High Copper Selectively Alters Lipid Metabolism and Cell Cycle Machinery in the Mouse Model of Wilson Disease*

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Dominik Huster1,2*, Tina D. Purnat1, Jason L. Burkhead1, Martina Ralle1, Oliver Fiehn1, Franziska Stuckert1, N. Erik Olson1, Daniel Teupser2**, and Svetlana Lutsenko1 3

From the 1Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, Oregon 97239, the 2Department of Medicine II and 3Institute of Laboratory Medicine, Clinical Chemistry, and Molecular Diagnostics, University of Leipzig, 04103 Leipzig, Germany, 4University of California Davis Genome Center, Davis, California 95616, and 5VizX Labs, Seattle, Washington 98119

Copper is essential for human physiology, but in excess it causes the severe metabolic disorder Wilson disease. Elevated copper is thought to induce pathological changes in tissues by stimulating the production of reactive oxygen species that damage multiple cell targets. To better understand the molecular basis of this disease, we performed genome-wide mRNA profiling as well as protein and metabolite analysis for Atp7b−/− mice, an animal model of Wilson disease. We found that at the presymptomatic stages of the disease, copper-induced changes are inconsistent with widespread radical-mediated damage, which is likely due to the sequestration of cytosolic copper by metallothioneins that are markedly up-regulated in Atp7b−/− livers. Instead, copper selectively up-regulates molecular machinery associated with the cell cycle and chromatin structure and down-regulates lipid metabolism, particularly cholesterol biosynthesis. Specific changes in the transcriptome are accompanied by distinct metabolic changes. Biochemical and mass spectroscopy measurements revealed a 3.6-fold decrease of very low density lipoprotein cholesterol in serum and a 33% decrease of liver cholesterol, indicative of a marked decrease in cholesterol biosynthesis. Consistent with low cholesterol levels, the amount of activated sterol regulatory-binding protein 2 (SREBP-2) is increased in Atp7b−/− nuclei. However, the SREBP-2 target genes are dysregulated suggesting that elevated copper alters SREBP-2 function rather than its processing or re-localization. Thus, in Atp7b−/− mice elevated copper affects specific cellular targets at the transcription and/or translation levels and has distinct effects on liver metabolic function, prior to appearance of histopathological changes. The identification of the network of specific copper-responsive targets facilitates further mechanistic analysis of human disorders of copper misbalance.

Copper plays an essential role in human physiology. It serves as a cofactor of key metabolic enzymes and is required for embryonic development, neuronal myelination, radical detoxification, and numerous other physiological processes. Mutations in copper-binding proteins have been linked to such devastating disorders as amyotrophic lateral sclerosis, Alzheimer disease, prion disease, and Menkes disease. In Wilson disease (WD), the direct link between elevated hepatic copper and development of liver pathology has been firmly established. The disease is caused by mutations of the copper-transporting ATPase ATP7B (Wilson disease protein) (1–3). ATP7B is expressed predominantly in the liver, where it transports copper from the cytosol into the lumen of the Golgi network for incorporation into ceruloplasmin, a copper-dependent ferridase. ATP7B is also required to export excess copper from the liver into the bile; this represents the major excretory route for copper in the body (4). In WD patients, both functions are disrupted, and copper accumulates to levels that are 10–20-fold higher than the norm (5). Gradual copper accumulation, most noticeable in the liver, induces marked changes in tissue structure and function.

Liver injury is the most common manifestation of WD, although neurological and psychiatric symptoms are also frequently observed (2, 4, 6). WD patients may show progressive hepatic cirrhosis, chronic active hepatitis, or rapidly developing liver failure (7). None of these clinical features is specific to WD, complicating its diagnosis. Remarkably, despite a long history (WD was described in 1912 (8)) and significant progress in characterization of its genetic basis (9–11), the molecular and metabolic changes that accompany initial stages of copper accumulation (and may serve as markers of disease progression) remain poorly characterized.

The effects of accumulated copper on lipid peroxidation, enzyme activity, and DNA stability have been reported in both patients and in animal models of WD (12–14), yet it is still unclear which of these manifestations are the primary effects of...
accumulated copper and which are the longer term consequences of the disease. Understanding the initial copper-specific changes is particularly important, not only because detecting such changes may serve as a useful diagnostic tool, but also because corrective or supportive treatment can be implemented to overcome observed metabolic alterations and further improve and supplement copper-chelation therapy.

To understand molecular events associated with the early stages of copper overload, we have utilized Atp7b<sup>−/−</sup> mice, an animal model of WD. We have shown previously that these animals accumulate copper to high levels and have several phenotypic features resembling WD (15). We have also observed that in the 6-week-old Atp7<sup>−/−</sup> mice, the hepatic copper is at its highest level, yet the pathology is still minimal (15). At this presymptomatic stage the changes in gene/protein expression as well as associated metabolic alterations in the liver are likely to represent specific responses to copper. In this study, we characterize changes in the hepatic mRNAs, proteins, and metabolites in response to copper accumulation in 6-week-old Atp7b<sup>−/−</sup> mice. We demonstrate that the effect of copper is selective and involves distinct metabolic pathways, organized in the interconnected network, although oxidative stress is not apparent. The most significant and unexpected are the up-regulation of cell cycle machinery and the down-regulation of cholesterol metabolism; the latter is accompanied by marked decrease of cholesterol in the liver. These novel findings pave the way for detailed mechanistic analysis of WD and other copper-induced pathologies.

**EXPERIMENTAL PROCEDURES**

**Animals**—The generation of the Atp7b<sup>−/−</sup> mouse has been described previously (16). Mice were maintained on strain C57BL × 129S6/SvEv, and female animals at 6 weeks of age were used for microarray and RT-PCR studies. The Atp7b<sup>−/−</sup> and control mice were housed at the Oregon Health & Science University animal facility according to the National Institutes of Health guidelines on the use of laboratory and experimental animals. Food and water were provided ad libitum, and no further treatment was carried out. Animals were euthanized at given time points and livers quickly removed for different tissue preparations.

**RNA Isolation for Microarray**—Immediately after removal, the liver pieces (~75 mg) were frozen in liquid nitrogen and stored until further use. The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol followed by an RNeasy cleanup procedure (Qiagen). The integrity of isolated RNA was electrophoretically verified by ethidium bromide staining and by absorbance ratio (A<sub>260 nm</sub>/A<sub>280 nm</sub> > 1.8). A total of six liver RNA samples (three biological replicates for WT and three for KO animals) were tested for RNA quality. Each of the six sample targets was hybridized to two different MOE430A Affymetrix GeneChip arrays (technical replicates). The Affymetrix chip contained 22,691 spots corresponding to 13,016 unique Unigene IDs (i.e. unique genes).

**Chip Performance Analysis**—Image processing and analysis were performed using Affymetrix MAS 5.0 software. The resulting intensities and coordinate information were saved in a CEL file format and then subjected to global scaling with an average target intensity of 350 to allow for direct comparison of hybridization values from different targets. Scaled results for each sample were saved as CHC files, and these data were used to evaluate overall chip performance (supplemental Table 1). The analysis indicated that the parameters describing the quality of RNA, hybridization, and detection were all within acceptable range.

The data in CEL format were imported into GeneSifter.net, a microarray data analysis application and subjected to RNA normalization using published algorithms (17). The RMA-normalized data set was then used to identify changed genes and determine the statistical significance and magnitude of changes (fold change). The significance was established using Wilcoxon t test with Benjamini and Hochberg adjustment for multiple comparisons. The data were annotated with NCBI Unigene IDs and with related Gene Ontology terms by using the Affymetrix NetAffx tool and NIAID-NIH DAVID (apps1.niaid.nih.gov/david/). Of the 310 significantly differentially expressed probe sets, 304 had Unigene IDs and were annotated in mouse Gene Ontology (GO). In a number of cases, when changes in the transcript were detected by more than one probe set, the sequences of these probe sets were manually compared with the sequence of the identified transcript to ensure that the probe sets indeed mapped to the same gene.

The GO analysis was carried out using GenMapp on-line tool (18, 19). For each identified GO term, Gene Map z-statistic was calculated. A positive z-statistic for any particular GO term indicated that there were more genes associated with this GO term than would be expected by random chance. The GO terms with positive z-statistic were then ranked based on the percent of changed genes that belong to the corresponding GO compared with the total number of genes on a chip with the same GO. This analysis identified those GO terms that were most strongly represented among the differentially expressed genes (probe sets). The results were independently confirmed by applying the GO Browser tool in NetAffx to the same set of differentially expressed genes. The gene expression data have been submitted to GEO data base (GEO number GSE5348).

The associations between altered genes or pathways were further evaluated using the Ingenuity Pathways Analysis software (Ingenuity® Systems). Affymetrix identifiers of the differentially expressed genes (the fold change of 1.5 or higher) and their corresponding expression values were loaded into the software and mapped to its corresponding gene object (so-called focus genes) in the Ingenuity Pathways Knowledge Base. The significance of the associations between the data set and the canonical pathway and functional annotations was calculated in two ways. First, the number of genes from the data set that map to the pathway was divided by the number of all known genes ascribed to the pathway. Second, the left-tailed Fisher exact test was used to calculate related p values and distinguish those functional/pathway annotations that had more focus genes than expected by chance. The networks of the focus genes were algorithmically generated based on their connectivity.

**Real Time PCR (RT-PCR)**—Total RNA was extracted from livers of 4-, 6-, and 32-week-old WT and KO mice (n = 6 per sample)
group) and checked for integrity as described above. One-step RT-PCR was performed with a LightCycler instrument (Roche Applied Science) in a total volume of 20 μl containing 50 ng of total RNA, 0.5 μM each primer, LightCycler RT-PCR reaction mix SYBR Green I (1×), and LightCycler RT-PCR Enzyme Mix (Roche Applied Science). Reverse transcription was performed at 50 °C for 20 min. The denaturation and amplification conditions were 95 °C for 15 s followed by up to 35 cycles of PCR. Each cycle of PCR included denaturation at 95 °C for 15 s and then 10 s of primer annealing at 55 °C and 20 s of extension/synthesis at 72 °C (20 s). The temperature ramp was 20 °C/s, except when heating to 72 °C, when it was 2 °C/s. At the end of the extension, step fluorescence of each sample was measured to allow quantification of the RNA. After amplification a melt-scanning of each sample was performed using serial dilutions of linearized plasmid cDNA to permit the calculation of the amount of copies. The amount of copies was calculated using the 2ΔΔCt method (20).

Human liver samples were obtained from eight Wilson disease patients who underwent liver transplantation and eight control livers from other patients who underwent liver resection. The study was approved by the institutional ethics committee of the University of Leipzig (registration number 236-2006) and followed ethical guidelines. RNA from small liver samples was isolated as described above. cDNA was synthesized from 2 μg of RNA by reverse transcription with SuperScript II RNase H− reverse transcriptase (Invitrogen) and random hexamer primers. Gene expression was determined by quantitative fluorogenic RT-PCR (ABI PRISM SDS 7900, Applied Biosystems). Primers and probes were selected to span two exons in order to prevent amplification of genomic DNA (supplemental Table 2). The PCR was prepared in a final volume of 12.5 μl of a reaction mixture containing 2.5 μl of cDNA (diluted 1:15), 5 mM MgCl2, 1.25 μl of 10× AmpliTaq buffer A, 200 μM dNTP (each), 0.3 unit of AmpliTaq Gold (Applied Biosystems), 200 nM 6-carboxyfluorescein-labeled oligonucleotide probe, and 900 nM of each oligonucleotide primer. The cycling conditions were as follows: 95 °C for 10 min and 40 two-step cycles of 95 °C for 15 s and 60 °C for 1 min. Standardization was performed using serial dilutions of linearized plasmid cDNA ranging from 10 to 106 copies. Data were analyzed with the ABI PRISM software. The mRNA expression results are given as the fold change of mRNA level compared with control and for HMG-CoA reductase as the copy number normalized to 106 copies of β-actin.

**Analysis of Soluble Proteins by Two-dimensional Gel Electrophoresis**—The liver tissue (50–100 mg wet weight) from control and Atp7b+/− mice was homogenized in 800 μl of buffer containing 10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 5 mM EDTA, 1 mM CaCl2, and 0.5 mM MgCl2 using a glass Dounce homogenizer with tight pestle. Soluble proteins were obtained by centrifuging samples at 100,000 × g for 30 min and collecting the supernatant. The lipids at the top of the supernatant were removed using a 0.22-μm centrifugal filter device (ultrafree-MC, Millipore). The soluble proteins were precipitated by adding 4 volumes of ice-cold acetone, 1 mM HCl. The mixture was vortexed and incubated at −20 °C for 1 h, and proteins were pelleted by centrifugation at 15,000 × g for 10 min, and the pellet was air dried for 5 min. Pellets were then dissolved by vortexing in 8 μl urea, and protein content was determined using a BCA assay (Pierce). 0.17 ml of this solution containing 2 mg of protein was mixed with 0.17 ml of 8 μl urea, 4% CHAPS, 100 mM DTT, 4% glycerol, and 4% pH 3–10NL IPG buffer (Amersham Biosciences). This solution was used for overnight reswelling of 18-cm pH 3–10NL IPG strips (Amersham Biosciences). The strips were focused using a Protein IEF Cell (Bio-Rad) under the following conditions: 20 °C, 0–500 V for 6 h (rapid), 500–3500 V for 3 h (linear), 3500 V for 8 h, with a 50-mA limit per gel. Following isoelectric focusing, the strips were reduced and alkylated, and the second dimension separation was performed using 12% gels. Gels were stained with Coomassie G-250 as described previously (21).

**Protein Identification by Mass Spectrometry**—The spots of interest were excised from Coomassie-stained gels, washed twice with doubly deionized water, and then twice with 50% (v/v) 50 mM NH4CO3, 50% acetonitrile. The gel pieces were then incubated in 100% acetonitrile for 2 min after which the liquid was removed, and the pieces were air dried until white in appearance. Rehydration of the gel pieces was performed with digestion buffer containing 0.01 μg/μl trypsin (Princeton Applied Research), 50 mM NH4CO3, and 50 mM CaCl2 followed by the addition of 60 μl of the same buffer without trypsin and incubation at 37 °C overnight. The reaction was stopped by adding 3 μl of 98% formic acid (Aldrich) to ~60 μl of digest. A 45-μl aliquot was analyzed by liquid chromatography-electrospray ionization-tandem mass spectroscopy using an ion trap mass spectrometer (LCQ Deca XP Plus, Thermo Electron Corp.). The analysis by liquid chromatography-electrospray ionization-tandem mass spectroscopy was performed using a capillary 180 μm × 12-cm column packed in-house with stable-bond C18 packing material (5 μm, ZORBAX, Agilent Technologies). The samples were applied to the column through a trap column (180 μm × 2.5 cm, packed in-house with the same packing material as above), and the peptides were separated using a linear gradient changing the solvent composition from 2% acetonitrile to 30% acetonitrile over a 30-min period with a constant flow rate of 1.5 μl/min. MS data were acquired in data-dependent mode in which a single survey scan (MS) was followed by up to four sequential data-dependent MS/MS scans on the four most intense peptide ions. A dynamic exclusion feature was used to extend the analysis to less abundant ions. The peptides were identified using SEQUEST (22), from within the Trans Proteomic Pipeline (version 2.71) (23). Scaffold (Pro-
Metabolic and mRNA Changes in Atp7b<sup>−/−</sup> Mice

teome Software, Portland, OR) was used to analyze protein identifications derived from MS/MS sequencing results. Scaffold validates peptide identifications using PeptideProphet (24) and derives corresponding protein probabilities using ProteinProphet (25). Proteins were considered as “identified” when probability scores were ≥0.99 (protein) and ≥0.90 (peptide) with a minimum of two peptides identified.

Serum Analysis—Blood was collected by cardiac puncture, and serum was separated by centrifugation after blood coagulation and used immediately for analysis. Serum lipids were quantified after isolation by sequential centrifugation as described previously (26).

Metabolic Profiling of Liver Tissue—2 mg of frozen tissue was homogenized for 30 s in an MM301 ball mill (Retsch, Germany). Extraction was carried using 1 ml of a one phasic mixture of isopropyl alcohol:acetonitrile:water (3:3:2, v/v) at −20°C for 5 min. After centrifugation, the supernatant was completely dried in a SpeedVac concentrator and then derivatized in two steps. First, carbonyl functions were protected by methoximation using 20 μl of a 40 mg/ml solution of methoxyamine hydrochloride in pyridine at 28°C for 30 min. The samples were then derivatized using 180 μl of N-methyl-N,N-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Düren, Germany) at 37°C for 30 min to increase the volatility. 0.5 μl of this solution was injected into an automatic liner exchange system unit (ALEX, Gerstel, Germany) at a 1:5 split ratio. For every sample, a fresh liner was used to avoid sample carryover. Each liner was deactivated and cleaned using a blank N-methyl-N,N-trimethylsilyltrifluoroacetamide injection followed by a flash heat ramp. The sample was introduced at 50°C using an MPS injector (Gerstel, Germany) and heated to 250°C using a 5°C/min ramp.

An Agilent 6890 gas chromatography oven (Hewlett-Packard, Atlanta, GA) was coupled to a Pegasus IV time of flight-mass spectrometer from Leco (St. Joseph, MI). An rtx-5-SilMS fused silica capillary column (Restek) of 30 m length, 0.25 mm inner diameter, and 0.25-μm film thickness was used for separation with the 0.25-mm inner diameter 10-m IntegraGuard column without film. For the analysis the gas chromatography oven was set to 85°C with duration of 210 s and a following ramp of 20°C/min. The target time was 330°C with duration of 5 min. The transfer line temperature was set to 250°C. Mass spectra were acquired with a scan range of 83–500 m/z and an acquisition rate of 20 spectra/s. The ionization mode was electron impact at 70 eV. The temperature for the ion source was set to 250°C. Chromatogram acquisition, data handling, automated peak deconvolution, library search, and retention index calculation was done by the Leco ChromaTOF software (version 2.32) using an in-house custom mass spectral library for compound identification.

Analysis of Mature SREBP-2 in Nuclear Extracts—Nuclear extracts were prepared essentially as described by Sheng et al. (27). Mouse livers (~0.7–1.0 g) were homogenized in 3 volumes of buffer 1 (10 mM HEPES, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1.0 mM EDTA, 1.0 mM EGTA, 20% glycerol, 1.0 mM DTT, Complete Protease Inhibitor Mixture. The suspension was centrifuged for 5 min at 2000 × g at 4°C. Pelleted nuclei were resuspended in 10 mM HEPES, pH 7.6, 100 mM KCl, 2.0 mM MgCl<sub>2</sub>, 1.0 mM EDTA, 10% glycerol, 1.0 mM DTT, Complete Protease Inhibitor Mixture. The one-tenth volume of 4.0 M ammonium sulfate, pH 7.9, was added, and the mixture was placed on a rocking shaker at 4°C for 45 min. The samples were then centrifuged at 20,000 × g at 4°C for 15 min. The supernatant contained nuclear extract. 10.0 μg of nuclear extract was then separated on a 7.5% Laemmli gel. Proteins were transferred to nitrocellulose in 10 mM CAPS, pH 11.0, 10% methanol. The membrane was blocked overnight in 50% Aquablock (EastCoastBio, North Berwick, ME). Anti-SREBP2 (Affinity Bioreagents, Golden, CO) was used at 1:5,000 dilution, and IRDye800-conjugated goat anti-rabbit (Rockland Immunochemicals, Gilbertsville, PA) was used at 1:20,000 dilution. Washes after both primary and secondary antibodies were in phosphate-buffered saline supplemented with 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, and 0.5% sodium deoxycholate as described in Sheng et al. (27). Immunodetection was visualized with an Odyssey infrared scanner (LI-COR, Lincoln, NE).

RESULTS

Evaluation of the Quality of the Array Data—To characterize molecular consequences of hepatic copper accumulation in the Atp7b<sup>−/−</sup> (KO) mice, we initially performed a comparative genome-wide analysis of transcripts isolated from livers of 6-week-old animals. At this age, the copper concentration in livers was elevated approximately 18-fold compared with control, but little if any pathology could be detected histologically (data not shown, see also Ref. 15). The mRNA was isolated from three of each control (WT) and Atp7b<sup>−/−</sup> livers (biological replicates), and for each of these samples two technical replicates were run. (Thus, the total number of samples used for hybridization with the Affymetrix arrays was 12.) The quality of generated data was initially verified by scatter plots, in which the overall distribution of normalized signal intensities was plotted for each pair of the biological replicates (supplemental Fig. 1). The signal intensities in all scatter plots fell along a 45° straight line in a symmetrical fashion, indicating that the data sets were appropriate for further analysis. More detailed pairwise comparisons revealed larger differences in signal distribution between the WT and KO samples compared with replicate samples (WT versus WT or KO versus KO) (supplemental Fig. 1, inset).

This result provided the first indication that there is a difference in gene expression between the control and Atp7b<sup>−/−</sup> livers even at the presymptomatic stage of disease. This conclusion was confirmed by quantitative evaluation of the data. The mean values, the range of signal intensities, and the patterns of mRNA signal distribution were compared for all WT and KO samples. These studies demonstrate that the mean and the range of signal amplitudes were very similar for all sam-
ples (Fig. 1A). However, the individual patterns of mRNA signal intensities fell into two distinct groups (WT and KO) corresponding to the sample genotype; the similarity between the members within each group was higher than between the groups (Fig. 1B). Altogether, the results in Fig. 1 and supplemental Fig. 1 demonstrate that the difference in the distribution of mRNA intensities between the WT and KO samples could be reliably attributed to the actual change in the individual gene expression rather than be caused by nonspecific variations between samples.

**Copper Accumulation Alters the Abundance of the Limited Number of Transcripts**—To identify significantly changed genes, a pairwise analysis of the data sets was performed using Wilcoxon t test. The resulting p value was adjusted for multiple comparisons by the method of Benjamini and Hochberg and then used to determine the significance of changes. Using the 2-fold change cutoff and the adjusted p value <0.05, 31 unique transcripts were found to be up-regulated (Table 1) and 45 down-regulated (Table 2). Among those, 15 transcripts were identified repeatedly as they were represented on the chip by more than one probe set (Tables 1 and 2). Repeated identification and similar fold change for these mRNAs provided an additional internal control and increased confidence in the results.

**TABLE 1**
The transcripts significantly up-regulated in *Atp7b*−/− livers (fold change >2, adjusted p value <0.05)

For the transcripts identified by different probe sets, all relevant Affymetrix probe set IDs and the highest of the found ratios are shown.

| Unigene cluster     | Ratio | Gene or protein name                          | Affymetrix ID      |
|---------------------|-------|-----------------------------------------------|--------------------|
| Cell cycle and proliferation |       |                                               |                    |
| Mm.14802            | 5.68  | H19 fetal liver mRNA                          | 1448194_a_at       |
| Mm.290830           | 2.62  | MAD2 (mitotic arrest deficient, homologue)-like 1| 1422460_at         |
| Mm.6856             | 2.48  | Pituitary tumor-transforming 1 (securin)       | 1424105_a_at       |
| Mm.231              | 2.16  | RAD51 homologue                               | 1418281_at         |
| Mm.2103             | 2.1   | Cyclin G1                                     | 1450016_at         |
| Mm.37801            | 2.13  | Shc SH2 domain-binding protein 1              | 1416299_at         |
| Mm.289747           | 2.04  | Cell division cycle 20 homologue             | 1439377_x_at; 1420827_a_at |
| Mm.275095           | 2.03  | Valosin-containing protein                    | 1429295_s_at; 1450017_at |
| Mm.3049             | 2.01  | CDC28 protein kinase 1b                       | 1416698_a_at       |
| Chromatin structure |       |                                               |                    |
| Mm.193539           | 3.64  | Histone H1c                                   | 1436994_a_at; 1416101_a_at |
| Mm.2999             | 3.55  | SMC2, structural maintenance of chromosomes 2 | 1448635_at         |
| Mm.206841           | 2.28  | SMC4, structural maintenance of chromosomes 4 | 1452197_at         |
| Mm.9870             | 2.28  | Centromere protein Q                          | 1423620_at         |
| Metabolism          |       |                                               |                    |
| Mm.246881           | 4.76  | Glycero-phosphodiester phosphodiesterase domain containing 3 | 1449526_a_at |
| Mm.338425           | 2.54  | Ectonucleotide pyrophosphatase/phosphodiesterase 3 | 1427302_at |
| Mm.371562           | 2.01  | Glutathione S-transferase, α1 (Ya)            | 1421041_s_at; 1439260_a_at |
| Metal ion binding   |       |                                               |                    |
| Mm.147226           | 2.52  | Metallothionein 2                             | 1428942_at         |
| Mm.192991           | 2.22  | Metallothionein 1                             | 1422557_s_at       |
| Transport           |       |                                               |                    |
| Mm.197518           | 2.05  | Lyososomal associated protein transmembrane 4B | 1436915_x_at       |
| Mm.325350           | 2.04  | Solute carrier family 37, member 2            | 1452492_a_at       |
| Regulation of translation |     |                                               |                    |
| Mm.132584           | 3.73  | Polyadenylate-binding protein-interacting protein | 11425521_at |
| Proteolysis         |       |                                               |                    |
| Mm.291569           | 2.85  | Serine/cysteine proteinase inhibitor clade A, member 3M | 1423867_at       |
| Cell redox homeostasis |     |                                               |                    |
| Mm.259293           | 2.82  | RIKEN cDNA 2700094K13 gene (selenoprotein H)   | 1436349_at; 1454686_at |
| Cholesterol metabolism |     |                                               |                    |
| Mm.316000           | 2.36  | Cytochrome P450, family 7, subfamily b, polypeptide 1 | 1421074_at; 1421075_s_at |
| Cell adhesion       |       |                                               |                    |
| Mm.289441           | 2.23  | Claudin 1                                     | 1450014_at; 1437932_a_at |
| mRNA export and processing |   |                                               |                    |
| Mm.28478            | 2.18  | Transmembrane protein 48                      | 1460355_at; 1424173_at |
| Heme biosynthesis   |       |                                               |                    |
| Mm.302724           | 2.17  | Aminolevulinic acid synthase 2, erythroid     | 1451675_a_at       |
| Unknown             |       |                                               |                    |
| Mm.317041           | 3.15  | RIKEN cDNA 6430706D22 gene                    | 1433685_a_at       |
| Mm.30387            | 2.06  | n-myc downstream regulated gene 1             | 1456174_x_at       |
| Mm.355125           | 2.00  | Mus musculus, clone IMAGE:3983821, partial mRNA | 1427320_at; 1454686_at |

**FIGURE 1.** Quality metrics for the control (WT) and *Atp7b*−/− (KO) data sets. A, distribution of RMA-normalized data for the WT and KO groups. The boxes depict the mean values of intensities, and the height of the boxes shows the range of signal amplitudes for 50% of data in each sample. The vertical bars illustrate the magnitude of variation within each group (WT and KO) for the remaining 50% of the data. B, hierarchical clustering of six samples showing good agreement between replicates and difference between the WT and KO groups.
The magnitude of changes as detected by the arrays was not very large. The H19 fetal liver mRNA was the most highly up-regulated (5.7-fold), and the cytochrome P450 and putative aci-reductone dioxygenase 1 were the most down-regulated (both about 5.4-fold). Several significantly changed mRNA were found to encode proteins with unknown function (supplemental Table 3). For example, mRNA for the hypothetical protein LOC381280 was up-regulated more than 3-fold, suggesting a role for this protein in response of the cell to copper accumulation. BLAST analysis revealed limited homology between LOC381280 and proto-oncogene tyrosine-protein kinase Fes/ Fps (c-Fes), an important regulator of cell growth and differentiation. Other up-regulated uncharacterized transcripts included those encoding selenoprotein H, which may have reductase/oxidase activity and protein C6orf139, a homologue of non-muscle myosin (both increased more than 2-fold). Two novel transcripts were down-regulated. The product of cDNA clone MGC:118117 has an unusual pI of 11.23 and could be a nuclear protein. Another down-regulated transcript (more than 5-fold decrease) corresponds to transcribed locus m.407081.

TABLE 2
The transcripts significantly down-regulated in Atp7b−/− livers (fold change >2, adjusted p value <0.05)

For the transcripts identified by different probe sets, all relevant Affymetrix probe set IDs and the highest of the found ratios are shown. The genes are grouped based on GO terms.

| Unigene ID | Ratio | Gene or protein name | Affymetrix cluster |
|------------|-------|----------------------|--------------------|
| **Lipid biosynthesis and lipid metabolism** | | |
| Mm.316652 | 5.16 | 3-Hydroxy-3-methylglutaryl-coenzyme A reductase | 1427229_at |
| Mm.57029 | 4.67 | Cytochrome P450, family 7, subfamily a, polypeptide 1 | 1422100_at; 1438743_at |
| Mm.741 | 4.45 | Fatty acid-binding protein 5, epidermal | 1416022_at; 1416021_a_at |
| Mm.15226 | 4.12 | 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 | 1433444_at; 1433443_a_at; 1433445_x_at |
| Mm.29847 | 3.93 | Isopentenyl-diphosphate δ-isomerase | 1423804_a_at; 1451122_at |
| Mm.28585 | 3.36 | Thyroid hormone-responsive SPOT14 homologue | 1422973_a_at |
| Mm.314113 | 3.0 | ELOVL family member 6, elongation of long chain fatty acids | 1417403_at; 1417404_at |
| Mm.140158 | 2.95 | Cytochrome P450, family 51 | 1422533_at; 1450646_at; 1424737_at |
| Mm.296169 | 2.88 | Squalene epoxidase | 1415993_at |
| Mm.2787 | 2.82 | α-Methylacyl-CoA racemase | 1417208_at |
| Mm.236443 | 2.53 | Fatty-acid synthase | 1423828_at |
| Mm.153625 | 2.36 | Lipin 1 | 1418288_at; 1426516_a_at |
| Mm.39472 | 2.4 | Farnesyl diphosphate synthase | 1423418_at |
| Mm.371560 | 2.2 | Farnesyl-diphosphate farnesyltransferase 1 | 1439459_x_at |
| Mm.282039 | 2.13 | ATP citrate lyase | 1422533_at; 1450646_at; 1424737_at |
| Mm.212789 | 2.09 | Peroxisome proliferator-activated receptor α | 1429951_at |
| Mm.296918 | 2.08 | Acetoacetyl-CoA synthetase | 1423797_at |
| Mm.30119 | 2.04 | Sterol-C4-methyl oxidase-like | 1423078_a_at |
| Mm.55075 | 2.04 | Lanosterol synthase | 1420013_s_at |
| Mm.335660 | 2.03 | Cytochrome P450, family 2, subfamily c, polypeptide 40 | 1423244_at |

| **Signaling** | | |
| Mm.28551 | 3.33 | Homologue of yeast RIO kinase 3 | 1460670_at |
| Mm.8534 | 2.12 | Epidermal growth factor receptor, transcript variant 2 | 1424932_at |
| Mm.295397 | 2.04 | DEP domain containing 6 | 1453517_at |
| Mm.29660 | 2 | SH3-binding kinase | 1451190_a_at |

| **Metabolism** | | |
| Mm.220358 | 2.76 | Glucokinase | 1425303_at |
| Mm.275979 | 2.58 | Dihydroxyacetonephosphate synthase | 1452689_at |
| Mm.200370 | 2.04 | Uridine phosphorylase 2 | 1451548_at |

| **Cell cycle progression** | | |
| Mm.223744 | 2.16 | Kinesin family member 5B | 1418429_at |
| Mm.210996 | 2.06 | Establishment of cohesion 1 homologue 1 | 1423234_at |
| Mm.33764 | 2.12 | Influenza virus NS1A-binding protein | 1450084_x_at |

| **Cell proliferation and apoptosis** | | |
| Mm.328931 | 2.35 | Proto-oncogene Ser/Thr protein kinase Pim-1 | 1435872_at |
| Mm.275071 | 2.12 | Jun oncogene | 1417409_at |
| Mm.3117 | 2.01 | Pleckstrin homology-like domain, family A, member 1 | 1418835_at |

| **Proteolysis** | | |
| Mm.272770 | 3.91 | Ubiquitin-specific protease 2 | 1417168_a_at |
| Mm.296022 | 2.27 | Membrane metalloendopeptidase | 1455961_at |

| **Electron transport** | | |
| Mm.220317 | 5.41 | Cytochrome P450, family 2. Subfamily c, polypeptide 37 | 1419094_at |

| **Solute transport** | | |
| Mm.125501 | 2.65 | Solute carrier family 26 (sulfate transporter), member 1 | 1451239_a_at |

| **Heme biosynthesis** | | |
| Mm.290578 | 2.2 | Aminolevulinic acid synthase 1 | 1424126_at |

| **Circadian rhythms** | | |
| Mm.33459 | 2.19 | D site albumin promoter-binding protein | 1418174_at |

| **Unknown** | | |
| Mm.291504 | 5.38 | Expressed sequence AL024120 | 1438758_at |
| Mm.22682 | 5.09 | Unknown protein, no BLAST matches | 1420062_at |
| Mm.362041 | 3.12 | CDNA clone MGC:118117 IMAGE:6399338 riken | 1426607_at |
| Mm.378235 | 2.82 | Transcribed locus similar to NP_036675.1 | 1438211_s_at |
| Mm.102470 | 2.27 | Fibronectin type 3 and ankyrin repeat domains 1 | 1453287_at |
| Mm.303115 | 2.22 | Ring finger protein 26 | 1417169_at |

The magnitude of changes as detected by the arrays was not very large. The H19 fetal liver mRNA was the most highly up-regulated (5.7-fold), and the cytochrome P450 and putative aci-reductone dioxygenase 1 were the most down-regulated (both about 5.4-fold). Several significantly changed mRNA were found to encode proteins with unknown function (supplemental Table 3). For example, mRNA for the hypothetical protein LOC381280 was up-regulated more than 3-fold, suggesting a role for this protein in response of the cell to copper accumulation. BLAST analysis revealed limited homology between LOC381280 and proto-oncogene tyrosine-protein kinase Fes/ Fps (c-Fes), an important regulator of cell growth and differentiation. Other up-regulated uncharacterized transcripts included those encoding selenoprotein H, which may have reductase/oxidase activity and protein C6orf139, a homologue of non-muscle myosin (both increased more than 2-fold). Two novel transcripts were down-regulated. The product of cDNA clone MGC:118117 has an unusual pI of 11.23 and could be a nuclear protein. Another down-regulated transcript (more than 5-fold decrease) corresponds to transcribed locus m.407081.
Verification of the Array Data Using Quantitative RT-PCR—To verify the results of oligonucleotide arrays, the RT-PCR analysis was performed for 21 transcripts encoding protein with known function (10 up-regulated and 11 down-regulated; for the list of RT-PCR primers see supplemental Table 2). The transcripts were selected to represent different metabolic pathways (proteins involved in cell cycle, metabolic enzymes, and metalloproteins) and different fold change. Altogether, we observed good correlation with respect to the direction of the change (up or down) (Fig. 2). For example, 9 of 10 tested transcripts, which were predicted to be up-regulated by the arrays, showed significant increase by RT-PCR, with the 10th transcript also showing an increase, which was less significant (Fig. 2A). Down-regulated transcripts also consistently showed a decrease in the RT-PCR analysis, although for 4 of 11 examined transcripts the change was small and/or not statistically significant (Fig. 2B). Most importantly, for 3 of 4 down-regulated transcripts that showed small or nonsignificant change at 6 weeks, very significant change was detected at the later time points (Fig. 2B), confirming the involvement of the corresponding genes in hepatic response to copper.

Correlation between the Extent of Changes in the Transcriptome and Proteome—The relatively small number of transcripts that was changed in response to copper accumulation pointed to specific effects of copper on gene expression/mRNA stability and presumably on liver proteins. To independently evaluate the magnitude of copper-induced changes in the liver proteome, the patterns of soluble proteins from control and Atp7b−/− samples were compared using two-dimensional gel electrophoresis. The overall protein patterns for control and Atp7b−/− samples were very similar (Fig. 3), consistent with the limited effect of copper on liver transcriptome. Several changes, however, were detected. The most obvious change was a marked increase in the amount of small proteins with the apparent molecular mass of 15 kDa in the KO samples (Fig. 3). Mass spectrometry analysis identified these proteins as metallothioneins 1 and 2. This finding was in full agreement with the results of arrays and RT-PCR, both of which showed significant up-regulation of metallothioneins 1 and 2 (Table 1 and Fig. 2).

Copper-induced formation of reactive oxygen species and resultant oxidative stress have been discussed in the literature as major factors in the development of WD pathology. Therefore, one of the unexpected outcomes of our gene array experiments was the apparent lack of significant changes in...
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A

![Graph showing gene expression analysis](image)

**FIGURE 4. Gene ontology analysis for significantly changed transcripts.** A, most significantly changed GO terms/pathways identified by canonical pathway analyses using the Ingenuity Pathways Knowledge Base. B, changes in various pathways as predicted by GeneSifter software. The dotted line indicates z-score of 2, which is a threshold for significant changes.

B

![Microscopy image](image)

**FIGURE 5. Electron microscopy of Atp7b<sup>−/−</sup> and control nuclei.** The images of the sections of the nuclei show nucleoli (thin arrow) clearly visible in both the Atp7b<sup>−/−</sup> and control samples and the electron-dense material (thick arrow) detected in the Atp7b<sup>−/−</sup> nuclei (magnification ×8,000).

protein machinery associated with oxidative stress. No change in mRNA level was detected for either catalase or copper zinc-dependent superoxide dismutase (SOD1), the key enzymes involved in peroxide and superoxide detoxification. Also, no up-regulation was detected for the heat shock proteins. A similar lack of changes was observed at the protein level, when SOD1 was examined. SOD1 is an abundant liver protein with known behavior on two-dimensional gels. It was easily detectable in our soluble samples (Fig. 3), and we confirmed the identity of SOD1 by mass spectrometry. No change in the amount of SOD1 in the Atp7b<sup>−/−</sup> liver was observed, supporting the results of the array experiments.

The Significantly Changed Genes Belong to Specific Metabolic Pathways—Although none of the utilized methods provides exhaustive coverage of either mRNA or proteins, altogether the gene array, RT-PCR, and protein analysis were consistent in showing that the effects of copper were restricted to a rather small population of mRNA/proteins. These limited changes suggested that specific metabolic pathways could be altered. To test this hypothesis, the gene ontology analysis was carried out initially for genes changed more than 2-fold (Tables 1 and 2) and, subsequently, for genes with more than 1.5-fold change (total 310 significantly changed probes; 137 up and 173 down-regulated; see supplemental Table 4). This second analysis was performed to account for the possibility that the actual fold change in mRNA abundance could have been significantly higher than that measured by the oligonucleotide arrays (see RT-PCR data). In both cases, the analysis of changed genes using different software packages (GenMapp, GeneSifter, NetAffx, or Ingenuity Pathway Analysis) identified the same gene ontology terms as mostly affected by copper accumulation (Fig. 4).

Consistently, the genes associated with mitotic cell cycle and lipid metabolism, particularly cholesterol biosynthesis, were among the most changed (Fig. 4). Steroids, glycerol, and alcohol metabolism as well as electron transport were also altered, whereas the transcripts encoding proteins involved in nucleic acid, amino acid, or carbohydrates metabolism were much less affected, if at all. (The complete list of the significantly changed GO terms is shown in supplemental Table 5.) The detailed hierarchical analysis of significantly changed GO terms also demonstrated that the up-regulated pathways were distinct from the down-regulated pathways and largely nonoverlapping.

By far, the largest fraction of up-regulated transcripts is associated with the cell cycle and more specifically with the regulation of mitosis, including chromosome segregation (Fig. 4; supplemental Table 6). These include SMC2 and SMC4 (structure chromosome maintenance), cyclins G1 and G2, and CDC20 and CDC28 protein kinases (for complete list see supplemental Table 6). The examination of hepatic nuclei from two pairs of WT and KO animals using electron microscopy showed that Atp7b<sup>−/−</sup> nuclei frequently contain electron-dense material that is less apparent in control nuclei (Fig. 5). This result pointed to structural and possibly functional changes in nuclear components of Atp7b<sup>−/−</sup> hepatocytes.

By contrast, the down-regulated transcripts were associated with lipid metabolism and particularly cholesterol biosynthesis (Fig. 4, Table 2, and supplemental Table 6). These include both the biosynthetic enzymes and regulatory proteins. For example, mRNA for nine proteins involved in cholesterol biosynthesis were all significantly down-regulated; these include cholesterol 7-α-monooxygenase, farnesyl-diphosphate farnesyltransferase, HMG-CoA-reductase, lanosterol synthase, and others. The mRNA levels for main transcription factors controlling lipid and fatty acid synthesis and oxidation, such as
sterol regulatory element-binding protein-1c and peroxisome proliferator-activated receptor-α, were decreased, although less strongly. Similarly, the mRNA for SPOT14 homologue (which can be involved in regulating the triglyceride pool and overall efficiency of de novo lipid synthesis) (29) was down-regulated.

**The Concentration and Distribution of Cholesterol Are Markedly Changed in Atp7b−/− Mice**—The notable dysregulation of genes associated with lipid metabolism strongly suggested significant changes in the levels of metabolites and particularly cholesterol in Atp7b−/− mice. This was confirmed by measurements of metabolite concentrations in the serum (Table 3). A significant decrease in the amount of triglycerides and cholesterol was detected; the latter was because of a decrease of high density lipoprotein- and VLDL-cholesterol, whereas LDL-cholesterol remained unchanged. The VLDL fraction was the most affected showing a 3.6-fold reduction in cholesterol concentration. This marked change of VLDL fraction suggested a significant decrease in the amount of triglycerides and cholesterol in the liver. Analysis of cholesterol in tissues using mass spectrometry demonstrated that liver cholesterol was indeed markedly decreased (by 33%, \( p = 0.014 \)) in all Atp7b knock-out samples (Fig. 6).

**Copper Does Not Inhibit Maturation or Nuclear Re-localization of SREBP-2**—To better understand how elevated copper induces such marked down-regulation of cholesterol metabolism, we compared the amount of activated SREBP-2 in hepatic nuclei of control and Atp7b−/− mice. SREBP-2 is a transcription factor that plays a key role in regulation of cholesterol biosynthesis. It is present in the inactive form in the endoplasmic reticulum; activation of SREBP-2 is controlled by the intracellular levels of cholesterol. When cholesterol is low, SREBP-2 is escorted to Golgi and then proteolytically cleaved. The mature 50-kDa product is released from the membrane, translocates to the nucleus, and binds to the sterol regulatory elements, stimulating cholesterol biosynthesis and restoring intracellular levels of cholesterol.

We have reasoned that elevated copper may inhibit SREBP-2 maturation or re-localization of activated SREBP-2 into the nuclei, thus blocking cholesterol biosynthesis. However, Western blot analysis of SREBP-2 in nuclear extracts from control and Atp7b−/− livers indicates that mature SREBP-2 is produced and present at higher levels in the nuclei of Atp7b−/− mice (Fig. 7A).

**The SREBP-2 Target Genes Are Misregulated in Atp7b−/− Mice and in Human Wilson Disease Livers**—To evaluate SREBP-2 activity, we measured the amount of mRNA for low density lipoprotein receptor (LDLR). The transcription of the LDLR gene is directly controlled by SREBP-2 via sterol-binding element 1 and is expected to be up-regulated in response to increased nuclear SREBP-2 (30, 31). Fig. 7B illustrates that despite high levels of mature SREBP-2 in Atp7b−/− nuclei, the

### Table 3

| Lipid, mM | Control | Atp7b−/− | t test (p) |
|----------|---------|----------|------------|
| Total cholesterol | 2.63 ± 0.19 | 2.16 ± 0.32 | 0.020 |
| Triglycerides | 1.98 ± 0.57 | 0.95 ± 0.14 | 0.004 |
| HDL-cholesterol | 1.71 ± 0.19 | 1.36 ± 0.18 | 0.018 |
| LDL-cholesterol | 0.61 ± 0.11 | 0.67 ± 0.06 | 0.310 |
| VLDL-cholesterol | 0.25 ± 0.07 | 0.07 ± 0.02 | 0.001 |

**FIGURE 6.** Mass spectrometry analysis of liver cholesterol. Overlay of chromatograms from 10 different samples, normalized to the intensity of the internal standard 66-cholesterol (mass spectrum, left inset in green) at extracted ion trace m/z 374. Endogenous cholesterol is shown at extracted ion trace m/z 368 (mass spectrum, right inset in red). WT animals (red lines; \( n = 4 \)) had significantly higher levels of cholesterol than KO animals (blue lines, \( n = 6 \), \( p = 0.003 \)).

**FIGURE 7.** Elevated copper does not inhibit metabolic regulation of SREBP-2 but affects SREBP-2 function. A, immunodetection of SREBP2 in nuclear extracts from control and Atp7b−/− livers. B, LDL receptor mRNA is down-regulated in livers of Atp7b−/− (mice) and Wilson disease patients (human) compared with corresponding controls. C, comparison of HMG-CoA reductase transcripts in Wilson disease patients (\( n = 8 \)) compared with control (\( n = 8 \), t test; \( p < 0.001 \)).
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amount of LDLR mRNA was significantly lower in KO samples compared with control. These data suggested that elevated copper inhibits SREBP-2 function in Atp7b−/− livers.

To verify the relevance of these observations to human disease, we compared the LDLR mRNA levels in liver samples from Wilson disease patients and control patients. The LDLR mRNA was significantly down-regulated (3.6-fold) in Wilson disease liver in agreement with observation in Atp7b−/− livers (Fig. 7B). HMG-CoA reductase, which plays a central role in cholesterol biosynthesis, is another important target of SREBP-2 that was down-regulated in Atp7b−/− mice (Table 2). Analysis of human HMG-CoA reductase mRNA by real time PCR demonstrated that HMG-CoA reductase transcript levels were markedly down-regulated (3.4-fold) in Wilson disease livers compared with control (Fig. 7C).

DISCUSSION

In this study, we have utilized the Atp7b−/− mice, an animal model for Wilson disease, and characterized changes in the transcriptome, proteome, and important metabolites associated with copper accumulation in the liver. We show that prior to development of obvious histopathology accumulated copper has a distinct and selective effect on liver gene expression and metabolism.

We conclude that the dysregulation of lipid metabolism and changes in the cell cycle machinery characterize presymptomatic stages of WD, whereas oxidative stress plays a minor role. Comparison of copper-induced mRNA changes with published mRNA profiles of liver responses to various stresses (32–35) illustrates that the overall pattern of changes in response to copper accumulation (i.e. the combination of most altered pathways) is distinct.

The Up-regulated Pathways—Although a large increase in the amount of metallothionein in response to copper accumulation was anticipated, the up-regulation of genes associated with cell cycle and chromosome structure was unexpected and very interesting. The up-regulated SMC2 and SMC4 mRNAs encode key components of the condensin complex, which is required for conversion of interphase chromatin into mitotic-like condensed chromosomes. Significantly, the level of up-regulation for SMC2 and SMC4 is similar, as would be expected for proteins that form heterodimers. The condensins association with chromosome and chromosome condensation is regulated via phosphorylation by CDC2 (36); the mRNA for mouse homologue of CDC2 is elevated in the Atp7b−/− liver (Table 1). Altogether, these transcriptional and presumably protein changes have a subtle but noticeable effect on nuclear structure. The Atp7b−/− nuclei show larger variation in size than control nuclei (our data and see Ref. 15) and frequently show electron-dense material, the exact nature of which remains to be established (Fig. 5).

In addition to these structural changes, up-regulation of cyclins G1 and G2, CDC28 kinase, as well as elevated levels of the fetal liver antigen and securin are all suggestive of increased cell proliferation. This conclusion is consistent with our earlier observation of increased nuclear staining with the proliferative marker Ki-67 in the KO livers (15). Up-regulation of cyclin G1 was also observed previously in the presymptomatic LEC rats, another animal model of WD (37), suggesting that the copper-dependent alteration of a cell cycle machinery is a species-independent phenomenon.

Pathways Down-regulated in Atp7b−/− Liver—The most significant effect of accumulated copper is on genes involved in cholesterol metabolism, although fatty acid and bile acid biosynthesis are also affected. The extent of these changes is illustrated in Fig. 8, which demonstrates that the enzymes involved in key steps of cholesterol biosynthesis are all affected (down-regulated) by copper overload. Indeed, direct metabolite measurements revealed a marked decrease of liver cholesterol. The impaired cholesterol biosynthesis is also clearly reflected in the reduction of the VLDL fraction of serum cholesterol, whereas the observed decrease in triglyceride levels can be attributed to increased expression of lipoprotein lipase. It is interesting that recent clinical chemistry measurements in a group of 45 WD patients found that in these patients total cholesterol, LDL cholesterol, and α-tocopherol levels were significantly lower compared with the control group (38), indicating that copper-induced alterations of cholesterol homeostasis are shared by WD patients and Atp7b−/− mice. It would be important to establish whether or not the observed changes in the serum lipid levels can serve as additional biochemical markers facilitating detection of the early stages of WD.

Inhibition of SREBP-2 Function Is Likely to Contribute to the Atp7b−/− Phenotype—The metabolic control of genes for the cholesterol, fatty acid, and lipid biosynthesis is well described. Low levels of metabolites up-regulate the rate-limiting biosynthetic enzymes through activation of specific transcription factors. For example, a decrease in intracellular cholesterol induces activation of SREBP-2, which in turn up-regulates genes involved in cholesterol metabolism. Our data indicate that this metabolic control is not disrupted by accumulated
Copper. Lower levels of cholesterol in \textit{Atp7b} \textsuperscript{−/−} livers are associated with increased levels of activated SREBP-2 in the \textit{Atp7b} \textsuperscript{−/−} nuclei, illustrating that neither processing of SREBP-2 nor its trafficking to nucleus are inhibited by copper overload. However, the target genes of SREBP-2 do not show expected up-regulation; in fact, they are markedly down-regulated. These results suggest that accumulated copper is likely to inhibit the SREBP-2 function either by directly binding and causing structural changes in SREBP-2 or by modulating SREBP-2 activity via other proteins. The precise mechanism for copper-dependent inhibition remains to be established; it may involve the known property of copper to compete with zinc and disrupt zinc fingers and/or copper-induced oxidation of Cys residues required for structure and function of transcription factors.

In addition to SREBP-2, other factors may contribute to the observed phenotype. The role of metallothioneins, greatly up-regulated in the \textit{Atp7b} \textsuperscript{−/−} livers, in cell differentiation and proliferation has been reported (39). Changes in hepatic glutathione levels were shown to alter the fatty-acid synthase expression, suggesting that lipogenesis may depend on the thiol redox state, which in turn could be greatly affected by copper overload (40). Increased levels of polyunsaturated fatty acids were previously linked to the \textit{in vivo} inhibition of expression of hepatic genes related to lipogenesis (41). Therefore, the detailed characterization of liver metabolites would provide important insight into copper-dependent regulation of lipid metabolism.

It is particularly significant that the effects on transcription/RNA stability that we observed in response to copper overload are opposite to the previously reported response to dietary copper deficiency. Specifically, in rats fed a copper-deficient diet, the fatty acid synthesis and assembly into triacylglycerols and phospholipids were up-regulated (42) in contrast to the down-regulation of fatty acid biosynthesis detected in our studies. These observations point to a tight connection between copper and lipid homeostasis, which is likely to be mediated through copper-dependent transcriptional control or modulation of RNA stability.

A Link between Lipid Metabolism and Cell Cycle—The effect of copper on a limited number of metabolic pathways suggests that the corresponding pathways could be functionally linked. Indeed, analysis of relationships between the significantly changed genes using the Ingenuity Pathways Analysis software reveals a highly scored network of 36 up- and down-regulated transcripts (Fig. 9). In this network, the connection is observed between lipid metabolism via sterol regulatory element-binding factor 1 and cell cycle via cyclin-dependent kinase inhibitor 1. Search for this link in the literature uncovered recent work in which lipid deprivation was shown to activate sterol regulatory element-binding factor 1 and induce cyclin-dependent kinase inhibitor 1 mRNA and protein (43). In our studies, the decrease in liver lipids also coincided with the elevated amount of cyclin-dependent kinase inhibitor 1 mRNA.

Implications for Understanding the WD Pathology—The relationship between copper and specific metabolic pathways revealed in our study has important implications for understanding the pathology of WD. The toxic role of copper has been commonly associated with the ability of the metal to
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induce formation of reactive oxygen species. We demonstrate that at the presymptomatic stage of copper accumulation oxidative stress does not appear to play the major role, as no significant up-regulation of transcript and/or proteins involved in oxidative stress response is observed. This observation is reminiscent of findings by Armendariz et al. (44). These investigators performed gene expression profiling of fibroblasts chronically accumulating copper because of ATP7A inactivation, and they found no evidence of oxidative stress-related gene expression despite significant copper accumulation. Thus, copper-induced radical formation, if it occurs, is unlikely to be central to the early stages of WD pathogenesis.

At the same time, some level of oxidative stress cannot be excluded. The expression of Rad51, which is cell cycle-dependent (45), can also be increased as a result of DNA damage, suggesting that accumulated copper may have some effect on DNA structure. Similarly, the up-regulation of glutathione S-transferase \(\alpha\) was noticeable and was also reported in the LEC rat (37). This enzyme metabolizes bilirubin and exhibits glutathione peroxidase activity thereby protecting cells from reactive oxygen species and the products of peroxidation (46). The up-regulated selenoprotein H may also have an antioxidant function (28). Finally, up-regulation of transcripts for proteosome subunit (macropain), ubiquitin-conjugating enzyme E2C, and glyoxalase 1 suggested the need for removal/detoxification of metabolic by-products, which may play a larger pathogenic role at later stages of the disease.

Previously, in the Atp7b\(^{-/-}\) animals nursed by homozygous knock-out dams, we observed microsteatosis at 6 weeks, which progressed to focal steatosis and lipid droplets in the nuclei of 12–20-week-old mice (15). In the 6-week-old Atp7b\(^{-/-}\) mice nursed by heterozygous dams, the steatosis is less severe and varies from apparent to nondetectable (data not shown). It appears that dysregulation of lipid biosynthesis is accompanied by accumulation of certain (perhaps intermediate) lipid metabolites and that the degree of accumulation may depend on the nutritional status of the animal. If variation in nutrition does play a role in the severity of hepatic response to copper overload, this may explain the larger variation between the RT-PCR and gene array data for metabolic genes compared with the cell cycle genes, measured at 6 weeks when the homeostatic capacity of liver is still significant. A better understanding of the extent of pathology induced by copper overload in livers lacking functional Atp7b. We demonstrate that the effect of copper is selective and involves specific metabolic pathways. This observation may have important practical implications, because specific cellular responses, particularly those involving metabolites, are more amenable to detection and possibly correction. We have also discovered that oxidative stress is not the major effect of copper at the early stages of the disease. Instead, lipid metabolism and cell cycle machinery are selectively affected by copper overload. The marked effect of copper on the specific metabolic pathways suggests that the extent of pathology may depend on the ability of tissue to correct/sustain these metabolic insults and thus be altered by “modifier” genes and nutritional status, which are different in individual individuals.

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