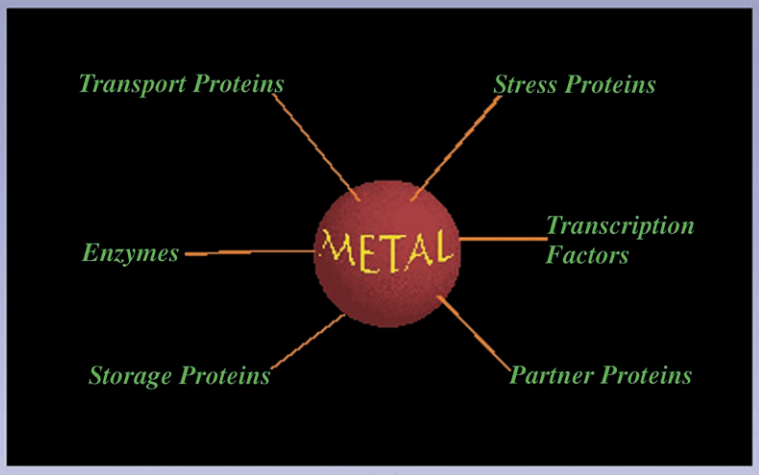
Proteomics of Metal Transport



Protein Mixture

IMAC enrichment
2D Gel Electrophoresis
Liquid Chromatography
+
ICP-MS, Radioactive metal assay,
Colorimetric metal detection, XAS,
Two photon microscopy,
micro-PIXE

Mass spectrometry
NMR, X-ray crystallography
+
EXFAS, XANES

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Proteomics of Metal Transport and Metal-Associated Diseases

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Abstract: Proteomics technology has the potential to identify groups of proteins that have similar biological function. However, few attempts have been made to identify and characterize metal-binding proteins by using proteomics strategies. Many transition metals are essential to sustain life. Copper, iron, and zinc are the most abundant transition metals relevant to biological systems. In addition to their important biological functions, metals can also catalyze the formation of damaging free radical species. Hence, their intracellular transport is tightly regulated. Despite recent insights into the intracellular transport of copper and other metals, our overall understanding of intracellular metal metabolism remains incomplete and it is likely that many metal-binding proteins remain undiscovered. Furthermore, the protein targets for metals during metal-associated disease states or during exposure to toxic levels of environmental metals are yet to be unravelled. A proteomics strategy for the analysis of metal-transporting or metal-binding proteins has the potential to uncover how a large number of proteins function in normal or metal-associated diseased states. Here we discuss the principal aspects of metal metabolism, and the recent developments in the area of the proteomics of metal transport.

Keywords: mass spectrometry · metabolic disorders · metal transport · proteomics · transition metals

Introduction

The completion of the human genome project has opened up many avenues for research in human health and disease. In order to understand the complex mechanism of biological processes and metal-associated diseases, one needs to look beyond the genes to the primary components of cellular events: the proteins. While the cell's genome is static, its proteome is as varied and dynamic as the different states and responses elicited by the cell. A genome encodes the protein sequence but does not provide any information about protein localization, structure, stability or interaction with other proteins. Recent advances in high-throughput separation techniques as well as mass spectrometric characterization of protein samples have provided a powerful tool for proteomics research. Efforts are now underway to categorize hundreds of newly discovered proteins according to their organelle location, structure, and function. This process has uncovered large, previously unknown networks of proteins and begun to outline how they interact with each other.

Among the known structurally characterized proteins, one in three contains a metal as a cofactor.[1] The majority of metalloproteins are enzymes (e.g., proteins containing Fe, Cu, Zn) or transcriptional factors (e.g., Zn). The ability of many these metals to exist in multiple oxidation states and different geometries allows them to promote complex biochemical reactions and participate in highly specialized biological functions.[1] Metalloproteins are involved in electron

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transport, oxygen storage, metal transport, chemical bond hydrolysis, redox processes, and synthesis of biochemical compounds. However, in spite of recent efforts to understand the components of intracellular metal transport and its disposition, the overall blueprint of metal metabolism remains elusive. Since metals are potentially toxic to cells, various specific defenses and disposal mechanisms for metals exist. Such mechanisms likely involve a large array of metal-stress proteins (e.g., metallothionein), regulatory proteins, and signaling proteins. Some are soluble whereas others are membrane-bound transporters. Current proteomics technology can be successfully employed to understand these metabolic processes involving metals. Metalloproteomics attempts to identify systematically large sets of proteins associated with metals and analyze their regulation, modification, interaction, structural assembly, and function as well as their involvement in diseases. Several groups of researchers, including our laboratory, are using proteomics technologies to study metal metabolism.[2–6] However, these efforts are still either in theoretical stages or are preliminary in nature. Therefore, a conceptual background of some of the aspects of metal metabolism along with the latest proteomics technologies is considered in this article. It is our hope such efforts will help to focus the future direction of proteomics studies related to metal metabolism.

Metals in the Environment

Today's environment consists of numerous natural as well as artificial metals. Metals have played a critical role in industrial development and technological advances. Most metals are not destroyed and accumulate in the environment.[7] As a result, biological life is exposed to high levels of various metals. The widespread distribution of metals in the environment is of great concern because of their toxicity; however, some metals are also essential for normal growth and development. Metals have been classified as essential, beneficial, and detrimental.[8] Trace elements essential for life include iron, zinc, copper, chromium, iodine, cobalt, molybdenum, and selenium. The beneficial metals include silicon, manganese, boron, and vanadium. Detrimental metals are those that are considered toxic, such as lead, cadmium, mercury, and arsenic. However, essential and beneficial metals can also have detrimental effects at high levels. The toxicity of metals is largely categorized into heavy metal toxicity, radiation effects of radioactive metals, carcinogenicity, and genotoxicity.[9]

Intracellular metal metabolism and the transport of copper, iron, and zinc: Intracellular concentrations of many transition metals are highly regulated due to their ability to catalyze the formation of harmful free radicals.[10] Detailed reviews of how cells achieve strict homeostatic control over copper, iron, and zinc are available.[11–14] In order to discuss proteomics of metal transport, it is necessary to describe what we know about the intracellular metabolism of metals. We therefore present a brief account of the intracellular metal transport of three important metals.

In the cell the delivery of metals to their cellular targets is accomplished by small soluble proteins called metallochaperones.[15] The nature of intracellular copper transport via these chaperone proteins is well known (Figure 1).[12,16] Copper is absorbed from the gastrointestinal tract by the P-type copper transporting ATPase, ATP7A, also known as the Menkes ATPase protein. In the circulation, copper joins the exchangeable pool where it is complexed to albumin and histidine.[17] Cu^{I} is transported into the cell by the human copper transporter (hCTR1) and is then shuttled to its cellular targets by a number of small metallochaperones that include Atox1, Ccs1, Cox17 and glutathione (GSH) for delivery to ATP7B (the Wilson ATPase protein), superoxide dismutase (SOD), cytochrome C oxidase and metallothionein, respectively. ATP7B transfers copper to the luminal side of the secretory network and is involved in incorporating copper into ceruloplasmin and in conveying copper to the bile canalicular membrane for biliary excretion. Both ATP7A and ATP7B transport copper specifically.[16,18] Although we have greatly increased our understanding of intracellular copper transport and how copper is distributed to different enzymes, many more proteins are likely to be involved in this process. Moreover, chaperone proteins for many other essential metals have not yet been discovered.[19]

The main iron transport protein in plasma is transferrin, which has two ferric iron-binding sites.[13] A number of proteins are involved in the cellular uptake of iron. These include the transferrin receptors 1 and 2 as well as the divalent metal transporter (DMT-1).[20–22] In the cell, excess iron is stored as ferritin, a multimeric protein that can bind upwards of 4000 iron atoms.[13] The regulation of gene products involved in iron metabolism is influenced by two cytoplasmic iron-regulatory proteins with mRNA-binding ability (IRP-1 and IRP-2).[23] Cellular export of iron is accomplished by ferroportin and export from non-intestinal cells requires ceruloplasmin.[11]

Zinc circulating in blood plasma is bound primarily to albumin and α_2-macroglobulin.[24] Mammalian zinc transporters fall into two main categories: the ZIP (ZRT, IRT-like protein) family and the CDF (cation diffusion facilitator) family.[14] The former group is responsible for zinc import into the cytoplasm, either from the extracellular space or from the lumen of intracellular organelles. The CDF family of transporters exports zinc from the cytoplasm out of the cell or into organelles. In the cytoplasm excess zinc is sequestered in metallothioneins.

Nature of metal binding sites: Metal-binding sites are heterogeneous and differ in the composition of their amino acid ligands, the number of ligands involved in binding and their coordination geometry. Amino acid ligands for metals can be in proximity to each other from the protein primary sequence or because of favorable positioning due to protein tertiary structure. Metal binding sites in proteins are generally located in highly hydrophobic regions.[1,25] Ligands for

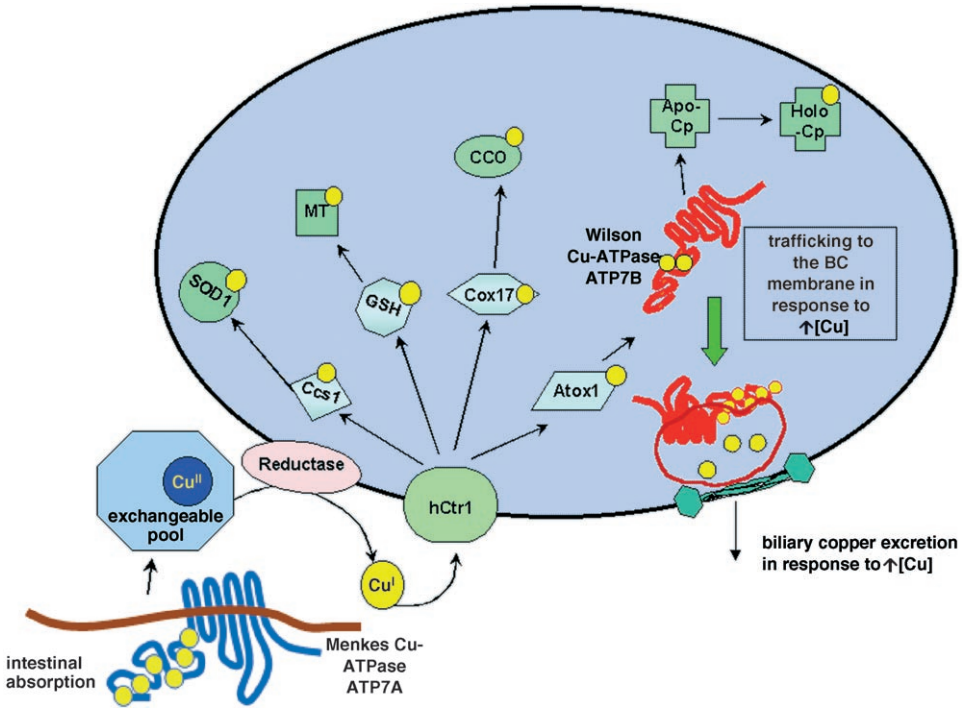

Figure 1. Normal intracellular copper transport in human hepatocyte. Copper is absorbed from the intestine by Menkes copper transporting ATPase (ATP7A). In the circulation, copper joins the exchangeable pool where it is complexed to albumin and histidine. Copper is then reduced by a hypothetical reductase and transported into the cell by human copper transporter (hCTR1). Copper is shuttled by a number of small metallochaperones such as Atox1, Ccs1, Cox17, and GSH for its delivery to ATP7B, SOD, cytochrome C oxidase, and metallothionein, respectively. ATP7B is involved in incorporating copper into ceruloplasmin as well as conveying copper to bile canalicular membrane for biliary excretion.

biologically important metal ions include imidazole nitrogen(s) from histidine residues, carboxylate oxygen from acidic residues such as aspartic acid/glutamic acid, thiol groups from cysteine or thio ether from methionine residues and amide nitrogens and carbonyl oxygens from the peptide backbone.[1,26] There are also specialized prosthetic groups such a porphyrin in hemoglobin.[27] The average interatomic metal–ligand coordination bond lengths in metalloproteins are: histidine, 2.02 Å; cysteine, 2.15 Å; and methionine 2.55 Å.[28] Ligand preference to a metal center is governed by the simple "hard/soft acid/base" (HSAB) principle.[1,29] Hard metals such as iron, chromium, and vanadium prefer hard donor such as carboxylate whereas soft metals such copper and zinc prefer combination with soft ligands such thiol, thio ether, and imidazole. The electronic properties of the metal ion bound to a polypeptide depend on the coordinating ligands but the coordination geometry is controlled by the protein structure.[30,31] Proteins that bind Cu^{I} adopt a linear (two-coordinate), trigonal (three-coordinate) or tetrahedral (four-coordinate) coordination geometry.[26,30] Cu^{II} prefers coordination number of 4, 5 or 6, and the coordination geometry is commonly square planar or octahedral with weak axial coordination.[32] The majority of iron proteins have four-coordinate tetrahedral or square planar sites.[33] Zinc has a preference for four-coordinate tetrahedral geometry but it can assume octahedral geometry as well.[34]

The majority of the catalytic metal centers in enzymes are located in hydrophobic pockets formed by tertiary protein structure. In contrast, metal centers in transport proteins or transcriptional factors are formed by closely spaced repeats of amino acids residues.[31] The most common metal binding motif is the CX_nC motif, consisting of two cysteines separated by two or more amino acids as found in metallothionein, transcriptional factors such as zinc finger motif, copper chaperone proteins, and heavy metal regulatory proteins.[35] ATP7A and ATP7B possess six heavy metal binding domains that each contains a copper-binding GMT/HCXXC motif. The copper chaperones Atox1 and Ccs possess N-terminal MTCXGC and MXCXXC domains, respectively. Metal centers can also form distinct arrangements such as Fe–S clusters, which are found in numerous proteins involved in electron transport mechanisms. In these clusters iron is usually bound by sulfur ligands from Cys and Met residues in various ratios.[36] Copper binding motif similar to the ATCUN motif in albumin is also found in many other proteins including proteins associated with prion and Alzheimer's disease.[37,38]

Metal-related diseases involving copper, iron or zinc: Most metal-associated diseases are related to defects in metal-transport proteins. These defects arise from mutations in the genes encoding for metal-transport proteins. Numerous mutations are often observed in the same gene resulting in a variety of pathological consequences.[9] Thus, it is not only important to identify the defective protein for a particular disease but also the consequences of this defect and the resulting pathophysiological states. Proteomics analysis of metal transport can play an important role in identifying the proteins involved in these processes.

Wilson and Menkes diseases are disorders of copper transport.[39] The Wilson ATPase gene[40–44] encodes a copper transporting P-type ATPase (ATP7B).[45] ATP7B binds six copper atoms with GMTCXXC repeat at the N-terminus of the molecule employing a cooperative mechanism.[46–48] Defects in this protein result in the toxic accumulation of copper in various tissues. Menkes disease is another genetic disorder of copper metabolism characterized by an impair-

ment of the absorption of dietary copper and severe disturbance in the intracellular transport of copper.[49,50] The Menkes ATPase gene encodes a copper-transporting P-type ATPase (ATP7A) with a high degree of homology with ATP7B.[51–53] This protein also binds copper in a 1:6 protein to copper ratio.[47] The defects in this protein lead to the clinical symptoms of classic Menkes disease. As a result, the supply of copper is prevented from reaching developmentally important copper enzymes such as lysyl oxidase, tyrosinase, cytochrome c oxidase, dopamine β-hydroxylase, superoxide dismutase, and amine oxidase.[54,55] Occipital Horn Syndrome is a mild allelic form of Menkes disease causing skeletal dysplasia but with little neurologic impairment except developmental delay.[56] The genetic basis is mutation in ATP7A.

Hereditary hemochromatosis is a disorder of iron metabolism where the uptake of iron from the digestive system is not downregulated, resulting in excessive iron uptake. The body continues to absorb high levels of dietary iron regardless of the levels of stored iron in the body. Hereditary hemochromatosis is an autosomal recessive disorder of iron metabolism. In aceruloplasminemia iron accumulates in various tissues including liver, brain, and pancreas. These findings are consistent with biochemical studies, which suggest direct involvement of ceruloplasmin in iron metabolism pathways. The ferroxidase activity of ceruloplasmin was first proposed in the 1960s.[57–59] The ceruloplasmin gene is located on chromosome 3, spanning approximately 36 kb and consisting of 19 exons.[60] Mutations in this gene lead to aceruloplasminemia demonstrating progressive neurodegeneration of the retinal and basal ganglia.[11] Ceruloplasmin is a secreted blue copper protein, which carries 95% of the circulating copper in the body.[61] The crystal structure shows that the holoprotein contains six copper atoms, three of which are in a trinuclear copper cluster.[62] The trinuclear copper cluster is responsible for activating oxygen during the catalytic cycle of the protein.

Acrodermatitis enteropathica is a rare autosomal recessive disease.[63] It was demonstrated that the disorder was caused by the inability to absorb sufficient zinc.[64] The linkage analysis revealed that the acrodermatitis enterpathica gene is located on chromosome 8q24.3 and the SLC39A4 gene has been found to be the cause for this disorder.[65–68] The SLC39A4 gene encodes for one member of a human zinc-iron regulated transporter-like protein (hZIP) family, hZIP4. Mutations in hZIP4 were found in patients with acrodermatitis enterpathica, which strongly suggest that the disorder is caused by a defect in this gene. The gene encodes a polypeptide of 647 amino acids, having eight transmembrane domains organized in two blocks of three and five. A histidine-rich region, which is considered to be the zinc-binding site, resides between the two blocks of transmembrane domains.[66,67,69] This gene is expressed abundantly in the small intestine, stomach, colon, and kidney. Little is known, however, about the function of hZIP4, although three other members of the ZIP family (hZIP1, hZIP2, and hZIP3) have been shown to transport zinc into the cytoplasm.[70,71]

There are several less common genetic disorders of metal metabolism.[56] TFR2-deficiency is a rare form of hemochromatosis caused by mutations in the transferrin receptor-2 gene, which maps to chromosome 7q22. Clinical features are related to mutations in HFE.[72] Ferroportin deficiency is related to hereditary hemochromatosis and is not related to the HFE locus. Ferroportin is the main iron export pump in macrophages and hepatocytes. Mutations are found in the SLC11A3 gene in chromosome 2q32.[73] Tricho-hepatic-enteric syndrome is a combination of hair abnormality, hepatic dysfunction with iron overload and diarrhea. This disorder may be related to perinatal hemochromatosis but the basis of the disease is unknown. GRACILE syndrome (Fellman syndrome) is caused by mutations in the BCS1L gene, which encodes a protein in the mitochondrial inner membrane required for the assembly of complex III in the mitochondrial respiratory chain.[74] Accumulation of iron in this disorder may be a secondary feature. Iron accumulation becomes less severe, as affected children get older.

The information available clearly indicates that there are systematic mechanisms of metal transport and disposition, most of which remain unknown. The proteomics research can therefore be employed to identify and characterize the members of these metal associated protein networks.

Proteomics Technology

Proteomics technology deals with the identification of large number of proteins from a particular organ, tissue, cell or organelle on the basis of structure or function. This involves protein isolation, separation, and identification. Technical developments in proteomics are led by mass spectrometry (MS), which encompasses the use of two fundamental separation technologies: two-dimensional gel electrophoresis (2DE-MS) and liquid chromatography (LC MS/MS). In a typical proteomics protocol (Figure 2), soluble proteins are first separated by 2D gel electrophoresis based on different isoelectric points (p*I*) and relative molecular masses (M_r). The protein spots are then excised and digested in gel with a site-specific cleavage enzyme, normally trypsin, into peptide fragments. The resulting products are identified by peptide mapping with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) and peptide sequencing with quadrupole tandem mass spectrometry (ion trap or QqTOF MS/MS). Alternatively, the total proteins isolated from a cell line can be directly digested in solution and subsequently analyzed by online LC MS/MS. The peptide mixture is separated by multi-dimensional LC columns, mainly strong cation exchange (SCX) and reversed-phase (RP), and identified by tandem mass spectrometry followed by database search. These mass spectrometry-based methods have been well established for general proteomics investigations which are described elsewhere.[75–78]

Differential protein expression is an important aspect of understanding intracellular metal-caused disease processes.

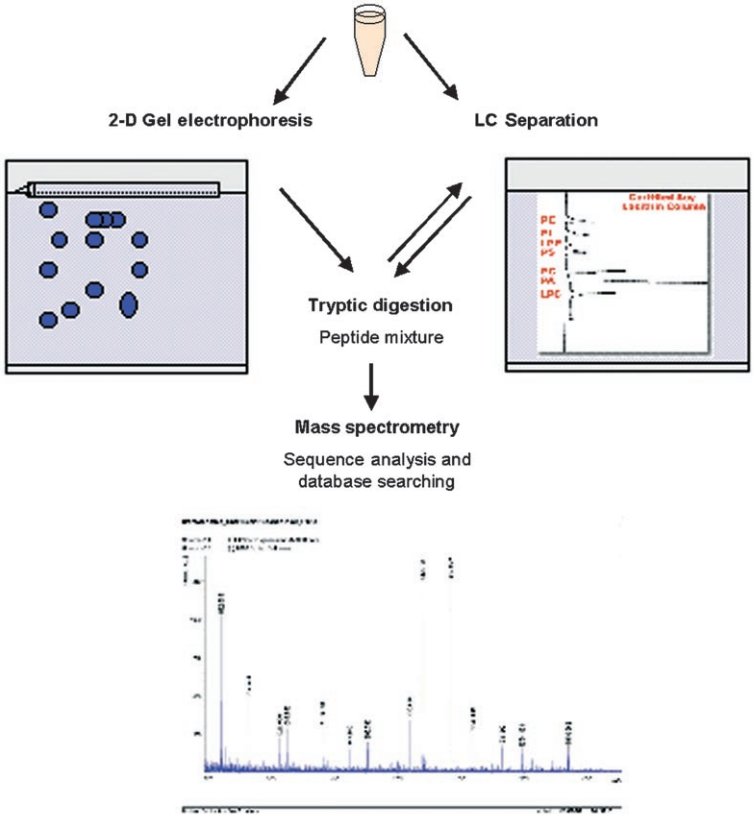

Figure 2. A typical protocol for proteomics analysis of a complex protein mixture.

Functional analysis on the proteome level requires simultaneous identification and quantification of individual proteins within complex mixtures.[79] Advances in mass spectrometric technologies have encouraged researchers to develop new analytical approaches to monitor the dynamic change of proteins. Quantitative profiling of the proteins expressed in a cell or tissue using certain chemicals and heavy-isotopic labeling is possible. The isotope-coded affinity tag (ICAT) reagent (which consists of three components: thiol-specific reactant, isotopically labeled linker and biotin group) was introduced by Gygi et al.[80] to modify Cys-containing proteins. The proteins derived from two different sets of cells are combined and subjected to tryptic digestion, affinity chromatography separation, and LC/MS-MS analysis. Quantitative analysis is performed based on the peaks with 8 Da difference between two identical peptides treated with light- (hydrogen) or heavy- (deuterated) ICAT. In a similar fashion, Cagney and Emili[81] adapted the MCAT (mass-coded abundance tagging) method involving the modification of C-terminal lysine residues of tryptic peptides with different guanidination. In this case the guanidination yielded 42 Da differences between modified and unmodified peptides for the relative quantification of proteins in a mixture. Stable isotopic labeling of proteins or peptides (e.g., ^{2}H, ^{13}C, ^{15}N, ^{18}O, etc.) has become a common method to determine relative protein abundance.[82–84] Recently Ong et al.[85,86] described a SILAC (stable-isotopic labeling in cell culture) approach in which a stably labeled amino acid (^{13}C or ^{15}N) in a cell-culture medium is incorporated into cells in culture. If cell culture conditions are modified, the relative quantification between protein populations in two conditions is achieved by comparing the signal intensities of labeled and unlabeled peptides. Owing to the large number of proteins involved, automatic online LC/MS-MS analysis is considered to be the preferential method for this application. However, it is not the only technique that can be used for such proteomics studies; off-line LC MALDI is an effective alternate choice. In this technique, the complex peptide mixture is separated by HPLC and the fractions are collected onto a MALDI target. Compared to multiple charged ions in ESI, singly charged ions generated from subsequent MALDI analyses simplify data interpretation. The flexibility and quality of MS/MS spectra are greatly improved by means of manual acquisition since samples can be stored on the MALDI target for a long time.

Although proteomics research has been widely used for the identification of novel proteins, unfortunately, there are very few studies related to proteomics of metal transport. We feel there are numerous areas where proteomics technologies can be applied to broaden our understanding of metal transport pathways and of proteins.

Identification of metal binding proteins and their interacting partners: As discussed above Wilson disease is caused by a gene mutation which causes the accumulation of large amounts of copper in the liver that leads to chronic liver damage.[87] The human hepatoma cell line Hep G2, which retains many fundamental characteristics of normal hepatocytes has been used to investigate metal accumulation in the liver and differential protein expression in response to metal toxicity. The immobilized metal affinity chromatography (IMAC) can be utilized to capture the metalloproteins or have metal-binding ability. In a recent study, Roelofen et al.[87] carried out a proteomics analysis of copper-induced changes in protein expression in Hep G2 cell lysates by surface enhanced laser desorption ionization (SELDI) mass spectrometry. Hep G2 cells were loaded with copper in culture and the expressed proteins were detected on weak

cation exchange (WCX) or copper-coated IMAC Ciphergen chips. According to SELDI mass mapping results, they found a number of ions changed intensities in profiling, and corresponded to 15 up-regulated and 6 down-regulated proteins in the cell lysates, and 21 proteins with increased abundance and 4 proteins with decreased abundance in the culture media. Eighteen copper-binding proteins appeared when the copper-depleted cell lysate was examined on a copper-coated IMAC chip by SELDI TOF MS. Although some of these proteins have been predicted to be likely metallothionein (MT) isoforms due to their measured masses, additional MS/MS experiments are required to confirm their identity.

In an initial study, 2DE-MS analysis of the protein fraction from an IMAC column allowed the identification of 38 high abundance copper- and zinc-binding proteins in Hep G2 cells.[2] We identified metal-binding proteins including albumin, enolase, S100 calcium-binding protein, grp78 and transferrin that all contain the following putative metal-binding motifs, $C(X)_nC$ ($n=2$–4) or $H(X)_mH$ ($m=0$–5) (Table 1). Furthermore, we detected several proteins with previously unknown metal-binding capability. For example, protein disulfide isomerase and peroxiredoxin contain similar copper-binding motifs as those in Wilson disease copper-transporting ATPase and the copper chaperone protein ATOX1.[2] In order to identify more proteins, 2DE resolution was improved by isolating Hep G2 subcellular (cytoplasmic and microsomal) fractions and applying them to IMAC. This eventually result-

Table 1. Copper-IMAC binding proteome of Hep G2 with putative metal binding motifs.[6]

Protein name (NCBI or SWISSPROT Accession No.)	Putative metal binding motif(s)
known divalent cation binding proteins	
α-actinin (P128167)	HXH, HX_4H, CX_3C
aldehyde dehydrogenase (P00352)	HH, HX_4H
aminoacylase 1 (Q03154)	HXH, HXXH, HX_4H
annexin IV (P09525)	HXXH
annexin V (Q8V69)	none
calreticulin precursor; calregulin (P27797)	none
α-enolase; 2-phosphopyruvate hydratase (P06733)	none
fructose-biphosphate aldolase A	HH
GRP78; BiP (P11021)	none
PDI (protein disulfide isomerase) (P07237)	2CXXC
PDI (A3) precursor (P30101)	2CXXC
pyruvate kinase, L-isozyme (P14786)	2HXXH
transketolase (P29401)	CX_3C
serum albumin (4389275)	17 disulfides
transferrin (NP_001054)	4CXXC, HH
redox proteins	
aldehyde reductase; alcohol dehydrogenase (P14550)	HH
cytochrome b5 reductase (P00387)	HH
GR (glutathione reductase) (P00390)	HXH, HX_4H, Interchain disulfide
peroxiredoxin (Prx) 1 (Q06830)	interchain disulfide, HXCH
peroxiredoxin 2 (P32119)	interchain disulfide
peroxiredoxin 6 (P30041)	none
chaperones	
co-chaperone p23	none
cyclophilin A; peptidylprolyl isomerase (10863927)	none
Hsp60 (heat shock 60 kD protein)	2HH, HXXH
Hsp70 (heat shock 70kD protein) (P11142)	none
Hsp90 (heat shock 90kD protein 1, α)	HX_4H
stress-induced phosphoprotein 1 (hsp70/hsp90 organizing protein)	HXH, CXXC, CX_3C
GRP78; BiP (P11021)	none
glycolytic enzymes	
GADPH; glyceraldehyde 3-phosphate-dehydrogenase (P04406)	HX_3H, CX_3C
GPI (glucose-6-phosphate isomerase) (P06744)	CXXC, HH, HXH, HX_4H
L-lactate dehydrogenase M chain (P00338)	HX_4H
phosphoglycerate kinase I (P00558)	HXXH
cytoskeletal proteins	
α-tubulin (Q13748)	CX_4C, HX_4H
β-tubulin (P07437)	none
cofilin (P23528)	none
γ-actin (JC5818)	HH
nucleic acid binding proteins	
40S ribosomal protein sa (34/67 kd laminin receptor) (P08865)	none
EEF-1A (euk. translation elongation factor 1 A) (Q96RE1)	HH, HXXH
EIF-3A; p27 BBP protein (NP 852134)	CXXC, CX_3C
EIF-4A (eukaryotic translation initiation factor 4A) (P04765)	CXXC
NDK A (nucleoside diphosphate kinase) (P15531)	none
40S ribosomal protein sa; 35/67 kDa laminin receptor (P08865)	none
40S ribosomal protein s4 (P15880)	HX_3H
ASF2; alternative splicing factor 2 (B40040)	HH
heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) (P22626)	HH, HX_4H
hnRNP H (P31943)	HXXH
hnRNP H3 (P31942)	2HXXH, HX_5H
hnRNP K (NP 112553)	none
hnRNP L (P14866)	CXXC, 2HXH, $5HX_5H$
LYAR	4CXXC, HH, HX_5H
Pro/Glu rich splicing factor; polypyramidine tract-binding protein (P23246)	2HH, HXH, HXXH, $2HX_3H$, $2HX_4H$
ribonucleoprotein	HX_4H
SAP 62; spliceosome-associated protein; splicing factor 3a subunit 2 (NP_009096)	CXXC
miscellaneous	
14-3-3 Protein	none
aspartate transaminase	HX_3H
glutathione synthetase (P48637)	HXH, HX_3H

Table 1. (Continued)

Protein name (NCBI or SWISSPROT Accession No.)	Putative metal binding motif(s)
HDGF (hepatoma-derived growth factor)	none
isocitrate dehydrogenase	HX_5H
nuclear chloride channel protein	none
PA28α (proteasome activator 28a subunit) (Q06323)	CX_4C
PEBP (phosphatidylethanolamine binding protein)	HH, HXXH
phosphogylycerate mutase I	none
RanBP1 (Ran binding protein 1) (P43487)	HXXH
UDP-glucose dehydrogenase	HXH
γ-actin (7441428)	HH
GADPH; glyceraldehydes-3-phosphate dehydrogenase (P04406)	HX_3H, CX_3C
RACK 1; guanine nucleotide binding protein (P25388)	HXH, HXXH

ed in the identification of a total of 67 copper-binding proteins (Figure 3).[6] Our analyses with high mass-accuracy MALDI QqTOF mass spectrometry had been extended to the copper metalloproteome on the other hepatoma cell lines Mz-Hep-1 and SK-Hep-1, and normal human liver, which showed similar profiles but with subtle differences.

Protein–protein interactions have been of increasing importance to address protein function involved in the regulation of cellular processes. Intensive efforts are required to identify the interacting partners of individual proteins on a large scale.[88] Common strategies to study protein–protein interaction are also known as "bait-and-fishing" techniques.[89] Large protein complexes are used as "bait" in a cell line to co-precipitate with associated proteins using affinity tags or antibodies, then the "fishing" proteins are separated by SDS-PAGE gel and identified by mass spectrometry.[89] To date no vigorous studies have been performed in the hepatic system.

Post-translational modifications: In addition to protein identification and quantitative analysis, a protein's post-translation modifications (PTMs) are also of particular interest to biologists. Metalloproteins include numerous metalloenzymes, metal-transport proteins, and metallochaperones that are involved in metal-dependent catalytic activity and regulatory roles with respect to reversible phosphorylation and dephosphorylation reactions. We have shown that post-translational modifications could be affected by the intracellular action of copper, in which the glycerylphosphoryl group at residue Glu-301 of elongation factor 1-alpha was lost in Hep G2 cells after copper depletion (Figure 4).[2] However, no detailed study of metal-transport phosphoproteomics, that is, how the proteins are catalyzed and regulated by kinases and phosphatases, has been reported to date.

Protein phosphorylation is a common modification, involving the addition of a phosphate ester group to the side chain of the hydroxyl amino acids (serine, threonine, and tyrosine), phosphoramidates of arginine, histidine, lysine, or acyl derivatives of aspartic and glutamic acid.[90,91] Although this post-translational modification cannot be predicted accurately from its translated gene sequence alone, mass spectrometry offers a straightforward approach to the identification of

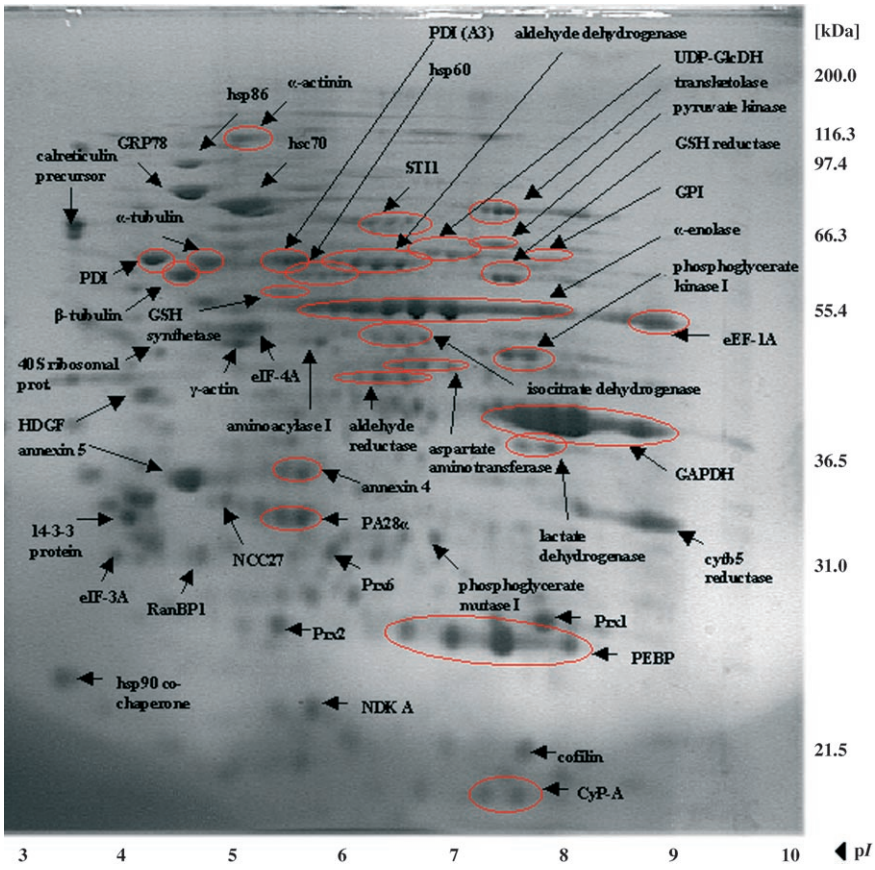

Figure 3. 2DE map of Hep G2 cytoplasmic proteins displaying copper-binding ability.[6] After elution from a Cu-IMAC column the proteins were separated on a pH 3–10 IPG strip and resolved on a vertical 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250 and protein spots were analyzed by MALDI-TOF MS. The identified proteins and their putative metal binding motifs are listed in Table 1.

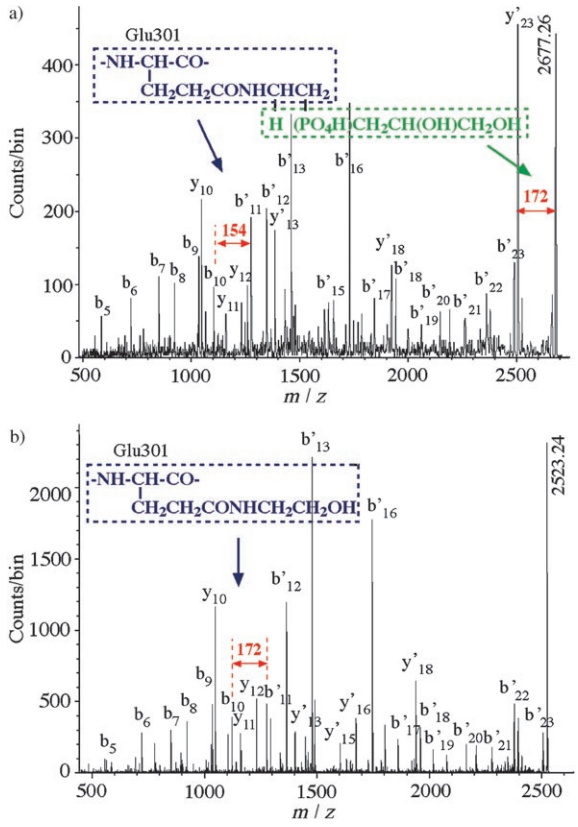

Figure 4. Peptide sequencing by MS/MS measurements.[2] Peptides were obtained from Cu-IMAC column in normal Hep diarrheaG2 cells (at *m*/*z* 2677.259) and copper-depleted Hep G2 cells (at *m*/*z* 2523.235). MALDI MS/MS spectra were acquired by Manitoba/Sciex prototype QSTAR QqTOF mass spectrometer, and assigned to the peptide sequence 291–313 (SVEMHHEALSEALPGDNVGFNVK) of elongation factor 1-alpha. The post-translational modification at residue Glu301 was identified and the corresponding chemical structures are shown in the insets ((a) glycerylphosphorylethanolamine and (b) ethanolamine).

the precise phosphorylation sites and quantitative comparison of the abundance of phosphorylated proteins in cells.[90–92] However, a direct MS analysis of phosphopeptides in a peptide mixture is often hindered by signal suppression of negatively charged phosphate groups in the positive ionization mode. The detection becomes more difficult when a phosphorylated peptide is at low stoichiometry (compared with nonphosphorylated peptide) and has multiple phosphorylation sites. Chemical or instrumental isolation of phosphopeptides prior to MS analysis improves experimental accuracy. The MS-based proteomics technologies can now be integrated with HPLC separation,[93] antibody-affinity purification,[94,95] IMAC enrichment[96,97] and tandem mass spectrometry (product ion scanning, precursor ion scanning, and neutral loss scanning).[98,99]

Immunoprecipitation using antibodies is an effective method to detect low abundance tyrosine phosphorylated proteins, but not well suitable for those with phosphorylated serine and threonine residues. IMAC-Fe^{3+} (or Ga^{3+}) facilitates a selective enrichment of phosphopeptides under acidic conditions.[100,101] However, the peptides rich in aspartic acids and glutamic acids also bind to these columns nonspecifically due to chemical similarity of carboxylate and phosphate groups, although this problem can be alleviated by converting carboxylic acid groups to methyl esters.[101] In some instances β-elimination of the phosphate group followed by introduction of an affinity tag such as biotin to capture phosphorylated species was also employed to detect phosphorylated peptides.[102,103] The weakness of this technique is that O-linked glycopeptide sites undergo the same elimination and interfere with the phosphorylation assignments. The tandem mass spectrometric technique used for characteristic fragments of a phosphopeptide by precursor ion scanning at *m*/*z* 79 (PO^{3-}) and 63 (PO_2^-) at negative ion mode simplifies analysis of peptide mixtures,[104] but it requires an additional peptide sequencing with MS/MS measurements (i.e., product ion scanning) at positive ion mode to identify the phosphorylation sites. Alternately, phosphotyrosine-specific immonium ion scanning at m/z 216 in positive ion mode has been successfully used to detect tyrosine-phosphorylated peptides. Similarly, a neutral loss of 98 and 80 Da corresponds to the loss of H_3PO_4 and HPO_3, respectively, which is commonly used for detection of phosphoserine and phosphothreonine peptides. The conventional methods using radioactive labeling with the incorporation of ^{32}P to identify phosphoproteins over a widespread-proteome scale have their own advantages and limitations. Currently, we are developing methods using mass spectrometry to understand the effects of metal ions on phosphorylation levels of cellular proteins.

Quantification of proteins and biomarker technology: Since metals play structural and catalytic roles in many biochemical reactions, metal-binding proteins may serve as biomarkers for many diseases. In this effort, some studies have shown that electrospray ionization (ESI) mass spectrometry can be effectively used to determine the metal-binding selectivity and stoichiometry in metal–protein complexes under physiological conditions.[105–109] Quantitative determination of the metals in various biological systems is also achieved by trace or ultra-trace elemental analysis using inductively coupled plasma mass spectrometry (ICP-MS), termed as "element-tagged" or "heteroatom-tagged" proteomics.[110,111] So far ICP-MS has been coupled to high-performance liquid chromatography (HPLC) to measure the metal contents in many metal transport proteins.

Protein biomarkers have attracted considerable attention from many clinical researchers. Similar to 2DE-MS and LC/MS-MS analysis, SELDI MS has demonstrated the ability for the early detection of changed protein profiles associated with disease states.[112–115] Approaches include the comprehensive proteomics analysis of very small amounts of normal and diseased tissues to identify aberrantly expressed proteins by mass spectrometry.[114] Such identification of new disease-specific targets will provide potential applications to protein-based diagnostics, and inevitably lead to drug discovery.[114]

Using an animal model, Simpson et al.[3] have conducted a copper dependant proteomics investigation in the liver of Cambridge breed sheep. Liver copper was accumulated over three months with a copper overload of up to two and four times of normal levels. The protein identification was performed by 2DE-MS peptide mass fingerprinting and a cross-species database search. Eighteen hepatic soluble proteins expressed differentially after copper overloading were identified, in which 16 proteins were upregulated whereas only apolipoprotein A-1 and serotransferrin were downregulated. The changes in abundance of a number of proteins, such as aldehyde dehydrogenase, carbonic anhydrase II, glutamate dehydrogenase, $NADP^+$-dependent isocitrate dehydrogenase, and protein disulfide isomerase, were interpreted as consistent with an early adaptive response to oxidative stress.

Limitations of current metalloproteomics strategies: Metalloproteomics focuses on the qualitative identification, quantitative analysis and structural characterization of metal-binding protein, and their structural metal-binding motifs using current proteomics technologies. This includes a number of studies on metal-transport proteins and the associated metals in a cellular process or a disease state. However, some metalloproteins are inherently unstable outside a narrow range of environmental conditions. In addition, a wide dynamic range of relative protein expression makes it hard to detect proteins expressed at low levels. Technical challenges are largely responsible for the slow evolution of proteomics research in this area.

Two-dimensional gel electrophoresis is a mature technique that has been commonly used for separating protein mixtures prior to the mass spectrometric analysis. A two-dimensional gel view provides a clear pattern of protein expression; it is capable of resolving thousands of proteins and allowing a straightforward comparison between different cellular states. Following high-resolution 2D separation, proteins of interest can be selected and then identified by mass spectrometry. Unfortunately, because of time-consuming processes in gel electrophoresis and in-gel enzymatic digestion, 2DE-MS is likely to remain fairly low-throughput. It also requires relatively large amount of starting material. This strategy often fails to detect low abundance proteins, proteins that are highly acidic or basic and large membrane proteins. This problem is extremely frustrating since many metalloproteins are membrane or membrane associated proteins as exemplified by the Wilson and Menkes ATPases (ATP7B and ATP7A).

The shortcomings of 2DE-MS have raised the need for developing a gel-free technology for proteomics analysis. LC-MS/MS is gaining acceptance and is amenable to automation. In principle, to detect low abundance proteins in a complicated peptide mixture, separation can be performed by using one-dimensional (RP), two-dimensional (SCX/RP) or three-dimensional (SCX/avidin/RP) chromatography. However, LC-MS/MS methods also have some inherent disadvantages. First, protein sequences must be in the database. Second, data validation (by manual inspection) is required on the low-quality spectrum, especially in the case of incorrect assignments by the mass spectrometric software. Third, despite the high-throughput nature of automated LC-MS/MS analysis, identification of unknown proteins or peptides with unusual modifications is very difficult.

Metalloproteins are sensitive to various experimental conditions and therefore difficult to isolate in a state of physiological conformation. The changes in their tertiary structure often results in the disturbance of the buried metal binding sites resulting in a loss of metal ions. The utilization of IMAC for proteomics analysis of metal-transport proteins takes advantage of the metal selective affinity to histidine-rich proteins and the proteins having potential metal-binding sites.[116,117] The cellular proteins bind to metals in vitro at physiological environment, which provides useful information to explore metal–protein binding properties in vivo. Nevertheless, formation of metal–protein complexes in IMAC cannot simulate the native metal-binding properties of proteins or the required geometry of the metal center in vivo. These proteins also compete with other proteins for column adsorption at neutral conditions. Thus, non-specific binding becomes an inevitable problem when isolating metalloproteins by IMAC alone. Although this can be avoided by converting proteins into peptides using on-column digestion as demonstrated in our previous experiments.[2] In the case of copper-IMAC, peptide binding prefers the order of residues histidine $>$ methionine $>$ cysteine. Even though we were able to detect protein disulfide isomerase (PDI) by our copper–IMAC approach (2DE-MS), the metal-binding peptides containing CXXC motifs can often be missed.[2] However, it is essential that the proteins obtained from IMAC and 2DE-MS tested for their specific metal-binding properties in follow-up experiments.[118]

Conclusions and Future Perspectives

Transition metals play a myriad of roles in biological systems. Major changes in environmental metal levels result in significant metal exposure to humans. Because of the environmental metal exposure, as well as diseases associated with metal transport in humans, there is an urgent need to understand the components of the human proteome involved in metal transport. This research seeks to identify the entire set of proteins that participate in metal binding, that are metal-induced as well as their partner proteins. The identification of novel metalloproteins, proteins that bind metals tightly and integrally, remains a major challenge, as does the detection of low abundance metal-binding proteins. Metal detection and its quantification directly in biological systems need to be combined efficiently with current proteomics strategies. The success of this approach will largely depend on the use of innovative protocols such as radioactive metal detection,[4] colorimetric assays[119] and protein enrichment with metal-loaded IMAC columns.[116] An efficient metal detection system integrated with novel separation

techniques will enhance the speed of detection. For example, ICP-MS coupled with LC can selectively detect metal containing proteins which can be analyzed by MS.[111] In addition, X-ray techniques such as X-ray absorption spectroscopy,[120] capillary electrophoresis with x-ray fluorescence spectroscopy,[121] micro-particle induced X-ray emission (PIXE),[122] and two photon microscopy[123] are capable of identifying and quantifying metals in biological systems. They can also be used to structurally characterize the metal binding sites.[124] The use of these techniques in concert with MS, X-ray crystallography, and NMR spectroscopy will improve the speed and efficiency of proteomics studies exploring metal transport in normal physiology and in metal-caused diseases.

Undoubtedly, proteomics as a way to delineate the function and activity of metal-transport proteins will continue to advance. Traditional 2DE-MS and LC-MS based approaches coupled with quantitative proteomics technologies will be further extended to improve the current comprehensive analysis of biological functions of metal-binding proteins expressed in different cell-types and tissue samples related to metal-caused diseases. The technical advances in proteomics will be accelerated by further developments of mass spectrometric instrumentation, protein separation, and purification techniques. We believe that current endeavors relating to proteomics of metal-transport will play an essential role in the identification of biomarkers for metal-associated diseases to improve clinical diagnosis and expedite development of novel treatment modalities.

Acknowledgements

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[1] R. H. Holm, P. Kennepohl, E. I. Solomon, *Chem. Rev.* **1996**, *96*, 2239–2314.
[2] Y. M. She, S. Narindrasorasak, S. Yang, N. Spitale, E. A. Roberts, B. Sarkar, *Mol. Cell. Proteomics* **2003**, *2*, 1306–1318.
[3] D. M. Simpson, R. J. Beynon, D. H. Robertson, M. J. Loughran, S. Haywood, *Proteomics* **2004**, *4*, 524–536.
[4] A. Katayama, A. Tsujii, A. Wada, T. Nishino, A. Ishihama, *Eur. J. Biochem.* **2002**, *269*, 2403–2413.
[5] F. Vanrobaeys, B. Devreese, E. Lecocq, L. Rychlewski, L. De Smet, J. Van Beeumen, *Proteomics* **2003**, *3*, 2249–2257.
[6] S. D. Smith, Y. M. She, E. A. Roberts, B. Sarkar, *J. Proteome Res.* **2004**, *3*, 834–840.
[7] B. Sarkar, *Heavy Metals in Environment*, Marcel Dekker, New York, **2002**.
[8] G. F. Nordberg, B. Sandstrom, G. Becking, R. A. Goyer, in *Heavy Metals in the Environment* (Ed.: B. Sarkar), Marcel Dekker, New York, **2002**.
[9] D. W. Cox, L. M. Cullen, J. R. Forbes, in *Heavy Metals in the Environment* (Ed.: B. Sarkar), Marcel Dekker, New York, **2002**.
[10] T. V. O'Halloran, V. C. Culotta, *J. Biol. Chem.* **2000**, *275*, 25057–25060.
[11] E. D. Harris, Y. Qian, M. C. Reddy, *Mol. Cell. Biochem.* **1998**, *188*, 57–62.
[12] D. L. Huffman, T. V. O'Halloran, *Annu. Rev. Biochem.* **2001**, *70*, 677–701.
[13] M. W. Hentze, M. U. Muckenthaler, N. C. Andrews, *Cell* **2004**, *117*, 285–297.
[14] T. Kambe, Y. Yamaguchi-Iwai, R. Sasaki, M. Nagao, *Cell. Mol. Life Sci.* **2004**, *61*, 49–68.
[15] R. A. Pufahl, C. P. Singer, K. L. Peariso, S. J. Lin, P. J. Schmidt, C. J. Fahrni, V. C. Culotta, J. E. Penner-Hahn, T. V. O'Halloran, *Science* **1997**, *278*, 853–856.
[16] N. Fatemi, B. Sarkar, *Environ. Health Perspect.* **2002**, *110 Suppl 5*, 695–698.
[17] P. Deschamps, P. P. Kulkarni, M. Gautam-Basak, B. Sarkar, *Coord. Chem. Rev.* **2005**, *249*, 895–909.
[18] Z. H. Lu, C. T. Dameron, M. Solioz, *Biometals* **2003**, *16*, 137–143.
[19] E. Luk, L. T. Jensen, V. C. Culotta, *J. Biol. Inorg. Chem.* **2003**, *8*, 803–809.
[20] Y. Cheng, O. Zak, P. Aisen, S. C. Harrison, T. Walz, *Cell* **2004**, *116*, 565–576.
[21] C. Camaschella, A. Roetto, A. Cali, M. De Gobbi, G. Garozzo, M. Carella, N. Majorano, A. Totaro, P. Gasparini, *Nat. Genet.* **2000**, *25*, 14–15.
[22] R. E. Fleming, *Curr. Opin. Gastroenterol.* **2005**, *21*, 201–206.
[23] G. Cairo, A. Pietrangelo, *Biochem. J.* **2000**, *352 Pt 2*, 241–250.
[24] J. G. Reyes, *Am. J. Physiol.* **1996**, *270*, C401–10.
[25] M. M. Yamashita, L. Wesson, G. Eisenman, D. Eisenberg, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5648–5652.
[26] S. Karlin, Z. Y. Zhu, K. D. Karlin, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14225–14230.
[27] J. Kuchar, R. P. Hausinger, *Chem. Rev.* **2004**, *104*, 509–525.
[28] L. Rulisek, J. Vondrasek, *J. Inorg. Biochem.* **1998**, *71*, 115–127.
[29] J. A. Cowan, *Inorganic Biochemistry, An Introduction*, 2nd ed., Wiley-VCH, New York, **1997**.
[30] K. A. Koch, M. M. Pena, D. J. Thiele, *Chem. Biol.* **1997**, *4*, 549–560.
[31] S. Karlin, Z. Y. Zhu, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8344–8349.
[32] E. I. Solomon, U. M. Sundaram, T. E. Machonkin, *Chem. Rev.* **1996**, *96*, 2563–2606.
[33] E. Y. Tshuva, S. J. Lippard, *Chem. Rev.* **2004**, *104*, 987–1012.
[34] S. Karlin, Z. Y. Zhu, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14231–14236.
[35] T. V. O'Halloran, *Science* **1993**, *261*, 715–725.
[36] P. Venkateswara Rao, R. H. Holm, *Chem. Rev.* **2004**, *104*, 527–559.
[37] C. Harford, B. Sarkar, *Acc. Chem. Res.* **1997**, *30*, 123–130.
[38] C. S. Burns, E. Aronoff-Spencer, G. Legname, S. B. Prusiner, W. E. Antholine, G. J. Gerfen, J. Peisach, G. L. Millhauser, *Biochemistry* **2003**, *42*, 6794–6803.
[39] B. Sarkar, *Chem. Rev.* **1999**, *99*, 2535–2544.
[40] A. M. Bowcock, L. A. Farrer, L. L. Cavalli-Sforza, J. M. Hebert, K. K. Kidd, M. Frydman, B. Bonne-Tamir, *Am. J. Hum. Genet.* **1987**, *41*, 27–35.
[41] A. M. Bowcock, L. A. Farrer, J. M. Hebert, M. Agger, I. Sternlieb, I. H. Scheinberg, C. H. Buys, H. Scheffer, M. Frydman, T. Chajek-Saul, B. Bonne-Tamir, L. L. Cavalli-Sforza, *Am. J. Hum. Genet.* **1988**, *43*, 664–674.
[42] V. Yuzbasiyan-Gurkan, G. J. Brewer, E. Boerwinkle, P. J. Venta, *Am. J. Hum. Genet.* **1988**, *42*, 825–829.
[43] P. C. Bull, G. R. Thomas, J. M. Rommens, J. R. Forbes, D. W. Cox, *Nat. Genet.* **1993**, *5*, 327–337.
[44] R. E. Tanzi, K. Petrukhin, I. Chernov, J. L. Pellequer, W. Wasco, B. Ross, D. M. Romano, E. Parano, L. Pavone, L. M. Brzustowicz, M. Devoto, J. Peppercorn, I. Bush, I. Sterlieb, M. Pirastu, J. F. Gusella, O. Evgrafov, G. K. Penchaszadeh, B. Honig, I. S. Edelman, M. B. Soares, I. H. Scheinberg, T. C. Gilliam, *Nat. Genet.* **1993**, *5*, 344–350.
[45] B. Sarkar, *J. Inorg. Biochem.* **2000**, *79*, 187–191.
[46] M. DiDonato, S. Narindrasorasak, J. R. Forbes, D. W. Cox, B. Sarkar, *J. Biol. Chem.* **1997**, *272*, 33279–33282.

[47] S. Lutsenko, K. Petrukhin, M. J. Cooper, C. T. Gilliam, J. H. Kaplan, *J. Biol. Chem.* **1997**, *272*, 18939–18944.
[48] P. Y. Jensen, N. Bonander, L. B. Moller, O. Farver, *Biochim. Biophys. Acta* **1999**, *1434*, 103–113.
[49] D. M. Danks, E. Cartwright, B. J. Stevens, R. R. Townley, *Science* **1973**, *179*, 1140–1142.
[50] S. M. Herd, J. Camakaris, R. Christofferson, P. Wookey, D. M. Danks, *Biochem. J.* **1987**, *247*, 341–347.
[51] J. Chelly, Z. Tumer, T. Tonnesen, A. Petterson, Y. Ishikawa-Brush, N. Tommerup, N. Horn, A. P. Monaco, *Nat. Genet.* **1993**, *3*, 14–19.
[52] J. F. Mercer, J. Livingston, B. Hall, J. A. Paynter, C. Begy, S. Chandrasekharappa, P. Lockhart, A. Grimes, M. Bhave, D. Siemieniak, T. W. Glover, *Nat. Genet.* **1993**, *3*, 20–25.
[53] C. Vulpe, B. Levinson, S. Whitney, S. Packman, J. Gitschier, *Nat. Genet.* **1993**, *3*, 7–13.
[54] D. M. Danks, in *Metabolic basis of inherited disease* (Ed.: M. G. Hill), New York, **1995**.
[55] M. DiDonato, S. Narindrasorasak, B. Sarkar, *Copper Transport and its Disorders: Molecular and Cellular Aspects*, Plenum Publishing, New York, **1999**.
[56] E. A. Roberts, in *Physician's Guide to the Treatment and Follow-up of Metabolic Diseases* (Eds.: N. Blau, G. F. Hoffman, J. Leonard, J. T. R. Clarke), Springer, Heidelberg, in press (**2005**).
[57] S. Osaki, D. A. Johnson, E. Frieden, *J. Biol. Chem.* **1966**, *241*, 2746–2751.
[58] G. R. Lee, S. Nacht, J. N. Lukens, G. E. Cartwright, *J. Clin. Invest.* **1968**, *47*, 2058–2069.
[59] S. Osaki, D. A. Johnson, E. Frieden, *J. Biol. Chem.* **1971**, *246*, 3018–3023.
[60] W. Takahashi, F. Yoshii, Y. Shinohara, *J. Neuroimmunol.* **1996**, *6*, 246–248.
[61] E. Frieden, *Clin. Physiol. Biochem.* **1986**, *4*, 11–29.
[62] V. N. Zaitsev, I. Zaitseva, M. Papiz, P. F. Lindley, *J. Biol. Inorg. Chem.* **1999**, *4*, 579–587.
[63] N. Danbolt, K. Closs, *Acta Derm. Venereol. Suppl. (Stockh.)* **1943**, *23*, 127–169.
[64] P. M. Barnes, E. J. Moynahan, *Proc. R. Soc. Med.* **1973**, *66*, 327–329.
[65] K. Wang, E. W. Pugh, S. Griffen, K. F. Doheny, W. Z. Mostafa, M. M. al-Aboosi, H. el-Shanti, J. Gitschier, *Am. J. Hum. Genet.* **2001**, *68*, 1055–1060.
[66] S. Kury, B. Dreno, S. Bezieau, S. Giraudet, M. Kharfi, R. Kamoun, J. P. Moisan, *Nat. Genet.* **2002**, *31*, 239–240.
[67] K. Wang, B. Zhou, Y. M. Kuo, J. Zemansky, J. Gitschier, *Am. J. Hum. Genet.* **2002**, *71*, 66–73.
[68] A. Nakano, H. Nakano, K. Nomura, Y. Toyomaki, K. Hanada, *J. Invest. Dermatol.* **2003**, *120*, 963–966.
[69] M. L. Guerinot, *Biochim. Biophys. Acta* **2000**, *1465*, 190–198.
[70] L. A. Gaither, D. J. Eide, *J. Biol. Chem.* **2000**, *275*, 5560–5564.
[71] L. A. Gaither, D. J. Eide, *J. Biol. Chem.* **2001**, *276*, 22258–22264.
[72] A. Roetto, F. Daraio, F. Alberti, P. Porporato, A. Cali, M. De Gobbi, C. Camaschella, *Blood Cells Mol. Dis.* **2002**, *29*, 465–470.
[73] O. T. Njajou, N. Vaessen, M. Joosse, B. Berghuis, J. W. van Dongen, M. H. Breuning, P. J. Snijders, W. P. Rutten, L. A. Sandkuijl, B. A. Oostra, C. M. van Duijn, P. Heutink, *Nat. Genet.* **2001**, *28*, 213–214.
[74] I. Visapaa, V. Fellman, J. Vesa, A. Dasvarma, J. L. Hutton, V. Kumar, G. S. Payne, M. Makarow, R. Van Coster, R. W. Taylor, D. M. Turnbull, A. Suomalainen, L. Peltonen, *Am. J. Hum. Genet.* **2002**, *71*, 863–876.
[75] R. Aebersold, M. Mann, *Nature* **2003**, *422*, 198–207.
[76] H. Steen, M. Mann, *Nat. Rev. Mol. Cell. Biol.* **2004**, *5*, 699–711.
[77] D. Figeys, *Anal. Chem.* **2003**, *75*, 2891–2905.
[78] T. J. Griffin, R. Aebersold, *J. Biol. Chem.* **2001**, *276*, 45497–45500.
[79] M. Hamdan, P. G. Righetti, *Mass Spectrom. Rev.* **2002**, *21*, 287–302.
[80] S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb, R. Aebersold, *Nat. Biotechnol.* **1999**, *17*, 994–999.
[81] G. Cagney, A. Emili, *Nat. Biotechnol.* **2002**, *20*, 163–170.
[82] Y. Oda, K. Huang, F. R. Cross, D. Cowburn, B. T. Chait, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6591–6596.
[83] D. R. Goodlett, A. Keller, J. D. Watts, R. Newitt, E. C. Yi, S. Purvine, J. K. Eng, P. von Haller, R. Aebersold, E. Kolker, *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1214–1221.
[84] X. Yao, A. Freas, J. Ramirez, P. A. Demirev, C. Fenselau, *Anal. Chem.* **2001**, *73*, 2836–2842.
[85] S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey, M. Mann, *Mol. Cell. Proteomics* **2002**, *1*, 376–386.
[86] S. E. Ong, I. Kratchmarova, M. Mann, *J. Proteome Res.* **2003**, *2*, 173–181.
[87] H. Roelofsen, R. Balgobind, R. J. Vonk, *J. Cell. Biochem.* **2004**, *93*, 732–740.
[88] C. von Mering, R. Krause, B. Snel, M. Cornell, S. G. Oliver, S. Fields, P. Bork, *Nature* **2002**, *417*, 399–403.
[89] A. Kumar, M. Snyder, *Nature* **2002**, *415*, 123–124.
[90] R. Aebersold, D. R. Goodlett, *Chem. Rev.* **2001**, *101*, 269–295.
[91] M. Mann, S. E. Ong, M. Gronborg, H. Steen, O. N. Jensen, A. Pandey, *Trends Biotechnol.* **2002**, *20*, 261–268.
[92] K. M. Loyet, J. T. Stults, D. Arnott, *Mol. Cell. Proteomics* **2005**, *4*, 235–245.
[93] B. A. Ballif, J. Villen, S. A. Beausoleil, D. Schwartz, S. P. Gygi, *Mol. Cell. Proteomics* **2004**, *3*, 1093–1101.
[94] H. Steen, B. Kuster, M. Fernandez, A. Pandey, M. Mann, *J. Biol. Chem.* **2002**, *277*, 1031–1039.
[95] M. Gronborg, T. Z. Kristiansen, A. Stensballe, J. S. Andersen, O. Ohara, M. Mann, O. N. Jensen, A. Pandey, *Mol. Cell. Proteomics* **2002**, *1*, 517–527.
[96] M. Hansson, A. V. Vener, *Mol. Cell. Proteomics* **2003**, *2*, 550–559.
[97] C. E. Haydon, P. A. Eyers, L. D. Aveline-Wolf, K. A. Resing, J. L. Maller, N. G. Ahn, *Mol. Cell. Proteomics* **2003**, *2*, 1055–1067.
[98] H. Steen, M. Fernandez, S. Ghaffari, A. Pandey, M. Mann, *Mol. Cell. Proteomics* **2003**, *2*, 138–145.
[99] A. Schlosser, R. Pipkorn, D. Bossemeyer, W. D. Lehmann, *Anal. Chem.* **2001**, *73*, 170–176.
[100] Y. M. She, Y. W. Huang, L. Zhang, W. S. Trimble, *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1123–1130.
[101] S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt, F. M. White, *Nat. Biotechnol.* **2002**, *20*, 301–305.
[102] Y. Oda, T. Nagasu, B. T. Chait, *Nat. Biotechnol.* **2001**, *19*, 379–382.
[103] T. S. Nuhse, A. Stensballe, O. N. Jensen, S. C. Peck, *Mol. Cell. Proteomics* **2003**, *2*, 1234–1243.
[104] G. Neubauer, M. Mann, *Anal. Chem.* **1999**, *71*, 235–242.
[105] X. Yu, M. Wojciechowski, C. Fenselau, *Anal. Chem.* **1993**, *65*, 1355–1359.
[106] P. Hu, Q. Z. Ye, J. A. Loo, *Anal. Chem.* **1994**, *66*, 4190–4194.
[107] Q. P. Lei, X. Cui, D. M. Kurtz, Jr., I. J. Amster, I. V. Chernushevich, K. G. Standing, *Anal. Chem.* **1998**, *70*, 1838–1846.
[108] R. Feng, A. L. Castelhano, R. Belledeau, Z. Yuan, *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 1105–1111.
[109] A. Urvoas, B. Amekraz, C. Moulin, L. Le Clainche, R. Stocklin, M. Moutiez, *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1889–1896.
[110] A. Sanz-Medel, M. Montes-Bayon, M. Luisa Fernandez Sanchez, *Anal. Bioanal. Chem.* **2003**, *377*, 236–247.
[111] J. Szpunar, *Analyst* **2005**, *130*, 442–465.
[112] N. Tang, P. Tornatore, S. R. Weinberger, *Mass Spectrom. Rev.* **2004**, *23*, 34–44.
[113] S. Hanash, *Nature* **2003**, *422*, 226–232.
[114] M. Merchant, S. R. Weinberger, *Electrophoresis* **2000**, *21*, 1164–1177.
[115] F. von Eggeling, K. Junker, W. Fiedle, V. Wollscheid, M. Durst, U. Claussen, G. Ernst, *Electrophoresis* **2001**, *22*, 2898–2902.
[116] E. S. Hemdan, Y. J. Zhao, E. Sulkowski, J. Porath, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 1811–1815.
[117] R. M. Chicz, F. E. Regnier, *Anal. Chem.* **1989**, *61*, 1742–1749.
[118] S. Narindrasorasak, P. Yao, B. Sarkar, *Biochem. Biophys. Res. Commun.* **2003**, *311*, 405–414.

[119] M. Hogbom, U. B. Ericsson, R. Lam, H. M. Bakali, E. Kuznetsova, P. Nordlund, D. B. Zamble, *Mol. Cell. Proteomics* **2005**.
[120] L. Banci, I. Bertini, S. Mangani, *J. Synchrotron Radiat.* **2005**, *12*, 94–97.
[121] S. E. Mann, M. C. Ringo, G. Shea-McCarthy, J. Penner-Hahn, C. E. Evans, *Anal. Chem.* **2000**, *72*, 1754–1758.
[122] R. Ortega, P. Moretto, A. Fajac, J. Benard, Y. Llabador, M. Simonoff, *Cell. Mol. Biol. (Paris)* **1996**, *42*, 77–88.
[123] C. J. Chang, E. M. Nolan, J. Jaworski, K. Okamoto, Y. Hayashi, M. Sheng, S. J. Lippard, *Inorg. Chem.* **2004**, *43*, 6774–6779.
[124] I. Ascone, W. Meyer-Klaucke, L. Murphy, *J. Synchrotron Radiat.* **2003**, *10*, 16–22.

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