
Session 8: Quantitative proteomics

Lectures

L8.1

Global quantification of cellular protein degradation kinetics

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Do young and old protein molecules have the same probability to be degraded? To answer this question we used metabolic pulse-chase labeling and quantitative mass spectrometry to obtain degradation profiles for thousands of proteins. We find that more than 10% of proteins are degraded non-exponentially in mouse fibroblasts. In all of these cases, proteins are less stable in the first few hours of their life and become more stable as they age. We find that degradation profiles are conserved between mouse and human and are similar in two different cell types. Many non-exponentially degraded (NED) proteins are subunits of multiprotein complexes that are produced in super-stoichiometric amounts relative to their exponentially degraded (ED) counterparts. Within complexes, NED proteins have larger interaction interfaces and assemble earlier than ED subunits. Amplifying genes encoding NED proteins increases their initial degradation. Consistently, our decay profiles can help to predict how DNA copy-number alterations affect protein levels. Together, our data show that non-exponential degradation is common, evolutionarily conserved and has important functional consequences for protein complex formation and aneuploidy.

L8.2

An integrated -omic approach to dissect diet-induced sterile inflammation and resolution

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Non-alcoholic steatohepatitis (NASH) is characterised by the accumulation of lipids (steatosis) with inflammation in the liver. Although steatosis is benign and reversible, inflammation can increase liver damage upon incorrect resolution. Therefore, it is of great importance to elucidate the molecular changes of the inflammatory responses and the dynamics thereof. Using a mouse model of diet-induced sterile inflammation, we aim to identify critical nodes of inflammation and subsequent resolution. Recently, feeding a western-type diet containing high cholesterol and high fat to *Ldlr*^{-/-} double knock-out mice has been shown to result in infiltration of inflammatory cells and pro-inflammatory gene expression in the liver. Thus, *Ldlr*^{-/-} mice fed a western diet represents a model for sterile inflammation associated with non-alcoholic fatty liver disease.

Ldlr^{-/-} mice were fed a western type diet (0.2% cholesterol/21% milk fat/46% carbohydrates/17% casein) for 14 or 28 days to induce 'metabolic inflammation'. To study the effect of diet change on the reversal of inflammatory responses, a separate group of mice was first fed a western type diet for 14 days and then switched back to regular chow for a further 3, 7 or 14 days. Upon sacrifice, the liver was harvested and processed for subsequent analyses. Tissue samples were homogenised in 2% SDS and processed by F.A.S.P. A 1×10-plex TMT experiment was performed to include two biological replicates per time point. Samples were separated by RPRP HPLC, pH 10, 20 fractions collected, acidified and analysed by LCMSMS on an Agilent/Q-Exactive.

In line with our expectations, western-diet feeding resulted in hyperlipidaemia and increased infiltration of inflammatory cells in the liver. Interestingly, these effects are completely reversed upon dietary switch after 7 days. The seminar will focus on our preliminary proteomic and transcriptomic findings obtained from the *Ldlr*^{-/-} mice fed on high-fat western diet *versus* a standard chow diet.

L8.3

Dynamic mapping of subcellular protein localization through quantitative proteomics

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Subcellular localization is a key determinant of protein function, and many cell biological processes involve movements of proteins between compartments. We have developed dynamic organellar maps, for the global determination of protein subcellular localization. The method combines rapid biochemical fractionation with quantitative mass spectrometry and cluster analysis. All major organelles are resolved; cross-validation of a 1000-member organellar marker set shows an estimated prediction accuracy of >92%. The absolute abundances of proteins are also obtained, allowing us to derive a quantitative model of cell architecture. We provide an interactive online database with subcellular localization information for 8700 proteins from HeLa cells. Furthermore, since organellar maps are highly reproducible, they are suitable for monitoring physiological changes in protein localization. We applied the method to investigate protein dynamics following EGF stimulation, revealing numerous known and novel translocations. Dynamic organellar maps are a versatile systems analysis tool, with many potential applications in cell biology.

L8.4

Proteomic approach to understand antipsychotic drug action

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Schizophrenia is a chronic psychiatric disease which emerges in early adulthood. Prevalence of schizophrenia approaches 0.7% of worldwide population; it has significant negative impact on patients' life quality and their lifespan. Unfortunately, there has been made a very modest progress in pharmacological treatment of schizophrenia over last 50 years. Clozapine appears to be the extraordinarily efficient drug; it displays exceptional effectiveness in suicide prevention and in treatment-resistant schizophrenia, however it also has severe metabolic side effects therefore it is prescribed as a drug of second/third choice. Clozapine binds many subtypes of membrane receptors thus application of a proteomic approach for evaluation clozapine mechanism of action is strongly justified. The aim of our study was to characterize proteomic alterations in different rat brain structures to extend the knowledge concerning clozapine mechanism of action. We performed comparative investigations in relation to other antipsychotic drug, risperidone, which is very common in schizophrenia treatment. Using two dimensional differential in gel electrophoresis (2D-DIGE) method and mass spectrometry-based technique (LC-MS/MS) with stable isotope labelling (iTRAQ) we had insight into about 4000–5000 proteins of given brain structure. We were able to determine drug-specific and tissue-specific as well as common changes in protein profiles upon drugs treatment. Extended literature survey and application of Ingenuity Pathway Analysis allowed us find functional connections between differential proteins and define upstream regulators as well as biochemical pathways and networks affected by drugs also these distinguishing clozapine. Clozapine influences actin cytoskeleton within cerebral cortex and in prefrontal cortex, whereas within the nucleus accumbens and striatum it diminishes oxidative stress. Moreover, risperidone strongly affects glucose homeostasis in hippocampus, whereas clozapine upregulates calcium sensors in cerebral cortex.

L8.5

MRM analysis of peptide panels for search of cancer-related cytokeratin-related biomarkers in body fluids

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High hopes for new proteomic biomarkers of a variety of diseases, including cancer diagnosis, prognosis and therapy monitoring were verified negatively quite fast, and it was pointed out that the published results were often not reproducible [1]. The necessity for further improvements and more systematic basic studies preceding the application in clinics was indicated, and the necessity to improve quantitative methods was stressed. In response Multiple Reaction Monitoring (MRM)-based [2], or more recently Parallell Reaction Monitoring (PRM) analytical platforms were worked out. They allow for the reliable protein/peptide concentration measurements in the background of extremely complex protein mixtures (e.g. blood plasma/serum). Moreover, in this methods, a panel of 10–100 proteins, represented by hundreds of peptides, their proteolytic fragments, can be subjected to a parallel analysis in the same experiment. Cytokeratins are established cancer markers and their potential is explored in a variety of cancer types using antibody-based analytical tools. The aim of the project is to explore the possibility to establish a MRM-based keratin panel for pleural fluid (PE) diagnostics, with the expectation to overcome the known disadvantages of antibody-based analyses and multiplex the analysis of a set of keratins of interest into one experiment. We have optimized sample preparation procedures to improve the sensitivity of MRM tests. We have shown [3] that a cancer-relevant panel of cytokeratin peptides can be quantitated on the background of PE and demonstrated its significant ability to identify cancer related PE samples. In parallel in frame of the global proteomic analyses of colon cancer tissue we have detected a strong neutrophil elastase proteolytic fingerprint marking the carcinoma tumor proteome and providing a vast reservoir of new potential biomarkers [4]. The new biomarker peptides can be included into the existing peptide panels increasing their sensitivity and specificity.

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L8.6

Understanding proteomic variability: an inter-laboratory study of hepatic ADME proteins

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Aim: To compare quantified protein levels of membrane transporters and enzymes relevant to drug disposition, using different proteomics methodologies.

Methods: Protein concentrations of hepatic drug transporters and metabolizing enzymes were quantified in ten identical human liver samples in six laboratories using their respective *in-house* methods. Nine phase I-metabolizing enzymes (CYPs), four phase II-metabolizing enzymes (UGTs), five uptake transporters (SLCs), and five efflux transporters (ABCs, and SLCs) were included in the analysis. The quantifications were performed using five targeted and one global, label free approach. Three laboratories determined the membrane proteins in the whole cell lysates while the other three laboratories analyzed proteins after subcellular fractionation. Enrichment factors were used to scale levels of proteins in subcellular fractions to protein levels in whole cell.

Results: The protein concentration levels of the higher expressed drug metabolizing enzymes, obtained by the different laboratories, were overall in good agreement. The protein levels obtained from the methods using whole tissue lysate differed on average two-fold, while levels quantified in subcellular fractions were four to seven-fold lower. The protein concentration levels of the lower expressed transporting proteins displayed a larger variation between the laboratories. The transporters were in general quantified with a three-fold average difference across five of the laboratories. However, scaled protein levels from plasma membrane fractions were on average ten to 38-fold lower compared to the other laboratories.

Conclusion: Despite large differences in methodologies, the enzymes were in good agreement between the laboratories. However, a higher variability was seen for the lower abundant transporters. This demonstrates that methodological differences, such as subcellular fractionation procedures, can influence protein quantification immensely.

Oral presentations

O8.1

Divisive iK-means algorithm as a tool for ROI selection in the search for MALDI-MSI based molecular signature of thyroid cancer

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Determination of cancer type is one of the crucial points in tumour treatment allowing for prognosis of cancer development and selection of proper personalized therapy. Features of HE stained tissue sample are most commonly used for that purpose. Although they may be sufficient for cancer classification in majority of cases, there are still some unsolved ones, when even the experienced pathologists cannot take a final decision. Having the molecular signature of each cancer type well defined would support clinicians in their work.

Fifteen tumour tissue samples with neighbouring healthy tissue from patients with five different types of thyroid cancer (anaplastic ATC, follicular FTC, medullary MTC, papillary classical PTC CV and papillary follicular PTC FV, with three samples for every type) were analysed by low spatial resolution MALDI-MSI technique with a raster width of 100 μm and m/z ranged from 600 to 4000 Da. Divisive iK-means algorithm applied to GMM based spectral features was used for identification of molecularly homogenous tissue regions on every sample. Each region was assigned "cancer" or "non-cancer" depending on its molecular similarity. Logistic regression models distinguishing between C and NC regions were built separately for every sample. The performance of every classifier was validated on two independent samples of the same cancer type and remaining 12 independent samples of different type (as negative control).

Medullary cancer tissue strongly separates from other cancers, allowing for reliable classification of independent MTC samples with the average accuracy of 93.34%. All three MTC classifiers were of the same quality (90.73%, 96.07% and 93.22%), also performing excellent on independent negative control samples – the average misclassification of 0.67% of other cancer tissue as MTC, and the correct classification of 95.96% of other cancer spectra as non MTC.

The classifiers built for other types of thyroid cancer did not distinguish that cancer types from remaining ones so well. Their signatures can be understood as pancancer indication. PTC trained classifiers recognized ATC tumour tissue with accuracy of 71.27% and specificity of 78.12%, while ATC trained one demonstrates accuracy of 60.74% on PTC samples and 66.39 on FTC samples. Our preliminary results show the potential of developed methodology in supporting identification of molecular biomarkers of thyroid tumours.

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Posters

P8.1

Label-free proteomic analysis reveals changes in red-ox and lipid homeostasis in the liver of cystathionine β -synthase deficient mice

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Homocysteine (Hcy) arises from the metabolism of the essential dietary protein amino acid methionine (Met). Cystathionine β -synthase (CBS) catalyzes the first step of the transsulfuration pathway that affords cysteine. Human CBS deficiency causes severe hyperhomocysteinemia (HHcy) and diverse clinical manifestations, including oxidative stress and fatty liver disease [1].

We hypothesize that CBS deficiency induces changes in gene expression that impair liver homeostasis. To identify the genes involved and gain an insight into hepatic functions of *Cbs* we analyzed the liver proteome of *Cbs*^{-/-} (n=14) and *Cbs*^{+/+} mice (n=16).

Using *Tg-I278T Cbs*^{-/-} mouse model [2] and label-free based relative quantitative proteomics we identified 101 liver proteins whose expression was significantly altered as a result of the *Cbs* gene inactivation. Among proteins fulfilling criteria (minimum of two identified peptides, fold-change *Cbs*^{-/-}/*Cbs*^{+/+} livers >1.5, and *p* values 0.05) 5 were up-regulated and 50 down-regulated. The most striking features were upregulation of glutathione *S*-transferases and down regulation of bleomycin hydrolase, in the *Cbs*^{-/-} mice. To find enriched annotations in the 101 identified proteins we used DAVID and PANTHER bioinformatic analysis tools. The GO analysis of the biological processes revealed that the differentially expressed proteins participated in diverse processes, including red-ox (Blvrb, Cyp2a5), glutathione (Gsta1, Gss), amino acid (Gldc, Agxt), carbon (Pgd, Pgam1) and lipid metabolism (Fasn, Ces1e). The analysis of physiological pathways using the KEGG resources showed that the most overrepresented pathways were detoxification by cytochrome P450, glutathione, amino acid, fatty acid, pyrimidine and nitrogen metabolism.

These findings suggest that *Cbs* interacts with diverse cellular processes, including red-ox reactions, detoxification, energy, and lipid metabolism, that are essential for normal liver homeostasis. Deregulation of genes involved in the metabolism of glutathione and red-ox reactions, suggests that *Cbs* deficiency increases oxidative stress. Deregulation of genes involved in detoxification and lipid metabolism provides a possible explanation for fatty liver disease associated with CBS deficiency in humans and mice.

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P8.2

Qualitative and quantitative mass spectrometry analysis of human follicular fluid (hFF) peptidome

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The interest in assisted reproductive technologies, including *in vitro* fertilization, has been growing recently due to increase in infertility rates. Currently there is no method for quality assessment of oocytes before fertilization, what results in embryo overproduction. Human follicular fluid (hFF), as the medium surrounding the oocyte during its development, may provide information about the quality of the oocyte. Therefore, there have been ongoing investigations concerning the composition of hFF and relations between the concentrations of its components and outcomes of the IVF procedure [1]. A great deal of attention has been drawn to proteomic content of hFF – in both qualitative [4] and quantitative studies [1]. Here, we focused on low molecular mass fraction (LMM, 10 kDa) resulting from ultrafiltration of hFF. The aim of this research was to characterize low molecular mass peptidomic fraction of human follicular fluid by mass spectrometry.

In this work we attempted the peptidomic approach [2] and SWATH-MS technique [3] to define a peptide map of LMMF of hFF. SWATH-MS allows for label-free qualitative and quantitative measurement of potentially all proteins in the sample in one run. Use of this method requires employment of reference spectral library built with measurements conducted in IDA mode. We performed measurements of samples in IDA mode in order to both, obtain a list of peptides present in hFF and build a spectral reference library. Subsequently, using obtained library, we carried out a pilot SWATH-MS study on peptidomic fraction of the preliminary set of hFF samples from women undergoing IVF.

In our preliminary study we identified and quantified peptides of the LMM fraction of hFF. Comprehensive examination of peptide/protein hFF composition of larger set of clinical samples may be beneficial in the future development of a method of the oocyte quality assessment before fertilization during the IVF procedure.

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P8.3

Influence of environmental and genetic factors on proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*

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Introduction and Aim: Many Gram-negative bacteria, especially pathogenic ones (such as *Neisseria meningitidis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Pseudomonas aeruginosa*, *Shewanella* spp., *Shigella* spp., *Salmonella* spp.), produce membrane vesicles (OMVs). Secretion of these vesicles to a medium is a naturally occurring phenomenon. Alterations in size, number and content of vesicles are frequently regarded as bacterial responses to changes in environmental conditions, such as pH, temperature, ionic strength, stress conditions or antibiotic pressure. OMV formation is also influenced by genetic factors.

The present study was aimed to determine the effects of genetic and environmental factors on *Campylobacter jejuni* OMV formation and quantitative protein composition.

Methods and Results: To identify proteins in the analyzed samples the routine LC-MS-MS/MS procedure was used. We analyzed the protein contents of the OMVs isolated from wild-type *C. jejuni* strain grown in optimal conditions and under stress conditions (increased atmospheric oxygen content – 15%, presence of polymyxin B). The OMVs from *C. jejuni* strains with mutations in *htrA* and *dsbI* genes were also examined. The significant quantitative difference in protein content between some OMVs were identified. Overrepresentation of proteins which are involved in flagellar assembly (FlgL, FlgE, FlgK) was observed in *C. jejuni dsbI* mutant strain.

Conclusions: Increased atmospheric oxygen content, presence of polymyxin B and mutation in *dsbI* gene have an influence on *C. jejuni* OMV protein content. We did not notice any difference between OMV isolated from wt strain and strain with *htrA* knockout.

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P8.4

Interaction of imatinib mesylate with human serum transferrin. The comparative spectroscopic studies

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Imatinib mesylate (Gleevec, Glivec) is a tyrosine kinase inhibitor mainly used in the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia (Ph+ CML). Human serum transferrin is the most abundant serum protein responsible for the transport of iron ions and many endogenous and exogenous ligands. In this study the mechanism of interactions between the imatinib mesylate and all states of transferrin (apo-Tf, Htf and holo-Tf) has been investigated by spectroscopic methods (fluorescence, UV-VIS, CD, zeta potential measurements). Based on the experimental results it was proved that under physiological conditions the imatinib mesylate binds to the each form of transferrin with a binding constant c.a. 10^5 M^{-1} . The thermodynamic parameters indicate that hydrogen bonds and van der Waals are involved in the interaction of apo-Tf with the drug or hydrophobic and ionic strength in the case of Htf and holo-Tf. Moreover, it was shown that common metal ions, Zn^{2+} and Ca^{2+} strongly influenced apo-Tf-Imt binding constant. The CD studies showed that there are no conformational changes in the secondary structure of the proteins. These results indicate that the ground state complex between protein and the drug was formed. No significant changes in secondary structure of the proteins upon binding with the drug and instability of apo-Tf-Imt system are the desirable effects from pharmacological point of view.

P8.5

Label free and iTRAQ-based proteomic analysis of plasma reveals differences in molecular mechanism of atherosclerosis related and non-related to chronic kidney disease

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The major cause of mortality in patients with chronic kidney disease (CKD) is atherosclerosis related to traditional and non-traditional risk factors. However, the understanding of the molecular specificity that distinguishes the risk factors for classical cardiovascular disease (CVD) and CKD-related atherosclerosis (CKD-A) is far from complete. In this study we investigated the alterations in plasma protein accumulation in patients with CKD and classical cardiovascular disease (CVD) without CKD. Plasma collected from patients in various stages of CKD, CVD patients without symptoms of kidney dysfunction and healthy volunteers (HVs), were analyzed by a coupled label-free and iTRAQ-based proteomic approaches. Dysregulated proteins were confirmed by ELISA and PRM analysis. One hundred twenty-two differentially expressed proteins were identified. By directly comparison the plasma proteomes from HVs and CKD and CVD patients in one study, we demonstrated that proteins involved in inflammation, blood coagulation, oxidative stress, vascular damage and calcification process exhibited greater alterations in patients with atherosclerosis related with CKD. These data indicate that the above nontraditional risk factors are strongly specific for CKD-A and appear to be less essential for the development of "classical" CVD. Moreover, the bioinformatics analysis revealed that 29 differentially expressed proteins were involved in lipid metabolism and atherosclerosis, 20 of which were apolipoproteins and constituents of high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Although dyslipidemia is common in CKD patients, we found that significant changes in apolipoproteins were not strictly associated with changes in plasma lipid levels. A lack of correlation between apoB and LDL concentration and an inverse relationship of some proteins with the HDL level was revealed. An increased level of apolipoprotein AIV, adiponectin, or apolipoprotein C, despite their anti-atherogenic properties, was not associated with a decrease in cardiovascular event risk in CKD patients. The presence of the distinctive pattern of apolipoproteins demonstrated in this study may suggest that lipid abnormalities in CKD are characterized by more qualitative abnormalities and may be related to HDL function rather than HDL deficiency.

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P8.6

Identification and quantification of high molecular weight fraction of human follicular fluid proteome by SWATH-MS

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Due to the increasing infertility rates in humans there has been a growing interest in assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF). However, low efficacy of the IVF procedure (~80% of embryos fail to be implanted) results in embryo overproduction. Therefore, there is an urgent need to develop a non-invasive method, which preferably would allow to assess the oocytes quality before IVF and reduce the number of produced embryos [1]. Proteins present in the human follicular fluid (hFF), the medium surrounding the oocyte during its development, may provide information about the quality of the oocyte and serve as biomarkers [2].

Therefore, in the present study we focused on the analysis of high molecular weight fraction (>10 kDa) of proteolytically digested proteins, isolated from hFF. We used microLC-MS/MS in the SWATH-MS (Sequential Window Acquisition of All Theoretical Fragment Ion Spectra) acquisition mode to perform label-free proteomic quantitative analysis [3].

We developed a SWATH-MS method for identification and quantification of high molecular weight proteins of hFF and applied it to preliminary set of hFF samples. As the SWATH-MS allows for fragmentation and detection of potentially all the constituents of the analyzed sample, our study provided a comprehensive map of high molecular weight fraction of hFF proteins. Among them 13 proteins have never been detected before in hFF. Future study of these proteins will enable to verify their role in the prognosis of development pregnancy, which could improve the selection of embryos with highest implantation competence for uterine transfer.

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P8.7

Cystathionine β -synthase deficiency elevates *N*-homocysteinylation at specific lysine residues in fibrinogen and serum albumin in mice

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Homocysteine (Hcy) is a non-coded amino acid, however, it can become a constituent of proteins in a process involving metabolic conversion to the thioester Hcy-thiolactone catalyzed by methionyl-tRNA synthetase, followed by non-enzymatic formation of isopeptide bonds with protein lysine residues in a process called *N*-homocysteinylation. This process alters protein's structure/function and has been associated with cardiovascular disease. We have previously reported that global protein *N*-homocysteinylation is elevated in mouse models of hyperhomocysteinemia (Jakubowski *et al.*, 2009, *EASEB J* **23**: 1721-1727).

Here we report that mouse fibrinogen and albumin carry *N*-linked Hcy attached to specific lysine residues. We studied cystathionine β -synthase (*Cbs*)-deficient *Tg-I287T Cbs*^{-/-} mice with severe hyperhomocysteinemia (plasma tHcy=272±50 μ M) and their *Tg-I287T Cbs*^{+/-} littermates (plasma tHcy=5.0±2.6 μ M). Mouse fibrinogen, purified by the glycine precipitation procedure, or plasma was digested with trypsin and *N*-Hcy-peptides were analyzed by liquid chromatography/mass spectrometry. Using *in vitro*-prepared mouse *N*-Hcy-albumin, we identified 21 lysine residues as *N*-Hcy attachment sites. Two of these, *N*-Hcy-Lys212 and *N*-Hcy-Lys525, were also identified *in vivo* in albumin from *Tg-I287T Cbs*^{-/-} (n=35) and *Tg-I287T Cbs*^{+/-} mice (n=38). The mean level of peptides containing *N*-Hcy-Lys212 or *N*-Hcy-Lys525 positively correlated with plasma tHcy ($r=0.38$, $p<0.05$) and was significantly elevated in *Tg-I287T Cbs*^{-/-} mice compared with their *Tg-I287T Cbs*^{+/-} littermates (409993±78749,2 and 147797±22643,7 for *Cbs*^{-/-} vs. 228983±19402,3 for *Cbs*^{+/-}, respectively, $p=0.0024$). Using *in vitro* prepared mouse *N*-Hcy-fibrinogen, we identified 2 *N*-Hcy-Lys residues in the α -chain, 17 *N*-Hcy-Lys residues in the β -chain and 19 *N*-Hcy-Lys residues in γ -chain. Six of these, β -chain *N*-Hcy-Lys 284 and *N*-Hcy-Lys 324, and γ -chain *N*-Hcy-Lys 273, *N*-Hcy-Lys 58, *N*-Hcy-Lys 120, and *N*-Hcy-Lys 88 were also identified in mouse fibrinogen *in vivo*. Levels of each of these *N*-Hcy-Lys-peptides were at least 2-fold elevated in fibrinogen from *Tg-I287T Cbs*^{-/-} mice relative to *Tg-I287T Cbs*^{+/-} animals.

Our results indicate that specific lysine residues are targeted for *N*-homocysteinylation in mouse albumin and fibrinogen. The physiological role of these modifications remains to be elucidated.

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