in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.6.3 Mitochondrial and peroxisomal β-oxidation of fatty acids

The liver plays an essential role in the metabolism of fatty acids. The degradation of the latter is mediated by three distinct enzyme systems located in mitochondria (β-oxidation), peroxisomes (α- and β-oxidation), and microsomes (monooxygenases, e.g. Cyp4A1). Figure 35 illustrates the biochemical reactions and proteins involved in β-oxidation of mitochondria and peroxisomes. Proteins identified via mass spectrometry are in bold. The mitochondrial β-oxidation pathway is further subdivided into functional groups comprising enzymes mediating continuous rounds of acetyl-CoA and acyl-CoA (n-2) formation, enzymes involved in the conversion of propionyl-CoA derived from fatty acids with odd C-atom numbers, enzymes involved in the degradation of unsaturated fatty acids, and enzymes mediating keton body formation.

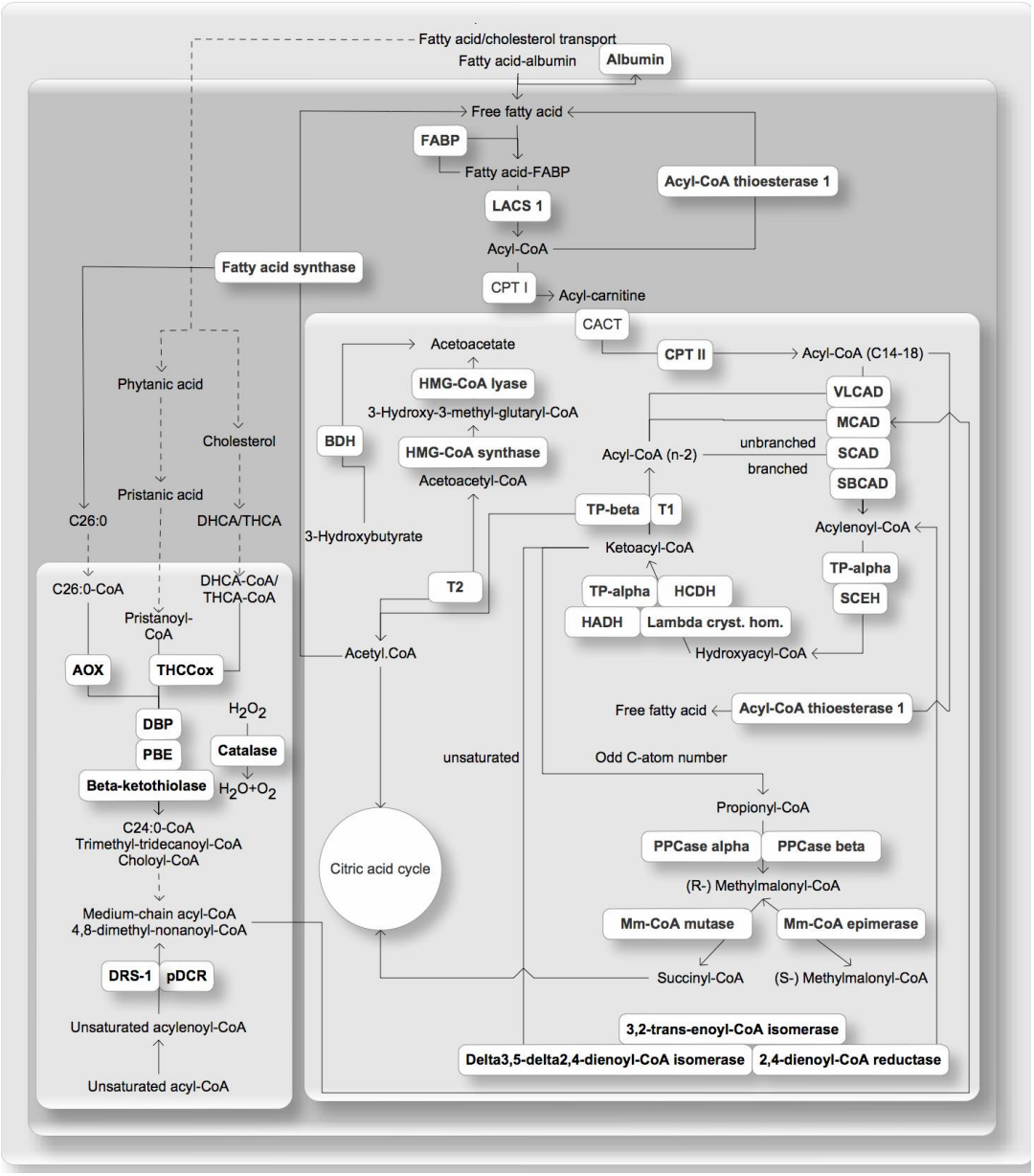


Figure 35: Proteins involved in fatty acid metabolism identified via mass spectrometry (in bold characters)

The alteration of the metabolism of long-chain fatty acids seems to be a common feature of tumors and is, indeed, observed in conjunction with hepatocellular carcinoma as well. It is reflected by increased plasma levels of free fatty acids observed e.g. in tumor-bearing rats [264], in mice with mammary tumors [265], and in patients with breast tumors [266]. In addition, the fatty acid composition of human non-tumorous and HCC tissue is significantly different with respect to alpha-linolenic acid and docosahexaenic acid, both being strongly reduced in liver cancer [267]. Moreover, free fatty acid levels were increased in ascites of patients with malignant abdominal tumors compared to that with liver cirrhosis and the ratio of unsaturated and saturated fatty acids was increased in patients with neoplasms [268, 269]. A similar result was obtained from patients suffering from primary and secondary liver tumors exhibiting a reduced ratio of stearic and oleic acid levels representing saturated and unsaturated C18-fatty acids, respectively [270]. Indeed, shotgun data supported alterations of the fatty acid metabolism at the protein expression level.

5.6.3.1 Mitochondrial proteins

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P43155	Carnitine O-acetyltransferase (**CAT**)	3	**3**	7,5	0	**0**	0	0,0
P23786	Carnitine O-palmitoyltransferase 2 (**CPT II**)	10	**11**	22,3	11	**14**	18,5	1,3
Q86TX2/ P49753	Acyl-coenzyme A thioesterase 1/2	8	**20**	30,4	5	**21**	17,8	1,1
P49748	Acyl-CoA dehydrogenase, very-long-chain specific; (**VLCAD**)	18	**27**	35,3	2	**3**	5,5	0,1
P11310	Acyl-CoA dehydrogenase, medium-chain specific; (**MCAD**)	14	**18**	36,1	7	**8**	28,3	0,4
P16219	Acyl-CoA dehydrogenase, short-chain specific, (**SCAD**)	10	**11**	32,5	3	**3**	8,5	0,3
Q16836	Short chain 3-hydroxyacyl-CoA dehydrogenase, (**HCDH**)	9	**15**	48,7	13	**17**	71,3	1,1
P45954	Acyl-CoA dehydrogenase, short/branched chain specific; (**SBCAD**)	19	**31**	50,2	11	**22**	33,3	0,7
P40939	Trifunctional enzyme alpha subunit, (**TP-alpha**)	29	**62**	51,1	29	**70**	46,5	1,1
P30084	Enoyl-CoA hydratase, (**SCEH**)	19	**60**	65,9	18	**48**	63,8	0,8
Q16836	Short chain 3-hydroxyacyl-CoA dehydrogenase, (**HCDH**)	9	**15**	48,7	13	**17**	71,3	1,1
Q99714	3-hydroxyacyl-CoA dehydrogenase type II; **HADH**	14	**38**	82,8	19	**36**	87,4	0,9
Q9Y2S2	**Lambda-crystallin homolog**	3	**5**	10,3	4	**6**	13,8	1,2
P55084	Trifunctional enzyme subunit beta; (**TP-beta**)	15	**28**	41,6	7	**10**	16	0,4
P42765	3-ketoacyl-CoA thiolase, (**T1**)	19	**42**	56,4	25	**70**	65,2	1,7

Table 18: Proteins participating in mitochondrial beta-oxidation identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Mitochondrial β-oxidation is based on continuous rounds of fatty acid chain shortening. The first step is the oxidation of fatty acids catalyzed by various acyl-CoA dehydrogenases with distinct chain length specificity. Their physiological impact is reflected by recessive autosomal diseases due to deficiencies of the various acyl-CoA dehydrogenases. The loss of VLCAD (Very-long-chain-specific acyl-CoA dehydrogenase; **P49748**) function, for instance, leads to impaired long-chain fatty acid β-oxidation. VLCAD metabolizes long-chain fatty acids such as palmitoyl-CoA, mysritoyl-CoA and stearoyl-CoA to acyl chain lengths of 12 carbons. MCAD (Medium-chain-specific acyl-CoA dehydrogenase, **P11310**) is specific for acyl chain lengths of 4 to 16 C-atoms and SCAD (Short-chain-specific dehydrogenase, **P16219**) oxidizes butanoyl-CoA to 2-butenoyl-CoA. Interestingly, VLCAD, MCAD, and SCAD were significantly less abundant in HCC compared to non-tumorous liver tissue. In contrast, all other enzymes involved in the formation of acyl-CoA and acetyl-CoA were more or less equally abundant in both tissues (Figure 36, table 18). This suggests that reduced VLCAD/MCAD/SCAD levels constitute a functional bottleneck concerning mitochondrial fatty acid degradation. These data are in accordance with immunohistochemical data (Figure 37). VLCAD, MCAD and SCAD show the tendency for downregulation in histological slices of HCC compared to liver tissue. Downregulation of acyl-CoA dehydrogenases was also published in EHCO. However, proteins with unaltered expression levels in shotgun were stated to be downregulated (e.g. 7 of overall 8 datasets compiled in EHCO claim enoyl-CoA hydratase (**P30084)** to be downregulated) (figure 37). This might indicate alternative ways for the modification of fatty acid metabolism realized by liver tumors.

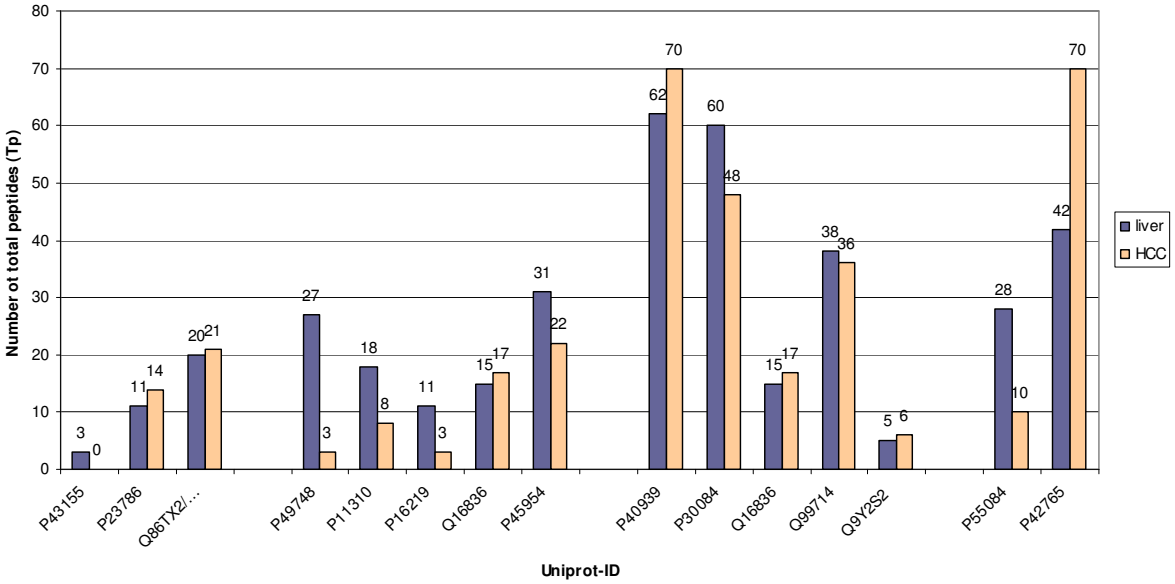
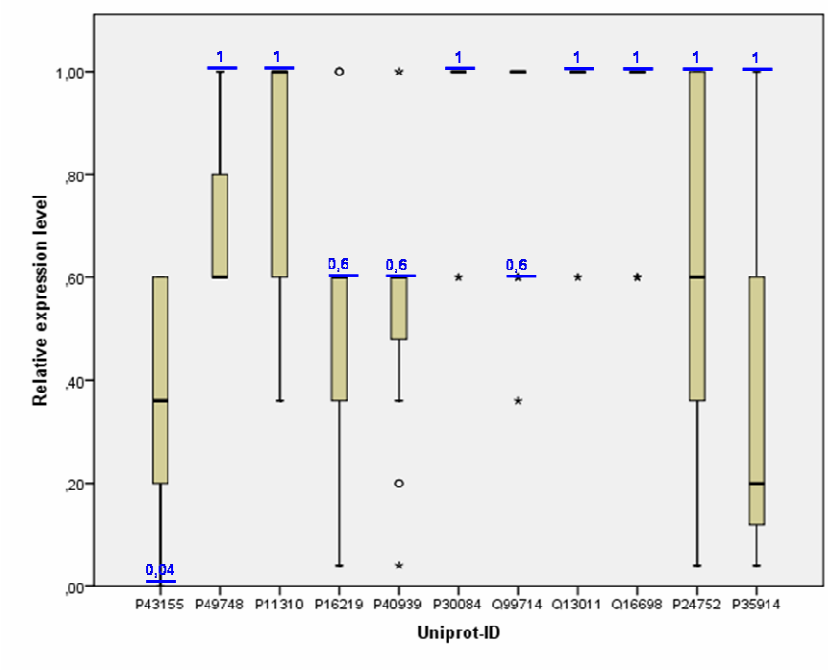


Figure 36: Abundance of enzymes of the mitochondrial β-oxidation in non-tumorous liver and HCC tissues determined by mass spectrometry



Uniprot-ID	up	down
P43155	0	1
P23786	0	1
Q86TX2/ P49753	0	1
P49748	0	4
P11310	0	2
P16219	0	5
Q16836	0	2
P45954	0	6
P40939	No results	
P30084	0	7
Q16836	0	2
Q99714	0	2
Q9Y2S2	0	2
P55084	0	2
P42765	0	3
Q13011	0	2
P42126	0	1
Q16698	0	3

Figure 37: Immunohistochemical (below left) and EHCO data (below right) concerning the relative abundance of mitochondrial enzymes involved in β-oxidation in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

The degradation of fatty acids with odd C-numbers finally results in the formation of propionyl-CoA (instead of acetyl-CoA) which is converted to succinyl-CoA. Three enzymes involved in this process have been identified: Propionyl-CoA carboxylase (PCC subunits alpha (**P05165**) and beta (**P05166**)), methylmalonyl-CoA mutase (**P22033**) and methylmalonyl-CoA epimerase (**Q96PE7**) (Table 19).

Odd number fatty acids		Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P05165	Propionyl-CoA carboxylase alpha chain; (**PCCase** subunit **alpha**)	9	9	19,3	0	0	0	0,0
P05166	Propionyl-CoA carboxylase beta chain, (**PCCase** subunit **beta**)	15	24	38,4	7	9	19,7	0,4
Q96PE7	**M**ethyl**m**alonyl-**CoA epimerase**	4	5	26,1	4	5	26,1	1,0
P22033	**M**ethyl**m**alonyl-**CoA mutase**	15	22	28,1	1	2	2,1	0,1

Table 19: Proteins for the conversion of propionyl-CoA identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

The impact of PCC on physiology is underlined by a hereditary disease leading to propionic acidemia due to PCC deficiency. Apart from its function in fatty acid metabolism it is also indispensable for the

breakdown of the essential amino acids valine, isoleucine, threonine, and methionine. Disruption of PCCA or PCCB prevents these acids from being metabolized leading to the accumulation of propionyl-CoA, propionic acid, ketones and other toxic compounds in the blood (Uniprot: P05165 and P05166). Methylmalonyl-CoA epimerase is also involved in the degradation of those amino acids as well as cholesterol via propionyl-CoA. Defects in this enzyme result in methylmalonic aciduria type mut. Methylmalonyl-CoA racemase catalyzes the interconversion of (R-) and (S-) methylmalonyl-CoA. Racemase deficiency causes methylmalonic aciduria type 3, an autosomal recessive disease leading to metabolic acidosis and secondary hyperammonaemia. PPCase subunits alpha and beta as well as methylmalonyl-CoA epimerase were significantly less abundant in HCC tissue indicating the tumor-associated deficiency in the conversion of propionyl-CoA from β-oxidation of odd-chain fatty acids, the degradation of amino acids valine, threonine, isoleucine, and methione as well as cholesterin (Figure 38, table 19). Unfortunately, immunohistochemical data for those proteins were not available and EHCO delivered contradictory data (Table 20).

Uniprot-ID	up	down
P05165	1	1
P05166	1	0
Q96PE7	1	0
P22033	0	2

Table 20: EHCO data for enzymes for the conversion of propionyl-CoA

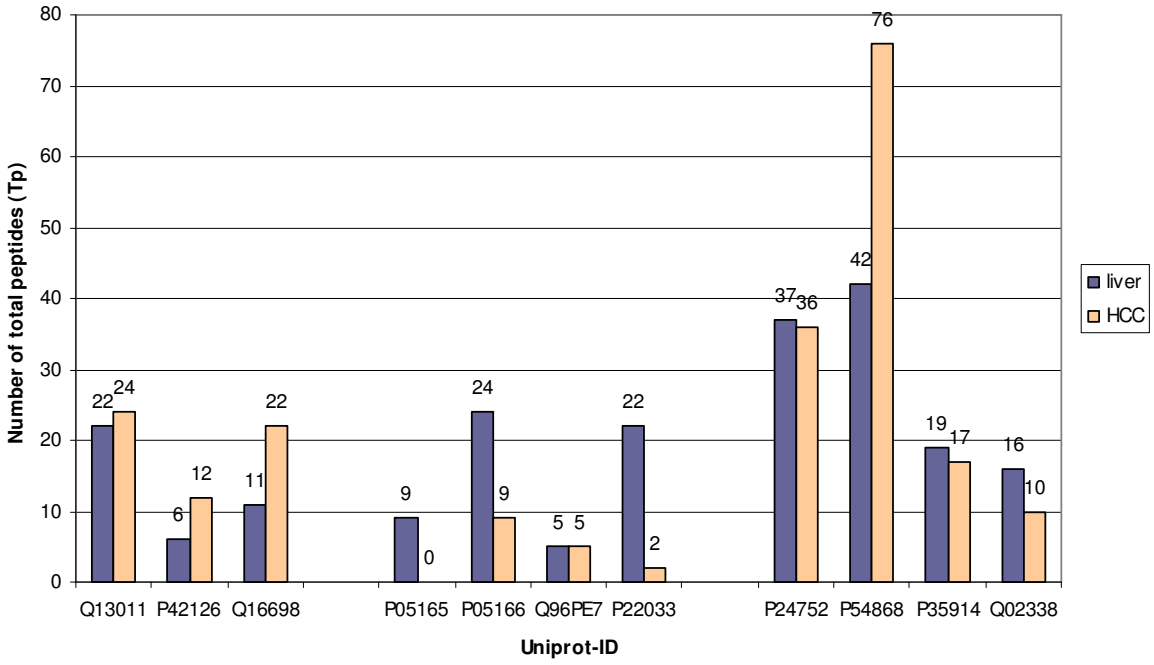


Figure 38: Relative abundance of enzymes involved in the degradation of unsaturated fatty acids, fatty acids with odd C-numbers, and keton body formation determined via mass spectrometry

The degradation of unsaturated fatty acids requires the enzymes delta3,5-delta2,4-dienoyl-CoA isomerase (**Q13011**), 3,2-trans-enoyl-CoA isomerase (**P42126**), and 2,4-dienoyl-CoA reductase (**Q16698**). The latter two were strongly upregulated in HCC tissue (Figure 38, table 21).

Unsaturated fatty acids		**Dp**	**Tp**	**Coverage (%)**	**Dp**	**Tp**	**Coverage (%)**	**Ratio of Tp (HCC/liver)**
Q13011	Delta3,5-delta2,4-dienoyl-CoA isomerase	10	22	35,7	15	24	57,3	1,1
P42126	3,2-trans-enoyl-CoA isomerase	5	6	25,2	9	12	36,8	2,0
Q16698	2,4-dienoyl-CoA reductase	6	11	26,3	10	22	35,8	2,0

Table 21: Proteins participating in the degradation of unsaturated fatty acids identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Histological data support the overexpression of 2,4-dienoyl-CoA reductase and the unaltered expression or slight upregulation of Δ3,5-delta2,4-dienoyl-CoA isomerase (Figure 39). Although both proteins show RELs of 1 for liver and tumorous tissue, there is indeed a significant difference in the expression levels (Figure 39). No data concerning the physiological impact of upregulation for these proteins were available. However, the impact of disruption of these enzymes on physiology is indicated by knock-out mouse models [271, 272].

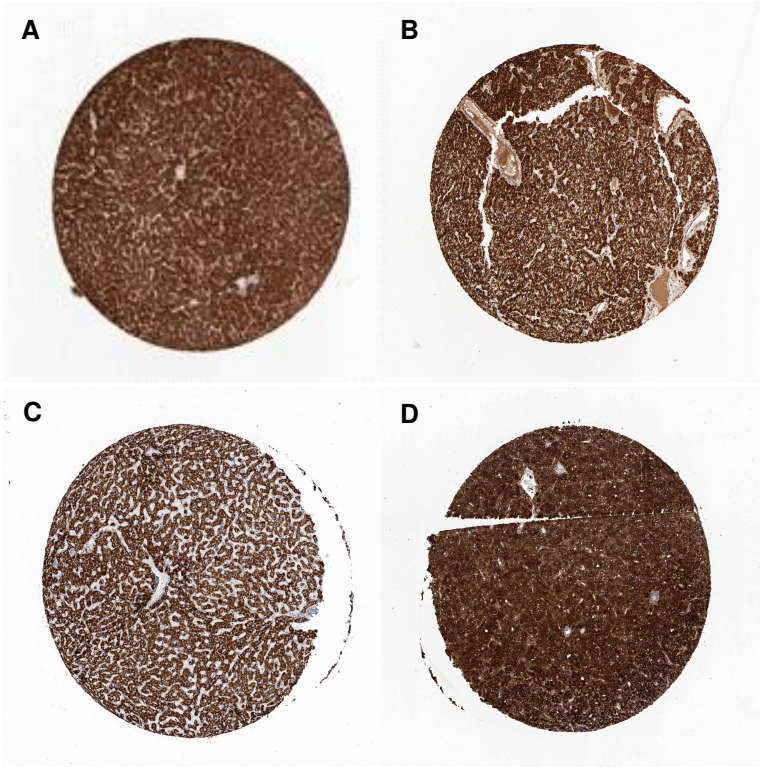


Figure 39: Immunohistochemical detection of Δ3,5-delta2,4-dienoyl-CoA isomerase (Q13011; A, B) and 2,4-dienoyl-CoA reductase (Q16698; C, D) of normal liver (A, C) and liver cancer tissue (B, D) (derived from www.proteinatlas.org)

Keton bodies (acetone, acetoacetic acid, and beta-hydroxybutyric acid) are produced as by-products of fatty acid beta-oxidation by hepatocytes and serve as energy source for heart and brain. The abundance of enzymes involved in keton body formation (acetyl-CoA acetyltransferase (T2), **P24752**); HMG-CoA synthase, **P54868**; HMG-CoA lyase, **P35914**; D-beta-hydroxybutyrate dehydrogenase (BDH), **Q02338**) was equal or just slightly different in non-tumorous vs. HCC tissue (Table 22).

Keton body formation		Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P24752	Acetyl-CoA acetyltransferase; (**T2**)	20	37	44,5	14	36	37,5	1,0
P54868	Hydroxymethylglutaryl-CoA synthase, (**HMG-CoA synthase**)	23	42	46,3	25	76	47	1,8
P35914	Hydroxymethylglutaryl-CoA lyase, (**HMG-CoA lyase**)	8	19	28,6	12	17	36,6	0,9
Q02338	D-beta-hydroxybutyrate dehydrogenase, (**BDH**)	9	16	28,3	9	10	32,9	0,6

Table 22: Proteins participating keton body formation identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

However, immunohistochemical protein expression data for P24752 and P35914 show the tendency for downregulation of those proteins (Figure 37) which is in accordance with EHCO-derived expression data (Table 23).

Uniprot-ID	up	down
P24752	0	4
P54868	0	3
P35914	0	2
Q02338	0	2

Table 23: EHCO data for enzymes for keton body formation

5.6.3.2 Peroxisomal proteins

Enzymes of peroxisomal β-oxidation identified via mass spectrometry are listed in table 24. The degradation of fatty acids in peroxisomes is limited to chain-shortening reactions producing fatty acids of reduced chain lengths which are consequently transferred to mitochondria in the form of carnitine esters. Nevertheless, peroxisomes contain enzymes of unique substrate specificity (figure 35, table 24). Hexacosanoic acid (C26:0) derived from diet or by endogenous synthesis is exclusively metabolized in peroxisomes by acyl-CoA oxidase 1 (AOX; **Q15067**). Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is also solely oxidized by peroxisomal acyl-CoA oxidase 2 (THCCox; **Q99424**). The latter additionally mediates the conversion of dihydroxycholestanoic and trihydroxycholestanoic acid, both formed in the liver from cholesterol, to chenodeoxycholate and cholate, respectively, followed by their excretion into bile canaliculi [273].

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver
Q15067	Acyl-coenzyme A oxidase 1, (**AOX**)	5	5	11,5	4	4	10,5	0,80
Q99424	Acyl-coenzyme A oxidase 2, (**THCCox**)	2	3	4,6	0	0	0	In liver only
P51659	Peroxisomal multifunctional enzyme type 2, (**DBP**)	18	30	28,3	6	10	10,3	0,33
Q08426	Peroxisomal bifunctional enzyme (**PBE**)	22	33	34	26	52	41,8	1,58
P09110	3-ketoacyl-CoA thiolase, (**Beta-ketothiolase**)	18	33	52,4	18	33	53,8	1,00
P04040	Catalase	22	33	48	27	51	56,5	1,55
Q9NUI1	Peroxisomal 2,4-dienoyl-CoA reductase (**pDCR**)	0	0	0	2	2	4,1	In HCC only
O75521	Peroxisomal 3,2-trans-enoyl-CoA isomerase; (**DRS-1**); (Hepatocellular carcinoma-associated antigen 88)	10	13	36,5	8	9	27,3	0,69

Table 24: Proteins of the peroxisomal degradation of fatty acids identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

AOX and THCCox are highly substrate-specific. Interestingly, AOX expression was unaltered whereas THCCox was exclusively detected in non-tumorous liver indicating an HCC-associated deficiency to metabolize pristanic acid and cholesterol derivatives including bile acids. The D-bifunctional protein (DBP; **P51659**) which is also indispensable for bile acid formation was significantly less abundant in HCC tissue. However, PBE (**Q08426**) upregulated in HCC exhibits slightly different substrate specificity and might compensate for the partial loss of DBP or reflect the altered abundance of fatty acid species. Interestingly, expression data for DBP are contradictory with downregulation via shotgun, EHCO (1 citation up, 2 citations down), and putative stable expression via immunohistochemistry (both tissues have REL 1). However, examination of histological slices revealed partially strong upregulation of DBP (as exemplified by figure 40). Shotgun data, immunohistochemical and EHCO data are compiled in figure 41.

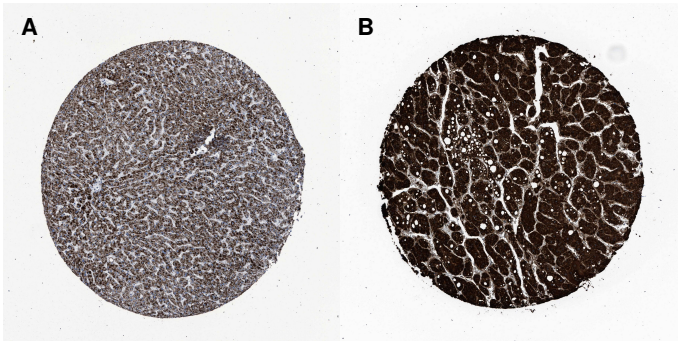
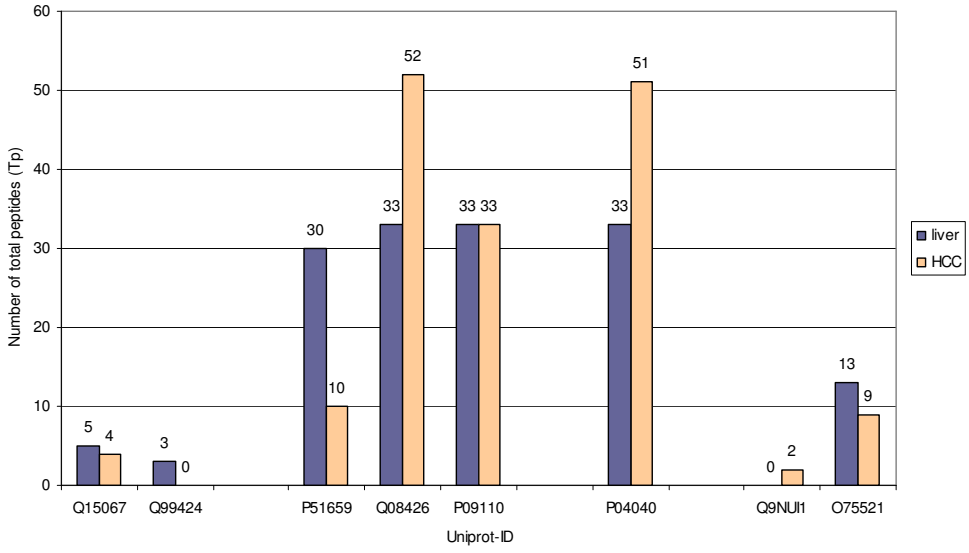
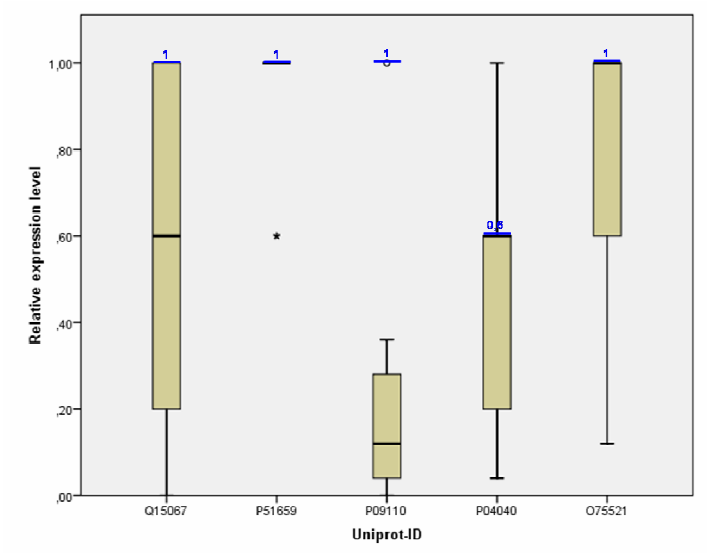


Figure 40: Immunohistochemical detection of DBP (P51659) in slices of normal liver (A) and liver cancer tissue (B) (derived from www.proteinatlas.org)





Uniprot-ID	up	down
Q15067	0	2
Q99424	0	3
P51659	1	2
Q08426	0	1
P09110	0	2
P04040	0	6
Q9NUI1	0	1
O75521	No results	

Figure 41: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of peroxisomal fatty acid-degrading enzymes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

In conclusion, shotgun data suggest

1. a pronouncedly reduced mitochondrial β-oxidation of fatty acids indicated by the low abundance of three distinct acyl-CoA dehydrogenases (VLCAD, MCAD, SCAD)
2. that the conversion of propionyl-CoA (derived from the degradation of fatty acids with odd C-number) to succinyl-CoA was strongly compromized in the investigated HCC sample. This assumption is based on three (of overall four) enzymes involved in this process found to be pronouncedly downregulated in the tumorous tissue in contrast to
3. auxiliary enzymes for the conversion of unsaturated fatty acids.
4. the impairment of the peroxisomal conversion of pristanc acid and other compounds mediated by the highly substrate-specific acyl-coenzyme A oxidase 2. In addition, the catalytically equivalent but potentially substrate-specifically distinct enzymes DBP and PBE were differentially expressed in the investigated tissue samples.

Taken together, those results point to the adaptation or reorganization of the fatty acid metabolism by HCC tumors and underline findings gained by alternative methods at the functional proteome level.

5.7 Comparison of lysosome-mediated functions of liver and HCC

The lysosome is primarily derived from the Golgi apparatus, secondarily from phagolysosomes and autophagolysosomes. It constitutes the principal digestive organ of the cell. Indeed, hepatocytes are capable to exhibit autophagy for e.g the removal and recycling of mitochondria. Moreover, endosomal vesicles end up in lysosomes resulting in the complete turn-over of the cell membrane within an hour. Lysosomes are acidified via H^+-ATPase to a pH of <5 and contain degrading enzymes for biological macromolecules such as acid esterases (cleavage of ester bonds via phosphate or sulfate, DNAses, RNAses, 1,4-glucosidase (mutated/inactive in lysosomal storage disease *Morbus Pompe*), cathepsins A, D, E, and carboxypeptidase for the recycling of amino acids for protein synthesis.

Overall 17 lysosomal proteins were identified in non-tumorous liver and HCC tissues. The proteins were assigned to four functional groups: Protein degradation, lipid degradation, polysaccharide degradation, and lysosomal acidification. The global comparison of those tissues revealed "loss of function" in protein and lipid degradation as well as "gain of function" in polysaccharide metabolism by lysosomes of tumor cells (Figure 42). Taking individual protein species within the functional groups into account a considerable metabolic diversification could be observed. The impact of enzymes mediating polysaccharide degradation is underlined by inherited storage disorders attributable to defects of the respective proteins. They frequently manifest in hepatomegaly in consequence of the deposition of excess non-degradable polysaccharides, lipids and/or metabolites thereof (table 25). Hence, HCC tissue might resemble liver tissue of patients suffering from storage diseases with respect to histologic and anatomical alterations.

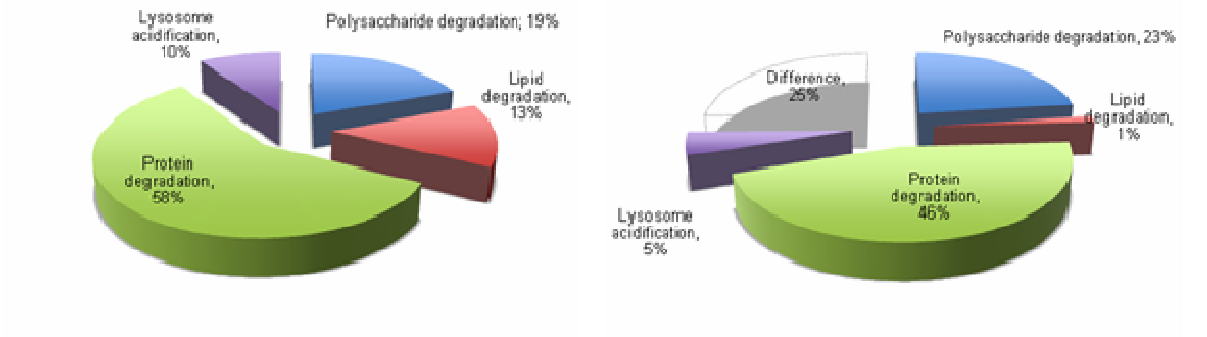


Figure 42: Functional comparison of liver (left) and HCC tissue (right). Percentage given for each functional group correspond to total peptide number of the respective group related to the total peptide number derived from lysosomal proteins of non-tumorous liver tissue.

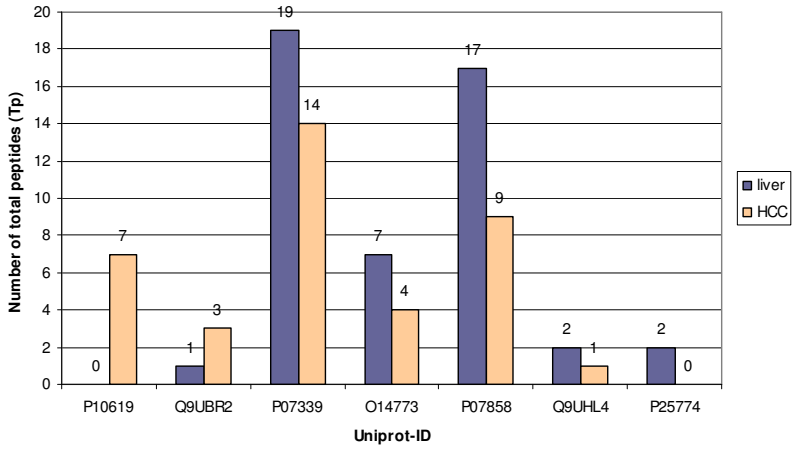
5.7.1 The lysosome as proteolytic organelle

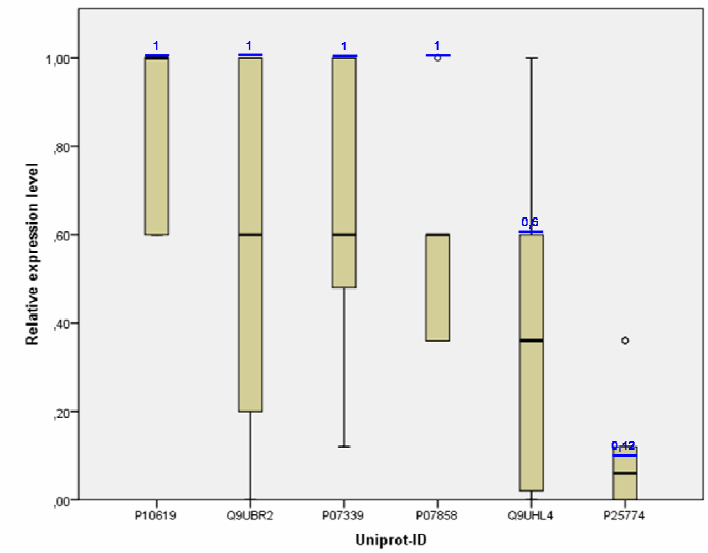
The abundance of lysosomal proteases and peptidases in non-tumorous liver and HCC tissue showed a pronounced diversification (Figure43, above). Cathepsins A (**P10619**) and Z (**Q9UBR2**) were solely detected or more abundant, respectively, in the tumor tissue. Cathepsin A is supposed to be essential for the activity and stability of β-galactosidase and neuraminidase. However, β-galactosidase (**P16278**) was significantly downregulated in HCC (see below). However, cathepsin A additionally

exerts carboxypeptidase-activity and can deamidate tachykinins. Tachykinin downregulation was implicated in gastric cancer and oesophageal cancer by hypermethylation of the TAC-1 promotor [274, 275]. Cathepsin Z, a matrix-degrading enzyme, was found to be overexpressed in aggressive melanoma [276].

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P10619	Cathepsin A	0	**0**	0	3	**7**	5	In HCC only
Q9UBR2	Cathepsin Z	1	**1**	4	2	**3**	7	3
P07339	Cathepsin D	9	**19**	24.8	11	**14**	39	0.7
O14773	Tripeptidyl-peptidase I	3	**7**	9.8	3	**4**	10	0.6
P07858	Cathepsin B	8	**17**	26.3	7	**9**	25	0.5
Q9UHL4	Dipeptidyl-peptidase 2	2	**2**	6.5	1	**1**	4	0.5
P25774	Cathepsin S	2	**2**	8.2	0	**0**	0	In liver only

Table 25: Lysosomal proteins participating in proteolysis identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.





Uniprot-ID	up	down
P10619	3	0
Q9UBR2	0	1
P07339	1	1
P07858	0	1
Q9UHL4	0	1
P25774	1	0

Figure 43: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of lysosomal proteins involved in proteolysis in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.7.2 The lysosome as polysaccharide and lipid degrading organelle

The abundance of polysaccharide- and lipid-degrading enzymes varied pronouncedly between liver and HCC tissue. Storage diseases associated with the malfunction of specific enzymes indicate their biological impact (table 26).

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)	Storage disease	Stored material	Hepato-megaly
O00754	Lysosomal alpha-mannosidase	0	**0**	0	1	**1**	1	In HCC only			
Q9HAT2	Sialate O-acetylesterase	0	**0**	0	1	**1**	3				
P07686	Beta-hexosaminidase beta chain	2	**2**	7.6	5	**8**	14	4.0			
P10253	Lysosomal alpha-glucosidase	5	**6**	6.6	6	**8**	10	1.3			
P16278	Beta-galactosidase	3	**6**	7.1	1	**1**	3	0.2	GM1-gangliosidosis	Ganglioside, keratan sulfate	+++
P08236	Beta-glucuronidase	2	**2**	5.1	0	**0**	0	In liver only	MPS VII	Dermatan sulfate	+++
Q13510	Acid ceramidase	6	**11**	25.3	1	**1**	7	0.1	Farber	Ceramid	+/-

Table 26: Lysosomal proteins participating in polysaccharide and lipid degradation identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted. For enzymes associated with lysosomal storage diseases, the stored materials and anatomical alterations are given.

The marked downregulation of β-galactosidase (**P16278**) and acid ceramidase (**Q13510**) is underlined by immunohistochemical data stating downregulation of beta-galactosidase and acid ceramidase by tendency. The inherited disturbance of acid ceramidase expression and activity manifests in Farber disease (syn. Farber lipogranulomatosis), a sphinoglipid disease characterized by subcutaneous lipid deposition, pain in joints and extremities and causes the early death of affected individuals. In addition, it is characterized by the marked accumulation of ceramide in lysosomes underlining the putative impact of ceramidase downregulation on histologically and analytically observable alterations of the lipid composition of HCC tumors and plasma. By the way, exogenous ceramide has been shown to exert a pro-apoptotic acitivity on a rat hepatoma cell line [277].

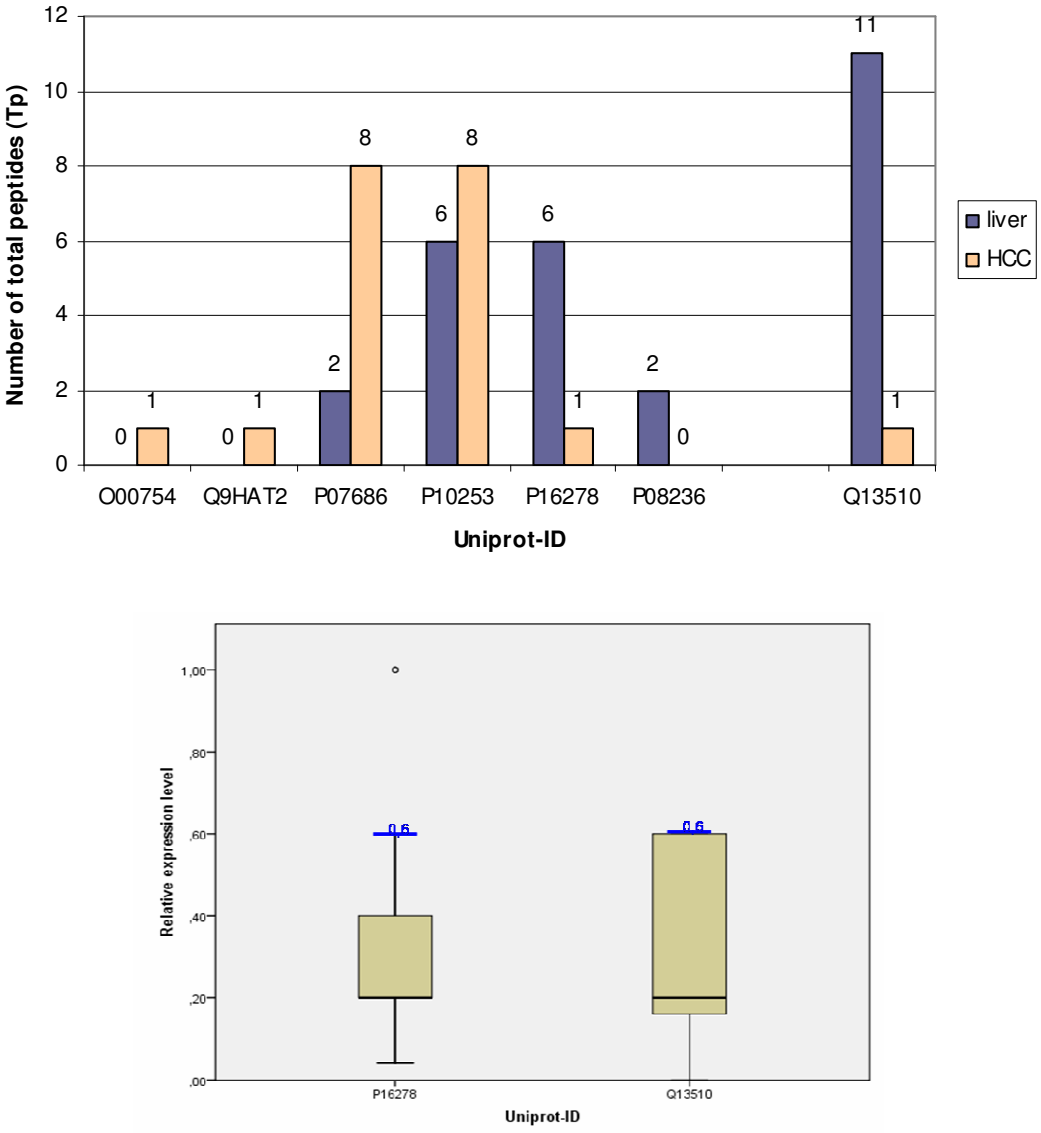


Figure 44: Shotgun data (above) and immunohistochemical data (below) concerning the relative abundance of lysosomal proteins involved in polysaccharide and lipid degradation in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.8 Proteins involved in detoxification of endobiotic and xenobiotic substances

Detoxifying enzymes are of special relevance by mediating resistance against anti-tumor drugs. Phase I reactions comprise hydrolysis, oxidation, and reduction of xenobiotics, phase II reactions include glucuronidation, sulfation, acetylation, methylation, glutathione conjugation, amino acid conjugation (glycine, taurine, glutamic acid). Apart from methylation and acetylation, hydrophilicity is increased and thence biliary or urinary excretion of xenobiotic or potentially harmful endogeneous substances is fostered. Phase II enzymes require cofactors (UDP-GA, PAPS, Acetyl-CoA, SAM, glutathione, taurine, glycine, glutamine) and are mainly located in the cytosol (except from UDP-glucuronosyltransferases).

5.8.1 Phase I- enzymes

Enzymes involved in hydrolysis, reduction and oxidation identified via mass spectrometry are listed in table 27 and table 28. Shotgun data, immunohistochemical and EHCO data are compiled in figure 46 and figure 48.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
O00748	Carboxylesterase 2 precursor	6	**7**	17.4	9	**24**	25.4	3.4
P23141	Liver carboxylesterase 1 precursor	32	**85**	58.9	27	**62**	50.1	0.7
P27169	Serum paraoxonase/arylesterase 1	2	**2**	4.2	5	**10**	20.8	5.0
P07099	Epoxide hydrolase 1	2	**3**	8.4	4	**9**	12.3	3.0
P34913	Epoxide hydrolase 2	7	**11**	18	2	**4**	7.4	0.4
P16152/O75828	Carbonyl reductase [NADPH] 1/3	10	**16**	45.8	16	**39**	60.3	2.4

Table 27: Phase I-enzymes identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

5.8.1.1 Hydrolysis

Hydrolysis of carboxylic acid esters, amides, thioesters as well as phosphoric acid esters and acid anhydrides are catalyzed by carboxylesterases, pseudocholinesterase, and paraoxonase. The first three reactions are mainly catalyzed by tissue-associated carboxylesterases and by two blood esterases (true acetylcholinesterase in the erythrocyte membrane, pseudocholinesterase in serum). Carboxylesterases are also involved in the conversion of xenobiotics to tumorigenic and toxic compounds (e.g. vinyl acetate) as well as in the activation of ester and amide prodrugs (e.g. lovastatin) and anti-tumor drugs (CPT-11). Two carboxylesterases were identified via shotgun, the liver carboxylesterase 1 (**P23141**), Carboxylesterase 2 (**O00748**), both located in the ER. Carboxylesterase 2 which is suggested to be highly expressed in small intestinum but just moderately in liver (Uniprot) was found to be significantly upregulated in HCC in contrast to liver tissue. Indeed,

carboxylesterase 2 expression was found to be high in normal liver tissue represented in “Human Protein Atlas” (corresponding to a REL of 1) but was consistently even higher in liver cancer tissue (exemplified in figure 45).

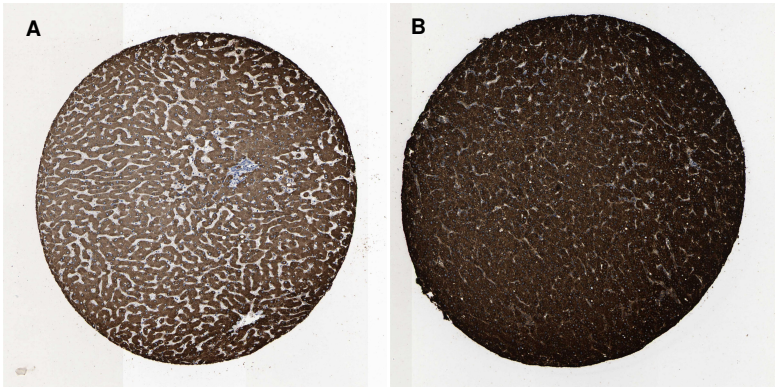


Figure 45: Immunohistochemical detection of carboxylesterase 2 (O00748) in liver (A) and liver cancer tissue (B) (derived from www.proteinatlas.org)

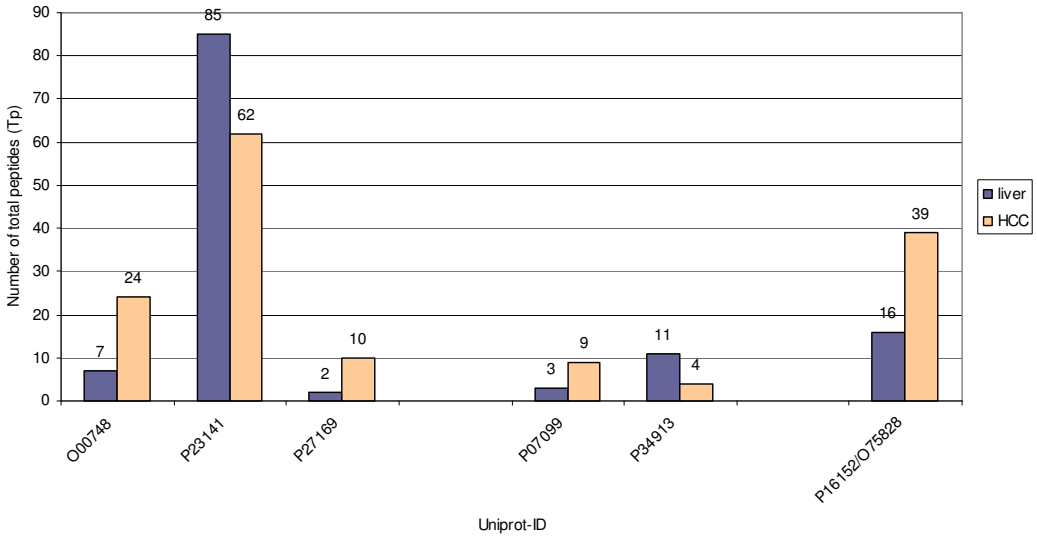
Liver carboxylesterase was slightly less abundant in HCC. Accordingly, histological data suggested slight downregulation of liver carboxylesterase expression levels by tendency. However, it has to be stated that normal liver as well as six (of overall 11 immunohistochemically investigated tissue samples) were assigned REL of 1. As it was described for carboxylesterase 2 (see above), no conclusions concerning the possibly even higher expression by liver cancer cells could be drawn from these data. Nevertheless, examination of the histological slices confirmed the abundance of liver carboxylesterase partially exceeding that of normal liver (data not shown; corresponding to figure 45). This result indicates a narrow-range inter-tumour variation of expression levels which might explain contradictory data delivered by EHCO.

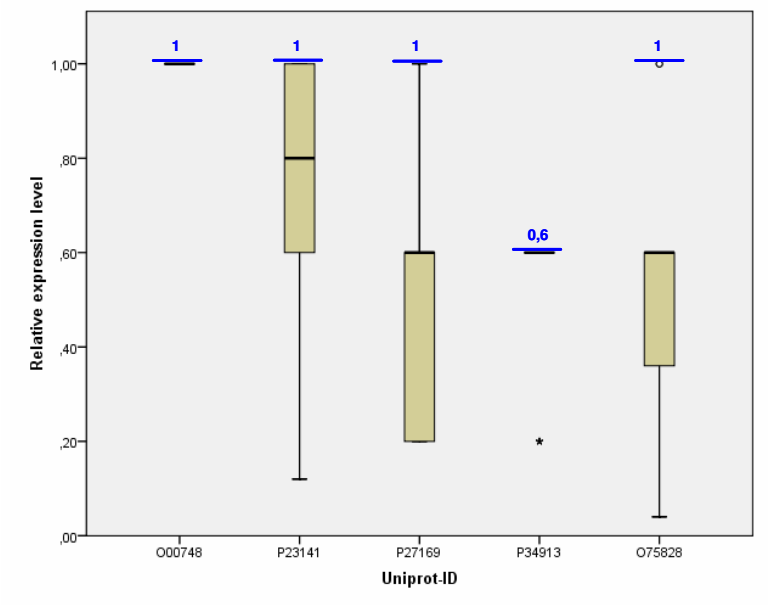
Serum paraoxonase (**P27169**), a liver-derived serum protein, was found to be pronouncedly more abundant in HCC tissue. However, EHCO as well as immunohistochemically-derived data suggest downregulation for this protein. This discrepancy might arise from the local enrichment of paraoxonase even at reduced expression levels (see 5.3 “The tumor secretome”).

Epoxide hydrolases catalyze water addition to epoxides and oxiranes resulting in trans-configurated vicinal diols. Among the five different forms of epoxide hydrolases (EH), the microsomal EH (mEH) (Epoxide hydrolase 1, **P07099**) and soluble EH (sEH) (Epoxide hydrolase 2, **P34913**) feature a broad substrate specificity and are involved in biotransformation of xenobiotics. In the liver, they colocalize with cytochrome P450 in the centrilobular region. Interestingly, mEH and sEH are not homologous and have different substrate specificities (e.g. oxirane almost exclusively by mEH). Microsomal EH is co-induced with cytochrome P450 (e.g. by phenobarbital, trans-stilbene oxide) and is a marker for pre-neoplastic alteration of induced foci and nodules. Epoxide hydrolase 1 and 2 were differentially expressed by being upregulated and downregulated, respectively, in HCC. The shotgun-derived expression data for sEH were in accordance to EHCO data. “Human Protein Atlas”, however, states unaltered expression of liver cancer compared to normal tissue.

5.8.1.2 Reduction

The reduction of aldehydes and ketones to the corresponding alcohols is catalyzed by alcohol dehydrogenases and carbonyl reductases. Xenobiotic substrates are e.g. acetohexamide, warfarin, and the anti-tumor drug daunorubicin, physiologic target is prostaglandin. Carbonyl reductases (are present in blood, liver, and other tissues. Carbonyl reductase(s) (carbonyl reductase 1/3, **P16152/O75828**)- the highly homologous isoforms could not be uniquely distinguished by mass spectrometry- was (were) pronouncedly more abundant in HCC tissue. However, immunohistochemical data suggest downregulation in liver cancer by tendeny. In addition, aldo-keto reductase family members were slightly but consistently more abandant in tumorous tissue (data not shown).





Uniprot-ID	up	down
O00748	0	1
P23141	1	2
P27169	0	5
P07099	0	1
P34913	0	4
P16152/O75828	No results	

Figure 46: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of hydrolytic and reducing phase I-enzymes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.8.1.3 Oxidation

The oxidation-reduction system for alcohols, aldehydes, and ketones comprises a variety of enzymes such as alcohol dehydrogenases, aldehyde dehydrogenases, carbonyl reductases, dihydrodiol dehydrogenase, aldehyde oxidase, and xanthine dehydrogenase/oxidase. For example, the metabolism of ethanol/methanol includes their conversion into the corresponding aldehydes by alcohol dehydrogenases followed by the aldehyde dehydrogenase-mediated oxidation to the respective carboxylic acids.

Alcohol dehydrogenases (ADHs) and aldehyde dehydrogenases (ALDHs)

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P07327/…	Alcohol dehydrogenase 1A/1B/1C	19	**106**	45.6	11	**75**	22.1	0.7
P08319	Alcohol dehydrogenase 4	25	**59**	78.2	15	**38**	55.5	0.6
P00352	Aldehyde dehydrogenase family 1 member A1, cytoplasmic	22	**56**	49.3	14	**40**	33.7	0.7
P05091	Aldehyde dehydrogenase, mitochondrial	29	**65**	60.2	30	**88**	59	1.4
P30837	Aldehyde dehydrogenase X, mitochondrial	14	**38**	39.1	12	**30**	29.8	0.8
P51648	Aldehyde dehydrogenase, microsomal	1	**1**	2.7	0	**0**	0	In liver only

Table 28: ADHs and ALDHs identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

The different isoforms of alcohol dehydrogenases are divided into four classes. Class I comprises the isozymes ADH1, ADH2, and ADH3 (**P07327**/..., 106/75), class II includes ADH4 (**P08319**, 59/38). Whereas class I ADHs catalyze the oxidation of ethanol, ADH4 preferentially uses larger aliphatic and aromatic alcohols as substrates. Alcohol dehydrogenases of class I and class II are expressed at high levels in liver. The slight to moderate downregulation of ADHs observed in shotgun was confirmed by immunohistochemical and EHCO data (Figure 46). Aldehyde dehydrogenases oxidize aldehydes to carboxylic acids using NAD as coenzyme and exhibit esterase activity. ALDH X (P30837) was found to be slightly less abundant in HCC which was in accordance with immunohistochemical data (exemplified in figure 47).

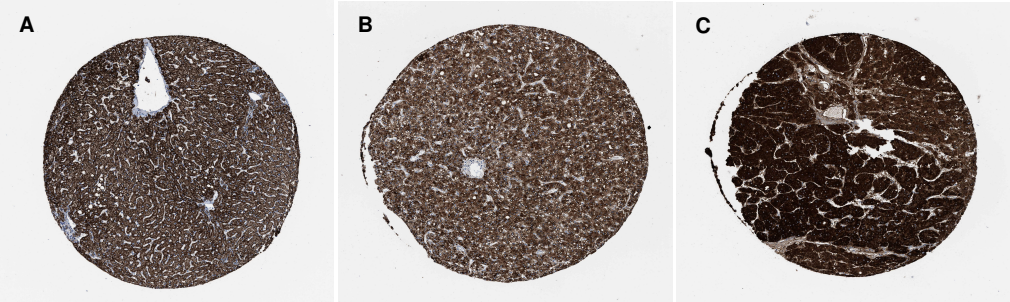
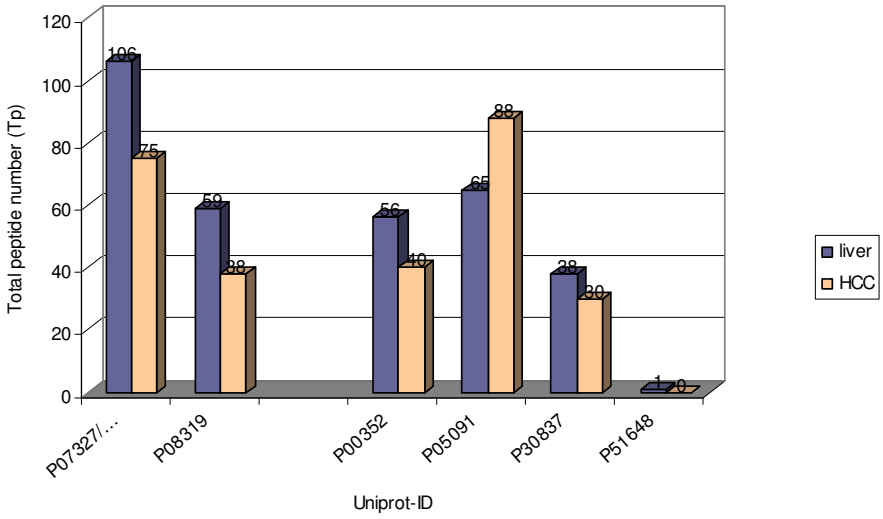
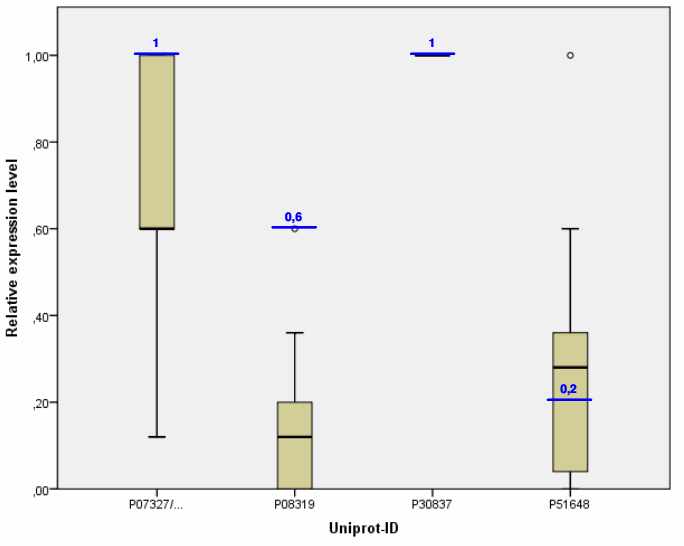


Figure 47: Immunohistochemical detection of ALDH X (P30837) in liver (A) and liver cancer (B and C, representing slightly different expression levels corresponding to REL of 1) (derived from www.proteinatlas.org)





Uniprot-ID	up	down
P07327/…	0	1
P08319	0	5
P00352	0	2
P05091	0	5
P30837	0	1
P51648	0	2

Figure 48: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of oxidative phase I-enzymes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

Molybdenum hydroxylases

Aldehyde oxidase (**Q06278**) and xanthine oxidase are molybdoenzymes which occur in significant amounts only in liver. They exhibit substrate specificity for aromatic aldehydes (e.g. benzaldehyde, tamoxifen aldehyde). Physiological substrates of these enzymes are homovanillyl aldehyde (from dopamine), 5-hydroxy-3-indolacetaldehyde (from serotonin), and retinal delivering retinoic acid, a regulator of cell proliferation and differentiation. Moreover, they are decisively implicated in the catabolism of biogenic amines and catecholamines and metabolize anti-cancer drugs such as O6-benzylguanine and methotrexate. In general, they exhibit substrate specificity complementary to cytochrome P450 enzymes in contrast to flavin monooxygenases (see below). Aldehyde oxidase (**Q06278**) was exclusively detected in liver tissue which is in accordance to EHCO data (not represented in "Human Protein Atlas").

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
Q06278	Aldehyde oxidase	2	**3**	2.2	0	**0**	0	In HCC only
P27338	Monoamine oxidase type B (MAO-B)	5	**7**	13.7	0	**0**	0	In HCC only
P31513	Hepatic flavin-containing monooxygenase 3	7	**11**	21.4	0	**0**	0	
P49326	Hepatic flavin-containing monooxygenase 5 (FMO 5)	1	**2**	2.8	0	**0**	0	In HCC only
P05177	Cytochrome P450 1A2	1	**2**	3.7	0	**0**	0	In HCC only
P11509/P20853/ Q16696	Cytochrome P450 2A6/2A7/2A13	1	**2**	1.8	1	**2**	4.5	1.0
P11712	Cytochrome P450 2C9	5	**9**	13.9	0	**0**	0	
Q02928	Cytochrome P450 4A11	3	**4**	9.6	0	**0**	0	In HCC only
P16435	NADPH--cytochrome P450 reductase	11	**15**	21.1	8	**13**	20.8	0.9
P22570	NADPH:adrenodoxin oxidoreductase, mitochondrial	5	**6**	20.8	3	**5**	14.5	0.8
P10109	Ferredoxin-1 (Hepatoredoxin)	2	**4**	14.1	1	**1**	4.9	0.3

Table 29: Aldehyde oxidase, monoamine oxidases, and cytochrome P450 monooxygenases identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

Monoamine oxidases (MAOs) and flavin monooxygenases (FMOs)

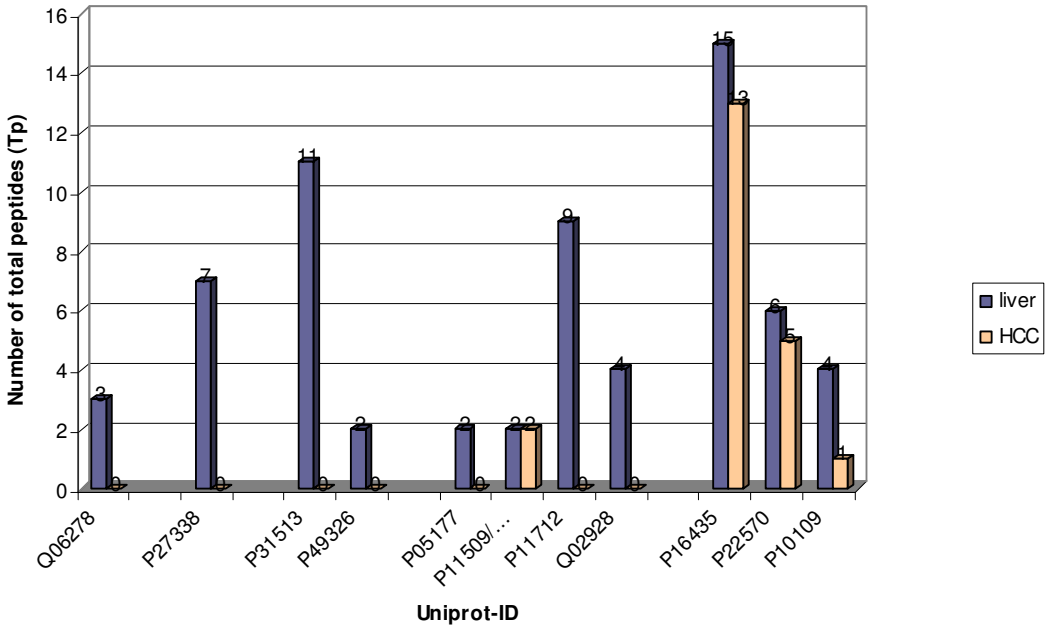
Monoamine oxidases (isozymes MAO-A and –B) are members of the flavin-containing amine oxidoreductases. (MAO-B, **P27338**) catalyzing the oxidative deamination of biogenic and xenobiotic amines (Wikipedia) such as serotonin (MAO-A) and dopamin (MAO-B). Although MAOs are implicated in neurological disorders, they are also expressed in others than nervous tissues as well. MAO-B is indeed highly expressed in liver tissue (REL 1) and is tendentially downregulated in liver cancer as indicated by immunohistochemical data (Figure 49). Indeed, MAO-B was solely detected in non-tumorous liver tissue by shotgun.

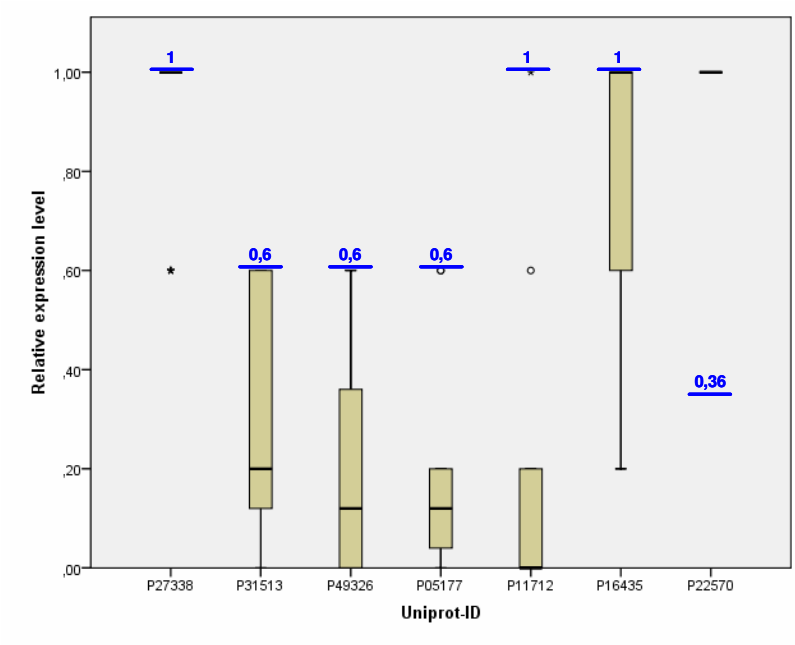
Flavin monooxygenases oxidize nitrogen, sulphur, and phosphorus of a variety of xenobiotics. They are located in microsomes and require NADPH and molecular oxygen. Many FMO reactions are also catalyzed by cytochrome P450. Two hepatic flavin-monooxygenases (**P31513**; **P49326**) were

identified by shotgun, both exclusively detected in liver tissue. Downregulation of those enzymes is in accordance to histological data indicating a loss of FMO-mediated function by liver cancer.

Cytochrome P450 (Cyt P450)

The cytochrome P450 superfamily comprises the most important phase I-biotransforming enzymes with respect to pharmako- and toxicokinetics. They catalyze the monooxygenation of their substrates delivering highly reactive epoxides as targets for phase II-enzymes. Although ubiquitously expressed, they are most prominent in liver microsomes. Microsomal and mitochondrial cytochrome P450 enzymes gain electrons from NADPH-cytochrome P450 reductase (**P16435**) and ferredoxin (**P10109**, 4/1 (**P16435**, 15/13)/ferredoxin reductase (**P22570**, 6/5), respectively. They exert key roles as electron carriers in the metabolism of e.g. steroid hormones, bile acids, fat-soluble vitamins, fatty acids, and eicosanoids. Cytochrome P450 proteins were almost consistently downregulated in HCC (detected only in liver except for cytochrome P450 2A enzyme(s)). Interestingly, the downregulation of ferredoxin (but not the reductase) indicates compromized reduction capacity of mitochondrial cytochromes (not necessarily or just partially involved in detoxification).





Uniprot-ID	up	down
Q06278	0	2
P27338	0	1
P31513	0	1
P49326	No results	
P05177	0	1
P11509/ P20853/ Q16696	0	3
P11712	0	7
Q02928	0	2
P16435	0	3
P22570	1	1
P10109	0	1

Figure 49: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of oxidative phase I-enzymes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.8.2 Phase II- enzymes

Enzymes involved in glucuronidation, sulfation, and methylation identified via mass spectrometry are listed in table 30. Shotgun data, immunohistochemical and EHCO data are compiled in figure 50.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P22309/P36509/ P22310/Q9HAW7	UDP-glucuronosyltransferase 1-1/1-2/1-4/1-7	5	**54**	17.8	0	**0**	0	In liver only
P54855/O75795	UDP-glucuronosyltransferase 2B15/2B17	1	**2**	1.7	0	**0**	0	
Q06520	Bile-salt sulfotransferase	7	**13**	29.8	9	**12**	42.8	0.9
P50225/P50226/ P50224	Sulfotransferase 1A1/1A2/1A3	5	**11**	27.1	1	**3**	6.1	0.3
P25325	3-mercaptopyruvate sulfurtransferase (MST)	7	**13**	39.1	8	**14**	42.1	1.1
P21964	Catechol O-methyltransferase	5	**7**	19.9	0	**0**	0	In liver only
P40261	Nicotinamide N-methyltransferase	2	**2**	13.6	0	**0**	0	
Q00266	S-adenosylmethionine synthetase isoform type-1 (MAT-I/III)	3	**3**	9.9	0	**0**	0	

Table 30: Phase II-enzymes identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

5.8.2.1 Glucuronidation

Glucuronidation constitutes the major pathway in xenobiotic biotransformation. It depends on the cofactor UDP-glucuronic acid which is derived from glucose-1-phosphate and is catalyzed by UDP-glucuronosyltransferases (UGTs) in the endoplasmic reticulum of liver and other tissues. The substrates of UGTs contain O, N, or S heteroatoms (e.g. aliphatic alcohols, phenols, carboxylic acids, primary and secondary aromatic aliphatic amines). Endogenous substrates comprise bilirubin, steroid hormones, and thyroid hormones. Glucuronide conjugates are polar, water-soluble and excreted via urine or bile depending on the size of the aglycon (<250 Da vs. >350 Da). However, glucuronic acid is the substrate for beta-glucuronidase (lysosomes, intestinal microflora) which reverts glucuronidation and hence fosters enterohepatic circulation of xenobiotics (delayed elimination). Moreover, co-factor availability limits the glucuronidation rate of drugs and xenobiotic glucuronidation can be impaired *in vivo* by factors depleting UDP-glucoronic acid levels (e.g. diethyl-ether, borneol, and galactosamin). UGTs as functional group (identified at the family level with **P22309/…, P54855/…**) were detected solely in liver tissue by shotgun.

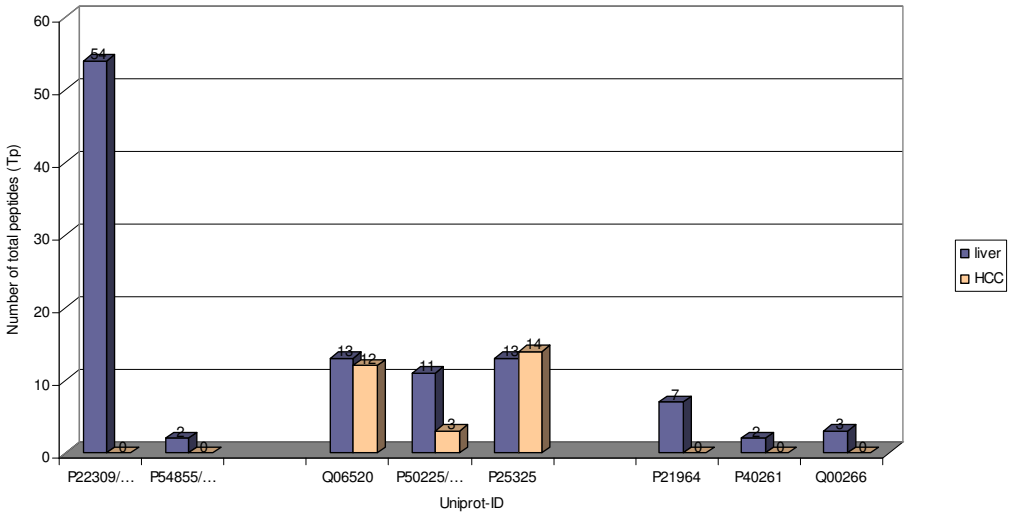
5.8.2.2 Sulfation

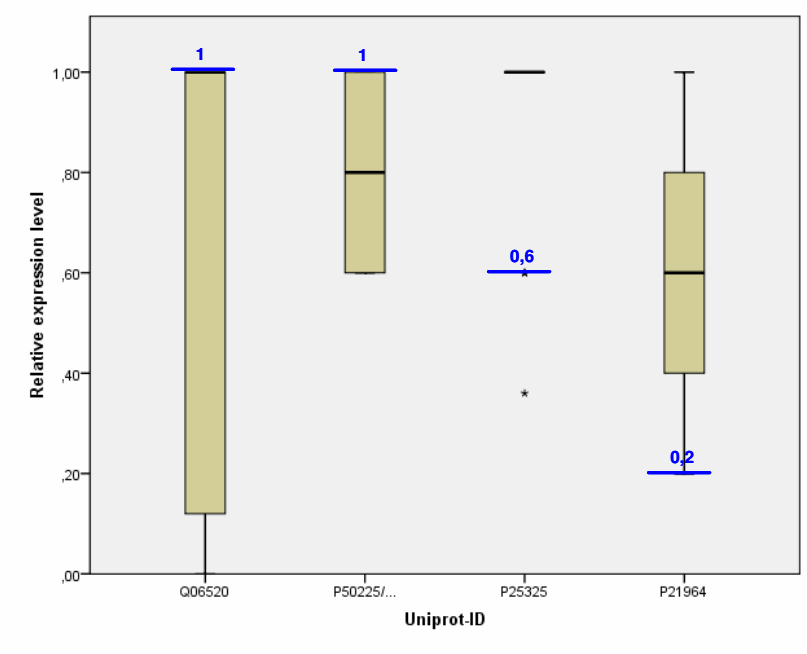
Many xenobiotics and endogenous substrates can be alternatively glucuronidated or sulfated. Cytosolic sulfur transferases in liver and other tissues catalyze the formation of highly water-soluble sulfuric acid esters. Sulfur transferases require the co-factor PAPS (3'-phosphoadenosine-5'-

phosphosulfate) for the transfer of sulfonat to xenobiotics, phenols, aliphatic alcohols, and some aromatic amins (but not carboxylic acids). Sulfated substances are excreted mainly in urine, those released into bile are frequently hydrolyzed by microfloral amylsulfatases retarding their elimination (see glucuronidation). Due to the commonly low concentrations of PAPS, sulfation is regarded as a low-capacity pathway in the elimination of toxic compounds. Three sulfotransferases were identified by shotgun. Bile-salt sulfotransferase (**Q06520**) and 3-mercaptopyruvate sulfurtransferase (**P25325**) catalyzing the sulfation of steroids, bile acids and the degradation of cyanide compounds or being involved in thiosulfate biosynthesis compounds, respectively, were equally abundant in liver and HCC. However, sulfotransferase 1A members (identified at the family level with **P50225/…**) were pronouncedly downregulated in HCC. E.g. sulfotransferase 1A1 catalyzes the sulfate conjugation of catecholamines and phenolic drugs. In addition, it mediates the sulfonation of 2-methoxyestradiol, a chemotherapeutic for e.g. the treatment of malignant glioma [278], esophageal adenocarcinoma [279], and breast cancer [280]. The downregulation of sulfotransferase 1A might indicate the modulation of the reactivity to chemotherapeutics in liver cancer cells.

5.8.2.3 Methylation

Methylation is a common but generally minor pathway in detoxification although it is indispensable in amino acid and cofactor biosynthesis. The attachment of methyl-groups decreases watersolubility of compounds and requires the co-factor SAM (S-adenosylmethionine) which is formed by S-adenosylmethionine synthetases from methionine, ATP and water. Phenols, catechols, aromatic and aliphatic amins, N-heterocyclics, and SH-containings compounds are the main substrates for methyltransferases such as phenol-O-methyltransferase and catechol-O- methlytransferase (**P21964**). The latter as well as nicotinamide N-methyltransferase (**P40261**) which is crucial for the biotransformation of drugs and xenobiotics by catalyzing the formation of pyridinium ions from pyridines (Uniprot) were solely detected in liver. Accordingly, S-adenosylmethionine synthetase (**Q00266**) which mediates the formation of SAM was also not detected in HCC. Taken together, these findings indicate a pronouncedly compromized methylation capacity of HCC cells at least with respect to detoxification.





Uniprot-ID	up	down
P22309/…	0	0
P54855/…	No results	

Q06520	0	2
P50225/…	0	2
P25325	No results	

P21964	1	1
P40261	1	6
Q00266	0	4

Figure 50: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of phase II-enzymes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

5.9 Proteins mediating cancer phenotypes

Proteins described in detail are listed in table 31 and table 32. Shotgun, immunohistochemically derived and EHCO data are compiled in figure 51.

5.9.1 Resistance against apoptosis

Sorcin (**P30626**), a calcium-binding protein, was found to be overexpressed in multi-drug-resistant cell lines, in gastric cancer tissue, and in gemcitabine resistant non-small cell lung cancer [281-283]. Indeed, its expression correlates with the expression of P-glycoprotein in leukemia patients [281]. In addition, overexpression of sorcin resulted in decreased intracellular Ca-levels and increased resistance to apoptosis correlating with the upregulation of Bcl-2, downregulation of Bax and by interfering with caspase-3 expression and activity [284, 285]. Sorcin was solely detected in HCC tissue. Unfortunately, sorcin was neither represented in “protein atlas” nor in EHCO, hence, data concerning its expression in HCC were not obtainable and were not reported thus far.

Cardiotrophin-1 (CT-1, **Q16619**) belongs to the IL-6 family of cytokines. It exerts anti-apoptotic effects on hepatocytes [286], cardiomyocytes and neurons by inducing the Jak-Stat pathway as well as other survival pathways [287, 288]. The Jak-Stat pathway mediates protection of hepatocytes against Fas-induced apoptosis, oxidative stress in the course of hypoxia/reoxigenation, and T-cell mediated liver injury [289-291]. Thereof this pathway is considered a basic and crucial component of the natural defense of liver tissue against apoptosis [292]. In addition, the Jak/Stat pathway as well as the Ras pathway are ubiquitously activated in HCC and are considered as promising point of attack for therapy [293]. The importance of the Jak/Stat pathway in development of HCC with respect to apoptosis inhibition and proliferation enhancement and thereof poor prognosis is indicated by Yoshikawa et al. and Schmitz et al. [294, 295] . However, expression of cardiotrophin-1 at the mRNA level is not significantly different between liver and HCC (data not shown, see Oncomine: CT-1). Taken together, the known activation of the Jak-Stat pathway and the exclusive detection of cardiotrophin-1 in HCC without a significant increase of the mRNA expression level might be an indication for the local enrichment of factors as determinants of the tumor microenvironment.

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
P30626	Sorcin (22 kDa protein)	0	**0**	0	3	**6**	17.2	In HCC only
Q16619	Cardiotrophin-1 (CT-1)	0	**0**	0	1	**4**	10.4	

Table 31: Proteins participating in the tumor phenotype “Resistance against apoptosis” identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

5.9.2 Unrestricted proliferation

NDRG2 (**Q9UN36**), a member of the N-Myc downstream-regulated gene family, was found to be downregulated in colorectal cancer tissue and to behave reversely to the expression of c-Myc. In addition, the decline of NDRG2 abundance correlated with the differentiation state of investigated samples [296]. Similar results were obtained for esophageal squamous-cell carcinoma [297] and oral squamous-cell carcinoma [298]. Constitutive expression of NDRG2 suppressed cell proliferation in a colon carcinoma cell line [299]. In addition, it exerts tumor metastasis-suppressive activity [300]. NDRG2 abundance was significantly decreased in HCC tissue compared to non-tumorous liver tissue which is in accordance to immunohistochemical data (Protein Atlas), EHCO, and data from literature [301].

Uniprot-ID	Protein name	Dp	Tp	Cov. (%)	Dp	Tp	Cov. (%)	Ratio of Tp (HCC/liver)
Q9UN36	Protein NDRG2	5	**9**	22.1	1	**1**	5.9	0.1
Q92945	Far upstream element-binding protein 2	0	**0**	0	2	**3**	4.1	In HCC only
O43598	c-Myc-responsive protein Rcl	3	**3**	16.7	4	**6**	25.3	2.0
P16949	Stathmin	1	**2**	8.7	3	**6**	21.5	3.0
Q86X76	Nitrilase homolog 1	0	**0**	0	3	**3**	14.4	In HCC only
P49789	Bis(5'-adenosyl)-triphosphatase	0	**0**	0	1	**1**	6.8	
P01112/ P01111/ P01116	GTPase H/N/KRas	0	**0**	0	2	**7**	13.8	In HCC only
Q15185	Prostaglandin E synthase 3	1	**1**	10.6	3	**5**	26.3	5.0
Q9H7Z7	Prostaglandin E synthase 2	2	**3**	8.8	6	**6**	24.7	2.0
Q04917	14-3-3 protein eta (Protein AS1)	0	**0**	0	3	**12**	12.2	In HCC only
P27348	14-3-3 protein theta	2	**2**	9.8	4	**15**	20	7.5
P61981	14-3-3 protein gamma (Protein kinase C inhibitor protein 1)	2	**3**	15.4	10	**20**	53	6.7
P31946	14-3-3 protein beta/alpha	3	**4**	20.7	9	**23**	50.8	5.8
P62258	14-3-3 protein epsilon (14-3-3E)	2	**3**	4.7	5	**12**	27.1	4.0
P63104	14-3-3 protein zeta/delta (KCIP-1)	5	**5**	31	8	**17**	42.9	3.4
Q9P032	HRPAP20	0	**0**	0	4	**8**	28.6	In HCC only
Q14696	Mesoderm development candidate 2	0	**0**	0	5	**5**	22.2	In HCC only
P55327	Tumor protein D52	1	**1**	7.6	2	**3**	17.9	3.0
P51858	Hepatoma-derived growth factor (HDGF)	0	**0**	0	1	**1**	6.7	In HCC only

Table 32: Proteins participating in the tumor phenotype "Unrestricted proliferation" identified by mass spectrometry. The number of distinct peptides (Dp), total peptides (Tp), and coverage (Cov. (%)) of proteins of non-tumorous liver (blue) and/or HCC tissue (orange) as well as the ratio of Tp (HCC/liver) are quoted.

The expression of c-Myc might be indirectly concluded from the sole detection and increased abundance of Far upstream element-binding protein 2 (FUSE, FBP2; **Q92945**), a transcriptional activator of c-Myc [302], and c-Myc-responsive protein Rcl (**O43598**) exhibiting tumorgenic potential [303], respectively. In addition, FBPs promote tumor-relevant functions via stathmin (**P16949**), a microtubule-destabilizing protein, and are considered as targets for HCC treatment [304]. The significant coregulation of stathmin was confirmed by shotgun, immunohistochemical and EHCO data.

Nitrilase 1 homolog (**Q86X76**) was found to be downregulated in esophageal adenomacarcinonma. In addition, it acts additively with Bis(5'-adenosyl)-triphosphatase (**P49789**) which shows also tumor-suppressive activity [305]. Interestingly, both proteins were solely detected in HCC.

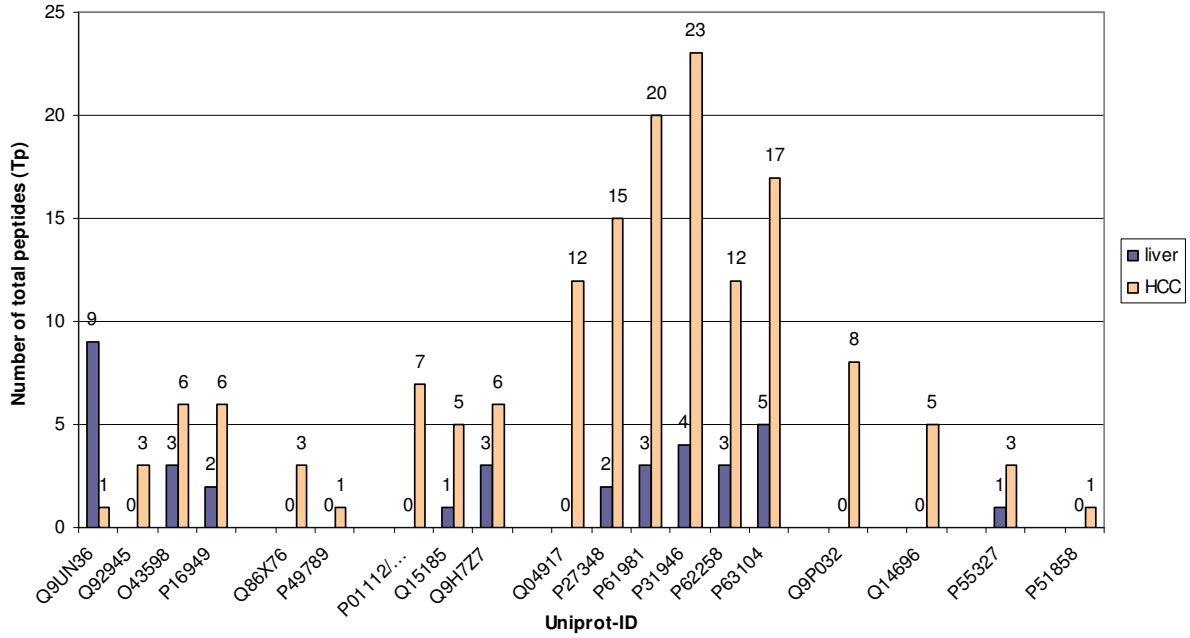
At least one isoform of GTPase Ras proteins (**P01112**/P01111/P0111)- the different highly homologous isoforms H-, N-, and K-Ras were not distinguishable by shotgun- was significantly more abundant in HCC tissue. The aberrant activation of Ras/MAPK-signaling, in particular involving K-Ras, in hepatocarcinogenesis is commonly accepted [175]. Interestingly, mutations of Ras occur at low frequency in HCC [306] in contrast to its overexpression [293, 307]. Another mechanism leading to the augmentation of proliferation is the increased synthesis of prostaglandin E synthase 2 (PGE2) since it has been shown that prostaglandins are able to enhance the effect of the growth factor EGF (epidermal growth factor) on hepatocytes. Again, upregulation of Akt and Ras pathways are involved [308]. Indeed, in HCC tissue prostaglandin E synthases 3 (**Q15185**) and 2 (**Q9H7Z7**) were upregulated 5-fold and 2-fold, respectively.

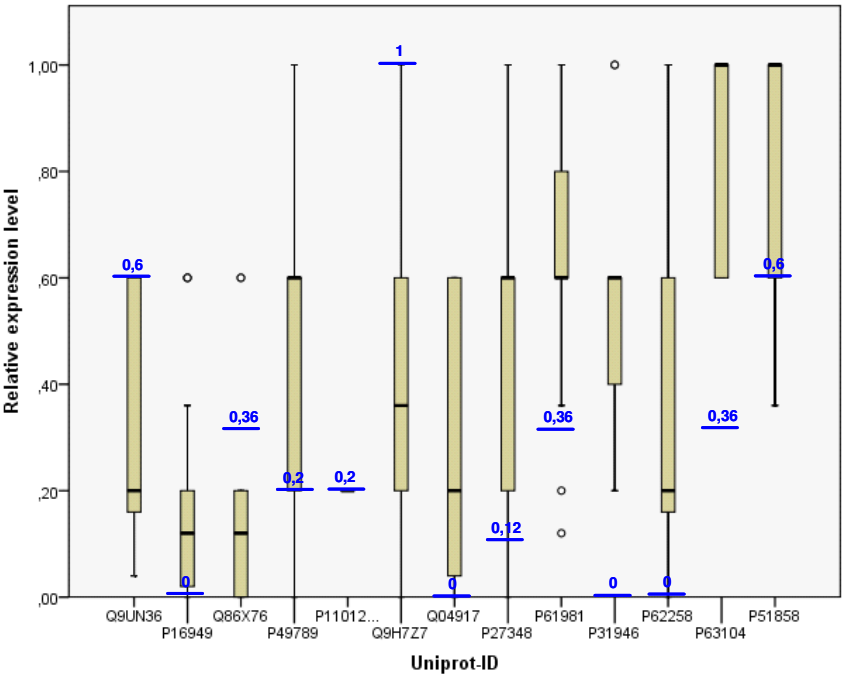
14-3-3 proteins (**Q04917**, **P27348**, **P61981**, **P31946**, **P62258**, **P63104**) are involved in a broad variety of cellular processes such as signal transduction, apoptosis, and cell-cycle regulation (reviewed in [309]). They are ubiquitous phospho-serine/threonine-binding proteins frequently dysregulated in cancer. Indeed, the expression of 14-3-3 proteins was found consistently and significantly upregulated in HCC. However, 14-3-3σ whose expression is regulated by p53 and is frequently downregulated in cancer was detected in neither of the tissues.

Former UPF0240 C6orf66, now designated as hormone-regulated proliferation-associated 20 kDa protein (**Q9P032**; HRPAP20), was detected only in HCC. HRPAP20 has been identified as phosphoprotein enhancing growth and survival of hormone-responsive tumor cells [310]. Furthermore, it is described as marker for tamoxifen resistance in breast cancer and as being associated with breast cancer cell invasion [311, 312]. Unfortunately, this protein was not covered by EHCO and “Human Protein Atlas”.

Mesoderm development candidate 2 (Mesd, NY-REN-61 antigen; **Q14696**) was detected solely in HCC tissue. Mesd is a chaperone for members of the LDLR-family and for co-receptors of canonical Wnt-signaling, LRP 5 and LRP6 [313, 314]. Mesd has recently been identified as potent inhibitor of Wnt-signaling in a prostate cancer cell line [315]. However, it has been shown that it is also indispensable for the modulation of Wnt-signaling via LRP6 since overexpression of the latter

enhanced Wnt-signaling only in the presence of co-expressed Msd [316]. This finding might provide an indication for participation of Wnt-signaling on hepatocarcinogenesis although LRP6 was not found in either tissue but protein LRP16 (Q9BQ69) which was upregulated in HCC. LRP16 is possibly involved in breast cancer suggested by its upregulation in a breast cancer cell line upon estrogen treatment [317]).





Uniprot-ID	up	down
Q9UN36	0	3
P16949	4	0
Q86X76	0	1
P49789	1	0
P01112/…	3	0
Q15185	1	0
Q9H7Z7	2	0
Q04917	2	0
P27348	No results	
P61981	No results	
P31946	1	0
P62258	No results	
P63104	2	0
P51858	4	0

Figure 51: Shotgun data (above), immunohistochemical (below left), and EHCO data (below right) concerning the relative abundance of proteins mediating cancer phenotypes in non-tumorous liver and liver cancer/HCC tissues. The blue lines and numbers depicted in the boxplot denote the expression level of the respective proteins in normal liver tissue.

Tumor protein D52 (**P55327**) is overexpressed in prostate and breast cancer as well as various other cancers and exerts a proliferation-stimulatory effect, serves as an anti-apoptotic and/or survival factor and is additionally capable to promote migration of tumor cells [318, 319]. It is significantly more abundant in HCC (3/2 with coverage of 17.9% vs. 1/1) which is in accordance to EHCO data. To my knowledge it is the first report concerning the overexpression of TPD52 in HCC and thereby indicating a putative role of this protein in hepatocarcinogenesis. Moreover this protein might be a useful target for anti-cancer treatment since shutting down expression leads to increased apoptosis as demonstrated in a TPD25-overexpressing prostate cancer cell line [318].

Hepatoma-derived growth factor (**P51858**) is frequently found upregulated in HCC [320] which is in accordance to data gained via shotgun. Furthermore, its expression correlates with poor prognosis for HCC patients [321] and seems to be the decisive factor downregulated by vitamin-K(2) which has been reported to suppress the growth of human hepatocellular carcinoma (HCC*) in vitro* and hepatocarcinogenesis in hepatitis C virus (HCV)-related cirrhosis *in vivo* [322].

6 References

1. Zwickl, H., et al., *A novel technique to specifically analyze the secretome of cells and tissues.* Electrophoresis, 2005. **26**(14): p. 2779-85.
2. Wilkins, M.R., et al., *From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis.* Biotechnology (N Y), 1996. **14**(1): p. 61-5.
3. Venter, J.C., et al., *The sequence of the human genome.* Science, 2001. **291**(5507): p. 1304-51.
4. Goodstadt, L. and C.P. Ponting, *Phylogenetic reconstruction of orthology, paralogy, and conserved synteny for dog and human.* PLoS Comput Biol, 2006. **2**(9): p. e133.
5. Pennisi, E., *Genetics. Working the (gene count) numbers: finally, a firm answer?* Science, 2007. **316**(5828): p. 1113.
6. Hillier, L.W., et al., *Genomics in C. elegans: so many genes, such a little worm.* Genome Res, 2005. **15**(12): p. 1651-60.
7. Stumpf, M.P., et al., *Estimating the size of the human interactome.* Proc Natl Acad Sci U S A, 2008. **105**(19): p. 6959-64.
8. Whisstock, J.C. and A.M. Lesk, *Prediction of protein function from protein sequence and structure.* Q Rev Biophys, 2003. **36**(3): p. 307-40.
9. Galperin, M.Y., *The Molecular Biology Database Collection: 2004 update.* Nucleic Acids Res, 2004. **32**(Database issue): p. D3-22.
10. Suzuki, M.M. and A. Bird, *DNA methylation landscapes: provocative insights from epigenomics.* Nat Rev Genet, 2008. **9**(6): p. 465-76.
11. Wang, Y., et al., *Linking covalent histone modifications to epigenetics: the rigidity and plasticity of the marks.* Cold Spring Harb Symp Quant Biol, 2004. **69**: p. 161-9.
12. Ho, L. and G.R. Crabtree, *Chromatin remodelling during development.* Nature. **463**(7280): p. 474-84.
13. Cowling, V.H., *Enhanced mRNA cap methylation increases cyclin D1 expression and promotes cell transformation.* Oncogene. **29**(6): p. 930-6.
14. Chen, M. and J.L. Manley, *Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches.* Nat Rev Mol Cell Biol, 2009. **10**(11): p. 741-54.
15. Wahl, M.C., C.L. Will, and R. Luhrmann, *The spliceosome: design principles of a dynamic RNP machine.* Cell, 2009. **136**(4): p. 701-18.
16. Kohler, A. and E. Hurt, *Exporting RNA from the nucleus to the cytoplasm.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 761-73.
17. Goldstrohm, A.C. and M. Wickens, *Multifunctional deadenylase complexes diversify mRNA control.* Nat Rev Mol Cell Biol, 2008. **9**(4): p. 337-44.
18. Fabian, M.R., et al., *Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation.* Mol Cell, 2009. **35**(6): p. 868-80.
19. Houseley, J., J. LaCava, and D. Tollervey, *RNA-quality control by the exosome.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 529-39.
20. Garneau, N.L., J. Wilusz, and C.J. Wilusz, *The highways and byways of mRNA decay.* Nat Rev Mol Cell Biol, 2007. **8**(2): p. 113-26.
21. Jackson, R.J., C.U. Hellen, and T.V. Pestova, *The mechanism of eukaryotic translation initiation and principles of its regulation.* Nat Rev Mol Cell Biol. **11**(2): p. 113-27.
22. Besse, F. and A. Ephrussi, *Translational control of localized mRNAs: restricting protein synthesis in space and time.* Nat Rev Mol Cell Biol, 2008. **9**(12): p. 971-80.
23. Goldberg, A.L., *Protein degradation and protection against misfolded or damaged proteins.* Nature, 2003. **426**(6968): p. 895-9.
24. Welchman, R.L., C. Gordon, and R.J. Mayer, *Ubiquitin and ubiquitin-like proteins as multifunctional signals.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 599-609.
25. Murray-Zmijewski, F., E.A. Slee, and X. Lu, *A complex barcode underlies the heterogeneous response of p53 to stress.* Nat Rev Mol Cell Biol, 2008. **9**(9): p. 702-12.
26. Takada, S., et al., *Cytoplasmic retention of the p53 tumor suppressor gene product is observed in the hepatitis B virus X gene-transfected cells.* Oncogene, 1997. **15**(16): p. 1895-901.
27. Zantema, A., et al., *Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies.* Virology, 1985. **142**(1): p. 44-58.
28. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-25.

29. Ow, Y.P., et al., *Cytochrome c: functions beyond respiration.* Nat Rev Mol Cell Biol, 2008. **9**(7): p. 532-42.
30. Colell, A., et al., *GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation.* Cell, 2007. **129**(5): p. 983-97.
31. Rondinelli, R.H., D.E. Epner, and J.V. Tricoli, *Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in late pathological stage human prostate cancer.* Prostate Cancer Prostatic Dis, 1997. **1**(2): p. 66-72.
32. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
33. Schek, N., B.L. Hall, and O.J. Finn, *Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human pancreatic adenocarcinoma.* Cancer Res, 1988. **48**(22): p. 6354-9.
34. Tamm, I., et al., *Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias.* Clin Cancer Res, 2000. **6**(5): p. 1796-803.
35. Cardoso, F., et al., *Evaluation of HER2, p53, bcl-2, topoisomerase II-alpha, heat shock proteins 27 and 70 in primary breast cancer and metastatic ipsilateral axillary lymph nodes.* Ann Oncol, 2001. **12**(5): p. 615-20.
36. Slany, A., et al., *Cell characterization by proteome profiling applied to primary hepatocytes and hepatocyte cell lines Hep-G2 and Hep-3B.* J Proteome Res. **9**(1): p. 6-21.
37. Gundacker, N.C., et al., *Cytoplasmic proteome and secretome profiles of differently stimulated human dendritic cells.* J Proteome Res, 2009. **8**(6): p. 2799-811.
38. Sagmeister, S., et al., *New cellular tools reveal complex epithelial-mesenchymal interactions in hepatocarcinogenesis.* Br J Cancer, 2008. **99**(1): p. 151-9.
39. Wimmer, H., et al., *Introducing the CPL/MUW proteome database: interpretation of human liver and liver cancer proteome profiles by referring to isolated primary cells.* Electrophoresis, 2009. **30**(12): p. 2076-89.
40. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.
41. Hsu, C.N., et al., *Detection of the inferred interaction network in hepatocellular carcinoma from EHCO (Encyclopedia of Hepatocellular Carcinoma genes Online).* BMC Bioinformatics, 2007. **8**: p. 66.
42. Leong, T.Y. and A.S. Leong, *Epidemiology and carcinogenesis of hepatocellular carcinoma.* HPB (Oxford), 2005. **7**(1): p. 5-15.
43. Llovet, J.M., A. Burroughs, and J. Bruix, *Hepatocellular carcinoma.* Lancet, 2003. **362**(9399): p. 1907-17.
44. Ramadori, G. and B. Saile, *Inflammation, damage repair, immune cells, and liver fibrosis: specific or nonspecific, this is the question.* Gastroenterology, 2004. **127**(3): p. 997-1000.
45. Bataller, R. and D.A. Brenner, *Liver fibrosis.* J Clin Invest, 2005. **115**(2): p. 209-18.
46. Drucker, C., et al., *Non-parenchymal liver cells support the growth advantage in the first stages of hepatocarcinogenesis.* Carcinogenesis, 2006. **27**(1): p. 152-61.
47. Nagasue, N., et al., *Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma.* Gastroenterology, 1993. **105**(2): p. 488-94.
48. Yamamoto, J., et al., *Recurrence of hepatocellular carcinoma after surgery.* Br J Surg, 1996. **83**(9): p. 1219-22.
49. Poon, R.T., et al., *Intrahepatic recurrence after curative resection of hepatocellular carcinoma: long-term results of treatment and prognostic factors.* Ann Surg, 1999. **229**(2): p. 216-22.
50. Thomas, M.B. and A.X. Zhu, *Hepatocellular carcinoma: the need for progress.* J Clin Oncol, 2005. **23**(13): p. 2892-9.
51. Bergsland, E.K. and A.P. Venook, *Hepatocellular carcinoma.* Curr Opin Oncol, 2000. **12**(4): p. 357-61.
52. Simonetti, R.G., et al., *Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials.* Ann Oncol, 1997. **8**(2): p. 117-36.
53. Hemming, A.W., et al., *Liver transplantation for hepatocellular carcinoma.* Ann Surg, 2001. **233**(5): p. 652-9.
54. Iwatsuki, S., et al., *Hepatic resection versus transplantation for hepatocellular carcinoma.* Ann Surg, 1991. **214**(3): p. 221-8; discussion 228-9.
55. Soreide, O., A. Czerniak, and L.H. Blumgart, *Large hepatocellular cancers: hepatic resection or liver transplantation?* Br Med J (Clin Res Ed), 1985. **291**(6499): p. 853-7.
56. Fong, Y., et al., *An analysis of 412 cases of hepatocellular carcinoma at a Western center.* Ann Surg, 1999. **229**(6): p. 790-9; discussion 799-800.
57. Poon, R.T., et al., *Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years.* Ann Surg, 2001. **234**(1): p. 63-70.
58. Vauthey, J.N., et al., *Factors affecting long-term outcome after hepatic resection for hepatocellular carcinoma.* Am J Surg, 1995. **169**(1): p. 28-34; discussion 34-5.

59. Vogl, T.J., et al., *Transarterial chemoembolization for hepatocellular carcinoma: volumetric and morphologic CT criteria for assessment of prognosis and therapeutic success-results from a liver transplantation center.* Radiology, 2000. **214**(2): p. 349-57.
60. Poon, R.T., et al., *Transarterial chemoembolization for inoperable hepatocellular carcinoma and postresection intrahepatic recurrence.* J Surg Oncol, 2000. **73**(2): p. 109-14.
61. Camma, C., et al., *Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials.* Radiology, 2002. **224**(1): p. 47-54.
62. Llovet, J.M. and J. Bruix, *Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival.* Hepatology, 2003. **37**(2): p. 429-42.
63. Raoul, J.L., et al., *Prospective randomized trial of chemoembolization versus intra-arterial injection of 131I-labeled-iodized oil in the treatment of hepatocellular carcinoma.* Hepatology, 1997. **26**(5): p. 1156-61.
64. Raoul, J.L., et al., *Randomized controlled trial for hepatocellular carcinoma with portal vein thrombosis: intra-arterial iodine-131-iodized oil versus medical support.* J Nucl Med, 1994. **35**(11): p. 1782-7.
65. Lau, W.Y., et al., *Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial.* Lancet, 1999. **353**(9155): p. 797-801.
66. Livraghi, T., et al., *Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection.* Radiology, 1995. **197**(1): p. 101-8.
67. Vilana, R., et al., *Tumor size determines the efficacy of percutaneous ethanol injection for the treatment of small hepatocellular carcinoma.* Hepatology, 1992. **16**(2): p. 353-7.
68. Ishii, H., et al., *Local recurrence of hepatocellular carcinoma after percutaneous ethanol injection.* Cancer, 1996. **77**(9): p. 1792-6.
69. Goldberg, S.N., et al., *Ablation of liver tumors using percutaneous RF therapy.* AJR Am J Roentgenol, 1998. **170**(4): p. 1023-8.
70. Elba, S., et al., *Randomized controlled trial of tamoxifen versus placebo in inoperable hepatocellular carcinoma.* Ital J Gastroenterol, 1994. **26**(2): p. 66-8.
71. Perrone, F., et al., *Tamoxifen in the treatment of hepatocellular carcinoma: 5-year results of the CLIP-1 multicentre randomised controlled trial.* Curr Pharm Des, 2002. **8**(11): p. 1013-9.
72. *Tamoxifen in treatment of hepatocellular carcinoma: a randomised controlled trial. CLIP Group (Cancer of the Liver Italian Programme).* Lancet, 1998. **352**(9121): p. 17-20.
73. Pignata, S., et al., *Endocrine treatment of hepatocellular carcinoma. Any evidence of benefit?* Eur J Cancer, 1998. **34**(1): p. 25-32.
74. Tan, C.K., et al., *Use of tamoxifen in hepatocellular carcinoma: a review and paradigm shift.* J Gastroenterol Hepatol, 2000. **15**(7): p. 725-9.
75. Kouroumalis, E., et al., *Treatment of hepatocellular carcinoma with octreotide: a randomised controlled study.* Gut, 1998. **42**(3): p. 442-7.
76. Lai, E.C., et al., *Postoperative adjuvant chemotherapy after curative resection of hepatocellular carcinoma: a randomized controlled trial.* Arch Surg, 1998. **133**(2): p. 183-8.
77. Yamamoto, M., et al., *Adjuvant oral chemotherapy to prevent recurrence after curative resection for hepatocellular carcinoma.* Br J Surg, 1996. **83**(3): p. 336-40.
78. Llovet, J.M., et al., *Cost effectiveness of adjuvant therapy for hepatocellular carcinoma during the waiting list for liver transplantation.* Gut, 2002. **50**(1): p. 123-8.
79. Muto, Y., et al., *Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group.* N Engl J Med, 1996. **334**(24): p. 1561-7.
80. Tennant, D.A., et al., *Metabolic transformation in cancer.* Carcinogenesis, 2009. **30**(8): p. 1269-80.
81. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis, 2009. **30**(7): p. 1073-81.
82. Thorgeirsson, S.S. and J.W. Grisham, *Molecular pathogenesis of human hepatocellular carcinoma.* Nat Genet, 2002. **31**(4): p. 339-46.
83. Farazi, P.A. and R.A. DePinho, *Hepatocellular carcinoma pathogenesis: from genes to environment.* Nat Rev Cancer, 2006. **6**(9): p. 674-87.
84. Lavanchy, D., *Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures.* J Viral Hepat, 2004. **11**(2): p. 97-107.
85. White, D.O. and F. Fenner, *Medical virology*. 3rd ed. 1986, Orlando: Academic Press. viii, 655 p.
86. Seeger, C. and W.S. Mason, *Hepatitis B virus biology.* Microbiol Mol Biol Rev, 2000. **64**(1): p. 51-68.

87. Matsubara, K. and T. Tokino, *Integration of hepatitis B virus DNA and its implications for hepatocarcinogenesis.* Mol Biol Med, 1990. **7**(3): p. 243-60.
88. Tokino, T., et al., *Chromosome deletions associated with hepatitis B virus integration.* Virology, 1991. **185**(2): p. 879-82.
89. Gozuacik, D., et al., *Identification of human cancer-related genes by naturally occurring Hepatitis B Virus DNA tagging.* Oncogene, 2001. **20**(43): p. 6233-40.
90. Murakami, Y., et al., *Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas.* Gut, 2005. **54**(8): p. 1162-8.
91. Tarn, C., et al., *Hepatitis B virus X protein differentially activates RAS-RAF-MAPK and JNK pathways in X-transforming versus non-transforming AML12 hepatocytes.* J Biol Chem, 2001. **276**(37): p. 34671-80.
92. Nijhara, R., et al., *Sustained activation of mitogen-activated protein kinases and activator protein 1 by the hepatitis B virus X protein in mouse hepatocytes in vivo.* J Virol, 2001. **75**(21): p. 10348-58.
93. Feitelson, M.A., *Hepatitis B virus in hepatocarcinogenesis.* J Cell Physiol, 1999. **181**(2): p. 188-202.
94. Yeh, C.T., *Hepatitis B virus X protein: searching for a role in hepatocarcinogenesis.* J Gastroenterol Hepatol, 2000. **15**(4): p. 339-41.
95. Wu, C.G., et al., *SAGE transcript profiles of normal primary human hepatocytes expressing oncogenic hepatitis B virus X protein.* FASEB J, 2002. **16**(12): p. 1665-7.
96. Ueda, H., et al., *Functional inactivation but not structural mutation of p53 causes liver cancer.* Nat Genet, 1995. **9**(1): p. 41-7.
97. Brechot, C., et al., *Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).* Semin Cancer Biol, 2000. **10**(3): p. 211-31.
98. Bouchard, M.J., L.H. Wang, and R.J. Schneider, *Calcium signaling by HBx protein in hepatitis B virus DNA replication.* Science, 2001. **294**(5550): p. 2376-8.
99. Chisari, F.V., et al., *Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice.* Proc Natl Acad Sci U S A, 1987. **84**(19): p. 6909-13.
100. Chisari, F.V. and C. Ferrari, *Hepatitis B virus immunopathology.* Springer Semin Immunopathol, 1995. **17**(2-3): p. 261-81.
101. Xu, Z., G. Jensen, and T.S. Yen, *Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum.* J Virol, 1997. **71**(10): p. 7387-92.
102. Kojima, T., *Immune electron microscopic study of hepatitis B virus associated antigens in hepatocytes.* Gastroenterol Jpn, 1982. **17**(6): p. 559-75.
103. Block, T.M., et al., *Molecular viral oncology of hepatocellular carcinoma.* Oncogene, 2003. **22**(33): p. 5093-107.
104. Galli, A., et al., *Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism.* Hepatology, 2005. **41**(5): p. 1074-84.
105. Lok, A.S., E.J. Heathcote, and J.H. Hoofnagle, *Management of hepatitis B: 2000--summary of a workshop.* Gastroenterology, 2001. **120**(7): p. 1828-53.
106. Chen, B.F., et al., *High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases.* Gastroenterology, 2006. **130**(4): p. 1153-68.
107. Williams, R., *Global challenges in liver disease.* Hepatology, 2006. **44**(3): p. 521-6.
108. Neumann, A.U., et al., *Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy.* Science, 1998. **282**(5386): p. 103-7.
109. Moradpour, D., F. Penin, and C.M. Rice, *Replication of hepatitis C virus.* Nat Rev Microbiol, 2007. **5**(6): p. 453-63.
110. Donato, F., et al., *Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women.* Am J Epidemiol, 2002. **155**(4): p. 323-31.
111. Andre, P., et al., *Hepatitis C virus particles and lipoprotein metabolism.* Semin Liver Dis, 2005. **25**(1): p. 93-104.
112. Pileri, P., et al., *Binding of hepatitis C virus to CD81.* Science, 1998. **282**(5390): p. 938-41.
113. Agnello, V., et al., *Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12766-71.
114. Scarselli, E., et al., *The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus.* EMBO J, 2002. **21**(19): p. 5017-25.
115. Evans, M.J., et al., *Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry.* Nature, 2007. **446**(7137): p. 801-5.
116. Blanchard, E., et al., *Hepatitis C virus entry depends on clathrin-mediated endocytosis.* J Virol, 2006. **80**(14): p. 6964-72.

117. Koutsoudakis, G., et al., *Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses.* J Virol, 2006. **80**(11): p. 5308-20.
118. Tscherne, D.M., et al., *Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry.* J Virol, 2006. **80**(4): p. 1734-41.
119. Moradpour, D., et al., *Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex.* Antiviral Res, 2003. **60**(2): p. 103-9.
120. Okamoto, T., et al., *Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90.* EMBO J, 2006. **25**(20): p. 5015-25.
121. Shiratori, Y., et al., *Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan.* Hepatology, 1995. **22**(4 Pt 1): p. 1027-33.
122. Ikeda, K., et al., *Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients.* J Hepatol, 1998. **28**(6): p. 930-8.
123. Hobert, O., *Gene regulation by transcription factors and microRNAs.* Science, 2008. **319**(5871): p. 1785-6.
124. Pedersen, I.M., et al., *Interferon modulation of cellular microRNAs as an antiviral mechanism.* Nature, 2007. **449**(7164): p. 919-22.
125. Konan, K.V., et al., *Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus.* J Virol, 2003. **77**(14): p. 7843-55.
126. Sklan, E.H., et al., *A Rab-GAP TBC domain protein binds hepatitis C virus NS5A and mediates viral replication.* J Virol, 2007. **81**(20): p. 11096-105.
127. Sklan, E.H., et al., *TBC1D20 is a Rab1 GTPase-activating protein that mediates hepatitis C virus replication.* J Biol Chem, 2007. **282**(50): p. 36354-61.
128. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. **442**(7098): p. 39-44.
129. Foy, E., et al., *Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2986-91.
130. Li, K., et al., *Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2992-7.
131. Szabo, G., S. Chang, and A. Dolganiuc, *Altered innate immunity in chronic hepatitis C infection: cause or effect?* Hepatology, 2007. **46**(4): p. 1279-90.
132. Dolganiuc, A., et al., *Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection.* J Immunol, 2006. **177**(10): p. 6758-68.
133. Tsubouchi, E., et al., *Infection and dysfunction of circulating blood dendritic cells and their subsets in chronic hepatitis C virus infection.* J Gastroenterol, 2004. **39**(8): p. 754-62.
134. Della Bella, S., et al., *Decrease and dysfunction of dendritic cells correlate with impaired hepatitis C virus-specific CD4+ T-cell proliferation in patients with hepatitis C virus infection.* Immunology, 2007. **121**(2): p. 283-92.
135. Dolganiuc, A., et al., *Myeloid dendritic cells of patients with chronic HCV infection induce proliferation of regulatory T lymphocytes.* Gastroenterology, 2008. **135**(6): p. 2119-27.
136. Janssens, V. and J. Goris, *Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.* Biochem J, 2001. **353**(Pt 3): p. 417-39.
137. Duong, F.H., et al., *Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A.* Gastroenterology, 2004. **126**(1): p. 263-77.
138. Christen, V., et al., *Activation of endoplasmic reticulum stress response by hepatitis viruses up-regulates protein phosphatase 2A.* Hepatology, 2007. **46**(2): p. 558-65.
139. Taylor, D.R., et al., *Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein.* Science, 1999. **285**(5424): p. 107-10.
140. Gale, M.J., Jr., et al., *Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein.* Virology, 1997. **230**(2): p. 217-27.
141. Kasprzak, A. and A. Adamek, *Role of hepatitis C virus proteins (C, NS3, NS5A) in hepatic oncogenesis.* Hepatol Res, 2008. **38**(1): p. 1-26.
142. Boulant, S., et al., *Structural determinants that target the hepatitis C virus core protein to lipid droplets.* J Biol Chem, 2006. **281**(31): p. 22236-47.
143. Asselah, T., et al., *Steatosis in chronic hepatitis C: why does it really matter?* Gut, 2006. **55**(1): p. 123-30.

144. Chen, C.M., et al., *Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-beta receptor modulates the signal pathway of the lymphotoxin-beta receptor.* J Virol, 1997. **71**(12): p. 9417-26.
145. Matsumoto, M., et al., *Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-beta receptor.* J Virol, 1997. **71**(2): p. 1301-9.
146. Kittlesen, D.J., et al., *Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation.* J Clin Invest, 2000. **106**(10): p. 1239-49.
147. Zhu, N., C.F. Ware, and M.M. Lai, *Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor.* Virology, 2001. **283**(2): p. 178-87.
148. Shrivastava, A., et al., *Ectopic expression of hepatitis C virus core protein differentially regulates nuclear transcription factors.* J Virol, 1998. **72**(12): p. 9722-8.
149. Erhardt, A., et al., *Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line.* Virology, 2002. **292**(2): p. 272-84.
150. Hayashi, J., et al., *Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha.* Hepatology, 2000. **32**(5): p. 958-61.
151. Tai, D.I., et al., *Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis.* Hepatology, 2000. **31**(3): p. 656-64.
152. Tai, D.I., et al., *Constitutive activation of nuclear factor kappaB in hepatocellular carcinoma.* Cancer, 2000. **89**(11): p. 2274-81.
153. Moriya, K., et al., *The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice.* Nat Med, 1998. **4**(9): p. 1065-7.
154. Gallinari, P., et al., *Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus.* J Virol, 1998. **72**(8): p. 6758-69.
155. Borowski, P., et al., *Nonstructural protein 3 of hepatitis C virus blocks the distribution of the free catalytic subunit of cyclic AMP-dependent protein kinase.* J Virol, 1997. **71**(4): p. 2838-43.
156. Errington, W., et al., *Subcellular localisation of NS3 in HCV-infected hepatocytes.* J Med Virol, 1999. **59**(4): p. 456-62.
157. Mottola, G., et al., *Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons.* Virology, 2002. **293**(1): p. 31-43.
158. Sakamuro, D., T. Furukawa, and T. Takegami, *Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells.* J Virol, 1995. **69**(6): p. 3893-6.
159. Zemel, R., et al., *Cell transformation induced by hepatitis C virus NS3 serine protease.* J Viral Hepat, 2001. **8**(2): p. 96-102.
160. Hassan, M., H. Ghozlan, and O. Abdel-Kader, *Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth.* Virology, 2005. **333**(2): p. 324-36.
161. Feng, D.Y., et al., *Effect of hepatitis C virus nonstructural protein NS3 on proliferation and MAPK phosphorylation of normal hepatocyte line.* World J Gastroenterol, 2005. **11**(14): p. 2157-61.
162. Kwun, H.J., et al., *p53-dependent transcriptional repression of p21(waf1) by hepatitis C virus NS3.* J Gen Virol, 2001. **82**(Pt 9): p. 2235-41.
163. Fujita, T., et al., *Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus.* Biochem Biophys Res Commun, 1996. **229**(3): p. 825-31.
164. Bataller, R., et al., *Hepatitis C virus core and nonstructural proteins induce fibrogenic effects in hepatic stellate cells.* Gastroenterology, 2004. **126**(2): p. 529-40.
165. Schulze-Krebs, A., et al., *Hepatitis C virus-replicating hepatocytes induce fibrogenic activation of hepatic stellate cells.* Gastroenterology, 2005. **129**(1): p. 246-58.
166. Girard, S., et al., *Hepatitis C virus NS5A-regulated gene expression and signaling revealed via microarray and comparative promoter analyses.* Hepatology, 2004. **40**(3): p. 708-18.
167. Asano, S., et al., *US3 protein kinase of herpes simplex virus protects primary afferent neurons from virus-induced apoptosis in ICR mice.* Neurosci Lett, 2000. **294**(2): p. 105-8.
168. Majumder, M., et al., *Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner.* J Virol, 2001. **75**(3): p. 1401-7.
169. Qadri, I., M. Iwahashi, and F. Simon, *Hepatitis C virus NS5A protein binds TBP and p53, inhibiting their DNA binding and p53 interactions with TBP and ERCC3.* Biochim Biophys Acta, 2002. **1592**(2): p. 193-204.
170. McClain, C.J., et al., *S-Adenosylmethionine, cytokines, and alcoholic liver disease.* Alcohol, 2002. **27**(3): p. 185-92.

171. Mandrekar, P. and G. Szabo, *Signalling pathways in alcohol-induced liver inflammation.* J Hepatol, 2009. **50**(6): p. 1258-66.
172. Hoek, J.B. and J.G. Pastorino, *Ethanol, oxidative stress, and cytokine-induced liver cell injury.* Alcohol, 2002. **27**(1): p. 63-8.
173. Bode, C. and J.C. Bode, *Activation of the innate immune system and alcoholic liver disease: effects of ethanol per se or enhanced intestinal translocation of bacterial toxins induced by ethanol?* Alcohol Clin Exp Res, 2005. **29**(11 Suppl): p. 166S-71S.
174. Chao, H.K., et al., *Evidence that mutational activation of the ras genes may not be involved in aflatoxin B(1)-induced human hepatocarcinogenesis, based on sequence analysis of the ras and p53 genes.* Mol Carcinog, 1999. **26**(2): p. 69-73.
175. Zender, L., et al., *Cancer gene discovery in hepatocellular carcinoma.* J Hepatol. **52**(6): p. 921-9.
176. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.
177. Meek, D.W., *Mechanisms of switching on p53: a role for covalent modification?* Oncogene, 1999. **18**(53): p. 7666-75.
178. Prives, C. and P.A. Hall, *The p53 pathway.* J Pathol, 1999. **187**(1): p. 112-26.
179. Giaccia, A.J. and M.B. Kastan, *The complexity of p53 modulation: emerging patterns from divergent signals.* Genes Dev, 1998. **12**(19): p. 2973-83.
180. Kruse, J.P. and W. Gu, *Modes of p53 regulation.* Cell, 2009. **137**(4): p. 609-22.
181. Carr, A.M., *Cell cycle. Piecing together the p53 puzzle.* Science, 2000. **287**(5459): p. 1765-6.
182. Hirao, A., et al., *DNA damage-induced activation of p53 by the checkpoint kinase Chk2.* Science, 2000. **287**(5459): p. 1824-7.
183. Sherr, C.J. and J.D. Weber, *The ARF/p53 pathway.* Curr Opin Genet Dev, 2000. **10**(1): p. 94-9.
184. Lowe, S.W. and A.W. Lin, *Apoptosis in cancer.* Carcinogenesis, 2000. **21**(3): p. 485-95.
185. Groth, A., et al., *Oncogenic Ras induces p19ARF and growth arrest in mouse embryo fibroblasts lacking p21Cip1 and p27Kip1 without activating cyclin D-dependent kinases.* J Biol Chem, 2000. **275**(35): p. 27473-80.
186. Jardine, L.J., et al., *Phosphorylation of murine p53, but not human p53, by MAP kinase in vitro and in cultured cells highlights species-dependent variation in post-translational modification.* Oncogene, 1999. **18**(52): p. 7602-7.
187. el-Deiry, W.S., *Regulation of p53 downstream genes.* Semin Cancer Biol, 1998. **8**(5): p. 345-57.
188. Yu, J., et al., *Identification and classification of p53-regulated genes.* Proc Natl Acad Sci U S A, 1999. **96**(25): p. 14517-22.
189. Chan, T.A., et al., *14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage.* Nature, 1999. **401**(6753): p. 616-20.
190. Laronga, C., et al., *Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression.* J Biol Chem, 2000. **275**(30): p. 23106-12.
191. Dellambra, E., et al., *Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes.* J Cell Biol, 2000. **149**(5): p. 1117-30.
192. Reed, J.C., *Dysregulation of apoptosis in cancer.* J Clin Oncol, 1999. **17**(9): p. 2941-53.
193. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**(5468): p. 1053-8.
194. Oda, K., et al., *p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53.* Cell, 2000. **102**(6): p. 849-62.
195. Lin, Y., W. Ma, and S. Benchimol, *Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis.* Nat Genet, 2000. **26**(1): p. 122-7.
196. Tlsty, T.D., *Genomic instability and its role in neoplasia.* Curr Top Microbiol Immunol, 1997. **221**: p. 37-46.
197. Wahl, G.M., et al., *Maintaining genetic stability through TP53 mediated checkpoint control.* Cancer Surv, 1997. **29**: p. 183-219.
198. Hendrix, M.J., *De-mystifying the mechanism(s) of maspin.* Nat Med, 2000. **6**(4): p. 374-6.
199. Teodoro, J.G., et al., *p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase.* Science, 2006. **313**(5789): p. 968-71.
200. Schwarte-Waldhoff, I., et al., *Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9624-9.
201. Gundacker, N., et al., *Knowledge-based proteome profiling: considering identified proteins to evaluate separation efficiency by 2-D PAGE.* Electrophoresis, 2006. **27**(13): p. 2712-21.

202. Rabilloud, T., et al., *A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels.* Proteomics, 2001. **1**(5): p. 699-704.
203. Mortz, E., et al., *Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis.* Proteomics, 2001. **1**(11): p. 1359-63.
204. Ponten, F., K. Jirstrom, and M. Uhlen, *The Human Protein Atlas--a tool for pathology.* J Pathol, 2008. **216**(4): p. 387-93.
205. Sahani, D.V., et al., *Advanced hepatocellular carcinoma: CT perfusion of liver and tumor tissue--initial experience.* Radiology, 2007. **243**(3): p. 736-43.
206. Jain, R., et al., *Quantitative estimation of permeability surface-area product in astroglial brain tumors using perfusion CT and correlation with histopathologic grade.* AJNR Am J Neuroradiol, 2008. **29**(4): p. 694-700.
207. Shweiki, D., et al., *Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis.* Nature, 1992. **359**(6398): p. 843-5.
208. Hashizume, H., et al., *Openings between defective endothelial cells explain tumor vessel leakiness.* Am J Pathol, 2000. **156**(4): p. 1363-80.
209. Pandharipande, P.V., et al., *Perfusion imaging of the liver: current challenges and future goals.* Radiology, 2005. **234**(3): p. 661-73.
210. Wang, Y., et al., *Evaluation of serum clusterin as a surveillance tool for human hepatocellular carcinoma with hepatitis B virus related cirrhosis.* J Gastroenterol Hepatol. **25**(6): p. 1123-8.
211. Ramagopal, S., *Are eukaryotic ribosomes heterogeneous? Affirmations on the horizon.* Biochem Cell Biol, 1992. **70**(5): p. 269-72.
212. Chappell, S.A., G.C. Owens, and V.P. Mauro, *A 5' Leader of Rbm3, a Cold Stress-induced mRNA, Mediates Internal Initiation of Translation with Increased Efficiency under Conditions of Mild Hypothermia.* J Biol Chem, 2001. **276**(40): p. 36917-22.
213. Ramagopal, S., *Induction of cell-specific ribosomal proteins in aggregation-competent nonmorphogenetic Dictyostelium discoideum.* Biochem Cell Biol, 1990. **68**(11): p. 1281-7.
214. Dresios, J., et al., *Yeast ribosomal protein L24 affects the kinetics of protein synthesis and ribosomal protein L39 improves translational accuracy, while mutants lacking both remain viable.* Biochemistry, 2000. **39**(24): p. 7236-44.
215. Dresios, J., et al., *Yeast ribosomal protein deletion mutants possess altered peptidyltransferase activity and different sensitivity to cycloheximide.* Biochemistry, 2001. **40**(27): p. 8101-8.
216. Dresios, J., et al., *A dispensable yeast ribosomal protein optimizes peptidyltransferase activity and affects translocation.* J Biol Chem, 2003. **278**(5): p. 3314-22.
217. Mauro, V.P. and G.M. Edelman, *The ribosome filter hypothesis.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12031-6.
218. Warner, J.R. and K.B. McIntosh, *How common are extraribosomal functions of ribosomal proteins?* Mol Cell, 2009. **34**(1): p. 3-11.
219. Liu, Y., et al., *Identification of differential expression of genes in hepatocellular carcinoma by suppression subtractive hybridization combined cDNA microarray.* Oncol Rep, 2007. **18**(4): p. 943-51.
220. Kim, J.H., et al., *Over-expression of the ribosomal protein L36a gene is associated with cellular proliferation in hepatocellular carcinoma.* Hepatology, 2004. **39**(1): p. 129-38.
221. Chung, E.J., et al., *Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray.* Mol Cells, 2002. **14**(3): p. 382-7.
222. Lau, W.Y., et al., *Differential gene expression of hepatocellular carcinoma using cDNA microarray analysis.* Oncol Res, 2000. **12**(2): p. 59-69.
223. Ma, X.J., et al., *Gene expression profiling of the tumor microenvironment during breast cancer progression.* Breast Cancer Res, 2009. **11**(1): p. R7.
224. Kasai, H., et al., *Differential expression of ribosomal proteins in human normal and neoplastic colorectum.* J Histochem Cytochem, 2003. **51**(5): p. 567-74.
225. Peng, S.F., L. Fu, and D.M. Tan, *[Heat-shock protein 27 linked to multi-drug resistance in human hepatic cancer cell lines].* Zhonghua Gan Zang Bing Za Zhi, 2007. **15**(5): p. 362-5.
226. Yang, Y.X., et al., *Proteome analysis of multidrug resistance in vincristine-resistant human gastric cancer cell line SGC7901/VCR.* Proteomics, 2006. **6**(6): p. 2009-21.
227. Zhang, D., et al., *Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer.* Mol Cell Proteomics, 2005. **4**(11): p. 1686-96.

228. Kassem, H., et al., *A potential role of heat shock proteins and nicotinamide N-methyl transferase in predicting response to radiation in bladder cancer.* Int J Cancer, 2002. **101**(5): p. 454-60.
229. Cornford, P.A., et al., *Heat shock protein expression independently predicts clinical outcome in prostate cancer.* Cancer Res, 2000. **60**(24): p. 7099-105.
230. Guo, K., et al., *Regulation of HSP27 on NF-kappaB pathway activation may be involved in metastatic hepatocellular carcinoma cells apoptosis.* BMC Cancer, 2009. **9**(1): p. 100.
231. Matsushima-Nishiwaki, R., et al., *Phosphorylated heat shock protein 27 represses growth of hepatocellular carcinoma via inhibition of extracellular signal-regulated kinase.* J Biol Chem, 2008. **283**(27): p. 18852-60.
232. Takai, S., et al., *Protein kinase C delta regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma.* Life Sci, 2007. **81**(7): p. 585-91.
233. Luk, J.M., et al., *Proteomic profiling of hepatocellular carcinoma in Chinese cohort reveals heat-shock proteins (Hsp27, Hsp70, GRP78) up-regulation and their associated prognostic values.* Proteomics, 2006. **6**(3): p. 1049-57.
234. Fang, C., et al., *Proteome analysis of human liver carcinoma Huh7 cells harboring hepatitis C virus subgenomic replicon.* Proteomics, 2006. **6**(2): p. 519-27.
235. Ciocca, D.R., et al., *Estrogen receptors, progesterone receptors and heat-shock 27-kD protein in liver biopsy specimens from patients with hepatitis B virus infection.* Hepatology, 1991. **13**(5): p. 838-44.
236. Feng, J.T., et al., *Heat-shock protein 27: a potential biomarker for hepatocellular carcinoma identified by serum proteome analysis.* Proteomics, 2005. **5**(17): p. 4581-8.
237. Lu, K.P. and X.Z. Zhou, *The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 904-16.
238. Young, J.C., et al., *Pathways of chaperone-mediated protein folding in the cytosol.* Nat Rev Mol Cell Biol, 2004. **5**(10): p. 781-91.
239. Skinner, M.M., et al., *Crystal structure of protein isoaspartyl methyltransferase: a catalyst for protein repair.* Structure, 2000. **8**(11): p. 1189-201.
240. Benyamini, P., P. Webster, and D.I. Meyer, *Knockdown of p180 eliminates the terminal differentiation of a secretory cell line.* Mol Biol Cell, 2009. **20**(2): p. 732-44.
241. Bai, J.Z., et al., *Alternatively spliced forms of the P180 ribosome receptor differ in their ability to induce the proliferation of rough endoplasmic reticulum.* Cell Biol Int, 2008. **32**(5): p. 473-83.
242. Michalak, M., et al., *Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum.* Biochem J, 2009. **417**(3): p. 651-66.
243. Mukhopadhyay, D. and H. Riezman, *Proteasome-independent functions of ubiquitin in endocytosis and signaling.* Science, 2007. **315**(5809): p. 201-5.
244. Ravid, T. and M. Hochstrasser, *Diversity of degradation signals in the ubiquitin-proteasome system.* Nat Rev Mol Cell Biol, 2008. **9**(9): p. 679-90.
245. Pickart, C.M. and D. Fushman, *Polyubiquitin chains: polymeric protein signals.* Curr Opin Chem Biol, 2004. **8**(6): p. 610-6.
246. Strous, G.J., et al., *Ubiquitin system-dependent regulation of growth hormone receptor signal transduction.* Curr Top Microbiol Immunol, 2004. **286**: p. 81-118.
247. Kavsak, P., et al., *Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation.* Mol Cell, 2000. **6**(6): p. 1365-75.
248. Di Guglielmo, G.M., et al., *Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover.* Nat Cell Biol, 2003. **5**(5): p. 410-21.
249. Zhang, Y., et al., *Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation.* J Neurosci, 2000. **20**(15): p. 5671-8.
250. Sigismund, S., et al., *Clathrin-independent endocytosis of ubiquitinated cargos.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2760-5.
251. Chen, H. and P. De Camilli, *The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2766-71.
252. Gupta-Rossi, N., et al., *Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor.* J Cell Biol, 2004. **166**(1): p. 73-83.
253. Lee, T.V., et al., *The E1 ubiquitin-activating enzyme Uba1 in Drosophila controls apoptosis autonomously and tissue growth non-autonomously.* Development, 2008. **135**(1): p. 43-52.
254. Kitagaki, J., et al., *Nitric oxide prodrug JS-K inhibits ubiquitin E1 and kills tumor cells retaining wild-type p53.* Oncogene, 2009. **28**(4): p. 619-24.
255. Feng, Q., et al., *UBE1L causes lung cancer growth suppression by targeting cyclin D1.* Mol Cancer Ther, 2008. **7**(12): p. 3780-8.

256. Pabarcus, M.K., et al., *CYP3A4 ubiquitination by gp78 (the tumor autocrine motility factor receptor, AMFR) and CHIP E3 ligases.* Arch Biochem Biophys, 2009. **483**(1): p. 66-74.
257. Miyoshi, Y., et al., *High expression of ubiquitin carboxy-terminal hydrolase-L1 and -L3 mRNA predicts early recurrence in patients with invasive breast cancer.* Cancer Sci, 2006. **97**(6): p. 523-9.
258. Rolen, U., et al., *Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines.* Mol Carcinog, 2006. **45**(4): p. 260-9.
259. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.
260. Christofk, H.R., et al., *The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth.* Nature, 2008. **452**(7184): p. 230-3.
261. Christofk, H.R., et al., *Pyruvate kinase M2 is a phosphotyrosine-binding protein.* Nature, 2008. **452**(7184): p. 181-6.
262. Altenberg, B. and K.O. Greulich, *Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes.* Genomics, 2004. **84**(6): p. 1014-20.
263. Asaka, M., et al., *Decreased serum aldolase B levels in patients with malignant tumors.* Cancer, 1988. **62**(12): p. 2554-7.
264. Frederick, G.L. and R.W. Begg, *A study of hyperlipemia in the tumor-bearing rat.* Cancer Res, 1956. **16**(6): p. 548-52.
265. Iguchi, T., et al., *Correlation between mammary tumor and blood glucose, serum insulin, and free fatty acids in mice.* Cancer Res, 1989. **49**(4): p. 821-5.
266. Kumar, K., P. Sachdanandam, and R. Arivazhagan, *Studies on the changes in plasma lipids and lipoproteins in patients with benign and malignant breast cancer.* Biochem Int, 1991. **23**(3): p. 581-9.
267. Hanai, T., et al., *Comparison of prostanoids and their precursor fatty acids in human hepatocellular carcinoma and noncancerous reference tissues.* J Surg Res, 1993. **54**(1): p. 57-60.
268. Greco, A.V., G. Mingrone, and G. Gasbarrini, *Free fatty acid analysis in ascitic fluid improves diagnosis in malignant abdominal tumors.* Clin Chim Acta, 1995. **239**(1): p. 13-22.
269. Jiang, J.T., et al., *Lipids changes in liver cancer.* J Zhejiang Univ Sci B, 2007. **8**(6): p. 398-409.
270. Wood, C.B., et al., *Reduction in the stearic to oleic acid ratio in human malignant liver neoplasms.* Eur J Surg Oncol, 1985. **11**(4): p. 347-8.
271. Janssen, U. and W. Stoffel, *Disruption of mitochondrial beta -oxidation of unsaturated fatty acids in the 3,2-trans-enoyl-CoA isomerase-deficient mouse.* J Biol Chem, 2002. **277**(22): p. 19579-84.
272. Miinalainen, I.J., et al., *Mitochondrial 2,4-dienoyl-CoA reductase deficiency in mice results in severe hypoglycemia with stress intolerance and unimpaired ketogenesis.* PLoS Genet, 2009. **5**(7): p. e1000543.
273. Wanders, R.J., et al., *Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases.* Biochem Soc Trans, 2001. **29**(Pt 2): p. 250-67.
274. David, S., et al., *Aberrant silencing of the endocrine peptide gene tachykinin-1 in gastric cancer.* Biochem Biophys Res Commun, 2009. **378**(3): p. 605-9.
275. Jin, Z., et al., *Hypermethylation of tachykinin-1 is a potential biomarker in human esophageal cancer.* Clin Cancer Res, 2007. **13**(21): p. 6293-300.
276. Rumpler, G., et al., *Identification of differentially expressed genes in models of melanoma progression by cDNA array analysis: SPARC, MIF and a novel cathepsin protease characterize aggressive phenotypes.* Exp Dermatol, 2003. **12**(6): p. 761-71.
277. Autelli, R., et al., *Divergent pathways for TNF and C(2)-ceramide toxicity in HTC hepatoma cells.* Biochim Biophys Acta, 2009. **1793**(7): p. 1182-90.
278. Kang, S.H., et al., *Antitumor effect of 2-methoxyestradiol in a rat orthotopic brain tumor model.* Cancer Res, 2006. **66**(24): p. 11991-7.
279. Kambhampati, S., et al., *2-methoxyestradiol inhibits Barrett's esophageal adenocarcinoma growth and differentiation through differential regulation of the beta-catenin-E-cadherin axis.* Mol Cancer Ther. **9**(3): p. 523-34.
280. Cho, J.K., et al., *Injectable delivery system of 2-methoxyestradiol for breast cancer therapy using biodegradable thermosensitive poly(organophosphazene) hydrogel.* J Drug Target.
281. Zhou, Y., et al., *Sorcin, an important gene associated with multidrug-resistance in human leukemia cells.* Leuk Res, 2006. **30**(4): p. 469-76.
282. Deng, L., et al., *Upregulation of soluble resistance-related calcium-binding protein (sorcin) in gastric cancer.* Med Oncol, 2009.

283. Qu, Y., et al., *Comparative proteomic profiling identified sorcin being associated with gemcitabine resistance in non-small cell lung cancer.* Med Oncol, 2009.
284. Qi, J., et al., *Overexpression of sorcin in multidrug resistant human leukemia cells and its role in regulating cell apoptosis.* Biochem Biophys Res Commun, 2006. **349**(1): p. 303-9.
285. Kawakami, M., et al., *Knock-down of sorcin induces up-regulation of MDR1 in HeLa cells.* Biol Pharm Bull, 2007. **30**(6): p. 1065-73.
286. Bustos, M., et al., *Protection against liver damage by cardiotrophin-1: a hepatocyte survival factor up-regulated in the regenerating liver in rats.* Gastroenterology, 2003. **125**(1): p. 192-201.
287. Lopez, N., J. Diez, and M.A. Fortuno, *Characterization of the protective effects of cardiotrophin-1 against non-ischemic death stimuli in adult cardiomyocytes.* Cytokine, 2005. **30**(5): p. 282-92.
288. Dolcet, X., et al., *Cytokines promote motoneuron survival through the Janus kinase-dependent activation of the phosphatidylinositol 3-kinase pathway.* Mol Cell Neurosci, 2001. **18**(6): p. 619-31.
289. Haga, S., et al., *Stat3 protects against Fas-induced liver injury by redox-dependent and -independent mechanisms.* J Clin Invest, 2003. **112**(7): p. 989-98.
290. Terui, K., et al., *Stat3 confers resistance against hypoxia/reoxygenation-induced oxidative injury in hepatocytes through upregulation of Mn-SOD.* J Hepatol, 2004. **41**(6): p. 957-65.
291. Klein, C., et al., *The IL-6-gp130-STAT3 pathway in hepatocytes triggers liver protection in T cell-mediated liver injury.* J Clin Invest, 2005. **115**(4): p. 860-9.
292. Marques, J.M., et al., *Cardiotrophin-1 is an essential factor in the natural defense of the liver against apoptosis.* Hepatology, 2007. **45**(3): p. 639-48.
293. Calvisi, D.F., et al., *Ubiquitous activation of Ras and Jak/Stat pathways in human HCC.* Gastroenterology, 2006. **130**(4): p. 1117-28.
294. Yoshikawa, H., et al., *SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity.* Nat Genet, 2001. **28**(1): p. 29-35.
295. Schmitz, K.J., et al., *Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection.* J Hepatol, 2008. **48**(1): p. 83-90.
296. Shi, H., et al., *Suppression of N-myc downstream-regulated gene 2 is associated with induction of Myc in colorectal cancer and correlates closely with differentiation.* Biol Pharm Bull, 2009. **32**(6): p. 968-75.
297. Choi, S.C., et al., *NDRG2 is one of novel intrinsic factors for regulation of IL-10 production in human myeloid cell.* Biochem Biophys Res Commun. **396**(3): p. 684-90.
298. Furuta, H., et al., *NDRG2 is a candidate tumor-suppressor for oral squamous-cell carcinoma.* Biochem Biophys Res Commun. **391**(4): p. 1785-91.
299. Kim, Y.J., et al., *NDRG2 suppresses cell proliferation through down-regulation of AP-1 activity in human colon carcinoma cells.* Int J Cancer, 2009. **124**(1): p. 7-15.
300. Lee, D.C., et al., *Functional and clinical evidence for NDRG2 as a candidate suppressor of liver cancer metastasis.* Cancer Res, 2008. **68**(11): p. 4210-20.
301. Park, M.Y., et al., *A quantitative analysis of N-myc downstream regulated gene 2 (NDRG 2) in human tissues and cell lysates by reverse-phase protein microarray.* Clin Chim Acta, 2008. **387**(1-2): p. 84-9.
302. Hsiao, H.H., et al., *Quantitative characterization of the interactions among c-myc transcriptional regulators FUSE, FBP, and FIR.* Biochemistry. **49**(22): p. 4620-34.
303. Ghiorghi, Y.K., et al., *The c-Myc target gene Rcl (C6orf108) encodes a novel enzyme, deoxynucleoside 5'-monophosphate N-glycosidase.* J Biol Chem, 2007. **282**(11): p. 8150-6.
304. Malz, M., et al., *Overexpression of far upstream element binding proteins: a mechanism regulating proliferation and migration in liver cancer cells.* Hepatology, 2009. **50**(4): p. 1130-9.
305. Sun, J., et al., *Nit1 and Fhit tumor suppressor activities are additive.* J Cell Biochem, 2009. **107**(6): p. 1097-106.
306. Karreth, F.A. and D.A. Tuveson, *Modelling oncogenic Ras/Raf signalling in the mouse.* Curr Opin Genet Dev, 2009. **19**(1): p. 4-11.
307. Newell, P., et al., *Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin in vivo.* J Hepatol, 2009. **51**(4): p. 725-33.
308. Choudhari, S.R., et al., *Deactivation of Akt and STAT3 signaling promotes apoptosis, inhibits proliferation, and enhances the sensitivity of hepatocellular carcinoma cells to an anticancer agent, Atiprimod.* Mol Cancer Ther, 2007. **6**(1): p. 112-21.
309. Morrison, D.K., *The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development.* Trends Cell Biol, 2009. **19**(1): p. 16-23.

310. Karp, C.M., et al., *Identification of HRPAP20: a novel phosphoprotein that enhances growth and survival in hormone-responsive tumor cells.* Cancer Res, 2004. **64**(3): p. 1016-25.
311. Karp, C.M., et al., *HRPAP20: a novel calmodulin-binding protein that increases breast cancer cell invasion.* Oncogene, 2007. **26**(12): p. 1780-8.
312. Tozlu-Kara, S., et al., *Oligonucleotide microarray analysis of estrogen receptor alpha-positive postmenopausal breast carcinomas: identification of HRPAP20 and TIMELESS as outstanding candidate markers to predict the response to tamoxifen.* J Mol Endocrinol, 2007. **39**(4): p. 305-18.
313. Kohler, C., et al., *The solution structure of the core of mesoderm development (MESD), a chaperone for members of the LDLR-family.* J Struct Funct Genomics, 2006. **7**(3-4): p. 131-8.
314. Hsieh, J.C., et al., *Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity.* Cell, 2003. **112**(3): p. 355-67.
315. Lu, W., et al., *Mesd is a universal inhibitor of Wnt coreceptors LRP5 and LRP6 and blocks Wnt/beta-catenin signaling in cancer cells.* Biochemistry. **49**(22): p. 4635-43.
316. Li, Y., et al., *Modulation of LRP6-mediated Wnt signaling by molecular chaperone Mesd.* FEBS Lett, 2006. **580**(22): p. 5423-8.
317. Han, W.D., et al., *Up-regulation of LRP16 mRNA by 17beta-estradiol through activation of estrogen receptor alpha (ERalpha), but not ERbeta, and promotion of human breast cancer MCF-7 cell proliferation: a preliminary report.* Endocr Relat Cancer, 2003. **10**(2): p. 217-24.
318. Ummanni, R., et al., *Altered expression of tumor protein D52 regulates apoptosis and migration of prostate cancer cells.* FEBS J, 2008. **275**(22): p. 5703-13.
319. Shehata, M., et al., *Tumor protein D52 overexpression and gene amplification in cancers from a mosaic of microarrays.* Crit Rev Oncog, 2008. **14**(1): p. 33-55.
320. Hu, T.H., et al., *Expression of hepatoma-derived growth factor in hepatocellular carcinoma.* Cancer, 2003. **98**(7): p. 1444-56.
321. Uyama, H., et al., *Hepatoma-derived growth factor is a novel prognostic factor for patients with pancreatic cancer.* Clin Cancer Res, 2006. **12**(20 Pt 1): p. 6043-8.
322. Yamamoto, T., et al., *Involvement of hepatoma-derived growth factor in the growth inhibition of hepatocellular carcinoma cells by vitamin K(2).* J Gastroenterol, 2009. **44**(3): p. 228-35.

7 Publication

7.1 A novel technique to specifically analyze the secretome of cells and tissues

Electrophoresis 2005, *26*, 2779–2785

Hannes Zwickl[1]
Elisabeth Traxler[1]
Stefan Staettner[2]
Wolfram Parzefall[1]
Bettina Grasl-Kraupp[1]
Josef Karner[2]
Rolf Schulte-Hermann[1]
Christopher Gerner[1]

[1]Department of Internal Medicine Clinic I, Institute of Cancer Research, Medical University of Vienna, Vienna, Austria
[2]Department of Surgery, Kaiser-Franz-Josef-Spital, Vienna, Austria

A novel technique to specifically analyze the secretome of cells and tissues

The secretome of cells and tissues may reflect a broad variety of pathological conditions and thus represents a rich source of biomarkers. The identity of secreted proteins, usually isolated from cell supernatants or body fluids, is hardly accessible by direct proteome analysis, because these proteins are often masked by high amounts of proteins actually not secreted by the investigated cells. Here, we present a novel method for the specific detection of proteins secreted by human tissue specimen as well as cultured cells and chose liver as a model. The method is based on the metabolic labelling of proteins synthesized during a limited incubation period. Then, the cell supernatant is filtered, precipitated, and subjected to two-dimensional gel electrophoresis. Whereas fluorography detected a large number of proteins derived from residual plasma and dead cells, the autoradiographs selectively displayed genuinely secreted proteins. We demonstrate the feasibility of this approach by means of the secretomes of the hepatocellular carcinoma-derived cell line HepG2 and human liver slices. The selective identification of cell- and tissue-specific protein secretion profiles may help to identify novel sets of biomarkers for wide clinical applications.

Keywords: Biomarker discovery / Liver / Metabolic labelling / Proteomics / Secretome
DOI 10.1002/elps.200410387

1 Introduction

Secreted proteins may determine, control and coordinate many biological processes in multicellular organisms, such as growth, division, differentiation and apoptosis at the cellular level, and development, immune defence, blood coagulation and tumourigenesis at the level of the whole organism. Their central role makes them hence a good target and source for therapeutical and drug-based intervention as well as a tool for diagnosis and prognosis of diseases like cancer. Thus, great interest is currently focused on the characterization of secreted proteins (the secretome) from isolated cells [1] and neoplastic tissues [2, 3] in order to find and identify novel biomarkers.

Several methods are available to investigate the secretome of normal and malignant cells, based on either computational analysis of genomes [4] or more directly by proteome analysis. While genetic analyses may provide a list of potentially secreted proteins, proteome analysis is indispensable to identify proteins actually secreted by cells and tissues under certain conditions.

Proteome analysis applied for biomedical research is often hindered by the high complexity of biological model systems [5]. Detection and identification of secreted proteins, indeed, has as well turned out to be a challenge for several reasons. Secreted proteins *in vivo* occur in interstitial fluids and blood plasma. Direct proteome analysis of these body fluids, however, is complicated by the high concentrations of main plasma constituents such as albumin, resulting in masking and dilution of the secreted proteins. For better accessibility of these proteins for analysis, they can be isolated from cell and tissue supernatants collected after cultivation *in vitro*. Freshly isolated and thoroughly washed cell and tissue samples, however, may contain residual fetal calf serum (FCS) or plasma constituents respectively. The amount of secreted proteins is usually very low, even minor contaminations with protein-rich plasma/FCS may easily mask proteins of interest. In addition, cultivation of cells and tissue preparation is unavoidably accompanied by cell death. Consequently, significant amounts of cytoplasmic proteins may be released into the supernatant, thereby concealing secreted proteins. Indeed, the majority of proteins listed

Correspondence: Prof. Christopher Gerner, Internal Medicine Clinic I, Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria
E-mail: christopher.gerner@meduniwien.ac.at
Fax: + 43-1-4277-9651

Abbreviation: RuBPS, Ruthenium II tris (bathophenanthroline disulfonate)

in secretome protein analyses from cells and tissues are not secreted proteins, but rather contaminants of cytoplasmic and plasmatic origin [1–3].

We have established a method, which allows the sensitive and selective detection of genuinely secreted proteins, applicable to cultured cells as well as tissue specimen. As model systems, we chose liver tissue and cultured hepatocarcinoma cells, because the secretome of these is well known and therefore well suited to test the applicability of the method. We have overcome the above-described technical limitations by a combination of *in vitro* metabolic labelling of cells and tissue slices, and subsequent detection of proteins by fluorescence analysis and autoradiography.

2 Materials and methods

2.1 Sample preparation and subcellular fractionation

Freshly resected human liver tissue was cut in slices of about 200 μm thickness, rinsed in Hank's buffered saline solution (HBSS) and incubated in serum-free, methionine- and cysteine-free William's E medium (ICN) in the presence of [^{35}S]-labelled methionine and cysteine (Trans35-Slabel, Biomedica, MP Biomedicals) for 6 h at 37°C. HepG2 cells were cultured at standard conditions, washed in HBSS and incubated in methionine- and cysteine-free RPMI-1640 for 2 h at 37°C. Supernatants were collected, filtered through a 0.22 μm filter (Millex-GP, Millipore, Billerica, MA, USA) to get rid of cell debris, soluble proteins were precipitated by the addition of ethanol. For the isolation of cytoplasmic proteins, all buffers were supplemented with protease inhibitors: PMSF (1 mM), aprotinin, leupeptin,and pepstatin A (each at 1 μg/mL). Cells were lysed in lysis buffer (10 mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100). The cytoplasmic fraction was separated from the nuclei by centrifugation and ethanol precipitated. Protein samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.5% SDS, 100 mM DTT).

2.2 Proteome analysis

Proteins were loaded by passive rehydration of IPG strips pH 5–8, 17 cm (BioRad, Hercules, CA, USA) at room temperature. IEF was performed in a stepwise fashion (1 h 0–500 V linear; 5 h 500 V; 5 h 500–3500 V linear; 12 h 3500 V). After IEF, the strips were equilibrated with 100 mM DTT and 2.5% iodacetamide according to the instructions of the manufacturer (BioRad). For SDS-PAGE using the Protean II xi electrophoresis system (BioRad), the IPG strips were placed on top of 1.5 mm 12% polyacrylamide slab gels and overlaid with 0.5% low-melting agarose. The gels were stained with a 400 nM solution of Ruthenium II tris (bathophenanthroline disulfonate) (RuBPS) as described by Rabilloud *et al.* [6]. Fluorography scanning was performed with the FluorImager 595 (Amersham Biosciences, Amersham, UK) at a resolution of 100 μm. After scanning, the gels were dried using the slab gel dryer SE110 (Hoefer, San Francisco CA, USA). Exposure of storage phosphor screens (Molecular Dynamics, Sunnyvale, CA, USA) occurred at room temperature for 24 h. Screens were subsequently scanned using the Phosphorimager SI (Molecular Dynamics) at a resolution of 100 μm. Proteins were identified by using the Swiss-Prot 2-D database [7] and by mass spectrometry analysis. Preparation of tryptic protein hydrolysates for the latter was carried out essentially as described by Katayama *et al.* [8]. Peptides were separated by nanoflow LC (1100 Series LC system, Agilent, Waldbronn, Germany) and identified by fragmentation analysis using an IT mass spectrometer (XCT-Plus, Agilent) equipped with an orthogonal nanospray ion source. Protein identification was accomplished with the Spectrum Mill MS Proteomics Workbench software.

3 Results and discussion

The analysis of cell and tissue supernatants collected after *in vitro* incubation is currently the most direct approach to identify secreted proteins. We have analyzed the secretome of human hepatocellular carcinoma cells, HepG2, by 2-DE and subsequent fluorescence detection (Figs. 1A and B). Actually, this protein fraction was found to contain not only the secreted proteins, but also cytoplasmic proteins such as chaperones and cytoskeleton components (Fig. 1A). Comparative analysis of the cytoplasmic protein fraction of the same cell experiment clearly identified these proteins as cytoplasmic contamination (Fig. 1C compared to Fig. 1A). Therefore, direct proteome analysis of cell supernatants may identify a lot of nonsecreted proteins. As *in vitro* cultivation of cells is unavoidably accompanied by cell necrosis, cytoplasmic proteins detected in cell supernatants will most plausibly be derived from such dead cells. Even a very small percentage of dead cells will be able to release protein amounts by far exceeding that of actually secreted proteins.

Metabolic labelling of cells allows the detection of proteins both by fluorescence analysis as well as autoradiography. While fluorescence analysis detects all proteins present in the investigated protein fraction, autoradiography detects only those proteins, which were

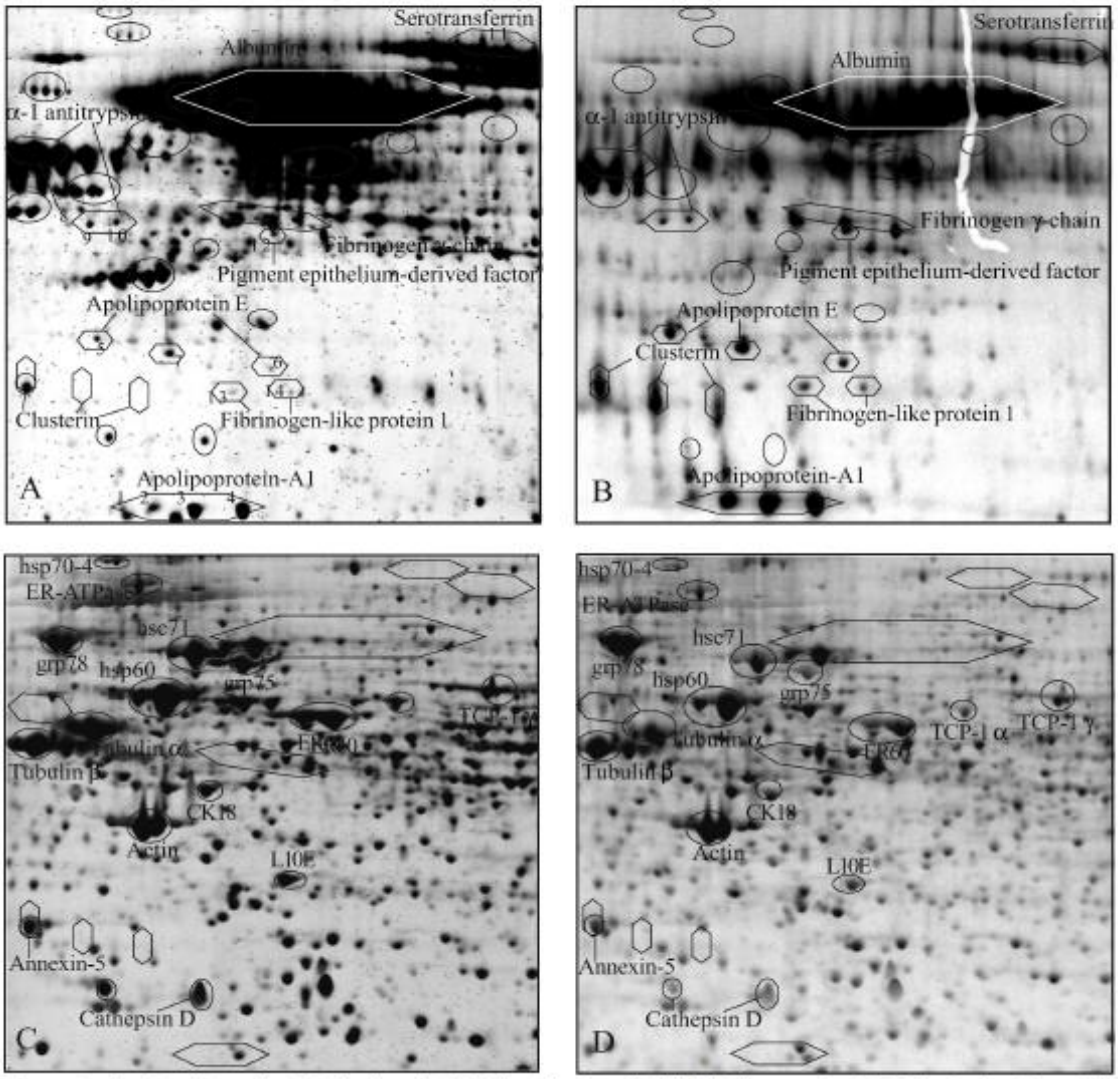


Figure 1. 2-D gel sections of protein fractions isolated from HepG2 cells. (A, B) Cell supernatant; (C, D) cytoplasmic protein fraction. (A, C) Fluorography of the RuBPS-stained gels; (B, D) ^{35}S-autoradiography. Genuinely secreted proteins are indicated by hexagons, cytoplasmic proteins are encircled. At our experimental conditions, cytoplasmic as well as secreted proteins were radiolabelled and hence became detectable by autoradiography (B, D). Note that most abundant proteins of the cytoplasmic protein fraction are detectable in the cell supernatants by fluorography, but not autoradiography. Secreted proteins are specifically identified in the autoradiograph of the cell supernatant. Spot numbers indicate those spots further investigated by MS (Table 1). RuBPS, fluorography of RuBPS-stained gels; AR, autoradiography of the same gels after drying and exposure to PhosphoImager screens.

synthesized by living cells during the metabolic labelling period [9]. When the cell supernatant of metabolically labelled HepG2 cells was analyzed by autoradiography, only genuinely secreted proteins, such as albumin, antitrypsin, fibrinogen chains, and apolipoprotein, were detectable (Fig. 1B). Autoradiography of the cytoplasmic protein fraction of HepG2 cells demonstrated that cytoplasmic proteins become as well metabolically labelled in intact cells (Fig. 1D). The absence of radiolabelled cytoplasmic proteins in cell supernatants therefore suggested

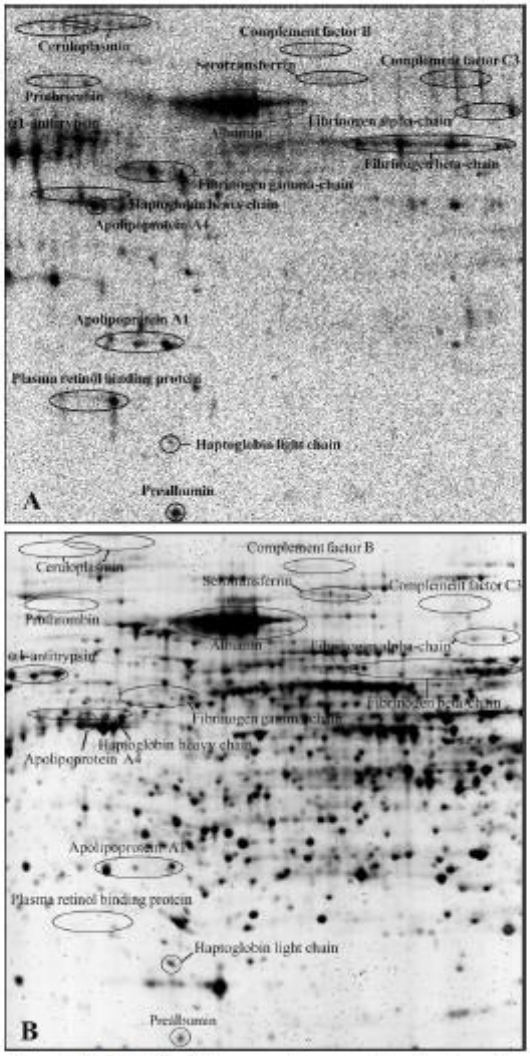


Figure 2. Analysis of the secretome protein fraction of a human liver sample. (A) ^{35}S autoradiography; (B) fluorography of the RuBPS-stained gel. Note that a large number of proteins known to be secreted by human liver is detectable in the autoradiograph without detection of nonsecreted proteins. In contrast, the fluorograph of the same gel is clearly dominated by cytoplasmic, and thus nonsecreted, proteins.

that intact and hence metabolically active cells contribute only proteins supposed to be secreted to the supernatant, but no cytoplasmic proteins. We detected cytoplasmic proteins in cell supernatants by fluorography, but not by autoradiography (Fig. 1A). In the case of HepG2 cells, up to more than 50% of the overall integrated fluorescence intensity associated with protein spots in the cell supernatant was contributed by secreted proteins. Obviously, unlabelled proteins did not originate from intact, metabolically active cells. As a conclusion, most plausibly, these proteins were derived from dead cells not performing active protein synthesis, which eventually released their cytoplasmic content into the supernatant.

We analyzed several selected spots from the HepG2 supernatant, which showed positive radiolabels, by mass spectrometric analysis of tryptic digests. Indeed, all identified 16 protein spots were found to be genuinely secreted proteins (Table 1). Most of them are known plasma proteins as expected to be secreted by hepatocytes. Interestingly, fibrinogen-like protein 1 (Q08830) has not yet been identified as secreted by HepG2 cells. The pigment epithelium-derived factor (P36955) has only recently been identified by Western analysis to be secreted by HepG2 cells [10].

A similar approach was applied to human liver tissue specimen. Freshly resected liver tissue samples were cut into slices and metabolically labelled for 6 h. As demonstrated in the case of HepG2 cells, the supernatant as well as the cytoplasmic protein fraction was isolated and subjected to 2-DE, fluorescence detection and autoradiography. Indeed, proteins were positively found radiolabelled, proving that cells making up the tissue were still alive during the incubation period. Only proteins known to be genuinely secreted by the liver were found radiolabelled in the supernatant fraction (Fig. 2A), which was again rich in cytoplasmic proteins displaying no radiolabel (Fig. 2B). Here, less than 5% of the overall integrated fluorescence intensity associated with protein spots in the cell supernatant was contributed by secreted proteins. This result demonstrates the problems associated with direct proteome analyses of tissue supernatants, identifying preferentially cytoplasmic proteins. The presently described method, however, proved to be applicable for freshly isolated tissue specimen as well as cultured cells.

In short terms, the described method consists of metabolic labelling of cultured cells or freshly isolated tissue slices followed by the analysis of the supernatant protein fraction by 2-DE. Only proteins detectable in the autoradiographs of these protein fractions are genuine members of the secretome of the investigated cells. Cutting these spots from nonlabelled material isolated and processed in parallel, enzymatic digest and mass spectrometric analysis of the resulting peptides eventually identifies the secreted proteins (Table 1). Quantification of autoradiography spot intensities may provide further information with respect to the quantitative secretion performance of cells and tissues (manuscript in preparation).

Table 1. Mass analysis of tryptic digests of protein spots isolated from HepG2 supernatant protein 2-D gels

Spot	Protein	Accession number	Mw	pI	Score	Sequence
1	Apolipoprotein A-I	P02647	28078.6	5.27	14.61	(K)DLATVYVDVLK(D)
					8.24	(K)DSGRDYVSQFEGSALGK(Q)
2	Apolipoprotein A-I	P02647	28078.6	5.27	12.22	(K)DLATVYVDVLK(D)
					11.49	(K)DLATVYVDVLK(D)
					7.23	(K)LLDNWDSVTSTFSK(L)
3	Apolipoprotein A-I	P02647	28078.6	5.27	19.42	(R)DYVSQFEGSALGK(Q)
					18.33	(R)EQLGPVTQEFWDNLEK(E)
					10.71	(K)LSPLGEEMR(D)
					9.14	(K)VQPYLDDFQKK(W)
4	Apolipoprotein A-I	P02647	28078.6	5.27	21.75	(R)DYVSQFEGSALGK(Q)
					20.18	(K)VSFLSALEEYTK(K)
					18.85	(K)DLATVYVDVLKDSGR(D)
					18.16	(K)LLDNWDSVTSTFSK(L)
					18.09	(K)DLATVYVDVLK(D)
					17.99	(R)VKDLATVYVDVLK(D)
					17.92	(K)DSGRDYVSQFEGSALGK(Q)
					16.47	(K)LHELQEKLSPLGEEMR(D)
					15.76	(K)DSGRDYVSQFEGSALGK(Q)
					15.46	(R)VKDLATVYVDVLK(D)
					14.50	(K)DLATVYVDVLKDSGR(D)
					14.02	(R)LEALKENGGAR(L)
					13.72	(R)VKDLATVYVDVLK(D)
					13.54	(R)THLAPYSDELR(Q)
					13.29	(K)ATEHLSTLSEK(A)
					13.24	(R)qKLHELQEK(L)
					12.38	(K)KWQEEmELYR(Q)
					12.16	(R)EQLGPVTQEFWDNLEK(E)
					11.79	(K)AKPALEDLR(Q)
					11.43	(K)LSPLGEEmR(D)
					10.99	(R)QEMSKDLEEVK(A)
					10.73	(R)LAEYHAKATEHLSTLSEK(A)
					10.44	(K)AKVQPYLDDFQK(K)
					10.16	(R)QGLLPVLESFK(V)
					9.87	(K)VQPYLDDFQKK(W)
					9.61	(K)LLDNWDSVTSTFSK(L)
					9.52	(K)ATEHLSTLSEK(A)
					9.50	(K)VQPYLDDFQKK(W)
					9.48	(R)VKDLATVYVDVLK(D)
					9.36	(K)WQEEmELYR(Q)
					9.09	(K)LSPLGEEMR(D)
					8.98	(R)VKDLATVYVDVLK(D)
					8.96	(K)VQPYLDDFQK(K)
					8.77	(K)WQEEMELYR(Q)
					8.64	(K)KWQEEMELYR(Q)
					8.44	(R)qKVEPLR(A)
					7.53	(R)THLAPYSDELR(Q)
					7.29	(K)LSPLGEEmRDR(A)
					6.20	(K)VEPLRAELQEGAR(Q)
5	Apolipoprotein E	P02649	34236.7	5.52	13.90	(R)VRAATVGSLAGQPLQER(A)
					13.18	(R)AATVGSLAGQPLQER(A)
6	Apolipoprotein E	P02649	34236.7	5.52	15.20	(K)AYKSELEEQLTPVAEETR(A)
7	Apolipoprotein E	P02649	34236.7	5.52	13.90	(R)VRAATVGSLAGQPLQER(A)
					13.18	(R)AATVGSLAGQPLQER(A)
					15.48	(K)TVVQLEGDNK(L)
					14.34	(K)VIQNEFTVGEECELETMTGEK(V)

Table 1. Continued

Spot	Protein	Accession number	Mw	pI	Score	Sequence
					11.78	(K)VIQNEFTVGEECELETmTGEK(V)
					10.44	(K)VIQNEFTVGEECELETMTGEKVK(T)
					9.68	(K)AIGLPEELIQK(G)
					8.14	(K)FTITAGSK(V)
8	α-1-Antitrypsin	P01009	44068.2	5.30	19.18	(K)VFSNGADLSGVTEEAPLK(L)
					18.80	(K)LQHLENELTHDIITK(F)
					15.54	(K)FNKPFVFLmIEQNTK(S)
					15.35	(K)SVLGQLGITK(V)
					14.94	(K)LYHSEAFTVNFGDTEEAK(K)
					14.54	(K)DTEEEDFHVDQVTTVK(V)
					14.51	(K)VFSNGADLSGVTEEAPLK(L)
					13.75	(K)LSITGTYDLK(S)
					12.72	(K)LSSWVLLMK(Y)
					12.50	(K)ITPNLAEFAFSLYR(Q)
					12.01	(K)qINDYVEKGTQGK(I)
					10.32	(R)LGMFNIQHCK(K)
					9.24	(K)GKWERPFEVK(D)
					8.20	(K)LVDKFLEDVK(K)
9	α-1-Antitrypsin	P01009	44068.2	5.30	15.32	(K)SVLGQLGITK(V)
					14.77	(K)LSITGTYDLK(S)
10	α-1-Antitrypsin	P01009	44068.2	5.30	18.69	(K)DTEEEDFHVDQVTTVK(V)
					17.81	(K)FNKPFVFLMIEQNTK(S)
					16.45	(K)SVLGQLGITK(V)
					14.81	(K)LSSWVLLMK(Y)
					13.47	(K)DTEEEDFHVDQVTTVK(V)
					11.89	(K)ELDRDTVFALVNYIFFK(G)
					11.62	(K)VFSNGADLSGVTEEAPLK(L)
					11.33	(K)VFSNGADLSGVTEEAPLK(L)
					11.22	(K)RLGMFNIQHCK(K)
					8.23	(R)TLNQPDSQLQLTTGNGLFLSEGLK(L)
					6.51	(K)WERPFEVK(D)
11	Serotransferrin (transferrin)	P02787	75181.4	6.70	18.57	(K)IECVSAETTEDCIAK(I)
					17.87	(K)IECVSAETTEDCIAK(I)
					12.12	(K)LCMGSGLNLCEPNNK(E)
12	Pigment epithelium-derived factor	P36955	44263.7	5.90	16.94	(K)TVQAVLTVPK(L)
					8.57	(K)ELLDTVTAPQK(N)
					6.38	(R)KTSLEDFYLDEER(T)
13	Fibrinogen-like protein 1	Q08830	36391.7	5.59	12.11	(K)IDLADFEK(N)
					9.12	(K)IRPNDFIPNVI(-)
14	Fibrinogen-like protein 1	Q08830	36391.7	5.59	17.11	(R)QYADCSEIFNDGYK(L)
					12.08	(K)IDLADFEK(N)
					10.37	(K)NLHFLTTQEDYTLK(I)
					8.36	(K)IDLADFEKNSR(Y)
	Fatty acid-binding protein (L-FABP)	P07148	14208.5	6.60	19.35	(K)YQLQSQENFEAFMK(A)
					12.10	(K)YQLQSQENFEAFMK(A)
	Fatty acid-binding protein (L-FABP)	P07148	14208.5	6.60	18.06	(K)YQLQSQENFEAFMK(A)
					16.90	(K)GVSEIVQNGK(H)
					15.48	(K)TVVQLEGDNK(L)
					14.34	(K)VIQNEFTVGEECELETMTGEK(V)
					11.78	(K)VIQNEFTVGEECELETmTGEK(V)
					10.44	(K)VIQNEFTVGEECELETMTGEKVK(T)

Table 1. Continued

Spot	Protein	Accession number	Mw	pI	Score	Sequence
					9.68	(K)AIGLPEELIQK(G)
					8.14	(K)FTITAGSK(V)
	Transthyretin (prealbumin)	P02766	15887.1	5.52	18.07	(K)TSESGELHGLTTEEEFVEGIYK(V)
					15.94	(K)AADDTWEPFASGK(T)
					14.26	(R)GSPAINVAVHVFR(K)
					12.59	(R)KAADDTWEPFASGK(T)
					12.07	(R)KAADDTWEPFASGK(T)
					9.45	(R)GSPAINVAVHVFRK(A)

Spots 1–14 are indicated in Fig. 1A, the other protein spots were outside the shown gel section. Peptide identification scores were determined by the Spectrum Mill software.

This method may hence open a wide field of applications. Proteins secreted by tissues may be assigned to their cell type of origin by the analysis of isolated cell types. In addition, specific secretome components may indicate well-defined physiological tissue states like growth, inflammation, malignant transformation or other pathologic abnormalities. Currently, we collect a comprehensive set of data derived from human liver tumour samples, this approach allows us to identify a relatively large number of proteins secreted by tumour tissue only, but not by normal liver tissue (manuscript in preparation). Thus, this technique will facilitate the search for a broad variety of biomarkers potentially applicable for prognosis, diagnosis and disease surveillance.

4 Concluding remarks

Here, we present a novel technique to specifically detect and identify proteins genuinely secreted by cells and freshly resected tissue specimen. This is the first successful approach to selectively investigate cell- and tissue-specific protein secretion profiles. Application of this technique to well-defined pathological tissue specimen compared to normal tissue may allow the identification of novel biomarkers of potentially high clinical relevance.

This study was supported by the Austrian GEN-AU project GZ200.058/6-VI/2/2002.

Received December 9, 2004

5 References

[1] Dupont, A., Tokarski, C., Dekeyzer, O., Guihot, A. L., Amouyel, P., Rolando, C., Pinet, F., *Proteomics* 2004, *4*, 1761–1778.

[2] Celis, J. E., Gromov, P., Cabezon, T., Moreira, J. M., Ambartsumian, N., Sandelin, K., Rank, F., Gromova, I., *Mol. Cell Proteomics* 2004, *3*, 327–344.

[3] Paweletz, C. P., Trock, B., Pennanen, M., Tsangaris, T., Magnant, C., Liotta, L. A., Petricoin III, E. F., *Dis. Markers* 2001, *17*, 301–307.

[4] Klee, E. W., Carlson, D. F., Fahrenkrug, S. C., Ekker, S. C., Ellis, L. B., *Nucleic Acids Res.* 2004, *32*, 1414–1421.

[5] Gerner, C., *Comb. Chem. High Throughput Screen.* 2004, *7*, 1–9.

[6] Rabilloud, T., Strub, J. M., Luche, S., van Dorsselaer, A., Lunardi, J., *Proteomics* 2001, *1*, 699–704.

[7] Sanchez, J. C., Appel, R. D., Golaz, O., Pasquali, C., Ravier, F., Bairoch, A., Hochstrasser, D. F., *Electrophoresis* 1995, *16*, 1131–1151.

[8] Katayama, H., Nagasu, T., Oda, Y., *Rapid Commun. Mass Spectrom.* 2001, *15*, 1416–1421.

[9] Gerner, C., Vejda, S., Gelbmann, D., Bayer, E., Gotzmann, J., Schulte-Hermann, R., Mikulits, W., *Mol. Cell Proteomics* 2002, *1*, 528–537.

[10] Sawant, S., Aparicio, S., Tink, A. R., Lara, N., Barnstable, C. J., Tombran-Tink, J., *Biochem. Biophys. Res. Commun.* 2004, *325*, 408–413.

8 Appendix

Table 33: Compilation of shotgun data, immunohistochemically derived and EHCO data including their compliance. Proteins are listed in the order of their Uniprot-ID. Numbers in the column “Shotgun proteomics” correspond to the number of total peptides (Tp), those listed in the column “EHCO II-database” correspond to the number of citations (of overall 8 datasets) stating up-or downregulation of the protein. The column “data compilation” summarizes immunohistochemically derived data (see chapter 4.6) expressed as percentage of histological samples showing unaltered, up- and downregulation for the respective proteins as well as the relative abundances derived from shotgun (S or numbers in blue and bold, numbers in blue indicate tendency for altered expression with less than factor 2 difference in terms of Tp) and EHCO (http://ehco.iis.sinica.edu.tw/) (E or underlined numbers). In the case of strong expression of proteins in liver and HCC (both with REL: 1, see chapter 4.6) no conclusions concerning a putative overexpression can be drawn (“???” in the “data compilation” column). The data compliance derived from comparisons of shotgun and EHCO (SE), Shotgun and immunohistochemically derived data (SP, P from “Protein Atlas”) as well as EHCO and histological data (EP).

Shotgun proteomics (Tp)			EHCO II- database		Data compilation			Data compliance		
Uniprot-ID	Liver	HCC	up	down	up	unaltered	down	SE	SP	EP
O00165	1	1	1	0	E	S		✗	n.d.	n.d.
O00217	4	4	1	1	54-36	36-55	9	✗	✔	✔
O00231	2	1	2	0	E		S	✗	n.d.	n.d.
O00232	1	0	No results		0-9	64	36-27	n.d.	✔	n.d.
O00264	10	7	1	2	???	25-33	75-67	✗	✔	✔
O00299	2	3	5	0	92	0	8	✗	✗	✔
O00303	2	0	No results				S	n.d.	n.d.	n.d.
O00305	0	1	No results		92-75	0-17	8	n.d.	✔	n.d.
O00499	0	1	No results		41	42-0	16-58	n.d.	✔	n.d.
O00625	0	2	3	0	0-9	55-45	45	✔	✔	✔
O00629	1	0	No results				S	n.d.	n.d.	n.d.
O00748	7	24	0	1	???	100	0	✗	✔	✗
O00754	0	1	No results		S			n.d.	n.d.	n.d.
O00764	4	4	No results			S		n.d.	n.d.	n.d.
O14558	1	0	No results				S	n.d.	n.d.	n.d.
O14561	0	1	No results		S			n.d.	n.d.	n.d.
O14602	2	11	No results		17-42	25-0	59	n.d.	✔	n.d.
O14618	0	1	0	1	0-17	0	100-83	✗	✔	✔
O14737	0	1	2	0	S			✔	n.d.	n.d.
O14745	0	3	2	0	25-42	58-0	17-58	✔	✔	✔
O14773	7	4	No results			S	s	n.d.	n.d.	n.d.
O14818	3	6	No results		S			n.d.	n.d.	n.d.
O14874	1	0	No results		90-80	0-10	10	n.d.	✔	n.d.
O14880	1	0	1	0	E		S	✗	n.d.	n.d.
O14933	1	0	No results		10-20	70-60	20	n.d.	✔	n.d.
O15116	1	1	No results		0	100	0	n.d.	✔	n.d.

O15145	4	4	2	1	33	**25-8**	42-58	✗	✔	✔
O15173	0	1	No results		**S**			n.d.	n.d.	n.d.
O15511	4	2	No results		0-8	58-67	**42-25**	n.d.	✔	n.d.
O43169	3	1	No results		???	92	**8**	n.d.	✔	n.d.
O43175	13	0	2	0	E		**S**	✗	n.d.	n.d.
O43181	3	0	No results		0-20	80-60	**20**	n.d.	✔	n.d.
O43324	1	0	No results				**S**	n.d.	n.d.	n.d.
O43399	0	1	1	1	**S**		E	✔✗	n.d.	n.d.
O43464	1	2	1	0	**S**			✔	n.d.	n.d.
O43488/O95154/ Q8NHP1	11	13	0	1		**S**	E	✗	n.d.	n.d.
O43598	3	6	No results		**S**			n.d.	n.d.	n.d.
O43615	0	1	No results		**S**			n.d.	n.d.	n.d.
O43617	4	2	No results				**S**	n.d.	n.d.	n.d.
O43708	0	1	0	3	**???**	45	55	✗	✔	✔
O43852	0	4	1	1	**S**		E	✔✗	n.d.	n.d.
O43895	0	3	No results		**45**	55	0	n.d.	✔	n.d.
O60218	4	18	2	1	**S**		E	✔✗	n.d.	n.d.
O60234	1	0	2	0	100	0	**0**	✗	✗	✔
O60493	2	0	No results				**S**	n.d.	n.d.	n.d.
O60613	1	3	No results		**S**			n.d.	n.d.	n.d.
O60701	15	15	1	0	E	**S**		✗	n.d.	n.d.
O60888	4	3	1	0	E	**S**		✗	n.d.	n.d.
O75197	1	0	No results		36	45	18	n.d.	✔	n.d.
O75208	2	3	No results			**S**		n.d.	n.d.	n.d.
O75223	0	3	No results		**75**	25	0	n.d.	✔	n.d.
O75340	10	5	1	0	0	25-58	**75-42**	✗	✔	✗
O75347	3	6	2	0	**S**			✔	n.d.	n.d.
O75356	10	4	0	1	???	0-10	**100-90**	✔	✔	✔
O75368	4	1	No results				**S**	n.d.	n.d.	n.d.
O75369	0	2	2	0	**100-91**	0-9	0	✔	✔	✔
O75380	0	1	No results		**S**			n.d.	n.d.	n.d.
O75390	3	1	1	0	E		**S**	✗	n.d.	n.d.
O75431	1	0	No results				**S**	n.d.	n.d.	n.d.
O75439	1	0	2	0	E		**S**	✗	n.d.	n.d.
O75489	12	6	1	0	8-33	50-25	**42**	✗	✔	✔
O75521	13	9	No results		???	**42-67**	58-33	n.d.	✔	n.d.
O75608	6	10	No results		**s**	**S**		n.d.	n.d.	n.d.
O75629	1	0	No results				**S**	n.d.	n.d.	n.d.
O75746/Q9UJS0	26	12	No results		???	33-50	**67-50**	n.d.	✔	n.d.
O75874	24	24	0	1		**S**	E	✗	n.d.	n.d.
O75891	49	17	0	3			**S**	✔	n.d.	n.d.
O75947	17	16	1	0	E	**S**		✗	n.d.	n.d.
O75955	1	0	No results		0	0	**100**	n.d.	✔	n.d.
O76054	3	1	0	1			**S**	✔	n.d.	n.d.
O94826	0	1	No results		**0-8**	0-75	100-17	n.d.	✔	n.d.

O94903	1	4	No results		**0**	83	17	n.d.	✗	n.d.
O95050	1	0	No results				**S**	n.d.	n.d.	n.d.
O95168	2	0	1	0	E		**S**	✗	n.d.	n.d.
O95202	0	1	No results		**100**	0	0	n.d.	✔	n.d.
O95210	1	3	No results		**17-33**	25-17	58-50	n.d.	✔	n.d.
O95299	2	0	1	0	E		**S**	✗	n.d.	n.d.
O95336	0	5	No results		**S**			n.d.	n.d.	n.d.
O95372	0	2	No results		**S**			n.d.	n.d.	n.d.
O95479	16	5	0	0	???	73	**27**	✗	✔	✔
O95563	2	0	2	1	E		**S**	✔✗	n.d.	n.d.
O95571	9	8	2	0	E	**S**		✗	n.d.	n.d.
O95777	2	0	No results		0	0-8	**100-92**	n.d.	✔	n.d.
O95831	19	23	0	0	50	**25**	25	✔	✔	✔
O95881	3	3	No results			**S**		n.d.	n.d.	n.d.
O95954	20	0	0	2	42-25	0-8	**58-67**	✔	✔	✔
O96000	5	2	No results				**S**	n.d.	n.d.	n.d.
O96008	3	2	No results		100-92	**0-8**	0	n.d.	✔	n.d.
P00167	11	9	0	4	???	**9-36**	91-64	✗	✔	✔
P00338	20	3	1	2	0	100	**0**	✔✗	✗	✗
P00352	56	40	0	2		**S**	E	✗	n.d.	n.d.
P00367/P49448	68	16	0	3			**S**	✔	n.d.	n.d.
P00390	2	0	0	0	60	40-0	**0-40**	✗	✔	✔
P00403	3	3	No results		???	**73-82**	27-18	n.d.	✔	n.d.
P00439	5	0	0	3			**S**	✔	n.d.	n.d.
P00441	17	18	0	4	0	**0**	100	✗	✗	✔
P00450	1	1	0	6	0-8	**34-42**	66-50	✗	✔	✔
P00480	24	15	0	2	???	**27-36**	73-64	✗	✔	✔
P00491	2	0	0	1	0-10	40-50	**60-40**	✔	✔	✔
P00492	2	0	1	0	???	0-30	**100-70**	✗	✔	✔
P00505	41	14	0	2	???	9-27	**91-73**	✔	✔	✔
P00558	17	18	3	0	E	**S**		✗	n.d.	n.d.
P00568/Q9Y6K8	8	11	0	0	0	**92**	8	✔	✔	✔
P00734	1	0	0	2	0-9	36	**64-55**	✔	✔	✔
P00738/P00739	34	36	0	6	0-8	**33-25**	67	✗	✔	✔
P00751	1	0	2	1	42-58	42-25	**17**	✔✗	✔	✔
P00915	8	11	0	1	9	**0**	91	✗	✗	✔
P00918	4	14	0	4	**0-18**	18-0	82	✗	✔	✔
P00966	19	5	0	3	???	8-17	**92-83**	✔	✔	✔
P01008	0	2	0	4	**0-11**	22-33	78-56	✗	✔	✔
P01009	17	0	1	3	60	20	**20**	✔✗	✔	✔
P01019	8	9	1	1	0-10	**80-70**	20	✗	✔	✔
P01023	3	0	0	5	0-18	18	**82-64**	✔	✔	✔
P01024	0	2	0	2	**0-27**	55-27	18-45	✗	✔	✔
P01112/P01111/ P01116	0	7	3	0	**0**	100	0	✔	✗	✗

P01591	0	1	0	3	**S**		E	✗	n.d.	n.d.
P01623/...	0	18	No results		**S**			n.d.	n.d.	n.d.
P01834	12	21	1	3	E**s**	**S**	E	✗	n.d.	n.d.
P01842	6	16	No results		**S**			n.d.	n.d.	n.d.
P01857	1	11	0	2	**S**		E	✗	n.d.	n.d.
P01871	2	0	0	2			**S**	✔	n.d.	n.d.
P02647	22	41	0	3	9	**91**	0	✗	✔	✗
P02649	4	10	0	1	**0-40**	80-60	20-0	✗	✔	✔
P02656	1	0	1	2	E		**S**	✔✗	n.d.	n.d.
P02671	2	0	0	4	58	42	**0**	✔	✗	✗
P02675	4	3	1	5	10-30	**40**	50-30	✗	✔	✔
P02679	1	1	1	5	E	**S**	E	✗	n.d.	n.d.
P02689	0	5	No results		**S**			n.d.	n.d.	n.d.
P02743	7	5	0	3	0-25	**42-33**	58-42	✗	✔	✔
P02753	8	20	1	3	**???**	20-50	80-50	✔✗	✔	✔
P02763	4	3	1	3	???	**0-9**	100-91	✗	✔	✔
P02766	5	13	0	5	**0**	30-40	70-60	✗	✗	✔
P02768	70	186	0	8	**17-25**	67-42	17-33	✗	✔	✔
P02774	3	1	0	3	36-55	45-0	**15-45**	✔	✔	✔
P02787	38	91	1	6	**45-64**	9-0	45-36	✔✗	✔	✔
P02790	2	0	0	2	11	11-0	**78-89**	✔	✔	✔
P02792	24	17	0	2	???	**0-30**	100-70	✗	✔	✔
P02794	8	10	1	0	???	**0-25**	100-75	✗	✔	✔
P04004	7	10	0	4	40	**10-0**	50-60	✗	✔	✔
P04040	33	51	0	6	0-8	**42-58**	58-33	✗	✔	✔
P04075	2	5	3	0	**S**			✔	n.d.	n.d.
P04080	5	4	1	1	E	**S**	E	✗	n.d.	n.d.
P04083	10	6	3	0	50-33	**0-17**	50	✗	✔	✔
P04179	63	36	0	1	???	**91**	9	✗	✔	✔
P04196	0	5	0	3	**S**		E	✗	n.d.	n.d.
P04217	4	11	0	1	**8**	92	0	✗	✔	✗
P04229	10	0	0	0		E	**S**	✗	n.d.	n.d.
P04264	16	0	No results				**S**	n.d.	n.d.	n.d.
P04406	23	30	4	0	0	**73**	27	✗	✔	✗
P04424	7	9	No results		???	**40**	60	n.d.	✔	n.d.
P04632	4	6	No results		89-78	**0**	11-22	n.d.	✗	n.d.
P04792	3	19	4	1	**0**	11	89	✔✗	✗	✔✗
P04844	1	0	0	2	60	40	**0**	✔	✗	✗
P05062	29	25	0	6	???	**0-11**	100-89	✗	✔	✔
P05089	31	16	0	6	???	**0-30**	100-70	✗	✔	✔
P05090	4	0	No results				**S**	n.d.	n.d.	n.d.
P05091	65	88	0	5	**s**	**S**	E	✗	n.d.	n.d.
P05109	1	0	0	2	0	100	**0**	✔	✗	✗
P05155	3	1	1	4	E		**S**	✔✗	n.d.	n.d.
P05165	9	0	1	1	E		**S**	✔✗	n.d.	n.d.

P05166	24	9	1	0	E		**S**	✗	n.d.	n.d.
P05177	2	0	0	1	0	0-27	**100-73**	✔	✔	✔
P05386	1	4	3	0	**70**	30	0	✔	✔	✔
P05386	1	4			**S**			n.d.	n.d.	n.d.
P05387	10	7	2	0	E	**S**	**s**	✗	n.d.	n.d.
P05388	7	6	3	0	67	**33**	0	✗	✔	✔
P05413	5	0	No results				**S**	n.d.	n.d.	n.d.
P05556	1	2	1	1	**???**	92	8	✔✗	✔	✔
P05783	17	0	No results				**S**	n.d.	n.d.	n.d.
P05787	30	0	1	2	E		**S**	✔✗	n.d.	n.d.
P05976	3	0	No results				**S**	n.d.	n.d.	n.d.
P06132	0	1	No results		**0-8**	0	100-92	n.d.	✔	n.d.
P06396	3	0	0	1			**S**	✔	n.d.	n.d.
P06576	48	69	No results		9-45	**82-18**	9-36	n.d.	✔	n.d.
P06702	1	0	No results		36	64	**0**	n.d.	✗	n.d.
P06733	47	73	0	2	0	**36-64**	64-36	✗	✔	✔
P06737	37	0	0	1	???	0-17	**100-83**	✔	✔	✔
P06744	7	3	No results		0-8	0	**100-92**	n.d.	✔	n.d.
P07029	2	0	No results				**S**	n.d.	n.d.	n.d.
P07099	3	9	0	1	**S**		E	✗	n.d.	n.d.
P07108	0	1	2	0	**75-83**	8	17-8	✔	✔	✔
P07148	45	16	3	5	???	0-9	**100-91**	✔	✔	✔
P07195	2	0	0	1			**S**	✔	n.d.	n.d.
P07203	18	15	1	1	0	**0**	100	✗	✗	✔✗
P07237	50	69	0	2	25	**75-67**	0-8	✗	✔	✔
P07327/P00325/ P00326	106	75	0	1	???	**22-36**	78-64	✗	✔	✔
P07339	19	14	1	1	???	**8-50**	92-50	✗	✔	✔
P07355	18	27	3	1	E**s**	**S**	E	✗	n.d.	n.d.
P07437/Q13885/ Q9BVA1/P68371/ Q13509/P04350	157	77	4	1	E		**S**	✔✗	n.d.	n.d.
P07686	2	8	No results		**S**			n.d.	n.d.	n.d.
P07737	24	19	No results			**S**	**s**	n.d.	n.d.	n.d.
P07741	12	10	1	0	91	**0**	9	✗	✗	✔
P07858	17	9	0	1	???	**0-13**	100-88	✗	✔	✔
P07900/P08238	67	43	3	0	50	**50**	0	✗	✔	✔
P07910	3	2	1	0	100-75	**0-25**	0	✗	✔	✔
P07954	18	37	1	1	**???**	33	67	✔✗	✔	✔
P08107/P54652/P17 066	31	58	2	1	67-42	**0-8**	33-50	✗	✔	✔
P08133	62	49	No results		30	**0**	70	n.d.	✗	n.d.
P08134	14	9	2	0	27	**73**	0	✗	✔	✔
P08236	2	0	0	1			**S**	✔	n.d.	n.d.
P08263/P09210/Q1 6772/Q7RTV2	249	69	1	2	???	0-17	**100-83**	✔✗	✔	✔

P08311	1	0	No results		0	100	**0**	n.d.	✗	n.d.
P08319	59	38	0	5	0	**0-8**	100-92	✗	✔	✔
P08559	1	0	No results				**S**	n.d.	n.d.	n.d.
P08574	2	5	2	0	**56-67**	33-22	11	✔	✔	✔
P08670	27	16	3	0	E	**S**	**s**	✗	n.d.	n.d.
P08697	1	0	0	3	0	91	**9**	✔	✔	✔
P08708	8	8	1	0	E	**S**		✗	n.d.	n.d.
P08758	18	10	2	0	E	**S**	**s**	✗	n.d.	n.d.
P08865	4	0	4	0	E		**S**	✗	n.d.	n.d.
P09110	33	33	0	2	0	**0**	100	✗	✗	✔
P09211	12	7	0	0	17-34	**17**	67-50	✔	✔	✔
P09382	4	2	0	0	8	0	**92**	✗	✔	✗
P09417	2	5	0	2	**S**		E	✗	n.d.	n.d.
P09429	0	1	1	1	**???**	67	33	✔✗	✔	✔
P09455	20	0	0	2			**S**	✔	n.d.	n.d.
P09467	27	25	0	4	???	**8-33**	92-67	✗	✔	✔
P09488/Q03013/ P46439	42	104	0	0	**0-9**	0	100-91	✗	✔	✗
P09493/P06753/ P67936/P07951	17	38	1	0	**0-11**	0	100-89	✔	n.d.	n.d.
P09496	0	1	1	0	**???**	55-64	45-36	✔	✔	✔
P09525	13	7	No results		0-20	**60-30**	40-50	n.d.	✔	n.d.
P09622	8	14	1	0	Es	**S**		✗	n.d.	n.d.
P09960	1	0	No results		73-45	0-27	**27**	n.d.	✔	n.d.
P09972	2	3	No results		42	**25**	33	n.d.	✔	n.d.
P0C0L4/P0C0L5	18	12	1	2	18	**64**	18	✗	✔	✔
P10109	4	1	0	1			**S**	✔	n.d.	n.d.
P10253	6	8	1	0	Es	**S**		✗	n.d.	n.d.
P10515	0	3	No results		**100**	0	0	n.d.	✔	n.d.
P10599	4	5	2	0	100	**0**	0	✗	✗	✔
P10606	7	10	1	**1**	Es	**S**	E	✗	n.d.	n.d.
P10619	0	7	3	0	**???**	58	42	✔	✔	✔
P10620	2	1	0	1			**S**	✔	n.d.	n.d.
P10768	4	2	0	1			**S**	✔	n.d.	n.d.
P10809	96	114	3	1	???	**89**	11	✗	✔	✔
P10909	1	7	2	2	**25-16**	8-17	67	✗✔	✔	✔
P11021	52	92	3	1	18	**82**	0	✗	✔	✗✔
P11142	46	47	4	0	0	**0-18**	100-82	✗	✔	✗
P11168	0	1	0	2	**0**	0	100	✗	✗	✔
P11177	3	9	No results		**S**			n.d.	n.d.	n.d.
P11182	8	5	1	0	91-73	**0-18**	9	✗	✔	✔
P11310	18	8	0	2	???	67-75	**33-25**	✔	✔	✔
P11498	64	32	0	1			**S**	✔	n.d.	n.d.
P11509/P20853/Q16696	2	2	0	3		**S**	E	✗	n.d.	n.d.

P11586	41	39	0	6	???	**0-9**	100-91	✗	✔	✔
P11712	9	0	0	7	???	0-10	**100-90**	✔	✔	✔
P12268	0	1	No results		**75**	25	0	n.d.	✔	n.d.
P12429	1	0	1	0	E		**S**	✗	n.d.	n.d.
P12694	7	0	0	1			**S**	✔	n.d.	n.d.
P12814/P35609/O43707	40	24	No results			**S**	**s**	n.d.	n.d.	n.d.
P12955	4	2	1	2	100-91	0-9	**0**	✗✔	✗	✗✔
P12956	1	0	No results				**S**	n.d.	n.d.	n.d.
P13489	2	0	No results				**S**	n.d.	n.d.	n.d.
P13639	24	4	1	0	91-82	0-9	**9**	✗	✔	✔
P13645	3	0	0	1			**S**	✔	n.d.	n.d.
P13667	23	29	No results		64	**27**	9	n.d.	✔	n.d.
P13674	0	3	No results		**67**	33	0	n.d.	✔	n.d.
P13693	5	9	0	4	42	**58**	0	✗	✔	✗
P13716	2	4	0	1	**0-9**	27	73-64	✗	✔	✔
P13796	28	9	0	1	0	100	**0**	✔	✗	✗
P13797	17	3	No results				**S**	n.d.	n.d.	n.d.
P13798	2	0	No results				**S**	n.d.	n.d.	n.d.
P13804	19	30	0	3	???	**82**	18	✗	✔	✔
P13861	2	0	No results		100-91	0-9	**0**	n.d.	✗	n.d.
P14174	2	1	2	1	20	40-20	**40-60**	✗✔	✔	✔
P14314	13	15	No results		???	**67**	33	n.d.	✔	n.d.
P14324	0	1	4	0	**S**			✔	n.d.	n.d.
P14550	7	11	0	1	33	**67**	0	✗	✔	✗
P14618	3	0	2	0	E		**S**	✗	n.d.	n.d.
P14625	102	110	1	3	100	**0**	0	✗	✗	✗✔
P14923	1	0	No results		18-9	18	**64-73**	n.d.	✔	n.d.
P14927	4	5	4	0	E**s**	**S**		✗	n.d.	n.d.
P15090	0	7	No results		**0**	**100**	**0**	n.d.	✗	n.d.
P15104	0	5	1	2	**50-58**	**17-0**	**33-41**	✗✔	✔	✔
P15121	1	0	1	0	30	20	**50**	✗	✔	✔
P15311	13	0	2	0			**S**	✔	n.d.	n.d.
P15374	0	3	1	0	**8**	92	0	✔	✔	✔
P15531/P22392	24	37	7	0	70	**30**	0	✗	✔	✔
P15880	3	0	1	1	E		**S**	✗✔	n.d.	n.d.
P16083/Q08257	12	10	No results		???	**0-17**	100-83	n.d.	✔	n.d.
P16152/O75828	16	39	No results		**???**	0-8	100-92	n.d.	✔	n.d.
P16219	11	3	0	5	0-33	75-33	**25-33**	✔	✔	✔
P16278	6	1	No results		0-25	17-8	**83-67**	n.d.	✔	n.d.
P16402	10	0	No results				**S**	n.d.	n.d.	n.d.
P16435	15	13	0	3	???	**60**	40	✗	✔	✔
P16455	0	1	0	0	**???**	0-11	100-89	✗	✔	✔
P16930	9	11	0	5	**s**	**S**	E	✗	n.d.	n.d.
P16949	2	6	4	0	**75**	25	0	✔	✔	✔

P17174	19	15	0	1		**S**	Es	✗	n.d.	n.d.
P18077	1	0	1	0	E		**S**	✗	n.d.	n.d.
P18124	4	4	2	0	E	**S**		✗	n.d.	n.d.
P18206	19	3	No results				**S**	n.d.	n.d.	n.d.
P18283	1	0	1	1	E		**S**	✗✔	n.d.	n.d.
P18621	2	0	No results				**S**	n.d.	n.d.	n.d.
P18669	12	19	No results		**s**	**S**		n.d.	n.d.	n.d.
P19013	1	0	0	0		E	**S**	✗	n.d.	n.d.
P19105	12	7	No results			**S**	**s**	n.d.	n.d.	n.d.
P19404	2	3	No results		0-27	**73-27**	27-45	n.d.	✔	n.d.
P19440	3	0	0	0		E	**S**	✗	n.d.	n.d.
P19971	8	11	No results		92	**8**	0	n.d.	✔	n.d.
P20073	3	0	0	1	83-67	0-17	**17**	✔	n.d.	n.d.
P20132	1	0	0	1			**S**	✔	n.d.	n.d.
P20290	1	1	No results		50-58	**42-33**	8	n.d.	✔	n.d.
P20339/P51148	4	8	No results		**45**	55	0	n.d.	✔	n.d.
P20340/Q9NRW1	8	0	0	1	18-55	9-0	**73-45**	✔	✔	✔
P20618	8	5	1	0	E	**S**	**s**	✗	n.d.	n.d.
P20674	4	5	0	1		**S**	E	✗	n.d.	n.d.
P20962	2	1	No results				**S**	n.d.	n.d.	n.d.
P21266	1	0	0	2	0	100	**0**	✔	✗	✗
P21281	4	0	1	0	27-36	9	**64-55**	✗	✔	✔
P21291	2	3	0	1	**s**	**S**	E	✗	n.d.	n.d.
P21333	1	0	No results		0	100	**0**	n.d.	✗	n.d.
P21399	23	33	0	3	45	**0**	**55**	✗	✗	✔
P21549	49	48	2	4	E	S	E	✗	n.d.	n.d.
P21695	8	16	0	1	**S**		E	✗	n.d.	n.d.
P21757	0	1	No results		**55**	45	0	n.d.	✔	n.d.
P21796	11	19	No results		???	**100**	0	n.d.	✔	n.d.
P21912	11	19	0	1	27-55	**27-0**	45	✗	✔	✔
P21953	3	2	0	1		**S**	Es	✗	n.d.	n.d.
P21964	7	0	1	1	75	25	**0**	✔✗	✗	✔✗
P22033	22	2	0	2			**S**	✔	n.d.	n.d.
P22061	0	5	1	1	**S**		**E**	✔✗	n.d.	n.d.
P22234	3	0	No results				**S**	n.d.	n.d.	n.d.
P22307	44	84	0	7	0	**0**	100	✗	✗	✔
P22309/P36509/P22310/Q9HAW7	54	0	0	0		E	**S**	✗	n.d.	n.d.
P22314	13	0	No results		18	82	**0**	n.d.	✗	n.d.
P22352	4	0	0	2			**S**	✔	n.d.	n.d.
P22570	6	5	1	1	100	**0**	0	✗	✗	✔✗
P22626	0	5	3	0	**80**	0	20	✔	✔	✔
P22695	10	3	No results		0-25	75-33	**25-42**	n.d.	✔	n.d.
P22792	0	2	0	2	**67**	25	8	✗	✔	✔
P22830	5	2	No results				**S**	n.d.	n.d.	n.d.

P23141	85	62	1	2	???	**45-55**	55-45	✗	✔	✔
P23284	24	37	3	1	0	**64-82**	36-18	✔✗	✔	✔
P23378	8	0	No results		0-29	0	**100-71**	n.d.	✔	n.d.
P23381	1	0					**S**	n.d.	n.d.	n.d.
P23396	1	1				**S**		n.d.	n.d.	n.d.
P23434	3	2	0	1		**S**	**Es**	✗	n.d.	n.d.
P23526	1	0	1	1		E	**S**	✗	n.d.	n.d.
P23528	25	23	No results			**S**		n.d.	n.d.	n.d.
P23786	11	14	0	1	**s**	**S**	E	✗	n.d.	n.d.
P24298	3	0	0	0		E	**S**	✗	n.d.	n.d.
P24534	1	2	No results		**67**	0	33	n.d.	✔	n.d.
P24539	4	0	1	0	E		**S**	✗	n.d.	n.d.
P24666	11	5	No results				**S**	n.d.	n.d.	n.d.
P24752	37	36	0	4	???	**9-55**	91-45	✗	✔	✔
P25311	2	0	0	3	0-8	33-50	**67-42**	✔	✔	✔
P25325	13	14	No results		75-83	**25-8**	0-8	n.d.	✔	n.d.
P25398	5	6	No results		25	**75**	0	n.d.	✔	n.d.
P25705	50	70	1	1	???	**83**	17	✗	✔	✔
P25774	2	0	1	0	17	33	**50**	✗	✔	✔
P25786	2	5	1	0	**S**			✔	n.d.	n.d.
P25787	7	4	No results		50-58	**50-17**	0-25	n.d.	✔	n.d.
P25788	3	1	0	1	0-8	75	**25-17**	✔	✔	✔
P25789	2	2	1	1	0-50	**90-0**	10-50	✗	✔	✔
P26038	24	10	No results		0	0-9	**100-91**	n.d.	✔	n.d.
P26373	5	1					**S**	n.d.	n.d.	n.d.
P26440	9	14	0	2	**s**	**S**	E	✗	n.d.	n.d.
P26599	5	3	1	0	82	**18**	0	✗	✔	✔
P26641	2	0	1	0	0-10	0-10	**100-80**	✗	✔	✔
P26885	7	8	No results		33	**67**	0	n.d.	✔	n.d.
P27105	2	0	0	2	0-42	33-0	**67-58**	✔	✔	✔
P27144	8	15	1	1	**Es**	**S**	E	✗	n.d.	n.d.
P27169	2	10	0	5	**???**	6	94	✗	✔	✔
P27338	7	0	0	1	???	75	**25**	✔	✔	✔
P27348	2	15	No results		**92-67**	0-17	8-16	n.d.	✔	n.d.
P27708	0	6			**18-27**	55-45	27	n.d.	n.d.	n.d.
P27797	10	17	2	0	67	**33-8**	0-25	✗	✔	✔
P27824	0	2	2	1	**56**	22	22	✔✗	✔	✔
P28062	3	0	0	1			**S**	✔	n.d.	n.d.
P28065	4	3	0	0	92	**8**	0	✔	n.d.	n.d.
P28066	3	1	No results				**S**	n.d.	n.d.	n.d.
P28070	2	2	3	0	0	**0-25**	100-75	✗	✔	✗
P28072	1	3	No results		**???**	8-33	92-67	n.d.	✔	n.d.
P28074	1	0					**S**	n.d.	n.d.	n.d.
P28331	19	6					**S**	n.d.	n.d.	n.d.
P28838	17	20	0	2		**S**	E	✗	n.d.	n.d.

P29401	14	6	No results				**S**	n.d.	n.d.	n.d.
P29692	5	8	2	0	Es	**S**		✗	n.d.	n.d.
P29966	2	0	1	0	27-18	18-9	**55-72**	✗	✔	✔
P30038	26	24	1	2	???	**36-55**	64-45	✗	✔	✔
P30039	10	4	0	2			**S**	✔	n.d.	n.d.
P30040	6	10	No results		**s**	**S**		n.d.	n.d.	n.d.
P30041	37	71	1	3	**???**	42-50	58-50	✔✗	✔	✔
P30042	8	18	0	1	**S**		E	✗	n.d.	n.d.
P30043	10	20	1	2	**S**		E	✔✗	n.d.	n.d.
P30044	29	27	No results		83	**17**	0	n.d.	n.d.	n.d.
P30046	5	5	0	3		**S**	E	✗	n.d.	n.d.
P30048	28	23	0	1	17-55	**75-18**	8-27	✗	✔	✔
P30049	12	4	0	1	8-25	92-58	**0-17**	✔	✔	✔
P30050	6	6	1	0	30-50	**40-10**	30-40	✗	✔	✔
P30084	60	48	0	7	???	**91**	9	✗	n.d.	n.d.
P30085	10	15	1	0	Es	**S**		✗	n.d.	n.d.
P30086	32	30	0	2	0	**0**	100	✗	✗	✔
P30101	33	41	3	0	100	**0**	0	✗	✗	✔
P30153	12	3	1	0	60-70	30-20	**10**	✗	✔	✔
P30405	5	1	No results				**S**	n.d.	n.d.	n.d.
P30613	0	2	1	0	**10**	90	0	✔	n.d.	n.d.
P30626	0	6	No results		**S**			n.d.	n.d.	n.d.
P30711	6	3	0	0			**S**	n.d.	n.d.	n.d.
P30837	38	30	0	1	???	**100**	0	✗	✔	✗
P31040	10	8	No results			**S**		n.d.	n.d.	n.d.
P31150/P50395	15	4			8-17	8-0	**83**	n.d.	✔	n.d.
P31327	257	150	1	4	???	**0-40**	100-60	✗	✔	✔
P31513	11	0	0	1	00	27-36	**73-64**	✔	✔	✔
P31930	12	7	1	0	90	**10**	0	✗	✔	✗
P31937	13	23	0	1	???	**58-67**	42-33	✗	✔	✔
P31946	4	23	1	0	**S**			✔	n.d.	n.d.
P31948	2	2	1	0	E	**S**		✗	n.d.	n.d.
P32119	24	42	No results		67	**33-0**	0-33	n.d.	✔	n.d.
P32754	13	9	0	6		**S**	Es	✗	n.d.	n.d.
P32929	3	2	0	2	???	**0-8**	100-92	✗	✔	✔
P32969	2	1	No results		64-45	0-18	**36**	n.d.	✔	n.d.
P33121	19	1	0	4	???	27-45	**73-55**	✔	n.d.	n.d.
P33316	0	10	1	0	**S**			✔	n.d.	n.d.
P34741	0	4	1	2	**18-27**	73-64	9	✔✗	✔	✔
P34896	8	4	0	3	???	42-50	**58-50**	✔	✔	✔
P34897	10	12	0	1	0-17	**33-25**	67-58	✗	✔	✔
P34913	11	4	0	4	0	83	**17**	✔	✔	✔
P35221	0	3	1	0	**???**	80	20	✔	✔	✔
P35222	1	0	3	1	???	8	**92**	✔✗	✔	✔
P35232	31	19	1	1	???	**100**	0	✗	✔	✔✗

P35268	7	8	1	0	E	**S**		✗	n.d.	n.d.
P35270	10	17	No results		**s**	**S**		n.d.	n.d.	n.d.
P35520	0	1	0	2	**0**	0	100	✔	✔	✔
P35542	2	2	0	2	11	**22**	67	✗	✔	✔
P35558	12	12	0	6	0-9	**0-9**	100-82	✗	✔	✔
P35573	2	0	1	1	E		**S**	✔✗	n.d.	n.d.
P35579	10	0	1	0	E		**S**	✗	n.d.	n.d.
P35590	1	0	0	0		E	**S**	✗	n.d.	n.d.
P35914	19	17	0	2	???	**0-22**	100-78	✗	✔	✔
P36269	8	0	0	1	0	0	**100**	✔	✔	✔
P36404	0	1	2	0	**S**			✔	n.d.	n.d.
P36406	1	0	0	1			**S**	✔	n.d.	n.d.
P36542	6	7	No results			**S**		n.d.	n.d.	n.d.
P36543	1	4	1	0	**0**	0-17	100-83	✔	✗	✗
P36551	0	3	No results		**50-33**	0-17	50	n.d.	✔	n.d.
P36578	5	0					**S**	n.d.	n.d.	n.d.
P36776	16	10	0	1	73	**27-9**	0-18	✗	✔	✔
P36871	37	14	0	1	???	0-25	**100-75**	✔	✔	✔
P36957	11	5	No results		???	20-30	**80-70**	n.d.	✔	n.d.
P36969	3	1			0	9	**91**	n.d.	✔	n.d.
P37235	4	1					**S**	n.d.	n.d.	n.d.
P37802	18	26	3	1	17	**83**	0	✗	✔	✔✗
P37837	5	4	2	0	E	**S**		✗	n.d.	n.d.
P37840	0	1	No results		**S**			n.d.	n.d.	n.d.
P38117	26	30	1	0	???	**100**	0	✗	✔	✔
P38159	2	0	No results				**S**	n.d.	n.d.	n.d.
P38606	3	0	No results		78	22	**0**	n.d.	✗	n.d.
P38646	34	67	2	0	E**s**	**S**		✔	n.d.	n.d.
P39019	11	8	3	0	E	**S**	**s**	✗	n.d.	n.d.
P39023	2	0	1	0	17-33	58-42	**25**	✗	✔	✔
P39687	0	1	No results		**80**	20	0	n.d.	✔	n.d.
P40121	3	1	3	0	100	0	**0**	✗	✗	✔
P40227	4	4	2	0	E	**S**		✗	n.d.	n.d.
P40261	2	0	1	6	E		**S**	✔✗	n.d.	n.d.
P40306	1	0	0	1			**S**	✔	n.d.	n.d.
P40429	0	1	No results		**S**			n.d.	n.d.	n.d.
P40925	5	6	1	0	E	**S**		✗	n.d.	n.d.
P40926	26	74	1	0	**???**	83	17	✔	✔	✔
P40939	62	70	No results		0-17	**75-58**	25	n.d.	✔	n.d.
P42126	6	12	0	1	**S**		E	✗	n.d.	n.d.
P42704	7	1	No results				**S**	n.d.	n.d.	n.d.
P42765	42	70	0	3	**s**	**S**	E	✗	n.d.	n.d.
P42766	3	0	2	1	83-58	0-25	**17**	✔✗	✔	✔
P43155	3	0	0	1	92	0	**8**	✔	✔	✔
P43487	0	1	No results		**S**			n.d.	n.d.	n.d.

P43490	9	9	0	3		**S**	E	✗	n.d.	n.d.
P43686	0	1	1	0	**30-40**	40-0	30-60	✔	n.d.	n.d.
P45880	2	4	1	0	**S**			✔	n.d.	n.d.
P45954	31	22	0	6		**S**	E**s**	✗	n.d.	n.d.
P46108	0	1	1	0	**0**	0	100	✔	✗	✗
P46459	1	0	1	0	75	25	**0**	✗	✗	✔
P46776	1	2	No results		**S**			n.d.	n.d.	n.d.
P46777	3	1	1	0	E		**S**	✗	n.d.	n.d.
P46779	0	3	No results		**S**			n.d.	n.d.	n.d.
P46782	5	5	2	0	E	**S**		✗	n.d.	n.d.
P46783	11	5	2	0	E		**S**	✗	n.d.	n.d.
P46940	3	0	1	0	50	0	**50**	✗	✔	✔
P46952	6	6	0	1		**S**	E	✗	n.d.	n.d.
P47914	1	1	2	0	E	**S**		✗	n.d.	n.d.
P47985	7	7	No results			**S**		n.d.	n.d.	n.d.
P48047	20	16			83	**17**	0	n.d.	✔	n.d.
P48147	1	0			0	27	**73**	n.d.	✔	n.d.
P48556	0	1	1	1	**0-30**	10-0	90-70	✔✗	✔	✔
P48637	0	1	No results		**S**			n.d.	n.d.	n.d.
P48643	3	0	5	0	90	10	**0**	✗	✗	✔
P48735	48	32	No results		91-82	**0-9**	9	n.d.	✔	n.d.
P49189	5	3	2	0	???	**33**	67	✗	✔	✔
P49207	0	1	No results		**S**			n.d.	n.d.	n.d.
P49326	2	0			0	0-25	**100-75**	n.d.	✔	n.d.
P49327	24	6	0	1	25-33	58-17	**17-50**	✔	✔	✔
P49411	7	9	0	2	42-50	**42-0**	17-50	✗	✔	✔
P49419	20	14	0	1	0	**58**	42	✗	✔	✗
P49588	1	0	No results				**S**	n.d.	n.d.	n.d.
P49593	0	1	2	0	**S**			✔	n.d.	n.d.
P49720	7	8	1	0	E	**S**		✗	n.d.	n.d.
P49721	8	7	No results		0	**0**	100	n.d.	✗	n.d.
P49748	27	3	0	4	???	25	**75**	✔	n.d.	n.d.
P49755	0	2	1	0	**S**			✔	n.d.	n.d.
P49773	9	3	1	1	E		**S**	✔✗	n.d.	n.d.
P49789	0	1	1	0	**67**	22	11	✔	✔	✔
P49821	2	0	No results				**S**	n.d.	n.d.	n.d.
P49914	0	2	0	1	**???**	64	36	✔	✔	✔
P50053	3	1	2	4	???	0-22	**100-78**	✔✗	✔	✔
P50213	4	8	0	1	**S**		E	✗	n.d.	n.d.
P50225/P50226/ P50224	11	3	0	2	???	50	**50**	✔	✔	✔
P50440	37	64	0	3	???	**0-17**	100-83	✗	✔	✔
P50502/Q8IZP2	4	0	1	0	0	55-64	**45-36**	✗	✔	✗
P50914	5	5	No results		9-27	**73-55**	18	n.d.	✔	n.d.

P50990	4	2			92-83	0-8	**8**	n.d.	✔	n.d.
P50995	6	0			80-90	10-0	**10**	n.d.	✔	n.d.
P51149	20	17			8	**33**	58	n.d.	n.d.	n.d.
P51452	7	5				**S**		n.d.	n.d.	n.d.
P51570	4	4	1	0	27-75	**64-0**	9-25	✗	✔	✔
P51571	5	1	No results				**S**	n.d.	n.d.	n.d.
P51648	1	0	0	2	36-45	27-0	**36-55**	✔	✔	✔
P51649	4	7	No results		**s**	**S**		n.d.	n.d.	n.d.
P51659	30	10	1	2	???	83	**17**	✔✗	✔	✔
P51687	4	3	No results			**S**		n.d.	n.d.	n.d.
P51857	1	0	0	2			**S**	✔	n.d.	n.d.
P51858	0	1	4	0	**50-60**	50-30	0-10	✔	✔	✔
P51884	16	3	No results		33	22	**44**	n.d.	n.d.	n.d.
P51888	6	0	0	1			**S**	✔	n.d.	n.d.
P51970	2	1	No results				**S**	n.d.	n.d.	n.d.
P52565	3	3	1	1	58	**42**	0	✗	✔	✔✗
P52566	4	1	0	1	0	100	**0**	✔	✗	✗
P52597	2	2	No results		67-50	**0-17**	33	n.d.	✔	n.d.
P52758	8	10	0	6	???	**17**	83	✗	✔	✔
P52815	2	9	1	0	**91**	0	9	✔	✔	✔
P52907	1	0	No results				**S**	n.d.	n.d.	n.d.
P52943	1	0					**S**	n.d.	n.d.	n.d.
P53597	3	5			**s**	**S**		n.d.	n.d.	n.d.
P54578	1	0	1	0	0	75	**25**	✗	✔	✗
P54819	14	34	No results		**82**	18	0	n.d.	✔	n.d.
P54855/O75795	2	0					**S**	n.d.	n.d.	n.d.
P54868	42	76	0	3	**s**	**S**	E	✗	n.d.	n.d.
P54886	1	0	2	0	0-8	8-0	**92**	✗	✔	✔
P54920/Q9H115	2	0	0	1	91	0	**9**	✔	n.d.	n.d.
P55072	28	52	3	0	92	**8**	0	✔	✔	✔
P55083	1	0	0	1			**S**	✔	n.d.	n.d.
P55084	28	10	0	2			**S**	✔	n.d.	n.d.
P55145	9	7	1	0	???	**58**	42	✗	✔	✔
P55157	15	0	0	1			**S**	✔	n.d.	n.d.
P55263	0	1	0	2	**S**		E	✗	n.d.	n.d.
P55327	1	3	2	0	**S**			✔	n.d.	n.d.
P55735	1	0	1	0	E		**S**	✗	n.d.	n.d.
P55957	4	7	No results		40-36	**50-9**	10-55	n.d.	✔	n.d.
P56134	1	0	1	0	E		**S**	✗	n.d.	n.d.
P56470	2	1	No results				**S**	n.d.	n.d.	n.d.
P56537	1	1				**S**		n.d.	n.d.	n.d.
P56556	2	1					**S**	n.d.	n.d.	n.d.
P57105	3	0			100-92	0-8	**0**	n.d.	✗	n.d.
P59190	4	1					**S**	n.d.	n.d.	n.d.
P60174	34	57	1	0	11-22	**56-22**	33-56	✗	✔	✔

P60604	0	1	No results		**58**	42	0	n.d.	✔	n.d.
P60660	19	19				**S**		n.d.	n.d.	n.d.
P60709/P63261	146	80	3	0	E	**S**		✗	n.d.	n.d.
P60842	2	0	0	2			**S**	✔	n.d.	n.d.
P60866	8	5	2	1	42	**42**	17	✗	✔	✔
P60900	5	4	No results		91	**0**	9	n.d.	✗	n.d.
P60953	18	16			80	**20**	0	n.d.	✔	n.d.
P60981	15	4	0	0		E	**S**	✗	n.d.	n.d.
P60983	6	2	1	1	100	0	**0**	✗✔	✗	✗✔
P61019	0	3	No results		**20**	80	0	n.d.	✔	n.d.
P61081	2	4	2	0	**55**	45	0	✔	✔	✔
P61088	8	6	1	0	0	**0-17**	100-83	✗	✔	✗
P61106	41	0	0	1	0-9	64-73	**36-18**	✔	✔	✔
P61158	1	1	1	0	42-67	**17-0**	42-33	✗	✔	✔
P61201	1	0	No results		8	0	**92**	n.d.	✔	n.d.
P61224/P62834	19	13			0	**100**	0	n.d.	✔	n.d.
P61247	0	1			**S**			n.d.	n.d.	n.d.
P61353	0	1	1	0	**???**	60	40	✔	✔	✔
P61457	0	1	0	2	**S**		E	✗	n.d.	n.d.
P61586	20	10	3	0	91	9	**0**	✗	✗	✔
P61604	2	3	1	0	100	**0**	0	✗	✗	✔
P61626	2	0	3	0	58	42	**0**	✗	✗	✔
P61916	3	2	No results		0	**0**	100	n.d.	✗	n.d.
P61956	6	9	1	0	**s**	**S**		n.d.	n.d.	n.d.
P61978	6	5	1	0	75	**25**	0	✗	✔	✔
P61981	3	20	No results		**92-83**	0-8	8	n.d.	✔	n.d.
P62081	10	9	2	0	E	**S**		✗	n.d.	n.d.
P62136/P62140	7	0	0	1	0	0	**100**	✔	✔	✔
P62158	7	14	1	0	**0**	67	33	✔	✗	✗
P62191	1	1	No results		0	**0-8**	100-92	n.d.	✔	n.d.
P62195	0	1	3	1	**42**	50	8	✗✔	✔	✔
P62241	6	2	2	0	E		S	✗	n.d.	n.d.
P62244	5	3	No results			**S**	**s**	n.d.	n.d.	n.d.
P62249	7	7	3	0	E	**S**		✗	n.d.	n.d.
P62256	0	1	No results		**0-30**	40-10	60	n.d.	✔	n.d.
P62258	3	12			**S**			n.d.	n.d.	n.d.
P62263	12	7			0	**73**	27	n.d.	✔	n.d.
P62266	0	2			**S**			n.d.	n.d.	n.d.
P62269	16	27	1	0	E**s**	**S**		✗	n.d.	n.d.
P62277	13	12	No results			**S**		n.d.	n.d.	n.d.
P62280	2	0					**S**	n.d.	n.d.	n.d.
P62318	1	0			100	0	**0**	n.d.	✗	n.d.
P62330	7	5	1	1	0	**80**	20	✗	✔	✗✔
P62333	4	0	No results				**S**	n.d.	n.d.	n.d.
P62424	6	2					**S**	n.d.	n.d.	n.d.

P62491/Q15907	8	0	2	0	17	67	**17**	✗	✔	✔
P62633	0	1	2	1	**S**		E	✗✔	n.d.	n.d.
P62701	4	4	1	0	80-90	**20-0**	0-10	✗	✔	✔
P62750	2	4	1	0	**S**			✔	n.d.	n.d.
P62753	2	3	1	0	58	**42-33**	0-8	✗	✔	✔
P62805	7	1	No results				**S**	n.d.	n.d.	n.d.
P62820/Q9H0U4/Q15286	45	6	1	0	???	82	**18**	✗	✔	✔
P62826	3	4	1	0	E	**S**		✗	n.d.	n.d.
P62829	4	4	No results		???	**18**	82	n.d.	n.d.	n.d.
P62837	0	4	1	0	**S**			✔	n.d.	n.d.
P62841	5	3	No results			**S**	**s**	n.d.	n.d.	n.d.
P62847	5	4	3	0	8	**92**	0	✗	✔	✔
P62851	5	4	No results			**S**		n.d.	n.d.	n.d.
P62854	5	3	0	1	E	**S**	**s**	✗	n.d.	n.d.
P62873/P62879	3	0	2	0	58	42	**0**	✗	✗	✔
P62888	6	3	4	0	88	13	**0**	✗	✗	✔
P62899	0	3	No results		**S**			n.d.	n.d.	n.d.
P62910	3	2				**S**		n.d.	n.d.	n.d.
P62913	6	5			89-56	**0-33**	11	n.d.	✔	n.d.
P62917	1	4	2	0	**S**			✔	n.d.	n.d.
P62937	24	23	5	0	67	**33**	0	✗	✔	✔
P62942	2	2	1	0	11	**89**	0	✗	✔	✔
P63000/P15153	25	21	1	0	18	**82**	0	✗	✔	✔
P63027	0	2	No results		**S**			n.d.	n.d.	n.d.
P63104	5	17	2	0	**100**	0	0	✔	✔	✔
P63208	0	4	No results		**42-33**	50-33	8-33	n.d.	✔	n.d.
P63241	18	16	No results		70-60	**0-10**	30	n.d.	✔	n.d.
P63244	6	5	2	0	100	**0**	0	✗	✗	✔
P68036	7	4	1	0	E	**S**	**s**	✗	n.d.	n.d.
P68104	11	9	0	1		**S**	E	✗	n.d.	n.d.
P68363/P68366	90	40	3	0	???	64-73	**36-27**	✗	✔	✔
P68402	1	0	No results				**S**	n.d.	n.d.	n.d.
P68871/...	54	84	1	1	E**s**	**S**	E	✗	n.d.	n.d.
P78371	4	1	1	0	E		**S**	✗	n.d.	n.d.
P78417	16	11	0	3		**S**	E**s**	✗	n.d.	n.d.
P78560	1	0	0	2	27	9	**64**	✔	✔	✔
P80404	55	50	1	1	E	**S**	E	✗	n.d.	n.d.
P82650	0	1	No results		**???**	38	63	n.d.	n.d.	n.d.
P82909	0	1			**S**			n.d.	n.d.	n.d.
P82980	3	3	0	1		**S**	E	✗	n.d.	n.d.
P83111	3	0	No results				**S**	n.d.	n.d.	n.d.
P83731	0	4	1	0	**S**			✔	n.d.	n.d.
P84077/P61204/P18085	48	67	1	0	0	**100**	0	✗	✔	✗

P84243/P68431	33	0	2	1	E		**S**	✗✔	n.d.	n.d.
P99999	12	12	No results		???	**100**	0	n.d.	✔	n.d.
Q00059	0	1			**S**			n.d.	n.d.	n.d.
Q00266	3	0	0	4			**S**	✔	n.d.	n.d.
Q00341	0	1	No results		**18**	82	0	n.d.	✔	n.d.
Q00796	10	14	0	2	**s**	**S**	E	✗	n.d.	n.d.
Q01082	1	0	No results				**S**	n.d.	n.d.	n.d.
Q01469	2	3	3	0	10	**90**	0	✗	✔	✔
Q01518	11	1	No results				**S**	n.d.	n.d.	n.d.
Q01581	7	7	1	0	E	**S**		✗	n.d.	n.d.
Q01995	25	5	0	1	18	82	**0**	✔	✗	✗
Q02218	2	0	No results		0	25	**75**	n.d.	✔	n.d.
Q02252	48	37	0	1		**S**	E	✗	n.d.	n.d.
Q02338	16	10	0	2		**S**	E**s**	✗	n.d.	n.d.
Q02818	5	4	1	2	75	**17**	8	✗	✔	✔
Q02878	5	1	2	0	E		**S**	✗	n.d.	n.d.
Q02928	4	0	0	2			**S**	✔	n.d.	n.d.
Q03154	8	8	0	3	0	**0**	100	✗	✗	✔
Q04760	10	17	No results		**s**	**S**		n.d.	n.d.	n.d.
Q04828/P52895/ P42330/P17516	28	36	0	0		**S**		✔	n.d.	n.d.
Q04837	14	19	1	0	75-88	**0**	25-13	✗	✗	✔
Q04917	0	12	2	0	**S**			n.d.	n.d.	n.d.
Q05639	5	0	No results				**S**	n.d.	n.d.	n.d.
Q05682	2	0			0	100	**0**	n.d.	✗	n.d.
Q06278	3	0	0	2			**S**	✔	n.d.	n.d.
Q06323	8	11	No results		10-50	**60-10**	30-40	n.d.	✔	n.d.
Q06520	13	12	0	2	???	**73**	27	✗	✔	✔
Q06830	48	59	2	3	60	**30-0**	10-40	✗	✔	✔
Q07020	5	3	No results			**S**		n.d.	n.d.	n.d.
Q07021	0	1	0	1	**S**		E	✗	n.d.	n.d.
Q07065	2	0	1	0	55-45	27	**18-27**	✗	✔	✔
Q07131	57	25	0	2			**S**	n.d.	n.d.	n.d.
Q08380	3	0	1	0	42-33	0	**58-66**	✗	✔	✔
Q08426	33	52	0	1	**s**	**S**	E	✗	n.d.	n.d.
Q10713	4	0	0	1	50	42	**8**	✔	✔	✔
Q12849	0	2	No results		**S**			n.d.	n.d.	n.d.
Q12931	11	7			0	**0-10**	100-90	n.d.	✔	n.d.
Q13011	22	24	0	2	???	**91**	9	✗	✔	✔
Q13126	1	2	0	1	**S**			n.d.	n.d.	n.d.
Q13162	37	39	0	1	0	**82**	18	✗	✔	✔
Q13228	18	37	0	3	???	**0-17**	100-83	✗	✔	✔
Q13268	0	35	0	0	**S**	E		✗	n.d.	n.d.
Q13404	14	7	No results				**S**	n.d.	n.d.	n.d.
Q13405	2	3	1	0	E**s**	**S**		✗✔	n.d.	n.d.
Q13409	1	0	No results				**S**	n.d.	n.d.	n.d.

Q13418	1	0	0	0	58	42	**0**	n.d.	✗	n.d.
Q13423	2	0	No results		27-36	27-18	**45**	n.d.	✔	n.d.
Q13510	11	1	No results		0	17-50	**83-50**	n.d.	✔	n.d.
Q13526	1	1	1	0	0	**100**	0	✗	✔	✗
Q13561	1	0	No results				**S**	n.d.	n.d.	n.d.
Q13576	2	0			78	22	**0**	n.d.	✗	n.d.
Q13813	15	3	1	0	E		**S**	✗	n.d.	n.d.
Q13907	2	6	3	0	**S**			✔	n.d.	n.d.
Q14011	0	3	0	0	**S**	E		✗	n.d.	n.d.
Q14019	11	2	0	1			**S**	✔	✔	✔
Q14032	9	9	0	1		**S**	E	✗	n.d.	n.d.
Q14103	3	5	No results		91	**9**	0	n.d.	✔	n.d.
Q14117	5	1	0	1	0	25-33	**75-67**	✔	✔	✔
Q14165	1	0	No results		0-9	18-27	**82-64**	n.d.	✔	n.d.
Q14197	1	2			**0**	91	9	n.d.	✗	n.d.
Q14203	2	0			100-83	0-17	**0**	n.d.	✗	n.d.
Q14353	2	5	0	4	**S**		E	✗	n.d.	n.d.
Q14696	0	5	No results		**S**			n.d.	n.d.	n.d.
Q14697	42	37			100-73	**0-27**	0	n.d.	✔	n.d.
Q14749	6	6	1	2	???	**33**	67	✗	✔	✔
Q14914	9	3	No results				**S**	n.d.	n.d.	n.d.
Q14964	3	0					**S**	n.d.	n.d.	n.d.
Q15056	0	1			**S**			n.d.	n.d.	n.d.
Q15067	5	4	0	2	???	**27**	73	✗	✔	✔
Q15084	22	25	0	1		**S**	E	✗	n.d.	n.d.
Q15121	1	0	3	0	E		**S**	✗	n.d.	n.d.
Q15126	3	3	No results			**S**		n.d.	n.d.	n.d.
Q15181	4	6			58-67	**42-25**	8	n.d.	✔	n.d.
Q15185	1	5	1	0	**S**			✔	n.d.	n.d.
Q15274	0	7	No results		**S**			n.d.	n.d.	n.d.
Q15293	1	2	0	3	**S**		E	✗	n.d.	n.d.
Q15363	2	0	3	1	50	50	**0**	✔✗	✗	✔✗
Q15365	1	0	0	1			**S**	✔	n.d.	n.d.
Q15370	1	0	1	1	E		**S**	✔✗	n.d.	n.d.
Q15404	0	3	No results		**S**			n.d.	n.d.	n.d.
Q15435	1	0	1	0	E		**S**	✗	n.d.	n.d.
Q15436	3	0	0	1			**S**	✔	n.d.	n.d.
Q15493	2	2	1	5	E	**S**	E	✗	n.d.	n.d.
Q15819	15	0	No results				**S**	n.d.	n.d.	n.d.
Q16134	12	6	0	1			**S**	✔	n.d.	n.d.
Q16540	1	3	No results		**S**			n.d.	n.d.	n.d.
Q16543	1	0	1	0	E		**S**	✗	n.d.	n.d.
Q16595	2	1	0	1	0	0-18	**100-82**	✔	✔	✔
Q16619	0	4	0	0	**S**	E		✗	n.d.	n.d.
Q16698	11	22	0	3	**???**	82	18	✗	✔	✔

Q16718	1	1	1	1	E	**S**	E	✗	n.d.	n.d.
Q16740	0	3	0	1	**100**	0	0	✗	✔	✗
Q16762	18	26	1	4	???	**83**	17	✗	✔	✔
Q16775	5	14	0	1	**S**		E	✗	n.d.	n.d.
Q16822	71	150	0	4	**0**	36	64	✗	✗	✔
Q16836	15	17	0	2		**S**	E	✗	n.d.	n.d.
Q16851	57	26	0	2			**S**	✔	n.d.	n.d.
Q16891	6	3	No results		0	9-45	**91-55**	n.d.	✔	n.d.
Q3LXA3	29	31	0	2		**S**		n.d.	n.d.	n.d.
Q4VC31	1	0	No results				**S**	n.d.	n.d.	n.d.
Q5HYK3	2	6			**S**			n.d.	n.d.	n.d.
Q5T2W1	2	1	2	0	0	0-27	**100-73**	✗	✔	✗
Q6IBS0	4	1	1	0			**S**	✔	✔	✔
Q6P587	5	6	No results		0-30	**30-20**	70-50	n.d.	✔	n.d.
Q7KZF4	2	0			100	0	**0**	n.d.	✗	n.d.
Q7Z4W1	23	37	0	3	???	**67-75**	33-25	✗	✔	✔
Q7Z5P4	9	21	No results		**S**			n.d.	n.d.	n.d.
Q86SX6	1	2			**S**			n.d.	n.d.	n.d.
Q86TX2/P49753	20	21	0	1		**S**	E	✗	n.d.	n.d.
Q86UP2	4	3	No results		100	**0**	0	n.d.	✗	n.d.
Q86VB7	1	0	1	3	0	100	**0**	✔✗	✗	✗
Q86WA6	4	19	No results		**S**			n.d.	n.d.	n.d.
Q86WU2	5	5	0	1		**S**	E	✗	n.d.	n.d.
Q86X76	0	3	0	1	**0-18**	0	100-82	✗	✔	✔
Q86X83	1	0	No results				**S**	n.d.	n.d.	n.d.
Q8IVH4	1	0					**S**	n.d.	n.d.	n.d.
Q8N4P3	0	4			**S**			n.d.	n.d.	n.d.
Q8N5N7	1	3			**???**	8-9	92-91	n.d.	✔	n.d.
Q8N655	0	1			**S**			n.d.	n.d.	n.d.
Q8N668	0	2			**S**			n.d.	n.d.	n.d.
Q8N983	2	1	2	0	E		**S**	✗	n.d.	n.d.
Q8NBJ7	4	5	No results		58-33	**8-17**	33-50	n.d.	✔	n.d.
Q8NBQ5	2	0	0	1	???	0-33	**100-67**	✔	✔	✔
Q8NBS9	8	8	1	0	E	**S**		✗	n.d.	n.d.
Q8NBX0	0	2	No results		**S**			n.d.	n.d.	n.d.
Q8NE62	7	0					**S**	n.d.	n.d.	n.d.
Q8NI60	1	2			**83-92**	17-0	0-8	n.d.	✔	n.d.
Q8TAA3	0	3			**25**	63	13	n.d.	✔	n.d.
Q8TCD5	1	1			50	**50**	0	n.d.	✔	n.d.
Q8TD30	0	1	0	1	**S**		E	✗	n.d.	n.d.
Q8TF76	1	1	No results			**S**		n.d.	n.d.	n.d.
Q8WUM4	1	0			9-45	36-0	**55**	n.d.	✔	n.d.
Q8WVJ2	2	0					**S**	n.d.	n.d.	n.d.
Q8WW59	14	23			**s**	**S**		n.d.	n.d.	n.d.
Q92506	6	7				**S**		n.d.	n.d.	n.d.
Q92558	0	3	1	0	**0-40**	30-0	70-60	✔	✔	✔

Q92688	0	1	No results		**S**			n.d.	n.d.	n.d.
Q92820	0	3	0	2	**???**	67	33	✗	✔	✔
Q92882	1	1	No results			**S**		n.d.	n.d.	n.d.
Q92945	0	3			**S**			n.d.	n.d.	n.d.
Q92947	7	4	0	1		**S**	**Es**	✔	n.d.	n.d.
Q93052	1	0	No results		45	55	**0**	n.d.	✗	n.d.
Q93077/...	96	0	3	0	E		**S**	✗	n.d.	n.d.
Q93079/...	78	0	No results				**S**	n.d.	n.d.	n.d.
Q93088	33	43	1	5	e	**S**	E	✗	n.d.	n.d.
Q93099	6	2	0	2			**S**	✔	n.d.	n.d.
Q969H8	5	7	No results			**S**		n.d.	n.d.	n.d.
Q96AB3	15	27				**S**		n.d.	n.d.	n.d.
Q96C23	0	1			**S**			n.d.	n.d.	n.d.
Q96CN7	2	0					**S**	n.d.	n.d.	n.d.
Q96DE0	0	3			**S**			n.d.	n.d.	n.d.
Q96EY1	0	1	1	0	**S**			✔	n.d.	n.d.
Q96EY8	4	7	1	0	**Es**	**S**		✔	n.d.	n.d.
Q96F10	1	0	0	1	0-17	50	**50-33**	✔	✔	✔
Q96FV2	1	0	No results		0	0-9	**100-91**	n.d.	✔	n.d.
Q96HR9	4	4	0	1	0-8	**67-58**	33	✗	✔	✔
Q96I99	17	14	0	1		**S**		n.d.	n.d.	n.d.
Q96IU4	11	18	No results		**s**	**S**		n.d.	n.d.	n.d.
Q96KP4	12	3	0	1	0	90	**10**	✔	✔	✔
Q96LJ7	5	7	0	1	???	**9**	91	✗	✔	✔
Q96PE7	5	5	1	0	E	**S**		✗	n.d.	n.d.
Q96R05	0	1	No results		**S**			n.d.	n.d.	n.d.
Q96RQ3	12	0	No results		36	64	**0**	n.d.	✗	n.d.
Q99424	3	0	0	3			**S**	✔	n.d.	n.d.
Q99439	0	1	0	1	**S**		E	✗	n.d.	n.d.
Q99471	1	0	No results		73-55	0	**27-45**	n.d.	✔	n.d.
Q99497	16	26	No results		9	**36**	55	n.d.	✔	n.d.
Q99611	0	1	1	1	**S**		E	✗✔	n.d.	n.d.
Q99623	9	10	No results		40-50	**60-40**	0	n.d.	✔	n.d.
Q99627	2	0					**S**	n.d.	n.d.	n.d.
Q99653	0	2			**0**	0-33	100-67	n.d.	✗	n.d.
Q99714	38	36	0	2	75-83	**25-8**	0-8	✗	✔	✔
Q99733	0	1	No results		**0-18**	73-55	27	n.d.	✔	n.d.
Q99798	23	38	1	0	100-52	**0**	0-47	✔	✔	✔
Q99807	3	4	No results			**S**		n.d.	n.d.	n.d.
Q99832	2	0	3	0	0-9	91-82	**0-9**	✗	✔	✔
Q9BPW8	4	14	0	1	**S**			n.d.	n.d.	n.d.
Q9BQ69	1	2	No results		**S**			n.d.	n.d.	n.d.
Q9BRA2	1	0	No results		67-60	17-40	**0**	n.d.	✗	n.d.
Q9BRG1	10	0					**S**	n.d.	n.d.	n.d.
Q9BRX8	0	4			**???**	18	82	n.d.	✔	n.d.
Q9BS26	3	3	2	0	E	**S**		✗	n.d.	n.d.

Q9BSE5	1	7	No results		**92-83**	0-8	8	n.d.	✔	n.d.
Q9BSH4	0	1			**???**	42-58	58-42	n.d.	✔	n.d.
Q9BTZ2	8	10	0	1	0	**0-17**	100-83	✗	✔	✔
Q9BU02	2	0	No results				**S**	n.d.	n.d.	n.d.
Q9BUE6	0	1			**S**			n.d.	n.d.	n.d.
Q9BUP3	0	2	2	1	**92**	0	8	✗✔	✔	✔
Q9BUR5	1	4	No results		**18-45**	27-18	55-36	n.d.	✔	n.d.
Q9BV57	8	5	0	2		**S**	E**s**	✗✔	n.d.	n.d.
Q9BVJ7	3	3	No results			**S**		n.d.	n.d.	n.d.
Q9BVK6	2	3	1	1	58	**42**	0	n.d.	✔	n.d.
Q9BVL4	1	0	No results				**S**	n.d.	n.d.	n.d.
Q9BWD1	4	2	0	0	???	0-10	**100-90**	✗	✔	✔
Q9BX68	13	16	No results		92-67	**8-17**	0-17	n.d.	✔	n.d.
Q9BXW7	3	3	No results		64	**36**	0	n.d.	✔	n.d.
Q9BY32	0	1			**0**	40-60	60-40	n.d.	✗	n.d.
Q9BYD1	0	4			**S**			n.d.	n.d.	n.d.
Q9BYV1	1	0					**S**	n.d.	n.d.	n.d.
Q9GZT3	3	4			50	**0**	50	n.d.	✗	n.d.
Q9H082	3	0					**S**	n.d.	n.d.	n.d.
Q9H0W9	10	3	0	1			**S**	✔	n.d.	n.d.
Q9H2L5	0	1	No results		**S**			n.d.	n.d.	n.d.
Q9H2U2	10	5					**S**	n.d.	n.d.	n.d.
Q9H2W6	0	2	0	1	**S**		E	✗	n.d.	n.d.
Q9H7Z7	3	6	2	0	**???**	0-8	100-92	✔	✗	✗
Q9H8H3	3	0	No results		0-8	0	**100-92**	n.d.	✔	n.d.
Q9H936/Q9H1K4	7	9			**s**	**S**		n.d.	n.d.	n.d.
Q9H9B4/Q9BWM7	4	5			???	**42-67**	58-33	n.d.	✔	n.d.
Q9H9J2	0	4			**S**			n.d.	n.d.	n.d.
Q9HAT2	0	1			**S**			n.d.	n.d.	n.d.
Q9HAV7	4	12			**S**			n.d.	n.d.	n.d.
Q9HCC0	11	11				**S**		n.d.	n.d.	n.d.
Q9HCN8	5	0	0	0	0-64	35-36	**65-0**	✗	✔	✔
Q9HDC9	3	4	0	2	???	**44-67**	56-33	✗	✔	✔
Q9NNW7	3	0	0	0	0	36-45	**64-55**	✗	✔	✔
Q9NP72	3	1	No results		???	9-36	**91-64**	n.d.	✔	n.d.
Q9NPJ3	8	5			???	**9-36**	91-64	n.d.	✔	n.d.
Q9NQ50	0	2	0	1	**20-60**	70-0	10-40	✗	✔	✔
Q9NQP4	0	1	No results		**83-67**	0-17	17	n.d.	✔	n.d.
Q9NQX3	1	0	0	1			**S**	✔	n.d.	n.d.
Q9NR28	8	6	0	0	100-80	**0-20**	0	✔	✔	✗
Q9NR31/Q9Y6B6	7	4	0	0	82-55	**9-18**	9-27	✔	✔	✔
Q9NR45	0	1	1	0	**67**	33	0	✔	✔	✔
Q9NR77	4	0	0	2			**S**	✔	n.d.	n.d.
Q9NRV9	3	6	0	1	**S**		E	✗	n.d.	n.d.
Q9NRX2	0	1	No results		**S**			n.d.	n.d.	n.d.
Q9NS69	1	0			???	90	**10**	n.d.	✔	n.d.

Q9NSE4	3	0	2	0	0-17	83-67	**17**	✗	✔	✔
Q9NTM9	0	1	No results		**S**			n.d.	n.d.	n.d.
Q9NUI1	0	2	0	1	**S**		E	✗	n.d.	n.d.
Q9NVI7	1	0	No results				**S**	n.d.	n.d.	n.d.
Q9NVS9	8	11	0	1	58-75	**17-8**	25-17	✗	✔	✔
Q9NX63	2	6	No results		**S**			n.d.	n.d.	n.d.
Q9NYL4	1	1	2	1	E	**S**	e	✗	n.d.	n.d.
Q9NYU2	1	0	No results		???	33	**67**	n.d.	✔	n.d.
Q9NZN3	2	0	0	1			**S**	✔	n.d.	n.d.
Q9P000	1	1	No results		33-58	**25-0**	42-25	n.d.	✔	n.d.
Q9P032	0	8			**S**			n.d.	n.d.	n.d.
Q9P0J0	2	0					**S**	n.d.	n.d.	n.d.
Q9P0Z9	3	2	0	2	???	**83**	16	✗	✔	✔
Q9P2E9	35	7	0	2	???	73	**27**	✔	✔	✔
Q9P2K3	0	2	No results		**S**			n.d.	n.d.	n.d.
Q9UBQ0	0	1			**S**			n.d.	n.d.	n.d.
Q9UBQ7	18	13	0	2	0-17	**8-0**	92-83	✗	✔	✔
Q9UBR1	4	0	0	1	0	0-18	**100-82**	✔	✔	✔
Q9UBR2	1	3	0	1	**???**	33-50	67-50	✗	✔	✔
Q9UBS4	1	2	No results		**9**	91	0	n.d.	✔	n.d.
Q9UDR5	25	18	0	1	0	**0**	100	✗	✗	✔
Q9UFN0	2	4	No results		**S**			n.d.	n.d.	n.d.
Q9UHK6	1	0	0	1			**S**	✔	n.d.	n.d.
Q9UHL4	2	1	0	1	0-17	25-17	**75-67**	✔	✔	✔
Q9UHV9	4	1	1	0			**S**	✗	n.d.	n.d.
Q9UI09	2	0	1	0	E		**S**	✗	n.d.	n.d.
Q9UI17	36	8	No results				**S**	n.d.	n.d.	n.d.
Q9UIJ7	9	33	1	1	**S**E		E	✔✗	n.d.	n.d.
Q9UJ68	1	1	No results		0-8	**42-33**	58	n.d.	✔	n.d.
Q9UJ70	1	0					**S**	n.d.	n.d.	v
Q9UJD0	1	2			**58-67**	33-25	8	n.d.	✔	n.d.
Q9UJM8	32	31	0	2		**S**	E	✗	n.d.	n.d.
Q9UJZ1	6	8	No results		82	**9**	9	n.d.	✔	n.d.
Q9UKK9	0	6	2	0	**0**	8-33	92-67	✔	✗	✗
Q9UL12	21	6	0	1			**S**	✔	n.d.	n.d.
Q9UL46	8	13	No results		**s**	**S**		n.d.	n.d.	n.d.
Q9ULZ3	6	4			36	**18**	45	n.d.	✔	n.d.
Q9UN36	9	1	0	3	0	36-45	**64-55**	✔	✔	✔
Q9UNM6	2	2	1	0	E	**S**		✗	n.d.	n.d.
Q9UPN3	4	0	No results				**S**	n.d.	n.d.	n.d.
Q9Y224	3	0	1	0	E		**S**	✗	n.d.	n.d.
Q9Y237	0	1	No results		**S**			n.d.	n.d.	n.d.
Q9Y277	3	3	0	0	0-18	**73-55**	27	✔	✔	✔
Q9Y2B0	10	12	1	0	E**s**	**S**		✗	n.d.	n.d.
Q9Y2Q3	11	25	No results		**???**	**50-58**	50-42	n.d.	✔	n.d.

Q9Y2Q9	1	0	1	0	E		**S**	✗	n.d.	n.d.
Q9Y2S2	5	6	0	2		**S**	E	✗	n.d.	n.d.
Q9Y2T3	2	2	No results		???	**67**	33	n.d.	✔	n.d.
Q9Y2V2	0	1			**S**			n.d.	n.d.	n.d.
Q9Y3A6	1	0					**S**	n.d.	n.d.	n.d.
Q9Y3B7	0	5			**S**			n.d.	n.d.	n.d.
Q9Y3B8	0	1			**S**			n.d.	n.d.	n.d.
Q9Y3C6	1	0					**S**	n.d.	n.d.	n.d.
Q9Y3D2	0	4			**S**			n.d.	n.d.	n.d.
Q9Y3D6	12	7			45	**55**	0	n.d.	✔	n.d.
Q9Y3D9	2	2	1	0	25-33	**58-50**	17-16	✗	✔	✔
Q9Y3E5	1	0	No results		???	91	**9**	n.d.	✔	n.d.
Q9Y3I0	1	0			100	0	**0**	n.d.	✗	n.d.
Q9Y490	31	5					**S**	n.d.	n.d.	n.d.
Q9Y4L1	14	18	No results		**s**	**S**		n.d.	n.d.	n.d.
Q9Y4Z0	1	0	2	0	E		**S**	✗	n.d.	n.d.
Q9Y5Z4	3	3	0	1	0	**50-67**	50-33	✗	✔	✔
Q9Y617	8	0	No results		0	0-38	**100-63**	n.d.	✔	n.d.
Q9Y6H1	0	3			**S**			n.d.	n.d.	n.d.
Q9Y6M9	2	0					**S**	n.d.	n.d.	n.d.
Q9Y6N5	4	0			???	100	**0**	n.d.	✗	n.d.

Table 34: List of functionally classified proteins identified in liver (blue) and HCC (orange) by mass spectrometry (see following pages). Proteins are assigned to metabolic pathways based on the databases Brenda (www.brenda-enzymes.org), Kegg pathway database (www.genome.jp/kegg/pathway.html) and Uniprot (www.uniprot.org) as well as biochemical and cell biological literature. In addition, the subcellular location is given for each protein based on informations from Uniprot and PubMed. (Abbreviations used in this table: b: biosynthesis, cytor c: cytoplasma (cytosol), d: degradation, ER: endoplasmic reticulum, lys: lysosome, m: metabolism, mito: mitochondrium, ns: non specified, p: peroxisome, rec: receptor, t: transfer, sec or s: secreted)

Uniprot-ID	Liver			HCC			Compartment	Energy metabolism (E)		Amino acid metabolism (A)													(U)	(Y)
	Dp	Tp	Coverage (%)	Dp	Tp	Coverage (%)		Respiratory chain	TCA	Ala, asp	Arg, pro	Cysteine	Glutamate	Gly, ser, thr	Histidine	Lysine	Methionine	Phenylalanine	Selenoamino	Val, leu, ile	Tryptophan	Tyr	Urea cycle	Pyruvate
O00165	1	1	8.6	1	1	8.6	ER/Mito/Nuc																	
O00217	3	4	15.2	2	4	9.5	Mito (im)	m																
O00231	2	2	5.9	1	1	3.1	Cyt																	
O00232	1	1	2.6	0	0	0	Cyt																	
O00264	6	10	37.9	5	7	28.2	ER (membrane)																	
O00299	2	2	12.4	2	3	12.4	Cyt/Nuc																	
O00303	1	2	5.3	0	0	0	Cyt																	
O00305	0	0	0	1	1	3.8	Mem																	
O00499	0	0	0	1	1	3.2	Nuc																	
O00625	0	0	0	1	2	4.1	Nuc																	
O00629	1	1	3.3	0	0	0	Cyt/Nuc																	
O00748	6	7	17.4	9	24	25.4	ER (lumen)																	
O00754	0	0	0	1	1	1.3	Lys																	
O00764	3	4	11.9	3	4	10.6	Cyt																	
O14558	1	1	8.1	0	0	0	Cyt																	
O14561	0	0	0	1	1	5.8	Mito	t																
O14602	1	2	6.9	2	11	15.3	Cyt																	
O14618	0	0	0	1	1	10.2	Cyt																	
O14737	0	0	0	1	1	10.4	ns																	
O14745	0	0	0	3	3	15.9	Cyt																	
O14773	3	7	9.8	3	4	9.8	Lys																	
O14818	2	3	12.1	5	6	23.4	Cyt																	
O14874	1	1	4.1	0	0	0	Mito (matrix)													r				
O14880	1	1	17.8	0	0	0	ER (membrane)																	
O14933	1	1	17.1	0	0	0	Cyt																	
O15116	1	1	12	1	1	12	Nuc																	
O15145	3	4	17.4	3	4	17.4	Cyt																	
O15173	0	0	0	1	1	4	Mem																	
O15511	4	4	33.8	1	2	7.9	Cyt																	
O43169	2	3	8.9	1	1	8.9	Mito (outer membrane)																	
O43175	8	13	19.9	0	0	0	Cyt							m										
O43181	1	3	6.3	0	0	0	Mito (im)	m																
O43324	1	1	5.7	0	0	0	Cyt																	
O43399	0	0	0	1	1	6.8	ns																	
O43464	1	1	6.1	2	2	5.7	Mito (intermembrane)																	
O43488/O95154/Q8NHP1	8	11	35.3	6	13	23	ER/Golgi																	
O43598	3	3	16.7	4	6	25.3	Nuc																	
O43615	0	0	0	1	1	6.2	Mito (inner membrane)																	
O43617	3	4	11.7	1	2	7.2	ER																	
O43708	0	0	0	1	1	9.3	Cyt															m		
O43852	0	0	0	1	4	6	Sec																	
O43895	0	0	0	3	3	7.1	Mem																	
O60218	4	4	19	10	18	42.1	Cyt																	
O60234	1	1	9.2	0	0	0	Cyt																	
O60493	2	2	17.9	0	0	0	Cyt																	
O60613	1	1	9.9	1	3	9.9	ER (lumen)																	
O60701	10	15	30.4	9	15	25.7	ns																	
O60888	3	4	33	2	3	17.9	mem																	

Uniprot-ID	Polysaccharide metabolism (P)			Monosaccharide metabolism (M)						Lipid metabolism (L)													
	Glycan	Glycosaminoglycan	Glycogen	Amino sugar	Fructose and mannose	Galactose	Glyc/Gluc	PPP	Pentose-glucuronate interconvers	Glyoxylate	Fatty acids	Keton bodies	Sterol/cholesterol	Steroids	Androgen/estrogen	Sphingolipid	Arachidonic acid	Bile acid	Glycerophospholipid	Glycerolipid	Retinol	Panthothenat and CoA	Estrogen
O00165																							
O00217																							
O00231																							
O00232																							
O00264														rec									
O00299																							
O00303																							
O00305																							
O00499																							
O00625																							
O00629																							
O00748																							
O00754	d																						
O00764																							
O14558																							
O14561											b												
O14602																							
O14618																							
O14737																							
O14745																							
O14773																							
O14818																							
O14874																							
O14880																							
O14933																							
O15116																							
O15145																							
O15173														rec									
O15511																							
O43169																							
O43175																							
O43181																							
O43324																							
O43399																							
O43464																							
O43488/O95154/Q8NHP1																							
O43598																							
O43615																							
O43617																							
O43708																							
O43852																							
O43895																							
O60218																							
O60234																							
O60493																							
O60613																							
O60701																							
O60888																							

Uniprot-ID	Nucleotide metabolism (N)			Cofactor metabolism ©															Detoxification (D)	
	Nucleotide sugars	Purine	Pyrimidine	Adenosylcobalamine	Betaine	Creatine	Nicotinate	One carbon-folate	Porphyrin	Taurine/hypotaurine	Tetrahydrobiopterin	Thiamin	Vitamin B6	Molybdopterin	Sarcosine	Putrescine	Carnitine	Ubiquinone	Glutathione	Detoxification: Drugs, chemicals, x
O00165																				
O00217																				
O00231																				
O00232																				
O00264																				
O00299																				
O00303																				
O00305																				
O00499																				
O00625																				
O00629																				
O00748																				m
O00754																				
O00764													m							
O14558																				
O14561																				
O14602																				
O14618																				m
O14737																				
O14745																				
O14773																				
O14818																				
O14874																				
O14880																				m
O14933																				
O15116																				
O15145																				
O15173																				
O15511																				
O43169																				
O43175																				
O43181																				
O43324																				
O43399																				
O43464																				
O43488/O95154/Q8NHP1																				m
O43598																				
O43615																				
O43617																				
O43708																				m
O43852																				
O43895																				
O60218																				m
O60234																				
O60493																				
O60613																				
O60701																				
O60888																				

O75197	1	1	0.7	0	0	0	mem																																																													
O75208	2	2	9.1	3	3	14.2	Mito																																																									b		
O75223	0	0	0	2	3	17.6	ns																																																										m	m
O75340	5	10	31.9	3	5	17.8	Mem(Nuc (membrane)/ER (membrane)																																																												
O75347	3	3	26.9	4	6	26.9	Cyt																																																												
O75356	9	10	27.3	3	4	10.7	ER (lumen)																																				m	m																		
O75368	4	4	36.8	1	1	11.4	Cyt																																																											
O75369	0	0	0	1	2	0.5	Cyt																																																											
O75380	0	0	0	1	1	12.1	Mito (im)	m																																																									
O75390	3	3	9.4	1	1	2.6	Mito (matrix)		m																									m																																	
O75431	1	1	8.4	0	0	0	Mito (outer membrane)																																																											
O75439	1	1	4.7	0	0	0	Mito (matrix)																																																											
O75489	8	12	44.3	5	6	26.5	Mito (im)	m																																																									
O75521	10	13	36.5	8	9	27.3	Per (matrix)																												m																																
O75608	5	6	28.3	7	10	43	Cyt																												m																																
O75629	1	1	9.5	0	0	0	Sec																																																											
O75746/Q9UJS0	19	26	37	6	12	11.9	Mito (inner membrane)																t																																												
O75874	17	24	54.8	16	24	46.4	Cyt/Per		m																																																							m	
O75891	22	49	35.7	10	17	15.6	Cyt																																																		m												
O75947	12	17	73.9	12	16	73.9	Mito (im)	m																																																									
O75955	1	1	3.5	0	0	0	mem																																																											
O76054	3	3	13.9	1	1	4.5	Nuc																																																											
O94826	0	0	0	1	1	3	Mito (outer membrane)																																																											
O94903	1	1	4.7	3	4	17.5	Cyt																																																											
O95050	1	1	8.7	0	0	0	Cyt														m																																														m
O95168	2	2	17.1	0	0	0	Mito (im)	m																																																									
O95202	0	0	0	1	1	2.8	Mito (membrane)																																																											
O95210	1	1	3.9	2	3	9.2	mem																																																											
O95299	2	2	3.9	0	0	0	Mito (matrix)	m																																																									
O95336	0	0	0	5	5	28.7	Cyt																								m																																				
O95372	0	0	0	2	2	19	Cyt																												m																																
O95479	11	16	18.2	2	5	4.2	ER (lumen)																								m																																				
O95563	2	2	17.3	0	0	0	Mito																																																											
O95571	7	9	37.4	7	8	44.1	Mito (matrix)/Cyt																																																											
O95777	2	2	27.1		0		n																																																											
O95831	15	19	35.9	16	23	33.3	Mito (inner membrane)/Nuc																																																											
O95881	3	3	22.1	3	3	22.1	ER (lumen)																																																											
O95954	17	20	42.5	0	0	0	Cyt/Golgi										m																																						m												
O96000	4	5	29.1	2	2	14	Mito (im)	m																																																									
O96008	3	3	8	2	2	5	Mito (outer membrane)																																																											
P00167	8	11	57.5	6	9	57.5	ER (membrane)/Cyt																																																											
P00338	9	20	33.1	3	3	10.8	Cyt					m												m							m																																					
P00352	22	56	49.3	14	40	33.7	Cyt																																							m																						
P00367/P49448	22	68	48.6	8	16	19.4	Mito (matrix)				m		m																																																						
P00390	2	2	6.9	0	0	0	Mito/Cyt						m																																																					m	m
P00403	2	3	15.9	2	3	13.2	Mito (im)	m																																																									
P00439	3	5	8.8	0	0	0	Cyt											b			b	b																																													
P00441	10	17	79.9	10	18	63	Cyt																																																											m
P00450	1	1	2	1	1	1.8	Sec																																																											
P00480	13	24	54	12	15	49.2	Mito				m												m																																												
P00491	2	2	15.2	0	0	0	Cyt																																								m	m				m													
P00492	2	2	10.6	0	0	0	Cyt																																								m																		d
P00505	24	41	57.9	7	14	18.8	Mito			m	m	m	m					bm			b	bm																																													
P00558	14	17	51.1	8	18	34.3	Cyt																							m																																					
P00568/Q9Y6K8	6	8	30.9	8	11	44.3	Cyt																																								m																		
P00734	1	1	3.1	0	0	0	s																																																											
P00738/P00739	17	34	39.9	17	36	42.4	s																																																											
P00751	1	1	1.8	0	0	0	s																																																											
P00915	5	8	24.9	9	11	49.8	Cyt																																																											
P00918	3	4	16.9	8	14	37.7	Cyt																																																											
P00966	12	19	37.4	4	5	18.4	Cyt			m	m												m																																												
P01008	0	0	0	1	2	2.6	s																																																											
P01009	9	17	26.6	0	0	0	s																																																											
P01019	3	8	11.3	3	9	11.3	s																																																											
P01023	2	3	3.1	0	0	0	s																																																											
P01024	0	0	0	2	2	2.2	s																																																											
P01112/P01111/P01116	0	0	0	2	7	13.8	c																																																											
P01591	0	0	0	1	1	8.8	s																																																											
P01623/...	0	0	0	3	18	22.9	s																																																											
P01834	6	12	65.1	6	21	65.1	s																																																											

P01842	2	6	28.6	4	16	57.1	s																																																												
P01857	1	1	4.2	3	11	13	s																																																												
P01871	1	2	3.5		0		s																																																												
P02647	15	22	46.8	23	41	62.5	s																																																												
P02649	4	4	14.8	7	10	25.2	s																																																												
P02656	1	1	16.2	0	0	0	s																																																												
P02671	2	2	3	0	0	0	s																																																												
P02675	3	4	8.6	1	3	2.6	s																																																												
P02679	1	1	5.1	1	1	4.4	s																																																												
P02689	0	0	0	1	5	9.1	s																																																												
P02743	5	7	20.6	4	5	20.2	s																																																												
P02753	5	8	27.4	11	20	64.7	s																																																												
P02763	3	4	18.9	3	3	19.4	s																																																												
P02766	4	5	49.7	7	13	49.7	s																																																												
P02768	32	70	57	52	186	80.8	s																																																												
P02774	1	3	5.5	1	1	5.5	Cyt																																																												
P02787	20	38	38.4	33	91	55.7	s																																																												
P02790	2	2	6.9		0		s																																																												
P02792	8	24	54.9	8	17	47.4	Cyt																																											m											
P02794	5	8	36.6	7	10	38.3	Cyt																																											m											
P04004	5	7	11.1	5	10	11.1	s																																																												
P04040	22	33	48	27	51	56.5	Per													m																																														m
P04075	1	2	5.5	3	5	12.9	Cyt																						m		m	m																																			
P04080	3	5	45.9	2	4	24.5	Cyt/Nuc																																																												
P04083	6	10	25.4	4	6	15.9	c																																																												
P04179	14	63	56.3	14	36	56.3	Mito (matrix)																																																												m
P04196	0	0	0	2	5	5.7	s																																																												
P04217	4	4	14.1	5	11	16	s																																																												
P04229	1	10	4.9	0	0	0	mem																																																												
P04264	7	16	15.5	0	0	0	Cyt																																																												
P04406	14	23	50.4	13	30	53.4	Cyt																						m		m																																					
P04424	5	7	18.8	6	9	21.1	Cyt			m	m												m																																												
P04632	3	4	19.4	4	6	26.9	Cyt/Mem																																																												
P04792	2	3	12.7	12	19	60.5	Cyt																																																												
P04844	1	1	1.9	0	0	0	ER (membrane)																		b																																										
P05062	15	29	41.2	13	25	38.7	Cyt																						m		m	m																																			
P05089	19	31	57.8	14	16	50	Cyt				m												m																																												
P05090	3	4	12.2	0	0	0	s																																																												
P05091	29	65	60.2	30	88	59	Mito (matrix)								m	d				d	m		m	m							m				m							b																									m
P05109	1	1	11.8	0	0	0	Cyt																																																												
P05155	2	3	5.4	1	1	3.4	s																																																												
P05165	9	9	19.3	0	0	0	Mito																												m																																
P05166	15	24	38.4	7	9	19.7	Mito												d																m																																
P05177	1	2	3.7	0	0	0	ER (membrane)																												m						m				m																						m
P05386	1	1	14	2	4	14	Cyt																																																												
P05386	1	1	14	2	4	14	Cyt																																																												
P05387	4	10	41.7	4	7	31.3	Cyt																																																												
P05388	4	7	16.1	3	6	11	Cyt																																																												
P05413	3	5	24.1	0	0	0	Cyt																												t																																
P05556	1	1	1.3	1	2	2.3	mem																																																												
P05783	10	17	32.6	0	0	0	Cyt																																																												
P05787	11	30	21.9	0	0	0	Cyt																																																												
P05976	1	3	8.2	0	0	0	Cyt																																																												
P06132	0	0	0	1	1	6	Cyt																																											m											
P06396	2	3	3.1	0	0	0	Cyt																																																												
P06576	24	48	64.8	24	69	66.5	Mito (im)	m																																																											
P06702	1	1	13.2	0	0	0	Cyt/Nuc																																																												
P06733	15	47	54.6	25	73	68	Cyt																								m																																					
P06737	16	37	23.6	0	0	0	Cyt																				m																																								
P06744	3	7	8.2	3	3	7.5	Cyt																				m				m	m																																			
P07029	1	2	6.2	0	0	0	n																																																												
P07099	2	3	8.4	4	9	12.3	ER (membrane)																																																												m
P07108	0	0	0	1	1	23	ns																												t																																
P07148	9	45	85	5	16	50.4	Cyt																												t																																
P07195	2	2	7.5	0	0	0	Cyt					m												m							m																																					
P07203	9	18	52.2	10	15	69.2	Cyt																																m																									m	m
P07237	27	50	51.8	29	69	60.6	ER (lumen)																																																												
P07327/P00325/P00326	19	106	45.6	11	75	22.1	Cyt														m										m				m							b			m																						m
P07339	9	19	24.8	11	14	38.6	Lys																																																												
P07355	11	18	46.6	15	27	50.7	mem																																																												

P07437/Q13885/Q9BVA1/P68371/Q13509/P04350	16	157	50.9	14	77	42.2	Cyt																																																												
P07686	2	2	7.6	5	8	14.2	Lys																		d	d																																							
P07737	11	24	64.3	10	19	68.6	Cyt																																																												
P07741	6	12	52.2	6	10	39.4	Cyt																																											m																		
P07858	8	17	26.3	7	9	24.8	Lys																																																												
P07900/P08238	19	67	29.8	10	43	17	Cyt																																																												
P07910	2	3	9.2	1	2	5.2	n																																																											
P07954	12	18	38.4	12	37	34.7	Mito/Cyt		m																																																									
P08107/P54652/P17066	15	31	30.4	17	58	28.4	Cyt																																																												
P08133	35	62	57.4	28	49	51.1	c																																																												
P08134	4	14	26.9	3	9	18.1	c																																																												
P08236	2	2	5.1	0	0	0	Lys																		d	d	m																												m												
P08263/P09210/Q16772/Q7RTV2	10	249	43.7	11	69	39.2	Cyt																																																										m	m
P08311	1	1	5.1	0	0	0	Mem																																																												
P08319	25	59	78.2	15	38	55.5	Cyt															m									m				m						b			m																						m
P08559	1	1	3.3	0	0	0	Mito (matrix)		m	m														m							m										m																									
P08574	2	2	12.3	3	5	13.2	Mito (im)	m																																																										
P08670	9	27	21.2	8	16	21.7	Cyt																																																												
P08697	1	1	4.9	0	0	0	s																																																												
P08708	6	8	51.1	5	8	34.8	Cyt																																																												
P08758	12	18	39.7	10	10	41.9	ns																																																												
P08865	4	4	23.7	0	0	0	Cyt																																																												
P09110	18	33	52.4	18	33	53.8	Per													d																	bm							b																									
P09211	6	12	42.9	4	7	22.9	Cyt																																																									m	m
P09382	3	4	31.9	1	2	11.1	s																																																											
P09417	2	2	13.1	3	5	16.8	c																																												b													
P09429	0	0	0	1	1	7.4	n																																																												
P09455	12	20	61.5	0	0	0	Cyt																																	t																								
P09467	15	27	53.3	9	25	36.7	Cyt																					m		m	m																																		
P09488/Q03013/P46439	12	42	55.5	21	104	68.3	Cyt																																																										m	m
P09493/P06753/P67936/P07951	5	17	17.6	9	38	33.1	Cyt																																																												
P09496			0	1	1	4.4	mem																																																												
P09525	7	13	33.9	6	7	30.4	c																																																												
P09622	6	8	12.4	5	14	12.2	Mito (matrix)		m					m						d				m							m																																				
P09960	1	1	2.8		0		Cyt																																						m																									
P09972	1	2	4.4	1	3	4.4	Cyt																					m		m	m																																		
P0C0L4/P0C0L5	5	18	5.9	3	12	4.2	s																																																												
P10109	2	4	14.1	1	1	4.9	Mito (matrix)																																																												
P10253	5	6	6.6	6	8	9.9	Lys																			m			m																																				
P10515	0	0	0	2	3	6	Mito (matrix)		m															m							m																																			
P10599	3	4	23.8	2	5	12.4	Cyt																																																												
P10606	5	7	24	5	10	24	Mito (im)	m																																																										
P10619	0	0	0	3	7	5	Lys																																																												
P10620	1	2	10.3	1	1	8.4	ER (membrane)/Mito (outer membrane)																																																											m	m
P10768	3	4	19.5	1	2	7.8	Cyt																																																											m
P10809	38	96	69.6	39	114	72.8	Mito																																																												
P10909	1	1	2.4	5	7	17.6	s																																																												
P11021	32	52	46.5	43	92	56.7	ER (lumen)																																																												
P11142	23	46	36.4	22	47	36.2	Cyt																																																												
P11168	0	0	0	1	1	2.1	mem																																																												
P11177	3	3	10.6	7	9	27	Mito (matrix)		m															m							m																																			
P11182	4	8	7.9	3	5	6	Mito													d																																															
P11310	14	18	36.1	7	8	28.3	Mito																														d																																
P11498	36	64	43.2	20	32	25.2	Mito		m															m																																											
P11509/P20853/Q16696	1	2	1.8	1	2	4.5	ER (membrane)														m																m						m				m																						m
P11586	27	41	38.9	25	39	31.9	Cyt																													m																					m													
P11712	5	9	13.9	0	0	0	ER (membrane)																																																											m
P12268	0	0	0	1	1	3.5	Cyt																																											m																		m
P12429	1	1	8	0	0	0	c																																																												
P12694	5	7	20.9	0	0	0	Mito													d																																															

P12814/P35609/O43707	17	40	24.1	5	24	7.1	Cyt																																																												
P12955	3	4	14.6	2	2	8.9	s																																																												
P12956	1	1	3	0	0	0	n																																																												
P13489	2	2	5.6	0	0	0	Cyt																																																												
P13639	14	24	22.6	2	4	5.8	Cyt																																																												
P13645	1	3	2	0	0	0	Cyt																																																												
P13667	20	23	38.8	17	29	28.2	ER (lumen)																																																												
P13674	0	0	0	2	3	6.4	ns																																																												
P13693	2	5	15.7	4	9	27.3	Cyt																																																												
P13716	2	2	8.8	3	4	12.1	Cyt																																											m											
P13796	23	28	52.2	6	9	15.5	Cyt																																																												
P13797	13	17	25.2	2	3	6.2	Cyt																																																												
P13798	2	2	3.4	0	0	0	Cyt																																																												
P13804	13	19	51.7	20	30	68.2	Mito (im)																																																												
P13861	1	2	4	0	0	0	mem																																																												
P14174	2	2	7.8	1	1	0	s																																																												
P14314	7	13	13.1	10	15	16.7	ER																	m																																									
P14324	0	0	0	1	1	5.1	Cyt																															b																												
P14550	7	7	27.1	8	11	35.4	Cyt															m											m				m								b			m																								m
P14618	2	3	5.3	0	0	0	Cyt																	m								m																		m																						
P14625	38	102	52.8	32	110	45	ER (lumen)																																																												
P14923	1	1	2.4	0	0	0	mem																																																												
P14927	4	4	43.2	4	5	34.2	Mito (im)	m																																																											
P15090	0	0	0	2	7	25	Cyt																											t																																		
P15104	0	0	0	3	5	12.9	Cyt						m																																																					
P15121	1	1	3.2	0	0	0	Cyt																	m						m	m																																						
P15311	3	13	6.5	0	0	0	Cyt																																																												
P15374	0	0	0	1	3	8.7	Cyt																																																												
P15531/P22392	8	24	56.6	12	37	78.3	Cyt/Nuc																																										m	m																		
P15880	2	3	8.5	0	0	0	Cyt																																																												
P16083/Q08257	10	12	41	9	10	39.5	Cyt																																																											m
P16152/O75828	10	16	45.8	16	39	60.3	Cyt																																		m																										m
P16219	10	11	32.5	3	3	8.5	Mito																											d																																		
P16278	3	6	7.1	1	1	3	Lys/Cyt																	d	d				m										m				m																							
P16402	1	10	6.8	0	0	0	n																																																												
P16435	11	15	21.1	8	13	20.8	ER (membrane)																																																												
P16455			0	1	1	8.7	Nuc																																																											m
P16930	7	9	27	5	11	18.4	Cyt															m																																												
P16949	1	2	8.7	3	6	21.5	Cyt																																																												
P17174	12	19	41.6	7	15	24.7	Cyt			m	m	m	m					bm			b	bm																																															
P18077	1	1	9.1	0	0	0	Cyt																																																												
P18124	2	4	10.1	4	4	21	Cyt																																																												
P18206	15	19	17.4	1	3	1.9	Cyt																																																												
P18283	1	1	5.8	0	0	0	Cyt																																		m																									m	m
P18621	2	2	15.8	0	0	0	Cyt																																																												
P18669	6	12	40.6	8	19	47.6	Cyt																							m																																							
P19013	1	1	1.7	0	0	0	Cyt																																																												
P19105	6	12	44.4	4	7	28.1	Cyt																																																												
P19404	2	2	9.2	3	3	13.7	Mito (im)	m																																																											
P19440	1	3	2.3	0	0	0	Mem												m																						m																m									m	m
P19971	5	8	18.9	5	11	16.6	Cyt																																										m	m																	m
P20073	3	3	8.8	0	0	0	c																																																												
P20132	1	1	4.3	0	0	0	Cyt					m		m																neo																																							
P20290	1	1	11.2	1	1	9.2	n																																																												
P20339/P51148	2	4	10.6	3	8	17.1	er																																																												
P20340/Q9NRW1	2	8	14.4	0	0	0	er																																																												
P20618	6	8	31.1	5	5	36.9	Cyt																																																												
P20674	3	4	20.7	4	5	30	Mito (im)	m																																																											
P20962	1	2	10.8	1	1	10.8	n																																																												
P21266	1	1	7.1	0	0	0	Cyt																																																										m	m
P21281	1	4	2.7	0	0	0	l																																																												
P21291	2	2	13.5	2	3	20.2	Cyt																																																												
P21333	1	1	0.6	0	0	0	Cyt																																																												
P21399	14	23	25	14	33	26	Cyt		x																									x																																			
P21549	20	49	61.7	20	48	57.4	Mito (matrix)/Per			m				m																																																						
P21695	4	8	21.8	12	16	49.9	Cyt																																				m																								
P21757	0	0	0	1	1	6	mem																																																												
P21796	8	11	37.8	13	19	59	Mito (outer membrane)																																																												
P21912	5	11	20.7	10	19	40.7	Mito (im)	m	m																																																										
P21953	2	3	7.7	1	2	4.8	Mito													d																																														

P21964	5	7	19.9	0	0	0	Cyt/Mem															m																																															m
P22033	15	22	28.1	1	2	2.1	Mito (matrix)													d														m																																			
P22061	0	0	0	4	5	27.3	Cyt																																																														
P22234	2	3	7.3	0	0	0	ns																																					m																				
P22307	26	44	52.8	33	84	60.7	Cyt/Mito/Per																											t																																			
P22309/P36509/P22310/Q9HAW7	5	54	17.8	0	0	0	ER (membrane)																			m						m							m						m											m											m
P22314	12	13	16.9	0	0	0	Cyt																																																													
P22352	2	4	11.9	0	0	0	s																																																													
P22570	5	6	20.8	3	5	14.5	Mito (matrix)																													m																															
P22626	0	0	0	4	5	14.7	n																																																													
P22695	6	10	19.2	1	3	3.1	Mito (im)	m																																																												
P22792	0	0	0	1	2	5	s																																																													
P22830	3	5	11.8	1	2	5.4	Mito (inner membrane)																																												m											
P23141	32	85	58.9	27	62	50.1	ER (lumen)																																																												m
P23284	16	24	69.2	20	37	63	ER (lumen)																																																													
P23378	4	8	7.1	0	0	0	Mito								m																																																						
P23381	1	1	3.4	0	0	0	Cyt																																																													
P23396	1	1	3.7	1	1	5.3	Cyt																																																													
P23434	2	3	17.3	1	2	5.8	Mito								d																																																						
P23526	1	1	2.5	0	0	0	Cyt										m	m																																																		
P23528	9	25	48.8	10	23	53	Cyt																																																													
P23786	10	11	22.3	11	14	18.5	Mito (inner membrane)																											t																																			
P24298	3	3	8.3	0	0	0	Cyt			m			m																																																								
P24534	1	1	6.7	2	2	12.4	Cyt																																																														
P24539	3	4	19.5	0	0	0	Mito (im)	m																																																												
P24666	8	11	43.7	4	5	21.5	Cyt																																																													
P24752	20	37	44.5	14	36	37.5	Mito																												m																																		
P25311	2	2	11.2	0	0	0	s																																																													
P25325	7	13	39.1	8	14	42.1	Cyt					m																																																								m
P25398	3	5	22.7	2	6	15.9	Cyt																																																													
P25705	24	50	42.1	34	70	60	Mito (im)	m																																																											
P25774	2	2	8.2	0	0	0	Lys																																																													
P25786	2	2	8.7	4	5	14.8	c																																																													
P25787	4	7	23.9	4	4	20.1	Cyt																																																													
P25788	1	3	5.5	1	1	3.5	Cyt																																																													
P25789	1	2	3.8	2	2	9.2	Cyt																																																													
P26038	11	24	23.2	4	10	7.6	Cyt																																																													
P26373	3	5	13.7	1	1	6.2	Cyt																																																													
P26440	6	9	25.1	7	14	27.2	Mito (matrix)													d																																																
P26599	2	5	7.7	1	3	4	n																																																													
P26641	2	2	5.3	0	0	0	Cyt																																																													
P26885	6	7	47.9	6	8	33.8	ER (lumen)																																																													
P27105	1	2	4.2	0	0	0	mem																																																													
P27144	5	8	26.5	10	15	39.9	Mito (intermembrane space)																																					m																				
P27169	2	2	4.2	5	10	20.8	s																																																													
P27338	5	7	13.7	0	0	0	Mito (outer membrane)								m	m		m			m	m	m																																														m
P27348	2	2	9.8	4	15	20	Cyt																																																													
P27708	0	0	0	2	6	0.9	Cyt/Nuc						m																																m																			
P27797	8	10	33.3	12	17	37.2	ER (lumen)																																																													
P27824	0	0	0	1	2	3	ER (membrane)																																																													
P28062	2	3	8.3	0	0	0	Cyt																																																													
P28065	3	4	12.3	3	3	12.8	Cyt																																																													
P28066	3	3	21.6	1	1	5	Cyt																																																													
P28070	1	2	8.3	2	2	11.7	Cyt																																																													
P28072	1	1	4.6	3	3	13.4	Cyt																																																													
P28074	1	1	6.3	0	0	0	Cyt																																																													
P28331	14	19	27.1	3	6	5.6	Mito (im)	m																																																											
P28838	14	17	38.3	16	20	46.4	Cyt				m																																																							m	
P29401	9	14	23.1	3	6	8	Cyt																								m																																					
P29692	4	5	19.2	5	8	18.5	Cyt																																																													
P29966	1	2	6.9	0	0	0	c																																																													
P30038	16	26	32.7	15	24	32.1	Mito (matrix)				m		m																																																							
P30039	6	10	36.5	4	4	23.3	ns																																																													
P30040	6	6	19.9	9	10	38.7	ER (lumen)																																																													
P30041	17	37	65.2	20	71	70.1	Cyt											m																																																	m
P30042	5	8	42.5	10	18	64.2	Mito																																																													
P30043	7	10	39.3	10	20	61.2	Cyt																																																												m
P30044	15	29	46.7	13	27	46.3	Mito/Cyt/Per																																																												m
P30046	4	5	33.1	4	5	32.2	Cyt															m																																													
P30048	11	28	43	9	23	40.6	Mito																																																											m	m

P30049	5	12	20.2	3	4	17.3	Mito (im)	m																																																										
P30050	6	6	47.3	5	6	43	Cyt																																																										
P30084	19	60	65.9	18	48	63.8	Mito									d				d	m															m																																
P30085	8	10	39.8	11	15	49.5	Cyt/Nuc																																											m																		
P30086	18	32	85	14	30	76.5	Cyt																																																										
P30101	23	33	50.5	24	41	43.8	ER (lumen)																																																										
P30153	6	12	16.3	2	3	7.3	Cyt																																																										
P30405	4	5	24.2	1	1	7.2	Mito																																																										
P30613	0	0	0	2	2	6.1	Cyt																m							m																			m																			
P30626	0	0	0	3	6	17.2	Cyt																																																										
P30711	5	6	24.6	3	3	15.4	Cyt																																																								m	m
P30837	14	38	39.1	12	30	29.8	Mito (matrix)																																																									m
P31040	6	10	12.8	5	8	9.6	Mito (im)		m																																																								
P31150/P50395	5	15	16.2	2	4	9.4	c																																																										
P31327	87	257	62.2	76	150	54.5	Mito				m		m										m																																															
P31513	7	11	21.4	0	0	0	ER (membrane)																																																									m
P31930	10	12	29.8	6	7	18.3	Mito (im)	m																																																									
P31937	10	13	34.2	14	23	51.5	Mito													m																																														
P31946	3	4	20.7	9	23	50.8	Cyt																																																										
P31948	1	2	2.4	1	2	2.9	Cyt																																																										
P32119	13	24	47	16	42	51.5	Cyt																																																								m	m
P32754	12	13	39.9	8	9	31	Cyt											m				m																																														
P32929	3	3	13.3	1	2	6.9	Cyt					m		m			m		m																																																
P32969	1	2	5.7	1	1	7.3	Cyt																																																										
P33121	12	19	22.2	1	1	3.7	Mito (outer membrane)/Per (membrane)/ER (membrane)																											m																																		
P33316	0	0	0	7	10	32.5	Mito/Nuc																																											m																		
P34741	0	0	0	1	4	10	mem																																																										
P34896	4	8	11.8	4	4	15.3	Cyt							m																																								m												
P34897	8	10	21.8	11	12	31.9	Mito							m																																								m												
P34913	7	11	18	2	4	7.4	ER (membrane)																																																									m
P35221	0	0	0	1	3	3.2	Cyt																																																										
P35222	1	1	3.6	0	0	0	Cyt																																																										
P35232	12	31	60.7	14	19	68.8	Mito (inner membrane)																																																										
P35268	5	7	39.8	5	8	39.8	Cyt																																																										
P35270	6	10	33.7	12	17	51	Cyt																																															m												
P35520	0	0	0	1	1	4.7	Cyt/Nuc							m			m		m																																																
P35542	1	2	8.5	2	2	15.4	s																																																										
P35558	6	12	11.9	4	12	6.4	Cyt		m															m																																												
P35573	2	2	2.5	0	0	0	Cyt																			m																																									
P35579	8	10	6.3	0	0	0	Cyt																																																										
P35590	1	1	1.9	0	0	0	mem																																																										
P35914	8	19	28.6	12	17	36.6	Mito													d															bd																																		
P36269	8	8	17.4	0	0	0	Mem												m																					m																m										m	m
P36404	0	0	0	1	1	13.6	c																																																										
P36406	1	1	1.7	0	0	0	Mem/Golgi (membrane)/Lys (membrane)																																																											
P36542	4	6	16.8	4	7	16.8	Mito (im)	m																																																										
P36543	1	1	6.2	1	4	6.2	l																																																										
P36551	0	0	0	2	3	7.3	Mito (intermembrane space)																																																m											
P36578	5	5	16.4	0	0	0	Cyt																																																										
P36776	14	16	21.8	6	10	8.3	Mito (matrix)																																																										
P36871	24	37	55.3	11	14	29.9	Cyt																			m			m	m	m																																						
P36957	5	11	12.1	5	5	11.3	Mito		m							d																																																			
P36969	2	3	12.7	1	1	7.1	Mito/Cyt																																																								m	m
P37235	2	4	10.4	1	1	6.2	mem																																																										
P37802	13	18	72.9	13	26	77.4	Cyt																																																										
P37837	4	5	14.5	3	4	11.3	Cyt																							m																																						
P37840	0	0	0	1	1	11.4	Cyt/Mem/Nuc																																																										
P38117	12	26	46.3	15	30	57.6	Mito (im)																																																										
P38159	2	2	6.9	0	0	0	n																																																										
P38606	2	3	6.2	0	0	0	l																																																										
P38646	18	34	37.1	33	67	55.2	Mito																																																										
P39019	8	11	36.6	5	8	27.6	Cyt																																																										
P39023	1	2	5.5	0	0	0	Cyt																																																										
P39687	0	0	0	1	1	8.4	n																																																										
P40121	2	3	10.1	1	1	4.6	Cyt																																																										
P40227	2	4	7.2	2	4	4.3	Cyt																																																										
P40261	2	2	13.6	0	0	0	Cyt																																													m													m
P40306	1	1	7.3	0	0	0	Cyt																																																										
P40429	0	0	0	1	1	5.4	Cyt																																																										
P40925	4	5	12.9	5	6	19.2	Cyt		m															m										m																																					

P40926	18	26	61.8	31	74	71.3	Mito (matrix)		m															m										m																																					
P40939	29	62	51.1	29	70	46.5	Mito									d				d	m															m																																				
P42126	5	6	25.2	9	12	36.8	Mito (matrix)																													m																																				
P42704	6	7	7.5	1	1	2.2	Nuc/Mito																																																																					
P42765	19	42	56.4	25	70	65.2	Mito													d																m							b																																	
P42766	3	3	26.8	0	0	0	Cyt																																																																				
P43155	3	3	7.5	0	0	0	Mito (inner membrane)/Per/ER																													t																																				
P43487	0	0	0	1	1	5.5	Nuc																																																																				
P43490	7	9	20.6	7	9	25.1	Cyt																																																		m														
P43686	0	0	0	1	1	3.3	Cyt																																																																				
P45880	2	2	8.9	2	4	6.3	Mito (outer membrane)																																																																				
P45954	19	31	50.2	11	22	33.3	Mito																													d																																				
P46108	0	0	0	1	1	5.6	Cyt																																																																				
P46459	1	1	1.9	0	0	0	c																																																																				
P46776	1	1	14.9	2	2	22.3	Cyt																																																																				
P46777	3	3	12.5	1	1	5.7	Cyt																																																																				
P46779	0	0	0	2	3	17.5	Cyt																																																																				
P46782	2	5	14.2	3	5	18.6	Cyt																																																																				
P46783	8	11	48.5	4	5	27.3	Cyt																																																																				
P46940	2	3	2.8	0	0	0	mem																																																																				
P46952	6	6	31.5	6	6	29	Cyt														m																																																						
P47914	1	1	9.4	1	1	9.4	Cyt																																																																				
P47985	4	7	18.6	5	7	19.7	Mito (im)	m																																																																			
P48047	10	20	44.6	11	16	51.6	Mito (im)	m																																																																			
P48147	1	1	2.4	0	0	0	Cyt																																																																				
P48556	0	0	0	1	1	5.8	Cyt																																																																				
P48637	0	0	0	1	1	4.4	Cyt						m																																																									m	m
P48643	2	3	6.1	0	0	0	Cyt																																																																				
P48735	21	48	46.9	18	32	45.8	Mito		m																																																																	
P49189	3	5	9.3	2	3	7.7	Cyt									d																																													b			
P49207	0	0	0	1	1	6.8	Cyt																																																																				
P49326	1	2	2.8	0	0	0	ER (membrane)																																																																			m
P49327	14	24	10.3	2	6	2	Cyt																													b																																				
P49411	7	7	26.3	8	9	23.5	Mito																																																																				
P49419	15	20	41.7	12	14	31.9	ns									bd																																																										
P49588	1	1	1.3	0	0	0	Cyt																																																																				
P49593	0	0	0	1	1	2.2	Cyt																																																																				
P49720	5	7	30.2	5	8	28.3	Cyt																																																																				
P49721	6	8	34.8	6	7	39.3	Cyt																																																																				
P49748	18	27	35.3	2	3	5.5	Mito																													d																																				
P49755	0	0	0	2	2	12.8	ER/Golgi (membrane)																																																																				
P49773	7	9	78.6	2	3	16.7	c																																																																				
P49789	0	0	0	1	1	6.8	Cyt																																										m																					
P49821	2	2	5.6	0	0	0	Mito (im)	m																																																																			
P49914	0	0	0	1	2	5.9	Cyt																																																m													
P50053	2	3	13.1	1	1	4	Cyt																						m																																													
P50213	2	4	7.9	5	8	21.9	Mito		m																																																																		
P50225/P50226/P50224	5	11	27.1	1	3	6.1	Cyt																																																																			m
P50440	19	37	49.6	24	64	53.9	Mito (inner membrane)				m			m									m																														b	b														
P50502/Q8IZP2	1	4	3.8		0		Cyt																																																																				
P50914	4	5	21.1	2	5	10.8	Cyt																																																																				
P50990	2	4	4.9	1	2	2.7	Cyt																																																																				
P50995	3	6	8.3	0	0	0	c																																																																				
P51149	10	20	55.6	9	17	54.6	er																																																																				
P51452	4	7	29.2	4	5	26.5	Nuc																																																																				
P51570	2	4	7.7	4	4	15.1	Cyt (supposed)																							m																																												
P51571	3	5	24.9	1	1	11	ER (membrane)																																																																				
P51648	1	1	2.7	0	0	0	ER (membrane)																																																																			m
P51649	3	4	7.7	6	7	15.1	Mito						m																																																													
P51659	18	30	28.3	6	10	10.3	Per									d				d	m															m																																				
P51687	4	4	10.5	1	3	2.7	Mito (intermembrane space)																																																																				
P51857	1	1	4.3	0	0	0	Cyt																																b				m																										
P51858	0	0	0	1	1	6.7	n																																																																				
P51884	6	16	21.3	1	3	5.3	s																																																																				
P51888	4	6	15.4	0	0	0	s																																																																				
P51970	1	2	12.2	1	1	0	Mito (im)	m																																																																			
P52565	3	3	25	3	3	22.1	c																																																																				
P52566	4	4	35.8	1	1	12.4	c																																																																				
P52597	1	2	4.6	1	2	4.1	n																																																																				
P52758	6	8	50.4	8	10	53.3	Cyt/Nuc																																																																				

P52815	2	2	11.6	5	9	23.2	Mito																																																										
P52907	1	1	5.6	0	0	0	Cyt																																																										
P52943	1	1	7.7	0	0	0	ns																																																										
P53597	2	3	9.3	4	5	17.7	Mito		m																																																								
P54578	1	1	4.9	0	0	0	Cyt																																																										
P54819	8	14	35.1	18	34	72.8	Mito (intermembrane space)																																					m																		
P54855/O75795	1	2	1.7	0	0	0	ER (membrane)																			m						m						m						m												m											m
P54868	23	42	46.3	25	76	47	Mito													d																bd																															
P54886	1	1	1.5	0	0	0	Mito (inner membrane)																m																																									
P54920/Q9H115	1	2	3.7	0	0	0	er																																																										
P55072	20	28	29.2	19	52	30.5	er																																																										
P55083	1	1	7.1	0	0	0	s																																																										
P55084	15	28	41.6	7	10	16	Mito								d					d	m														m																																
P55145	6	9	33.5	5	7	23.5	s																																																										
P55157	11	15	16.7	0	0	0	ER																												t																																
P55263	0	0	0	1	1	4.4	Cyt																																					m																		
P55327	1	1	7.6	2	3	17.9	ER (lumen)/Cyt																																																										
P55735	1	1	0	0	0	0	er																																																										
P55957	3	4	22.1	4	7	21.5	Cyt/Mito (membrane)																																																										
P56134	1	1	11.7	0	0	0	Mito (im)	m																																																									
P56470	2	2	9.6	1	1	5.3	s																																																										
P56537	1	1	10.2	1	1	7.3	Cyt																																																										
P56556	1	2	8.6	1	1	8.6	Mito (im)	m																																																									
P57105	1	3	8.3	0	0	0	Mito (outer membrane)																																																										
P59190	2	4	10.4	1	1	5.2	er																																																										
P60174	14	34	54.2	20	57	79.5	Cyt																																																										
P60604	0	0	0	1	1	14.5	Cyt																																																										
P60660	8	19	58.3	6	19	53	Cyt																																																										
P60709/P63261	19	146	56.8	11	80	44.3	Cyt																																																										
P60842	1	2	2.5	0	0	0	Cyt																																																										
P60866	2	8	19.3	3	5	25.2	Cyt																																																										
P60900	4	5	13	4	4	16.3	Cyt																																																										
P60953	4	18	22.5	2	16	16.8	mem																																																										
P60981	7	15	48.5	4	4	32.1	Cyt																																																										
P60983	4	6	33.1	1	2	7.7	Cyt																																																										
P61019	0	0	0	2	3	12.7	er																																																										
P61081	2	2	10.9	2	4	9.3	Cyt																																																										
P61088	6	8	48.7	6	6	42.1	Cyt																																																										
P61106	3	41	19.1	0	0	0	er																																																										
P61158	1	1	2.6	1	1	2.6	Cyt																																																										
P61201	1	1	5.6	0	0	0	Cyt/Nuc																																																										
P61224/P62834	4	19	20.7	5	13	28.3	mem																																																										
P61247	0	0	0	1	1	6.4	Cyt																																																										
P61353	0	0	0	1	1	6.6	Cyt																																																										
P61457	0	0	0	1	1	15.4	Cyt/Nuc																																														b									
P61586	6	20	42	4	10	24.4	c																																																										
P61604	1	2	13.7	2	3	23.5	Mito																																																										
P61626	2	2	16.2	0	0	0	s																																																										
P61916	3	3	31.8	2	2	21.2	s																																																										
P61956	1	6	12.6	1	9	12.6	Nuc																																																										
P61978	6	6	21.4	5	5	13.2	n																																																										
P61981	2	3	15.4	10	20	53	Cyt																																																										
P62081	8	10	27.3	6	9	22.7	Cyt																																																										
P62136/P62140	3	7	14.7	0	0	0	Cyt																																																										
P62158	5	7	32.2	7	14	37.6	Cyt																																																										
P62191	1	1	3.4	1	1	3.4	Cyt																																																										
P62195	0	0	0	1	1	3.9	Cyt																																																										
P62241	4	6	24.5	2	2	12	Cyt																																																										
P62244	5	5	38.5	2	3	16.9	Cyt																																																										
P62249	6	7	30.8	6	7	41.1	Cyt																																																										
P62256	0	0	0	1	1	8.2	Cyt																																																										
P62258	2	3	4.7	5	12	27.1	Cyt																																																										
P62263	7	12	41.7	6	7	41.7	Cyt																																																										
P62266	0	0	0	2	2	15.4	Cyt																																																										
P62269	9	16	43.4	12	27	53.9	Cyt																																																										
P62277	6	13	28.5	6	12	32.5	Cyt																																																										
P62280	2	2	19.6	0	0	0	Cyt																																																										
P62318	1	1	7.9	0	0	0	n																																																										
P62330	4	7	28.6	2	5	17.7	c																																																										
P62333	2	4	7.7	0	0	0	Cyt																																																										

P62424	3	6	15	1	2	4.9	Cyt																																																												
P62491/Q15907	3	8	15.7	0	0	0	er																																																												
P62633	0	0	0	1	1	8.5	c																																																												
P62701	3	4	13.3	2	4	4.6	Cyt																																																												
P62750	2	2	8.3	3	4	21.8	Cyt																																																												
P62753	2	2	6.4	3	3	18.1	Cyt																																																												
P62805	4	7	41.7	1	1	7.8	n																																																												
P62820/Q9H0U4/ Q15286	3	45	18.5	2	6	13.2	er																																																												
P62826	3	3	17.1	4	4	21.3	Cyt/Nuc																																																												
P62829	2	4	27.1	3	4	34.3	Cyt																																																												
P62837			0	2	4	23.8	Cyt																																																												
P62841	3	5	22.1	2	3	22.1	Cyt																																																												
P62847	3	5	29.3	2	4	20.3	Cyt																																																												
P62851	3	5	24	2	4	16	Cyt																																																												
P62854	2	5	20.9	2	3	20.9	Cyt																																																												
P62873/P62879	2	3	6.8	0	0	0	Mem																																																												
P62888	4	6	51.3	3	3	34.8	Cyt																																																												
P62899	0	0	0	3	3	18.4	Cyt																																																												
P62910	2	3	17	1	2	9.6	Cyt																																																												
P62913	6	6	23	5	5	21.9	Cyt																																																												
P62917	1	1	4.3	2	4	10.5	Cyt																																																												
P62937	13	24	67.9	13	23	67.9	Cyt																																																												
P62942	2	2	13	2	2	13	Cyt																																																												
P63000/P15153	5	25	29.7	3	21	17.2	mem																																																												
P63027	0	0	0	1	2	14.7	er																																																												
P63104	5	5	31	8	17	42.9	Cyt																																																												
P63208	0	0	0	3	4	20.2	Cyt/Nuc																																																												
P63241	6	18	46.1	6	16	46.1	Cyt																																																												
P63244	4	6	16.4	4	5	15.1	mem																																																												
P68036	5	7	48.7	4	4	31.2	Cyt																																																												
P68104	5	11	15.6	4	9	13.4	Cyt																																																												
P68363/P68366	13	90	34.6	8	40	23.5	Cyt																																																												
P68402	1	1	8.3	0	0	0	Cyt																																																												
P68871/...	16	54	89.8	17	84	88.4	s																																																												
P78371	3	4	8.4	1	1	3.7	Cyt																																																												
P78417	7	16	28.6	9	11	32	Cyt																																																								m	m
P78560	1	1	0	0	0	0	Cyt (intracellular)																																																												
P80404	26	55	55.8	22	50	48.8	Mito			m			m																																																						
P82650	0	0	0	1	1	3.3	Mito																																																											
P82909	0	0	0	1	1	15.5	Mito																																																												
P82980	2	3	6.7	2	3	6.7	Cyt																																		t																								
P83111	1	3	2.7	0	0	0	Mito																																																												
P83731	0	0	0	3	4	19.1	Cyt																																																												
P84077/P61204/P 18085	12	48	67.4	11	67	56.4	c																																																												
P84243/P68431	4	33	43.4	0	0	0	n																																																												
P99999	6	12	43.8	6	12	49.5	Mito (matrix)	m																																																											
Q00059	0	0	0	1	1	4.9	Mito																																																												
Q00266	3	3	9.9	0	0	0	ns										m		m																																														
Q00341	0	0	0	1	1	1	Cyt																													m																													
Q00796	9	10	43.7	10	14	42	Cyt (supposed)																					m																																						
Q01082	1	1	0.6	0	0	0	Cyt																																																												
Q01469	2	2	25.9	3	3	31.9	Cyt																											m																																
Q01518	4	11	12.6	1	1	4.8	mem																																																												
Q01581	2	7	2.5	2	7	2.5	Cyt												d																bd																															
Q01995	13	25	71.6	4	5	25.4	Cyt																																																												
Q02218	2	2	2.4	0	0	0	Mito (matrix)		m							d					m																																												
Q02252	25	48	44.1	23	37	46.7	Mito												m																																														
Q02338	9	16	28.3	9	10	32.9	Mito (matrix)																												bd																															
Q02818	5	5	11.5	4	4	11.7	ER/Golgi/Cyt																																																											
Q02878	5	5	24.3	1	1	5.2	Cyt																																																												
Q02928	3	4	9.6	0	0	0	ER (membrane)																											m						m				m																								m
Q03154	8	8	33.1	7	8	28.9	Cyt																m																																											
Q04760	8	10	42.4	10	17	51.6	Cyt																	m																																									m
Q04828/P52895/P 42330/P17516	7	28	29.4	7	36	35.3	Cyt																																																												m
Q04837	9	14	51.4	12	19	66.9	Mito																																																												
Q04917	0	0	0	3	12	12.2	Cyt																																																												
Q05639	3	5	7.8	0	0	0	Cyt																																																												
Q05682	1	2	1.4	0	0	0	Cyt																																																												
Q06278	2	3	2.2	0	0	0	Cyt												m	m	m																															m						m							m
Q06323	6	8	36.1	8	11	42.2	Cyt																																																												

Q06520	7	13	29.8	9	12	42.8	Cyt																																																													m
Q06830	16	48	49.7	18	59	73.4	Cyt																																																											m	m
Q07020	4	5	20.2	3	3	19.7	Cyt																																																												
Q07021	0	0	0	1	1	4.6	Mito (matrix)																																																												
Q07065	2	2	5.6	0	0	0	Cyt																																																												
Q07131	12	57	34.6	10	25	28.7	Cyt																						m			m			m															m																			
Q08380	2	3	5.3		0		s																																																												
Q08426	22	33	34	26	52	41.8	Per								d				d	m																bm																																			
Q10713	1	4	3.2	0	0	0	Mito (matrix)																																																												
Q12849	0	0	0	2	2	9.6	Cyt																																																												
Q12931	8	11	14.2	4	7	8	Mito																																																												
Q13011	10	22	35.7	15	24	57.3	Mito/Per																													m																																			
Q13126	1	1	6.4	2	2	14.5	Cyt										m																																																						
Q13162	11	37	39.9	10	39	41.3	Cyt																																																												m	m
Q13228	10	18	28	16	37	43.4	Cyt																																																													sensin
Q13268	0	0	0	13	35	51.2	Nuc																																			x																														
Q13404	5	14	19	4	7	16.3	Cyt																																																												
Q13405	2	2	16.3	3	3	23.5	Mito																																																												
Q13409	1	1	3.3	0	0	0	Cyt																																																												
Q13418	1	1	2.4	0	0	0	mem																																																												
Q13423	2	2	3	0	0	0	Mito (im)		m																																														m													
Q13510	6	11	25.3	1	1	6.6	Lys																																					m																											
Q13526	1	1	11.7	1	1	11.7	Nuc																																																												
Q13561	1	1	4.7	0	0	0	Cyt																																																												
Q13576	1	2	1.8	0	0	0	mem																																																												
Q13813	13	15	8.2	3	3	2.3	Cyt																																																												
Q13907	2	2	18.1	5	6	33.5	Per																																			b																														
Q14011	0	0	0	1	3	0	Nuc																																																												x
Q14019	7	11	38	2	2	16.9	Cyt																																																												
Q14032	4	9	11.2	3	9	7.4	Cyt																																							b																									
Q14103	2	3	6.8	2	5	6.8	n																																																												
Q14117	4	5	13.3	1	1	3.9	Cyt																																											m				m																	m
Q14165	1	1	4.1	0	0	0	ER (membrane)																																																												
Q14197	1	1	5.8	1	2	5.8	Cyt																																																												
Q14203	1	2	1	0	0	0	Cyt																																																												
Q14353	2	2	9.7	4	5	25.4	Cyt				m			m																																																							
Q14696	0	0	0	5	5	22.2	ER																																																												
Q14697	25	42	37.7	20	37	29.6	ER/Golgi																		b																																																
Q14749	5	6	24.4	4	6	16.9	Cyt							m																																																								
Q14914	6	9	24	2	3	6.7	Cyt																																						m																										
Q14964	1	3	5.1	0	0	0	er																																																												
Q15056	0	0	0	1	1	9.3	Cyt																																																												
Q15067	5	5	11.5	4	4	10.5	Per																													bm																																			
Q15084	12	22	39.3	12	25	37.7	ER (lumen)																																																												
Q15121	1	1	7.7	0	0	0	Cyt																																																												
Q15126	2	3	12.5	2	3	12.5	Per																																			b																														
Q15181	1	4	3.1	6	6	27	Mito	m																																																											
Q15185	1	1	10.6	3	5	26.3	Cyt																																						m																										
Q15274	0	0	0	4	7	15.8	Cyt																																																	m													
Q15293	1	1	4.8	2	2	10.6	ER (lumen)																																																												
Q15363	1	2	6	0	0	0	ER/Golgi (membrane)																																																												
Q15365	1	1	3.7	0	0	0	Nuc/Cyt																																																												
Q15370	1	1	10.2	0	0	0	n																																																												
Q15404	0	0	0	3	3	24.2	c																																																												
Q15435	1	1	5	0	0	0	Cyt/Nuc																																																												
Q15436	2	3	4.7	0	0	0	er																																																												
Q15493	1	2	4.3	1	2	5.7	Cyt																																																												
Q15819	6	15	29	0	0	0	Cyt																																																												
Q16134	10	12	23.5	4	6	10.7	Mito (im)																																																												
Q16540	1	1	9.8	2	3	24.2	Mito																																																												
Q16543	1	1	3.7	0	0	0	Cyt																																																												
Q16595	2	2	15.7	1	1	5.7	Mito																																																												
Q16619	0	0	0	1	4	10.4	s																																																												
Q16698	6	11	26.3	10	22	35.8	Mito																													m																																			
Q16718	1	1	8.6	1	1	8.6	Mito (im)	m																																																										
Q16740	0	0	0	2	3	9.7	Mito																																																												
Q16762	9	18	45.8	12	26	49.8	Mito (matrix)																																																												m
Q16775	4	5	18.5	10	14	51.9	c																m																																															m
Q16822	30	71	46.1	35	150	50.5	Mito		m															m																																														
Q16836	9	15	48.7	13	17	71.3	Mito (matrix)								d				d	m																m																																			

Q16851	13	57	37.6	11	26	30.9	Cyt																			m			m																																						
Q16891	5	6	12.1	3	3	7.7	Mito (inner membrane)																																																													
Q3LXA3	17	29	45.7	18	31	49.7	Cyt																																																												
Q4VC31	1	1	6.3	0	0	0	ns																																																												
Q5HYK3	2	2	9.2	4	6	19.3	Mito																																																				b		
Q5T2W1	1	2	2.7	1	1	2.1	Cyt																																																												
Q6IBS0	1	4	3.7	1	1	3.7	Cyt																																																												
Q6P587	2	5	13.4	4	6	33.5	ns																																																												
Q7KZF4	2	2	3.1	0	0	0	n																																																												
Q7Z4W1	11	23	64.8	14	37	74.6	Mem																									m																																			
Q7Z5P4	5	9	25	9	21	33.3	s																																																												
Q86SX6	1	1	8.9	2	2	14	Mito																																																												
Q86TX2/P49753	8	20	30.4	5	21	17.8	Mito/Cyt																											b																																		
Q86UP2	3	4	2.5	3	3	2.9	er																																																												
Q86VB7	1	1	1	0	0	0	s																																																												
Q86WA6	4	4	10.3	10	19	30.9	Mito																																																											m
Q86WU2	3	5	9.1	3	5	8.9	Mito																	m																																															
Q86X76	0	0	0	3	3	14.4	Cyt/Nuc																																																												
Q86X83	1	1	9.5	0	0	0	Nuc/Cyt																																																												
Q8IVH4	1	1	4.1	0	0	0	Mito																																									b																
Q8N4P3	0	0	0	3	4	17.9	ns																																																												
Q8N5N7	1	1	7	3	3	28.5	Mito																																																												
Q8N655	0	0	0	1	1	0.5	ns																																																												
Q8N668	0	0	0	2	2	11.6	Nuc/Cyt																																																												
Q8N983	1	2	5.1	1	1	5.1	Mito																																																												
Q8NBJ7	4	4	12.6	5	5	18.3	ER (lumen)																																																												
Q8NBQ5	1	2	5.3	0	0	0	s																																																												
Q8NBS9	5	8	14.1	5	8	12.5	ER (lumen)																																																												
Q8NBX0	0	0	0	1	2	6.8	Mito									d																																																								
Q8NE62	7	7	22.1	0	0	0	Mito							m																																																											
Q8NI60	1	1	2.5	2	2	4.6	Mito																																																												
Q8TAA3	0	0	0	2	3	9.8	Cyt																																																												
Q8TCD5	1	1	9	1	1	9	Cyt																																																												
Q8TD30	0	0	0	1	1	1.9	Cyt			m			m																																																											
Q8TF76	1	1	2.1	1	1	2.1	n																																																												
Q8WUM4	1	1	1.8	0	0	0	c																																																												
Q8WVJ2	2	2	7	0	0	0	ns																																																												
Q8WW59	7	14	40.1	14	23	58	Nuc																																																												
Q92506	4	6	20.3	4	7	20.3	ns																																					b																				
Q92558	0	0	0	1	3	1.6	Cyt																																																												
Q92688	0	0	0	1	1	9.6	n																																																												
Q92820	0	0	0	2	3	11.9	s																																																												
Q92882	1	1	5.6	1	1	5.6	Cyt																																																												
Q92945	0	0	0	2	3	4.1	Nuc/Cyt																																																												
Q92947	5	7	17.1	3	4	13.2	Mito									d					m														m																																				
Q93052	1	1	2.5	0	0	0	Cyt/Nuc/Mem																																																												
Q93077/...	4	96	35.9	0	0	0	n																																																												
Q93079/...	2	78	19.8	0	0	0	n																																																												
Q93088	12	33	40.9	13	43	43.8	Cyt							m			m																																																					
Q93099	5	6	17.3	2	2	8.3	Cyt															m																																																	
Q969H8	3	5	20.8	5	7	30.1	s																																																												
Q96AB3	8	15	55.6	9	27	56.6	Mito/Nuc																																																												
Q96C23	0	0	0	1	1	4.1	Cyt																								m																																							
Q96CN7	2	2	4.7	0	0	0	Per																																																												
Q96DE0	0	0	0	3	3	24.6	Nuc																																																												
Q96EY1	0	0	0	1	1	4.8	Mito (matrix)																																																												
Q96EY8	2	4	10.4	4	7	20.8	Mito																																													m											
Q96F10	1	1	13.5	0	0	0	ns																m																																																	
Q96FV2	1	1	4.7	0	0	0	ns																																																												
Q96HR9	2	4	12.5	2	4	12.5	mem																																																												
Q96I99	14	17	42.1	9	14	29.6	Mito		m																																																															
Q96IU4	6	11	29	7	18	44.3	Cyt/Nuc																																																											m
Q96KP4	8	12	32.4	3	3	8.8	Cyt																																																												
Q96LJ7	3	5	16	5	7	26.5	ER/Golgi/Nuc																																																												
Q96PE7	4	5	26.1	4	5	26.1	Mito													d															m																																					
Q96R05	0	0	0	1	1	7.5	Cyt																																				t																							
Q96RQ3	10	12	22.6	0	0	0	Mito (matrix)													d																																																			
Q99424	2	3	4.6	0	0	0	Per																																																												
Q99439	0	0	0	1	1	5.5	Cyt																																																												
Q99471	1	1	11.7	0	0	0	Cyt																																																												

Q99497	9	16	61.9	15	26	76.2	n																																																									
Q99611	0	0	0	1	1	3.3	ns											m																																														
Q99623	8	9	33.4	8	10	33.8	Mito (inner membrane)/Cyt/Nuc																															x																														
Q99627	2	2	13.9	0	0	0	Cyt/Nuc																																																								
Q99653	0	0	0	2	2	11.8	c																																																								
Q99714	14	38	82.8	19	36	87.4	Mito									d				d	m														m																																				
Q99733	0	0	0	1	1	2.9	n																																																							
Q99798	18	23	31.7	23	38	39.5	Mito		m																									m																																		
Q99807	3	3	8.8	4	4	17.5	Mito																																																					b		
Q99832	1	2	4.6	0	0	0	Cyt																																																							
Q9BPW8	4	4	28.9	11	14	43.3	c																																																							
Q9BQ69	1	1	4.3	1	2	4.3	ns																															x																														
Q9BRA2	1	1	8.1	0	0	0	c																																																							
Q9BRG1	6	10	37.5	0	0	0	er																																																							
Q9BRX8	0	0	0	2	4	11.8	s																																																							
Q9BS26	3	3	11.6	2	3	6.4	ER (lumen)																																																							
Q9BSE5	1	1	7.7	4	7	19	Mito															m																																				b				
Q9BSH4	0	0	0	1	1	3.4	ns																																																							
Q9BTZ2	5	8	19.2	7	10	33.1	Per																																							m																							
Q9BU02	1	2	12.2	0	0	0	Cyt																																																	m								
Q9BUE6	0	0	0	1	1	11.6	Mito																																																							
Q9BUP3	0	0	0	2	2	9.5	Cyt/Nuc (envelope)																																																							
Q9BUR5	1	1	5.6	3	4	17.2	s																																																							
Q9BV57	7	8	38	4	5	23.5	Mem										b																																													
Q9BVJ7	3	3	27.3	3	3	21.3	Cyt/Nuc																																																							
Q9BVK6	2	2	9.8	3	3	14	ER/Golgi (membrane)																																																								
Q9BVL4	1	1	3.9	0	0	0	ns																																																							
Q9BWD1	2	4	6.8	1	2	2.8	Cyt									d				d	m			m											m	bd																																
Q9BX68	6	13	54.6	6	16	60.1	s																																																							
Q9BXW7	2	3	9.7	3	3	13	Mito																																																							
Q9BY32	0	0	0	1	1	10.8	Cyt																																										m	m																m
Q9BYD1	0	0	0	3	4	21.3	Mito																																																							
Q9BYV1	1	1	2.9	0	0	0	Mito			m				m																																																			
Q9GZT3	3	3	34.9	4	4	31.2	m																																																							
Q9H082	1	3	4.8	0	0	0	er																																																							
Q9H0W9	8	10	39.7	3	3	9.5	Nuc																																																							m
Q9H2L5	0	0	0	1	1	7.5	Cyt																																																							
Q9H2U2	5	10	21	5	5	20.7	Mito	m																																																							
Q9H2W6	0	0	0	2	2	13.3	Mito																																																							
Q9H7Z7	2	3	8.8	6	6	24.7	ER/Golgi																																			m																										
Q9H8H3	3	3	17.2	0	0	0	ns																																																							m
Q9H936/Q9H1K4	4	7	16.2	4	9	15.9	Mito (inner membrane)						t																																																			
Q9H9B4/Q9BWM7	3	4	14.6	2	5	16.5	Mito (membrane)																																																							
Q9H9J2	0	0	0	1	4	3.9	Mito																																																							
Q9HAT2	0	0	0	1	1	2.9	Lys																																																							
Q9HAV7	4	4	15.2	7	12	31.8	Mito																																																							
Q9HCC0	8	11	19	9	11	21.8	Mito (matrix)													d																																												
Q9HCN8	2	5	5.9	0	0	0	ER (lumen)																																																							
Q9HDC9	3	3	12.7	2	4	6.7	mem																																																							
Q9NNW7	2	3	6.5		0		Mito																																											m																m
Q9NP72	3	3	16.5	1	1	5.3	er																																																							
Q9NPJ3	5	8	40.7	4	5	37.9	Cyt																																																							
Q9NQ50	0	0	0	2	2	16.5	Mito																																																							
Q9NQP4	0	0	0	1	1	9	Cyt																																																							
Q9NQX3	1	1	2.7	0	0	0	Cyt																																																		b						
Q9NR28	5	8	25.5	5	6	18.4	Mito/Cyt																																																							
Q9NR31/Q9Y6B6	4	7	34.3	4	4	34.3	er																																																							
Q9NR45			0	1	1	5	ns																				m																																									
Q9NR77	2	4	10.8	0	0	0	Per (membrane)																																																							
Q9NRV9	2	3	16.4	6	6	34.9	Cyt																																																							m
Q9NRX2			0	1	1	8.6	Mito																																																							
Q9NS69	1	1	7.7	0	0	0	Mito (outer membrane)																																																							
Q9NSE4	2	3	3.9	0	0	0	Mito																																																							
Q9NTM9	0	0	0	1	1	8.1	Cyt/Nuc																																																							
Q9NUI1	0	0	0	2	2	4.1	Per																												d																																	
Q9NVI7	1	1	2.2	0	0	0	ns																																																							
Q9NVS9	5	8	25.7	7	11	41	Cyt																																																	m							
Q9NX63	2	2	13.2	6	6	28.6	Mito																																																							
Q9NYL4	1	1	9	1	1	9	Mem																																																							
Q9NYU2	1	1	1.1	0	0	0	ER (lumen)																	b																																													

Q9NZN3	1	2	3.1	0	0	0	mem																																																										
Q9P000	1	1	10.6	1	1	10.6	Nuc/Cyt																																																										
Q9P032	0	0	0	4	8	28.6	Mito	x																																																							
Q9P0J0	2	2	15.3	0	0	0	Mito (im)	m																																																							
Q9P0Z9	2	3	6.9	2	2	6.9	Per							m																																																	
Q9P2E9	18	35	16.9	6	7	5.9	ER (membrane)																																																										
Q9P2K3	0	0	0	1	2	4.8	n																																																										
Q9UBQ0	0	0	0	1	1	7.1	er																																																										
Q9UBQ7	13	18	58.2	9	13	35.4	Cyt																	m										m																															
Q9UBR1	2	4	8.6	0	0	0	Cyt																																	m				m																	m
Q9UBR2	1	1	4	2	3	7.3	Lys																																																										
Q9UBS4	1	1	3.1	2	2	7.8	ER (lumen)																																																										
Q9UDR5	17	25	25.5	11	18	17.6	Mito									bd																																																		
Q9UFN0	2	2	10.5	1	4	5.7	Cyt																																																										
Q9UHK6	1	1	2.6	0	0	0	Mito/Per																											m							m																									
Q9UHL4	2	2	6.5	1	1	4.1	Lys																																																											
Q9UHV9	3	4	29.9	1	1	7.8	Cyt																																																										
Q9UI09	2	2	21.4	0	0	0	Mito (im)	m																																																		b		
Q9UI17	19	36	33.7	5	8	9.7	Mito (matrix)							m																																	d															
Q9UIJ7	8	9	44.5	17	33	77.5	Mito (matrix)																																					m																	
Q9UJ68	1	1	6.4	1	1	6.4	Cyt										r																																															
Q9UJ70	1	1	4.7	0	0	0	ns						m															m																																						
Q9UJD0	1	1	3.9	1	2	3.9	ns																																																										
Q9UJM8	17	32	51.4	17	31	59.5	Per																												m																																
Q9UJZ1	5	6	18.3	4	8	12.4	mem																																																										
Q9UKK9	0	0	0	5	6	26	ns																																					m																		
Q9UL12	12	21	18.7	4	6	6.9	Mito (matrix)							m																																							d										
Q9UL46	6	8	39.3	8	13	51	Cyt																																																										
Q9ULZ3	4	6	22.6	4	4	21.5	Cyt																																																										
Q9UN36	5	9	22.1	1	1	5.9	Cyt																																																										
Q9UNM6	2	2	8.8	1	2	4	Cyt																																																										
Q9UPN3	1	4	0.2	0	0	0	Cyt																																																										
Q9Y224	2	3	14.8	0	0	0	Cyt/Nuc																																																										
Q9Y237	0	0	0	1	1	10.7	Mito																																																										
Q9Y277	2	3	10.6	2	3	10.6	Mito (outer membrane)																																																										
Q9Y2B0	7	10	39.6	8	12	44	ER (lumen)																																																										
Q9Y2Q3	8	11	44.7	13	25	70.8	Per																																																								m	m
Q9Y2Q9	1	1	4.8	0	0	0	Mito																																																										
Q9Y2S2	3	5	10.3	4	6	13.8	ns																											m																																
Q9Y2T3	1	2	4.2	1	2	4.2	Cyt																																					m																		
Q9Y2V2	0	0	0	1	1	10.9	Cyt																																																										
Q9Y3A6	1	1	10	0	0	0	ER/Golgi (membrane)																																																										
Q9Y3B7	0	0	0	2	5	19.3	Mito																																																										
Q9Y3B8	0	0	0	1	1	7.6	Mito/Nuc																																																										
Q9Y3C6	1	1	10.2	0	0	0	Nuc																																																										
Q9Y3D2	0	0	0	4	4	14.9	Mito																																																									m
Q9Y3D6	5	12	32.2	5	7	32.2	Mito (outer membrane/per (membrane)																																																										
Q9Y3D9	2	2	13.7	2	2	13.2	Mito																																																										
Q9Y3E5	1	1	8.4	0	0	0	Mito																																																										
Q9Y3I0	1	1	3.8	0	0	0	Cyt																																																										
Q9Y490	18	31	13.8	4	5	4	Cyt																																																										
Q9Y4L1	8	14	13.3	11	18	18.3	ER (lumen)																																																										
Q9Y4Z0	1	1	11.5	0	0	0	Nuc																																																										
Q9Y5Z4	3	3	17.6	2	3	13.2	Cyt																																																									m
Q9Y617	6	8	18.9	0	0	0	Cyt							m																																						m								
Q9Y6H1	0	0	0	3	3	33.8	Mito																																																										
Q9Y6M9	1	2	10.6	0	0	0	Mito (im)	m																																																								
Q9Y6N5	4	4	12.2	0	0	0	Mito																																																									m

9 Curriculum Vitae

Personal data

Name:	Hannes Zwickl
Date, place of birth:	October 10, 1970; Tulln, Austria
Marital state:	married
Address:	3470 Kirchberg/Wagram, Kirchenplatz 1, Austria

Education and employment

1987-1994: Working in the parental winery

1994: *Studienberechtigungsprüfung*

1994-2002: Study of biology, branch of study: Microbiology, Diploma thesis: “Investigation of the endocytosis mechanism of Human Rhinovirus Serotype 2 (HRV2)" in the lab of Prof. Blaas (Institute of Medical Biochemistry, Vienna Biocenter)

2003-2006: Doctoral studies at the Institute of Cancer Research in the lab of Prof. Gerner

2004-2006: University course for postgraduates "Toxicology" at the Medical University of Vienna

since 2006: Collaborating in the establishment of the "Center for Regenerative Medicine" (Head of the center: Prof. Nehrer) dedicated to orthopaedics research focussing on osteoarthritis and tissue engineering; Responsible for ISO-certification, financial issues, acquisition of project fundings, project management. Scientific focus on the pharmacological effect of hyaluronan on chondrocytes and synoviocytes and bioreactor development for the optimization of transplants for the treatment of cartilage lesions

2009: Training to "Certified Project Manager" at the Danube-University Krems

Publications

Investigation of Collagen Transplants Seeded with Human Autologous Chondrocytes at the Time of Transplantation
Hannes Zwickl, Eugenia Niculescu-Morzsa, and Stefan Nehrer
Cartilage July 2010 1: 194-199

Investigation of bone allografts representing different steps of the bone bank procedure using the CAM-model.
Holzmann P, Niculescu-Morzsa E, Zwickl H, Halbwirth F, Pichler M, Matzner M, Gottsauner-Wolf F, Nehrer S.
ALTEX. 2010;27(2):97-103.

Cell characterization by proteome profiling applied to primary hepatocytes and hepatocyte cell lines Hep-G2 and Hep-3B.
Slany A, Haudek VJ, Zwickl H, Gundacker NC, Grusch M, Weiss TS, Seir K, Rodgarkia-Dara C, Hellerbrand C, Gerner C.
J Proteome Res. 2010 Jan;9(1):6-21.

Introducing the CPL/MUW proteome database: interpretation of human liver and liver cancer proteome profiles by referring to isolated primary cells.
Wimmer H, Gundacker NC, Griss J, Haudek VJ, Stättner S, Mohr T, Zwickl H, Paulitschke V, Baron DM, Trittner W, Kubicek M, Bayer E, Slany A, Gerner C.
Electrophoresis. 2009 Jun;30(12):2076-89.

New cellular tools reveal complex epithelial-mesenchymal interactions in hepatocarcinogenesis.
Sagmeister S, Eisenbauer M, Pirker C, Mohr T, Holzmann K, Zwickl H, Bichler C, Kandioler D, Wrba F, Mikulits W, Gerner C, Shehata M, Majdic O, Streubel B, Berger W, Micksche M, Zatloukal K, Schulte-Hermann R, Grasl-Kraupp B.
Br J Cancer. 2008 Jul 8;99(1):151-9.

Knowledge-based proteome profiling: considering identified proteins to evaluate separation efficiency by 2-D PAGE.
Gundacker N, Bayer E, Traxler E, Zwickl H, Kubicek M, Stöckl J, Gerner C.
Electrophoresis. 2006 Jul;27(13):2712-21.

A novel technique to specifically analyze the secretome of cells and tissues.
Zwickl H, Traxler E, Staettner S, Parzefall W, Grasl-Kraupp B, Karner J, Schulte-Hermann R, Gerner C.
Electrophoresis. 2005 Jul;26(14):2779-85.

Human rhinovirus type 2 is internalized by clathrin-mediated endocytosis.
Snyers L, Zwickl H, Blaas D.
J Virol. 2003 May;77(9):5360-9.