Wilson’s disease (WD) is a severe hepato-neurologic disorder that affects primarily children and young adults. WD is caused by mutations in \textit{ATP7B} and subsequent copper overload. However, copper levels alone do not predict severity of the disease. We demonstrate that temporal and spatial distribution of copper in hepatocytes may play an important role in WD pathology. High-resolution SXRF imaging \textit{in situ} indicates that copper does not continuously accumulate in \textit{Atp7b}⁻ hepatocytes, but reaches a limit at 90-300 femtomoles. The lack of further accumulation is associated with the loss of copper transporter Ctr1 from the plasma membrane, and the appearance of copper-loaded lymphocytes and extracellular copper deposits. The WD progression is characterized by changes in sub-cellular copper localization and transcriptome remodeling. The SXRF imaging and mRNA profiling both point to the key role of nucleus in the initial response to copper overload and suggest time-dependent sequestration of copper in deposits as a protective mechanism. The metabolic pathways, upregulated in response to copper, show compartmentalization that parallels changes in sub-cellular copper concentration. In contrast, significant down-regulation of lipid metabolism is observed at all stages of WD irrespectively of copper distribution. These observations suggest new stage-specific as well as general biomarkers for WD. The model for the dynamic role of copper in WD is proposed.

Wilson’s disease is a severe disorder of copper metabolism with varied manifestations and, if untreated, invariably lethal outcome. In WD, copper excretion from the liver is blocked due to genetic inactivation of the copper transporter ATP7B. Copper overload, which follows, induces marked changes of liver morphology and function (1). Although WD is unquestionably caused by copper accumulation, the precise role of copper in inducing pathological changes remains poorly understood. Increased oxidation of lipids, DNA damage, and enzymes’ inactivation has been reported for WD livers (2,3). These effects are typically observed when pathology is fully developed; therefore it is uncertain whether the detected changes represent the cause of the disease or its consequences. Copper content in selected cell compartments of WD hepatocytes, such as lysosomes, has been measured (4-7);
however, comparative quantitative analysis of copper distribution within liver tissue and individual cells is not available. In diseased liver, large amounts of accumulating copper are found in the cytosol, where copper is bound to metallothioneins, high-affinity metal chelating proteins. Whether this sequestered copper is exchangeable and active is unclear. Furthermore, the severity and the on-set of WD do not directly correlate with copper concentrations in the liver (6,8,9), suggesting that additional factors, such as spatial distribution of copper in the liver as well as environmental and genetic modifiers may play an important role in WD pathology. To begin addressing these unresolved issues, we have utilized the Atp7b−/− mice, an animal model of WD. We demonstrate that the disease progression is associated with specific changes in the intracellular distribution of accumulating copper, rather than merely an increase in hepatic copper content. We show that distinct cellular compartments become progressively involved in response to copper overload. The involvement of cellular compartments in cellular response appears to correlate with copper concentration in these compartments. We also identified potential mechanisms that protect cells against copper overload. Our results begin to explain the lack of simple correlation between the total amount of copper in the liver and severity of WD manifestations.

**EXPERIMENTAL PROCEDURES**

**Tissue preparation.** Mice were housed according to the NIH guidelines; the procedures were approved by the OHSU IACUC. Mice were fed with Rodent Diet 5001 (Lab Diet, St Louis, MO), containing 13 ppm Cu, 70 ppm Zn, and 270 ppm Fe. At given time points, the animals were euthanized and perfused with 10 mL 0.9% saline injected in the left ventricle. For the SXRF analysis, liver pieces were embedded with Cryochrome (ThermoShandon) on an aluminum block, snap-frozen in dry ice-cooled iso-pentane, and cryo-sectioned (Leica CM1850). The 10 µm sections were transferred onto a 0.001” mylar-window (Hyde, Chicago, IL) attached to a lucite sample holder (Huffstutter Machining, Hillsboro, OR), dried and stored in a desiccator.

**Serum cholesterol analysis.** Blood was collected by cardiac puncture and serum was separated by centrifugation; the concentration of cholesterol was determined after fractionation by sequential centrifugation as described by Teupser et al (10).

**Microarray analysis.** Animal ages were selected based on histology to represent stage I (6 weeks), stage II (46 weeks) and stage III (60 weeks) of the disease. Immediately after removal of livers, total RNA was isolated using TRIzol reagent (Invitrogen) followed by an RNaesy cleanup procedure (Qiagen). RNA integrity was electrophoretically verified by ethidium bromide staining and optical densities ratio (OD260/280nm>1.8). Each sample (n = 3 per age group – biological replicates) was hybridized to two MOE430A Affymetrix arrays (technical replicates). Image processing and analysis were performed using Affymetrix MAS 5.0 software. The data were saved in a CEL format, imported into GeneSifter data analysis software (Geospiza), and subjected to RNA normalization using published algorithms (11). The RMA-normalized dataset was then used to identify changed genes and determine statistical significance and magnitude of changes. Significance was established using Wilcoxon t-test with Benjamini and Hochberg adjustment for multiple comparisons. The hierarchical gene ontology analysis was performed by GeneSifter software using “biological function” and “cellular component” option. To confirm the mRNA profiling data, changes in several transcripts were verified using real-time PCR with GAPDH as internal standard (iCycler, Biorad). The list of primers used for the experiments is given in Suppl. Table 1. The relative expression of target mRNA in the Atp7b−/− mouse liver compared to the wt mouse liver was quantified using the 2ΔΔct method(12).

**SXRF imaging** was performed on beamlines 2-ID-E and 2-ID-D at the Advanced Photon Source at the Argonne National Laboratory at incident X-ray energy of 10 keV. X-rays were monochromatized by a double-crystal Si(111) (Kohzu); a Fresnel zone plate focused beam to a spot of 0.5 (h) x 0.5 (v) µm² (13). Samples were raster-scanned and the resulting fluorescence of each element was measured simultaneously at
each pixel (dwell time: 1 sec per pixel) using an energy-dispersive silicon drift detector (SII NanoTechnology). For quantitation, the entire X-ray fluorescence spectrum was collected at every position during the scan. The spectra were then individually fitted using modified Gaussians for fluorescence peak descriptions, and a version of the SNIP algorithm for background determination (14). Conversion of fluorescence counts to 2-dimensional densities of elements (µg/cm²) was done using a calibration curve based on the X-ray fluorescence of the thin film NBS standards 1832 (Cu) and 1833 (Zn), National Institute of Standards and Technology, Gaithersburg, MD. The concentrations of copper (in mM or µg/g wet weight) were calculated from 2D densities (µg/cm²) by taking into account the atomic mass of Cu and using 1.035 g/cm³ as samples density.

For average elemental content or larger areas, spectra were extracted from original dataset, added up, fitted and quantified as above. To estimate the amount of copper in individual cells, cellular volume was calculated considering hepatocytes as cylinders with identical height and diameter. During disease progression the diameter of hepatocytes increases dramatically (from 18 up to 62 µm), but whether the cell height increases proportionally is unknown. Consequently, we calculated a maximum cell volume (assuming the diameter and the height of cells were equal) and a minimum cell volume (assuming the cell height remains the same as at 6 weeks). Nuclei in a healthy hepatocyte were considered spheres. A nucleus is smaller than 10 µm (the tissue thickness) in diameter, therefore it comprises only a fraction of the total amount of copper in the corresponding area. Consequently, we calculated the volume of the nucleus (approximately 10% of the total cell volume) and subtracted it from the total cylindrical volume of the corresponding region. We then determined the amount of copper in the remaining (cytoplasmic) volume using cytoplasmic Cu concentration, subtracted this contribution from the total amount of copper in the cylinder to yield the amount and subsequently the concentration of copper in the nuclei. The calculations were performed for each scan (n=6) individually. Data ranges (min and max) were used for samples with n ≤ 10; and standard deviation (SD) - for all others. SDs were calculated from the mean averages; statistical significance was calculated using Student’s t-test with two-tailed distributions in either paired or, where applicable, un-paired mode. Three-dimensional copper concentrations were calculated from the area concentrations and tissue thickness of 10 µm. Shrinking of the samples in the x-y plane was determined to be negligible.

Immunohistochemistry. Mounted 5-µm tissue sections were hydrated in phosphate-buffered saline (PBS), blocked for 30-60 min in blocking buffer (5% goat serum, 0.2% Triton X-100, 1% bovine serum albumin (BSA) in PBS) and then incubated with rabbit anti-hCTR1 (1:100 dilution in PBS containing 0.25% BSA, 0.2% Triton X-100) for 2 hrs at room temperature. Following 3x10 min washes with PBS slides were incubated with goat anti-rabbit Cy3 labeled antibody (at 1:10000) for 2 hrs at room temperature. Sections were washed 3 x for 10 min with PBS, mounted with Vectashield®-DAPI (Vector, Burlingame, CA) and analyzed by confocal microscopy (LSM 5 Pascal, Carl Zeiss Microimaging, Thornwood, NY). The immunostaining of Na,K-ATPase was done using the same protocol except the sections were incubated with the rabbit anti-mouse Na, K ATPase (1:1000 dilution, kind gift from Jack Kaplan, University of Illinois at Chicago) at 4ºC overnight. The sections were washed 4 x for 15 min with 1 ml PBS each, incubated with Cy3 labeled goat anti-rabbit IgG (1:15,000 dilution) in the dark for 2 hrs. After incubation and two additional washes (2 x 10min) with PBS the sections were mounted onto slides using Vectashield with DAPI and visualized.

RESULTS
Quantitative imaging of copper in the liver using SXRF microscopy. To understand the mechanisms of pathology development in WD, we have utilized the Atp7b−/− mice, an animal model for hepatic course of WD (6). In Atp7b−/− liver, the disease progresses in three major stages: (I) minor/absent morphological changes (up to 6-8 weeks); (II) inflammation, necrosis, and swelling of hepatocytes (most pronounced at 13-20 weeks), and (III) liver regeneration with marked proliferation of bile ducts (after 30-46 weeks) (6). While copper is elevated at all three stages of the disease, no correlation is observed between the
total copper content and the \textit{Atp7b}\(^{-/-}\) liver morphology/function, similarly to human WD patients. Consequently, to better understand the role of copper in WD pathology we examined copper distribution within the \textit{Atp7b}\(^{-/-}\) hepatocytes in physiologically relevant tissue context.

Until recently, such measurements have been very difficult, because available methods lack sufficient sensitivity and/or spatial resolution. Histological staining with rhodanine is not quantitative (15) and does not detect all forms of copper in the livers. Electron microprobe is highly sensitive and quantitative, but does not allow simultaneous comparison of individual cells and larger areas of tissue. SXRF has emerged as a promising technology to overcome these problems (16-22). Third generation synchrotron sources provide sufficient flux in a focused spot to measure the inherent fluorescence of metals with exceptional sensitivity (down to the attomolar \(10^{-18}\) M range) and subcellular resolution. At the time of our experiments, SXRF had not yet been used for a single cell analysis \textit{in situ}. Consequently, we have developed a necessary protocol (Suppl. Fig.1) and applied it to the \textit{Atp7b}\(^{-/-}\) liver. Since SXRF measurements and animal work depend on instrumentation/animal availability, we initially focused on three time points in the disease progression: 6 weeks (stage I), 13 weeks (early stage II) and 20 weeks (mid-late stage II). It should be noted that the \textit{Atp7b}\(^{-/-}\) animals are born with greatly reduced levels of copper in the liver compared to wild-type (8.1 ± 1.3 compared to 249.5 ± 54.3 \(\mu\)g/g dry weight at 2-4 days (23)) and all copper accumulation occurs after birth.

\textit{Changes in total copper content in the \textit{Atp7b}\(^{-/-}\) liver differ from the kinetics of copper accumulation in individual hepatocytes.} It is assumed that during the course of WD, copper continues to accumulate in the liver facilitating pathology development. However, our analysis of \textit{Atp7b}\(^{-/-}\) livers revealed a more complex picture. Integrated SXRF scans of tissue areas demonstrated that copper concentration in the \textit{Atp7b}\(^{-/-}\) livers is high at 6 weeks (228 - 530 \(\mu\)g/g, or 6.4 ±2.3 mM, \(n=5\)), but decline subsequently to 60 - 159 \(\mu\)g/g, or 2.3±1.1 mM, \(n=5\)) at 20 weeks (Fig. 1A). Atomic absorption spectroscopy (AAS) analysis of liver pieces confirmed this observation (Suppl. Fig 2).

This time-dependent decrease could be due to the replacement of hepatocytes with other cell types that accumulate less copper (fibroblasts, for example). Alternatively, unlimited copper accumulation in the liver can be prevented through regulation of copper uptake into hepatocytes or stimulation of copper efflux. To distinguish between these two scenarios (cell loss versus limited accumulation) we measured copper content of individual cells using SXRF (Fig. 1B). In contrast to tissue sections, the amount of copper in individual hepatocytes not only did not decline, but increased 3 -10 fold in the period from 6 to 13 weeks (Fig. 1B). Thus, during this time copper continues to accumulate in hepatocytes, and the decrease in copper content in the liver tissue must be due to a decrease in the number of hepatocytes and the appearance of non-hepatic cells. (A lower number of hepatocytes at 13 weeks compared to 6 weeks is apparent in the H&E staining, Suppl. Fig.3BC). However, after 13 weeks, no further increase of copper in individual hepatocytes was observed indicating that copper accumulation had reached its upper limit (90 - 300 femtomoles or 50 - 200 billion copper atoms per hepatocyte). Thus, the pathology development from stage I (6 weeks) to early stage II (13 weeks) is associated with the elevation of cellular copper, while subsequent events in disease progression cannot be due to copper increase and can be caused by intracellular copper re-distribution.

The decrease in copper accumulation is associated with the down-regulation of copper transporter Ctr1. To better understand the molecular mechanism behind limited copper accumulation, we examined the plasma membrane levels of the major copper uptake transporter Ctr1 before and after hepatic copper had reached its limit, i.e. at 6 and 20 weeks, respectively (Fig. 2A). At 6 weeks, Ctr1 was easily detected at the plasma membrane of hepatocytes in either control or \textit{Atp7b}\(^{-/-}\) livers; and the patterns of Ctr1 staining were very similar (Fig 2Aa,b). At 20 weeks, the staining of Ctr1 in the control liver was the same as at 6 weeks (Suppl. Fig.4) while the \textit{Atp7b}\(^{-/-}\) liver showed weaker and uneven staining (Fig.
2Ac,d). Closer examination of individual cells revealed the almost complete lack of Ctrl at the plasma membrane of enlarged hepatocytes. Smaller hepatocytes that had retained cell-cell contacts and non-hepatic cells still showed some Ctrl staining. The loss of Ctrl appeared specific, because another plasma membrane protein, Na,K-ATPase, was present at the plasma membrane of all hepatocytes (Fig. 2B).

Further analysis of mRNA levels demonstrated that the Ctrl transcript was decreased in the Atp7b−/− liver compared to control at the stage I of WD and was significantly down-regulated at the stage II (Fig. 2C), thus providing possible explanation for the decrease of the Ctrl protein. (The Ctrl mRNA down-regulation was verified by real-time PCR, Suppl. Fig 5). The mRNA levels for another copper transporter, Ctr2, and for Na,K-ATPase were not changed, further suggesting specificity of Ctrl down-regulation.

Decreased hepatic accumulation of copper is accompanied by the appearance of copper-loaded inflammatory cells and extracellular copper deposits. The decrease of Ctrl at the plasma membrane is likely to diminish copper uptake thus protecting liver against further copper overload. Coincidentally, in 20-weeks Atp7b−/− livers (but not earlier), we observed yellowish extracellular deposits (Fig 3A), which are visible in stained and even unstained tissue (Fig 3A). These extracellular deposits are highly enriched in Cu (maximum concentration of 43 mM), S and Fe. The co-clustering of three elements is not observed within cells, where Cu distribution does not parallel the distribution of Fe and show only partial correlation with S. This result suggests that the extra-cellular copper deposits are not merely a product of necrotic hepatocytes, but are more likely produced due to the diminished uptake by hepatocytes.

It is not known whether in WD liver cells other than hepatocytes accumulate copper. Since the decrease in copper uptake by hepatocytes coincides with the appearance of inflammatory cells, we measured copper concentration in these cells (Fig. 3C). Lymphocytes and macrophages express homologous copper-transporting ATPase, ATP7A, but not ATP7B, and a priori are not expected to accumulate copper. At 20-weeks, the copper concentration in these cells was indeed lower than in neighboring Atp7b−/− hepatocytes (1.4 mM compared to 3.8 mM), but ~12 times higher compared to hepatocytes in healthy tissue. Thus, copper that is no longer taken by Atp7b−/− hepatocytes is re-directed, at least partially, to other cells in the liver.

Hepatic nuclei contain copper, which became highly elevated at the stage I of WD. So far, our results indicated that the development of WD pathology (stage I and early stage II) was associated with the increase of hepatic copper content, but the data offered little information about the site of Cu action. Also, the late stage II showed no further copper elevation raising question about the role of copper, if any, at this stage of the disease. Consequently, to gain additional insight we compared the distribution of copper within control and Atp7b−/− hepatocytes at all three time points. At 6 weeks, in control hepatocytes, copper was highest in the cytosol (which contains abundant Cu,Zn-superoxide dismutase and mitochondria). Unexpectedly, significant amount of copper was also detected in the nucleus (Fig. 4A), even after the contribution of cytosolic copper was subtracted. The nuclear copper concentration was in the range of 20-69 µM (n=6), and the average concentration (40 µM) was only 3 times lower than copper concentration in the cytosol (average: 120 µM; range: 60-170 µM, n=6). Considering that the volume of mouse nuclei is approximately 340 µm³, the total amount of copper in hepatic nucleus equals 10 million atoms. This high amount of copper in healthy nuclei suggests an important but currently unknown role for copper in mammalian nuclei.

In 6-weeks Atp7b−/− hepatocytes, a massive entry of copper into the nucleus was observed (Figure 4A,B). The average copper concentration in this compartment was increased by about 100 fold (to 7.2 mM; range 3.9 – 10.2 mM, n=3) compared to control. The concentration of copper in the cytosol and nucleus became similar; the Cuncn/Cucyt ratio was close to 1.02 (0.80 – 1.44, n=8). This observation suggests that excess copper is present in a form that easily shuttles between the cytosol and nucleus leading to equilibrium. It further implies that this form of...
copper is not present in normal hepatocytes, where no such equilibrium is observed. (We examined by Western blotting the levels of two potential copper carriers, metallothionein and Atox, in Atp7b-/- nuclei. Neither metallothionein nor Atox1, both detectable in a cytosol, were elevated in nuclei, data not shown).

The concentration of copper in cellular compartments changes with disease progression. Further time-course studies demonstrated that control liver had a fairly constant copper concentration in either tissue sections (100-120 µM, n=3 for each age group) or individual hepatocytes (range: 90-133 µM, n=3). In contrast, we observed distinct changes in the cellular and sub-cellular copper concentrations when we compared the 6 and 13 week-old Atp7b-/- hepatocytes (Fig. 4B). In older mice, the average nuclear and cytosolic copper concentration decreased from initially 7.2 mM to 2.1 mM (range 0.9 - 3.8 mM, n=3). The decrease in concentration was observed despite elevation of total copper content in a cell. This counter-intuitive phenomenon was caused by two factors. One was a marked increase in a cellular volume: hepatocytes expanded from 4,641±10 µm³ (n=24) at 6 weeks to 19,059±1,814 µm³ (n=20) at 13 weeks. (Nuclei increased from 340±2 µm³, n=106, to 8,329±264 µm³, n=50). Another important factor was the appearance of numerous “hot spots” (localized deposits enriched in copper, Fig. 4B). At 6 weeks, only few small (~ 0.5µm) hot spots were detected. The copper concentration in these spots was slightly higher compared to surrounding cytosol (5.7- 10.5 mM; average 8.5 mM versus average 7.2 mM). At 13 weeks, hot spots became larger (1-6 µm, average 2 µm) and more abundant (Fig. 4B), although their copper concentration on average did not change very significantly (5.0-15.8 mM, average 9.7 mM, n=3, Fig 4.C).

At 20 weeks, copper concentration in the cytosol and nucleus decreased further (Fig. 4B, Suppl. Table 3), while their volumes showed no further increase (in fact, some decrease was detected). Thus, at 20 weeks copper was leaving these compartments. In contrast, copper accumulation within the hot spots became truly pronounced, and the concentration of copper in hot spots reached 10.0 – 35.7 mM (average 22.4 mM, n=3). (For the summary of all changes, see Suppl.Table 2).

Physiological processes up-regulated in WD liver show time-dependent compartmentalization that parallels copper distribution. Time-dependent changes in local copper concentrations suggested that copper may trigger distinct cellular responses at different stages of the disease. Previously, we found that the initial stage of WD is characterized by selective up-regulation of machinery associated with cell cycle and marked down-regulation of cholesterol metabolism (8). To determine whether these pathways remained permanently altered during pathology development (or change along with the changes in copper distribution), we performed mRNA profiling using livers from Atp7b-/- mice at the advanced stage II of the disease and from the regenerating portion of Atp7b-/- liver at stage III. The profiles were compared with the age-matched controls and our earlier data collected at Stage I. The accuracy of the gene array measurements was verified by the real-time PCR analysis of several altered transcripts (Suppl. Fig. 6).

The summary of Gene Ontology for the most significantly up-regulated and down-regulated processes at all three stages is shown in Table 1A and 1B, respectively. We found that the unregulated processes differ considerably depending on the stage of the disease (Table 1A). Cell cycle machinery is most affected at stage I; stage II is characterized by marked changes in cytoskeleton and cell adhesion, as well as upregulation of glycerol and organic ether metabolism; and stage III shows the hallmarks of increased immune response (Table 1A). Most striking observation is an apparent time-dependent switch between cellular compartments that house the up-regulated metabolic pathways (Fig.5). Specifically, nucleus is most noticeably involved at the stage I but is not a significant player at the stage III of the disease. In contrast, the processes in the lysosomes and endocytic vesicles are not activated at the stage I but appear to play the major role at the stage III (Fig. 5). The switch in the compartmentalization of up-regulated pathways parallels changes in copper concentration in these compartments suggesting...
that intracellular copper localization may have significant effect on cellular response.

Lipid metabolism is the major down-regulated process in WD. In contrast to up-regulated pathways that are time and compartment specific, lipid metabolism is most significantly down-regulated at all stages of WD (Table 1B). Two other pathways down-regulated at all stages of WD, although less significantly, were alcohol metabolism and reductive/oxidative processes. This observation suggests that lipid metabolism is exquisitely sensitive to copper misbalance and that monitoring lipid metabolism may provide useful information about liver function in WD.

To verify that changes in the liver transcripts for enzymes involved in cholesterol biosynthesis (Suppl. Table 3) are accompanied by metabolic changes, we compared cholesterol levels in the serum of control and \( \text{Atp7b}^{-/} \) animals. These measurements revealed significant cholesterol misbalance (Fig. 6). Specific changes in different fractions of serum cholesterol were similar at 6 weeks and 30 weeks. As observed previously for Stage I (6), the VLDL cholesterol was markedly decreased in \( \text{Atp7b}^{-/} \) serum at either 6 or 30 weeks, representing the most sensitive measure of cholesterol misbalance. The HDL and LDL cholesterol levels were not changed significantly at 30 weeks, although a downward was detected for the HDL fraction.

DISCUSSION
The goal of this study was to better understand the role of copper in pathology development in WD. The consequences of complete \( \text{Atp7b} \) inactivation provided by a gene knockout can be more severe or manifest sooner compared to those of a single site mutation. However, the role of copper in triggering the disease is likely to be the same. Specifically, we demonstrate that the status of the \( \text{Atp7b}^{-/} \) liver is defined by the levels of copper in individual hepatocytes, the intracellular copper distribution, and the upregulation of metabolic pathways in specific cell compartments. Our experiments identify lipid metabolism as the pathway most significantly down-regulated in WD and therefore a likely contributing factor to WD pathogenesis. The serum VLDL cholesterol could be a useful marker to monitor the functional state of the WD liver.

Using high-resolution imaging in situ, we have determined the copper content of individual hepatocytes, showed that different cell compartments are preferentially involved in response to copper overload, and discovered the existence of a limit for copper accumulation in individual hepatocytes.

These results begin to explain the lack of simple correlation between the copper levels and the functional state of WD liver. We propose the following model for the role of copper in WD progression (Fig. 7). Early on, the inactivation of \( \text{Atp7b}^{-/} \) is associated with copper accumulation by hepatocytes. At this stage copper enters the hepatic nuclei and triggers the remodeling of liver transcriptome, which involves up-regulation of cell cycle and down-regulation of lipid metabolism. Once copper levels are 250-800 fold above the norm, copper uptake is ceased and/or additional copper-export mechanisms are activated to prevent further copper accumulation. Our data suggest that the lack of continued copper accumulation could be due to down-regulation of \( \text{Ctr}1 \).

Copper which is no longer absorbed by hepatocytes, has three potential routes of further distribution: it may be absorbed by the inflammatory cells, deposited in extracellular aggregates, or exported through filtering into urine (Fig. 7). Together, these processes may lessen the copper load in hepatocytes and thus facilitate hepatic regeneration. High copper levels in lymphocytes compared to healthy hepatocytes support the proposed role for elevated copper in inflammation as a response to tissue injury (24-26). Given the observed up-regulation of transcripts associated with inflammatory processes observed at the later stages of the disease it is tempting to speculate that the redirection of copper uptake from hepatocytes to other cell types may facilitate inflammation/contribute to activation of non-hepatic cells. Yet, the copper levels in lymphocytes are lower than in the age-matched \( \text{Atp7b}^{-/} \) hepatocytes likely due to compensatory
effects mediated by another copper-transporting ATPase, ATP7A, expressed in these cells (27).

At the stage II, copper concentration in the nuclei and cytosol decreases and the intracellular deposits appear (Fig. 7). The driving force for copper accumulation in deposits is unknown. One possibility is a time-dependent polymerization of copper-bound metallothioneins (28), which are highly up-regulated in Atp7b<sup>−/−</sup> liver (8), with a subsequent phagocytosis of oligomers into lysosomes. This scenario is consistent with the previous reports finding copper in electron dense lysosome-like compartments at the late stage of WD (29). We assume that the “hot spots” represent copper within the lysosomes-like compartments because granular copper accumulation in lysosomes has been reported in WD patients (5,30); although this remains to be formally established.

It should be emphasized that the observed copper compartmentalization reflects preferential rather than exclusive distribution. Elevated copper can be found (and presumably act) in various cellular locations particularly at stage II when the disease phenotype is most severe. Nevertheless, the change in local copper concentrations is very distinct and, at the late stages of the disease, is likely to represent an important protective mechanism. As the result of copper redistribution, the “mobile” and likely active form of copper is sequestered minimizing the range of copper activity.

The mechanism of further copper decrease in hepatocytes and liver recovery remains to be characterized. The involvement of the ABC-type transporter MRP2 in copper excretion was suggested based on the measurements of copper export in the MRP2-deficient rats (31). It is interesting that several ABC transporters are up-regulated in Atp7b<sup>−/−</sup> livers (our gene array data, not shown). Determining the molecular nature of the mechanism that facilitates loss of copper from hepatocytes may open new avenues for treatment of WD.

**Acknowledgements**

We thank Cara Poage and Carolyn Gendron (OHSU Histology Core) for help with tissue sectioning and staining, Dr. Milton Finegold, Dr. Ann Hubbard, and Dr. Jack H Kaplan for critical reading of this manuscript and helpful comments. Furthermore, the authors acknowledge assistance by Wolfgang Wilfert with lipid measurements. This work was supported by the National Institutes of Health Grant PO1 GM067166 to SL and by the German Research Foundation Grant Hu932/3-2 to DH. The use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science Contract DE-AC-02-06CH11357.

**FIGURE LEGENDS**

**Figure 1.** Time dependent changes of copper levels in the Atp7b<sup>−/−</sup> liver differ from changes in individual hepatocytes. SXRF measurements were performed for liver tissue sections (left) and individual hepatocytes (right) at three different time points (n=5 for each time point).

**Figure 2.** Ctr1 levels decrease in 20 weeks old Atp7b<sup>−/−</sup> hepatocytes. (A) Confocal images of liver sections immunostained with rabbit anti-Ctr1 antibody; (a) 6-week old wild-type, (b) 6-week old Atp7b<sup>−/−</sup>, and (c,d) 20-week old Atp7b<sup>−/−</sup> mice. The right panel shows magnified views of areas in white rimmed boxes 1-3. (B) Confocal images of 20 weeks old control (a) and Atp7b<sup>−/−</sup> (b) liver sections immunostained with anti-Na,K-ATPase antibody. The staining at plasma membrane is apparent in even enlarged hepatocytes. (C) The mRNA levels in Atp7b<sup>−/−</sup> samples compared to control (taken as 1) are down-regulated. The average RMA normalized fluorescence intensity of RNA hybridization signal from 3 biological replicates for Ctr1, another copper transporter Ctr2 and unrelated plasma membrane protein
Na,K-ATPase (the time-dependent down-regulation of Ctr1 mRNA was additionally verified and confirmed by real-time PCR).

**Figure 3. Accumulation of copper in extracellular deposits (A,B) and inflammatory cells in 20 weeks old Atp7b<sup>−/−</sup> liver.** (A) Extra-cellular deposits (indicated by arrows) in the unstained SXRF section (left) and an H&E stained tissue (right). The scale bar is 100 µm. (B) H&E of the scan area (50 x 50 µm; bar = 10µm) illustrates the absence of nuclei/cells in the area of deposit (circled). Elemental maps of sulfur (S), iron (Fe) and copper (Cu) illustrate an increased concentration (lighter spots) of each element in the same area. Concentrations are displayed using red temperature false coloring (on a logarithmic scale) (C) Inflammatory cells were identified by phosphorus map (left) and by H&E staining (middle) and the copper concentration in these areas was measured by SXRF (right).

**Figure 4. Intracellular copper distribution in control and Atp7b<sup>−/−</sup> hepatocytes.** (A) The 2D fluorescence maps demonstrate the distribution of two elements (P and Cu) within the same scanned area of control or Atp7b<sup>−/−</sup> livers at 6 weeks. Concentrations are displayed using red temperature false coloring (on a logarithmic scale) with lighter color corresponding to higher concentrations. The phosphorous concentration in the nuclei (circled by white dotted line) is higher than in the cytosol in either control or Atp7b<sup>−/−</sup> sample. Copper concentrations in the nucleus is lower compared to cytosol in control livers and equal to cytosolic copper in Atp7b<sup>−/−</sup> sample. (B) SXRF scans of Atp7b<sup>−/−</sup> livers at three time points demonstrate progressive increase in copper concentration in hot spots (marked by arrows) along with the decrease of average copper concentration in the remainder of cytosol (given in numerical values above the images). (C) Quantitation of copper concentrations in hepatic nuclei, cytosol, and “hot spots” at different ages (data are generated using 2 livers per each age, 2-3 scans per each sample).

**Figure 5. Association of the significantly upregulated pathways with cellular compartments.** The compartmentalization of significantly upregulated pathways was examined using the GeneSifter software. The Z-score above 2 (green dotted line) indicates significant over-representation of proteins/pathways in given compartment compared to average change per compartment.

**Figure 6. Sterol metabolism is disregulated at different stages of WD.** Analysis of serum cholesterol fractions demonstrates a significant decrease of VLDL cholesterol in Atp7b<sup>−/−</sup> animals at either 6 weeks or 30 weeks (n=6 per group, *p<0.05).

**Figure 7. Illustration of the proposed role of copper in WD progression.** The inactivation of Atp7b<sup>−/−</sup> results in disrupted copper export from hepatocytes and copper accumulation. Metallothionein (MT) is upregulated as an early response to copper overload and sequesters copper in the cytosol. Excess copper enters the nucleus and triggers the remodeling of liver transcriptome. Subsequently, copper leaves the nuclei decreasing nuclear involvement and concentrates in the intracellular deposits. When copper reaches the limit of accumulation, the uptake stops (or/and the efflux is activated); and more intracellular copper is sequestered in the deposits (presumably lysosomes). The copper that is not taken by hepatocytes is absorbed by the inflammatory cells and is deposited in extracellular aggregates. Metabolic pathways in compartments other than nucleus became activated.
Table 1A. The most significantly up-regulated processes in Atp7b−/− livers

<table>
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<th>Stage I</th>
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<th>Stage III</th>
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<td>Acylglycerol metabolism</td>
<td>5.06</td>
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<td>B cell apoptosis</td>
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<td>Processing, presentation of peptide antigen</td>
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<td>Lymphocyte activation during immune response</td>
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<td>Positive regulation of phagocytosis</td>
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<td>Glycerol ether metabolism</td>
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<td>4.72</td>
<td>Cell-matrix adhesion</td>
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<td>Response to chemical stimulus</td>
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<td>Positive regulation of endocytosis</td>
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Table 1B. The most significantly down-regulated processes in Atp7b−/− livers

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<thead>
<tr>
<th>Stage I</th>
<th>Score</th>
<th>Stage II</th>
<th>Score</th>
<th>Stage III</th>
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<td>Organic anion transport</td>
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<td>Regulation of fat cell differentiation</td>
<td>4.84</td>
<td>Fatty acid metabolism</td>
<td>3.19</td>
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</table>
REFERENCES

Figure 1
Figure 2

A  Ctr1

a) WT  b) Atp7b/-

c) Atp7b/-  d) Atp7b/-

B  Na, K-ATPase

WT  Atp7b/-

C

![Chart showing intensity comparison for different groups]

- Stage I
- Stage II

CTR1  CTR2  Na,K-ATPase
Figure 3

A

B

C
Figure 4

A

Control

Atp7b−/−

B

<table>
<thead>
<tr>
<th></th>
<th>6 weeks</th>
<th>13 weeks</th>
<th>20 weeks</th>
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<tr>
<td>Av (mM)</td>
<td>7.47</td>
<td>2.03</td>
<td>1.37</td>
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</table>

C

- **Nucleus**
- **Cytosol**
- **Hot Spots**

![Graph showing copper levels over time]
Figure 6

- HDL cholesterol (mg/dl)
  - WT
  - Atp7b−/−

- LDL cholesterol (mg/dl)
  - WT
  - Atp7b−/−

- VLDL cholesterol (mg/dl)
  - WT
  - Atp7b−/−

Age (weeks): 6, 30
Wilson's disease at a single cell level: intracellular copper trafficking activates compartment-specific responses in hepatocytes
Martina Ralle, Dominik Huster, Stefan Vogt, Wiebke Schirrmeister, Jason L. Burkhead, Tony R. Capps, Lawrence Gray, Barry Lai, Edward Maryon and Svetlana Lutsenko

J. Biol. Chem. published online July 20, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.114447

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