Novel perspectives in mammalian copper metabolism through the use of genome-wide approaches

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ABSTRACT
The transition metal copper plays an essential role in many biological processes but is highly toxic in excess. Recent studies have characterized a highly conserved set of proteins that mediate cellular copper import, distribution, sequestration, utilization, and export. Nevertheless, the pathogenesis of copper overload and copper deficiency disorders is not well understood, and we are only beginning to comprehend the results of mild copper overload or deficiency in relation to nutritional uptake and common diseases at the population level. Technological advances open the possibility to dissect the complete genome for genetic variants predisposing to copper overload or depletion and for variations in gene expression generated by either reduced or excessive copper intake. We discuss the potential of integrated genome-wide applications to advance our knowledge of copper homeostasis and to develop molecular biomarker profiles as indicators of copper status. Am J Clin Nutr 2008;88(suppl):821S–5S.

INTRODUCTION
Copper is an essential micronutrient, which plays a critical role in various biological processes. Both shortage and excess of this trace metal can lead to serious abnormalities, as illustrated by the inherited disorders Menkes disease and Wilson disease. A tight balance of copper uptake, excretion, storage, and utilization is therefore crucial and is maintained by several proteins that appear to be largely conserved in evolution. Several of these proteins have been characterized in the past decades via a variety of studies, including positional cloning and heterologous complementation of copper-related phenotypes in yeast (1).

Systems biology can be defined as an approach to understand all the genome-wide changes in a biological system in a specified condition by integrating the studies of different biological organization levels. Systems biology therefore offers unique possibilities to identify novel regulatory mechanisms and adaptive responses involved in copper metabolism. Differences in genomic, transcriptomic, proteomic, and metabolic profiles due to variations in copper exposure will characterize new genes, proteins, signaling cascades, and metabolites that have a role in copper biology. The integration of these techniques will help to dissect the precise regulatory mechanisms that underlie these profiles. Individual profiles may also be used as a fingerprint for variations in gene expression generated by either reduced or excessive copper intake. We discuss the potential of integrated genome-wide applications to advance our knowledge of copper homeostasis and to develop molecular biomarker profiles as indicators of copper status. Am J Clin Nutr 2008;88(suppl):821S–5S.

ACHIEVEMENTS IN SYSTEMS BIOLOGY APPROACHES TO UNRAVEL COPPER METABOLISM
Advances in genome-wide screening technologies have enabled us to examine copper-dependent changes of the genome, the transcriptome, the proteome, and the metabolome in a systematic and high-throughput fashion. The contribution of each of the “-omics” approaches to dissecting copper metabolism and the most prominent studies in these areas are discussed below. The strategies used to gain novel insights into the mechanisms of copper homeostasis and these strategies are described below and are summarized in Figure 1. The mostly used techniques and analysis methods are summarized in Table 1.

Genetics/genomics
Inherited disorders of copper homeostasis have rendered valuable insights on genes that critically regulate copper metabolism. Positional cloning of the ATP7A gene and the highly homologous ATP7B in Menkes disease and Wilson disease, respectively, established the pivotal role of copper transporting P-type ATPases in the biosynthesis of cuproproteins in the secretory pathway and in the cellular excretion of copper. More recently, a positional cloning approach in the Bedlington terrier dog breed affected with copper toxicosis, lead to the identification of COMMD1 as a novel gene involved in copper excretion (1). Indian childhood cirrhosis, Tyrolean infantile cirrhosis, and idiopathic copper toxicosis are human copper overload diseases that originate from a

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combination of high dietary copper intake and genetic predisposition. Despite some attempts, the underlying genetic defects of the latter disorders have not been identified yet. In the near future, high-density small-nucleotide polymorphism arrays should help to reveal novel genes that play a role in copper metabolism in these and other diseases of aberrant copper homeostasis both in humans and in several sheep and dog breeds. Subsequently, genetically engineered mouse models will be studied to clarify the precise functions of these genes in copper homeostasis.

Functional genomics also holds great potential to identify new genes that are important in copper homeostasis. It is now possible to individually over-express or silence genes in mammalian cell lines by using cDNA libraries or libraries encoding short hairpin RNAs, respectively. To assess the effects of such treatments on copper metabolism, highly sensitive indicators of cellular copper status are necessary, preferably at the single cell level. Several laboratories are currently developing chemical or genetically encoded copper sensors that may aid in functional genomic screens (7).

Transcriptomics
Recent advances in microarray-based transcription analysis have increased the sensitivity, reproducibility and accuracy to a level that the expression of virtually the whole genome can be

FIGURE 1. Flow chart of systems biology approaches in copper metabolism.
monitored. At this moment, transcriptomics is therefore the most powerful “-omics” approach for complete genome coverage. Extended sets of differentially expressed genes have been identified in various cell types and in tissues of animals that are either exposed to variable copper concentrations or that have inappropriate copper concentrations due to inherited gene mutations. Genome-wide time-series gene expression analyses in HepG2 cells showed a rapid response of genes induced by copper that exclusively comprised metallothionein genes. Copper overload also resulted in a delayed induction of genes directly or indirectly involved in oxidative stress (8). The main hepatic transcriptional response in Atp7b−/− mice or mice exposed to a high-copper diet represented genes involved in cholesterol synthesis, cell death, and cell cycle progression (8, 9). Fibroblasts or macrophages that were exposed to copper similarly revealed enrichment in expression of genes involved in one or more of these processes, although the individual genes differed (10, 11). Strikingly, however, none of these experiments showed a differential expression of genes directly involved in copper import or export, implying that transcriptional regulation of copper homeostasis in mammals is limited. Regulatory mechanisms involved in maintaining intracellular copper concentrations therefore probably result from posttranslational events. Although genome-wide transcriptional profiling will not identify novel “homeostasis” genes, this approach will prove essential to elucidating the adaptive responses of cells to chronic copper deficiency or copper overload and possibly will identify novel gene regulatory networks involved in these responses. This technique might also be applied to generate patient-specific gene signatures to assist in disease diagnosis and prognosis. Interestingly, Kim et al investigated (12) the gene expression profiles of cirrhotic liver tissue of Wilson disease patients. The gene expression signature of Wilson disease liver biopsies resembled the gene signature in livers of patients with hepatocellular carcinoma. From these signatures, a set of 12 genes, which encode secreted proteins, were identified as markers for prognosis toward hepatocellular carcinoma.

### Proteomics

The effects of copper on proteins are broad and comprise variations in protein expression, structure, localization, and posttranslational modifications. Specifically, they also include variations in the metal affinity of copper binding proteins and variations in copper-dependent protein-protein interactions. MALDI-TOF mass spectrometry plays a central role in the identification of proteins and protein modifications. Mass spectrometry is often preceded by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) to visualize changes in protein expression or by affinity purification to select for proteins with specific properties. SELDI-TOF mass spectrometry provides a quick and high-throughput method to analyze protein expression, but is not followed by protein identification. Protein microarrays offer an alternative sensitive way to screen for protein expression, without using mass spectrometry. In contrast with transcriptomics, proteomics techniques are subject to detection limitations because the physicochemical properties of individual proteins differ manifold more than those of nucleic acids.

Copper-induced expression changes in several proteins have been determined in North-Ronaldsay sheep, which are highly sensitive to increased dietary copper. These changes mainly comprise proteins involved in the protection against the toxic effects of copper, such as metallothioneins and other metal-binding proteins. These changes are associated with the induction of an adaptive cellular response to maintain intracellular copper homeostasis. The expression of these proteins is rapidly induced (within 24 hours) in response to an increase in dietary copper, and their expression levels return to normal when dietary copper is reduced. This rapid and adaptive response to copper overload provides evidence for an acute protective response to toxic copper levels. However, the long-term effects of chronic copper overload on protein expression and their role in the development of copper-induced liver disease remain to be elucidated.

### Table 1

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*For more information about these techniques, we refer to the following review articles (3–6). PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; SNP, single-nucleotide polymorphism; GC, gas chromatography; LC, liquid chromatography; NMR, nuclear magnetic resonance.*
effects of copper overload (13). Interestingly, livers of rats subjected to a low-copper diet showed changes in expression of proteins involved in cholesterol metabolism, consistent with the transcriptional changes observed in response to copper overload in other studies (14). In addition, copper deficiency resulted in the increased expression of several protein chaperones that promote folding, which suggests that in the absence of copper, copper-containing proteins are misfolded.

Specific purification methods (Table 1) have proven useful in identifying novel copper-binding proteins, novel copper-binding motifs, and copper-associated protein modifications in human hepatoma cell lines (15–17), which may serve as biomarkers for disease states. Of specific interest in the context of copper-proteomics is the study of (copper-dependent) protein-protein interactions, also termed interactomics. As an example, yeast-two-hybrid studies with ATP7B as a bait revealed interactions of GRX1 (glutaredoxin), PLZF (promyelocytic leukemia zinc finger), and p62 (dynactin subunit p62) with ATP7B (18–20). The interactions of ATP7B with p62 and GRX1 are copper-dependent and affect the localization or the ATPase function of ATP7B. These data illustrate the successful potential of interactomics to identify novel proteins involved in the regulation of copper homeostasis.

**Metabolomics**

Although individual metabolites have been studied for centuries, the metabolomics field is the youngest research approach in the “-omics” family. It studies, in a high-throughput and systematic manner, changes in metabolite profiles and quantities in an organism or cellular system secondary to disease state or environmental parameters. By combining one of the various extraction methods with mass spectrometric analysis (see Table 1), many different metabolites can be accurately measured. Nevertheless, a combination of multiple analysis methods is necessary to reach significant coverage of the complete metabolome. Changes in enzyme function greatly affect the variety and quantity of several metabolites. Because copper is a catalyst in several enzymatic reactions, changes in metabolite quantity and variety are to be expected on copper challenge or depletion. In addition, copper-mediated oxidative stress results in peroxidation of biomolecules that can subsequently act as signal transducers and affect metabolite quantities and diversity. Although copper-dependent metabolite profiles have not been examined extensively yet, some studies indicated that the abundance of metabolites involved in carbohydrate metabolism, dopamine metabolism, and detoxification of oxidative stress vary in response to changing copper concentrations (21–23). In Wilson disease patients, proton MR (magnetic resonance) spectroscopy was conducted and provided a noninvasive way to determine metabolic changes in the brain of these patients (24), thus illustrating the potential of extensive metabolome profiling for clinical studies.

The development of metabolomics faces many challenges. The number of metabolites and their chemical diversity vastly exceeds the proteome. Metabolite extraction from several biological matrices, separation before mass spectrometry, data preprocessing, metabolite identification, and data analysis all need further development. More fundamentally, because many of the metabolomics techniques rely on the calculation of mass differences that are to be expected on the basis of known reactions or prediction models, uncommon reactions and reactions with large mass differences cannot be reliably measured. Future research in which the whole metabolome can be monitored for copper-dependent variations will probably unravel new and highly important metabolome networks that will help in the understanding of copper metabolism and may serve as potential biomarkers.

**CONCLUSIONS**

Will systems biology completely replace recent and current methods that appeared so successful in the characterization of the mechanisms of copper homeostasis? Obviously, this is not the case. Because genome-wide assessment of copper metabolism is a relatively new field, it needs to be developed parallel to more conventional approaches. In that respect, further development of system biology approaches to unravel copper metabolism will appear highly dependent on existing well-characterized and genetically homogeneous yeast and animal models. In this context, the incorporation of bioinformatics and biostatistics in this area will also be highly necessary. In addition, as an unbiased approach, systems biology will generate rather than test hypotheses; novel genes and novel perspectives identified in a systems biology approach need to be rigorously functionally tested.

In conclusion, the rapid evolution of genome-wide profiling techniques set the first careful and exciting steps and will pave the way for investigating novel aspects of copper metabolism. The individual profiles established by any of the systems biology approaches to unraveling copper metabolism may serve as a fingerprint for disease state and nutrition state. Using an integrated, multidisciplinary, systems biology approach, we will better understand the role of copper in a variety of human diseases, including cardiovascular and neurodegenerative disorders, which might provide novel therapeutic avenues.

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**REFERENCES**


