Published in final edited form as:

Metallomics. 2012 July ; 4(7): 660–668. doi:10.1039/c2mt20017a.

A systems approach implicates nuclear receptor targeting in the *Atp7b*^{-/-} mouse model of Wilson's Disease

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Abstract

Wilson's disease (WD) is an inherited disorder of copper metabolism characterized by liver disease and/or neurologic and psychiatric pathology. The disease is a result of mutation in *ATP7B*, which encodes the ATP7B copper transporting ATPase. Loss of copper transport function by ATP7B results in copper accumulation primarily in the liver, but also in other organs including the brain. Studies in the *Atp7b*^{-/-} mouse model of WD revealed specific transcript and metabolic changes that precede development of liver pathology, most notably downregulation of transcripts in the cholesterol biosynthetic pathway. In order to gain insight into the molecular mechanisms of transcriptomic and metabolic changes, we used a systems approach analysing the pre-symptomatic hepatic nuclear proteome and liver metabolites. We found that ligand-activated nuclear receptors FXR/NR1H4 and GR/NR3C1 and nuclear receptor interacting partners are less abundant in *Atp7b*^{-/-} hepatocyte nuclei, while DNA repair machinery and the nucleus-localized glutathione peroxidase, SelH, are more abundant. Analysis of metabolites revealed an increase in polyol sugar alcohols, indicating a change in osmotic potential that precedes hepatocyte swelling observed later in disease. This work is the first application of quantitative Multidimensional Protein Identification Technology (MuDPIT) to a model of WD to investigate protein-level mechanisms of WD pathology. The systems approach using "shotgun" proteomics and metabolomics in the context of previous transcriptomic data reveals molecular-level mechanisms of WD development and facilitates targeted analysis of hepatocellular copper toxicity.

Keywords

copper; Wilson's Disease; ATP7B; liver; nuclear receptor; lipid metabolism; DNA repair; proteomics; metabolomics; transcriptomics

Introduction

Wilson's disease (WD) is an autosomal recessive disorder that results from mutation in the *ATP7B* gene and a defective ATP7B copper transporter. Copper transport by ATP7B is essential for maintenance of copper homeostasis, and the protein is expressed in several tissues including the liver, lung, kidney, intestine and mammary gland ¹. ATP7B expression in the liver is essential for maintenance of systemic copper homeostasis, where the protein has both biosynthetic function as a Golgi-resident protein and a copper export function in a vesicular transport system ². In hepatocytes, ATP7B maintains cytosolic copper levels by transporting excess copper to vesicles for excretion from the cell through the canalicular membrane into the bile ³. Thus, WD may be caused by mutations in *ATP7B* ^{4, 5}, which can alter cellular trafficking or copper transport by the ATP7B protein and, critically, disruption of copper export from hepatocytes. Hepatic Cu accumulation in WD is typically in excess of 250 µg/g of dry weight, and pathology that can range from mild inflammation to cirrhosis and fulminant liver failure due to attenuated biliary copper export ^{1, 6}. Onset of WD symptoms is usually in childhood or adolescence, though many patients have been diagnosed much later in life ⁷. Thus, there is a latent period when copper is accumulating, but no pathology is yet observed. It appears that the liver can accommodate high copper level for some time until the compensatory response is overwhelmed and pathology develops. Currently, the molecular level response to copper accumulation in WD, and the cellular systems that may lead to observable pathology are not well understood.

Animal models of WD are essential tools for understanding how copper accumulation leads to WD. The *Atp7b*^{-/-} mouse model of WD has established its utility in studies of copper hepatotoxicity associated with WD. The hepatic pathology in these animals is consistent with a WD phenotype and includes hepatic Cu accumulation reaching peak levels around six weeks of age, low serum oxidase activity and decrease in cholesterol levels ^{2, 8}. Hepatic Cu levels decline as the animals age. Interestingly, at six weeks of age, there is no visible evidence of pathology and little evidence of hepatotoxicity despite high hepatic copper. We have focused our studies on disease mechanisms at this age—using the highest level of Cu, but lowest pathology to investigate what are most likely Cu-specific effects rather than secondary effects of disease progression and inflammation.

Although hepatic copper accumulation is the key influence in WD development, and treatment of WD is focused on limiting this accumulation, it is still not entirely clear which cellular machinery is altered by copper accumulation and how those changes lead to development of liver disease. Recent advances in data-driven “Systems Biology” approach have led to significant insights in understanding the molecular targets of Cu accumulation in WD by identifying the transcript, protein and elemental changes in rodent models of WD ⁹⁻¹¹. These systems approaches have revealed the key pathways targeted at the mRNA level, particularly at the six weeks stage and include increases in expression of cell cycle machinery, downregulation of lipid metabolism and cholesterol biosynthesis ^{12, 13}. Connecting observed changes in the transcriptome with changes in other cellular machinery to define factors that lead to pathology is challenging. The subcellular distribution of copper has been analysed and it was discovered that nuclei are early and specific targets of Cu accumulation, and despite liver Cu reaching peak concentrations at six weeks of age, Cu measured in individual hepatocytes continues to increase to 13 weeks, when histologic changes appear ^{8, 11, 14}. Analysis of targeted proteins is challenging due to sample complexity and varied physiochemical properties of proteins, though instrumentation and data analysis continues to improve. Likewise, analyses of cellular metabolites are still in early development compared to transcriptomics, as each metabolite must be independently standardized and validated. Recent work has, however, confirmed significant attenuation of

sterol metabolism at all stages of disease in these animals¹⁵, though amino acid metabolism and β -oxidation are not affected.

In this study, we use a multidimensional liquid chromatography and mass spectrometry approach to analyse the nuclear fraction from six-week-old wild type and *Atp7b*^{-/-} animals as well as metabolite analysis to probe Cu-mediated changes to cell machinery that may lead to WD pathology. We observed attenuation of key transcription factors, and resulting altered lipid metabolism and up-regulation of specific DNA repair machinery. This indicated Cu-induced DNA damage and cellular response to oxidative stress that may later lead to observed cellular phenotypes.

Experimental

Animal Handling

The generation of *Atp7b*^{-/-} mice has been described previously¹⁶. *Atp7b*^{-/-} and wild type mice were housed at the Oregon Health & Science University Department of Comparative Medicine according to the National Institutes of Health Office of Lab Animal Welfare guidelines on the use of laboratory and experimental animals¹⁷. Wild type and *Atp7b*^{-/-} Mice were maintained on strain C57BL x 129S6/SvEv. Housing was in shoebox cages on Rodent Diet 5001 (Lab Diet, St. Louis, MO), containing 13 ppm Cu, 70 ppm Zn, and 270 ppm Fe and water ad libitum, with a 12-hour light/dark cycle. Mice of both sexes were used for metal measurements and proteomic studies. Animals were euthanized at six weeks of age by CO₂ asphyxiation followed by cervical dislocation. Animals were immediately exsanguinated and perfused with 0.9% saline. Livers placed on ice before isolation of hepatic nuclei as described in¹⁴. Protein concentration in nuclei was determined by BCA assay. Trypsin digestion and 2D LC-MS/MS was performed as described¹⁸.

Multidimensional Protein Identification

The 8 purified nuclei samples from mouse liver generated 1,457,456 total MS2 spectra. DTA files were created using BioWorks 3.2 (ThermoFisher) with a molecular weight range of 550 to 4,000, an absolute intensity threshold of 500, group scan setting of 1, a minimum group count of 1, a minimum of 25 ions, and charge state analysis using the ZSA algorithm. A mouse species subset of the Sprout protein database (version 2010.07; 16,293 sequences) was prepared with concatenated reversed entries (and common contaminants) and searched with SEQUEST (ThermoFisher). Parent ion and fragment ion tolerances of 2.5 and 1.0 Da were used with calculated average and monoisotopic masses, respectively. Cysteine had a static modification mass of +57 Da, methionine had a variable modification mass of +16 Da, and trypsin enzyme specificity was chosen. An in-house suite of programs¹⁹ was used to calculate discriminant function values from SEQUEST scores to identify “correct” peptides and discard “incorrect” peptides using sequence-reversed matches to estimate false discovery rates. Of the 1,457,456 mouse liver spectra, 292,369 passed thresholds, with 3,054 matches to reversed sequences, giving an estimated peptide false discovery rate of 1.1%. A protein summary with peptide subset removal (Parsimony principle) was performed where proteins were required to have two or more fully-tryptic peptides with distinct sequences. Different charge states of the same peptide sequence were not considered as unique peptides. There were 1,390 non-redundant proteins identified with 17 protein matches to reversed sequences (1.2% protein FDR).

Semi-quantitative protein abundances were estimated using spectral counts²⁰. One of the samples had unusually low total numbers of MS2 spectra indicating an experimental malfunction and was excluded from the quantitative analysis. A partitioning of shared peptide counts amongst the proteins containing shared peptides on the basis of relative total unique counts to the associated proteins was performed to provide more accurate spectral

count estimates²¹. The corrected spectral counts from the 7 samples were normalized using quantile normalization²² before applying two-sample t-Tests and a 21-protein sliding window Z-score calculation (for fold-change estimates). The logarithm (base 2) of the average spectral counts for wild type mice to *Atp7b*^{-/-} mice ratios were used in the Z-score calculations. Proteins were sorted from highest to lowest average spectral count before calculating Z-scores of the log₂ ratios.

The distribution of spectral counts based on gene ontology was performed using PIPE2²³. Clustering of proteins with altered abundance was with the Heatmap2 function in the R statistical package (www.r-project.org).

Association between altered protein abundance and cellular pathways was evaluated by Ingenuity Pathways Analysis (Ingenuity® Systems, Redwood City, CA). Uniprot IDs were converted to Entrez Gene IDs and uploaded into the application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. A Z-score cutoff of ± 1.645 was set to identify molecules whose expression was significantly differentially abundant. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the molecules in the network.

Immunodetection of FXR

Nuclear proteins were extracted from purified nuclei as described in¹⁴. 10 μ g of protein for each sample was separated by 10% SDS-PAGE and transferred to PVDF in 10% methanol, 10% CAPS pH 11.0. Membranes were blocked with 5% nonfat dry milk. Rabbit anti-FXR α /NR1H4 (Millipore, Billerica, MA) was diluted to 1:20,000 in PBS-T. Secondary immunodetection was by anti-rabbit horseradish peroxidase ECL and imaging with charge-coupled device digital darkroom. The blot was stained with coomassie brilliant blue to confirm equal loading. Quantitation was with ImageJ Gel Analysis tools and normalization was to a non-specific coomassie stained band.

Determination of serum corticosterone

Serum corticosterone in four wild type and four *Atp7b*^{-/-} animals was determined by ELISA specific to corticosterone (Enzo Life Sciences, Farmingdale, NY). Each serum sample was run in triplicate and compared to a standard curve per the manufacturer's instructions. Equal numbers of male and female animals were used for each genotype.

Tissue metabolic profiling

Small liver tissue pieces were collected when mice were euthanized and used for tissue metabolite profiling. Tissue analysis was performed as described for cholesterol in¹³. Metabolites in *Atp7b*^{-/-} animals compared to wild type with a fold-change of ± 1.5 and a p-value of ≤ 0.05 for a two-tailed Student's T-test were considered significantly different.

Results

Nuclear proteins predominate the distribution of identified proteins

Evaluation of the quality of label-free MuDPIT for nuclear proteins—The range of total spectral counts for each analyzed sample was from 34,225 to 42,433 spectra. Contaminant counts were between 140 and 480, indicating a low amount of potential interfering contaminants. From these samples and the defined criteria, 1388 valid proteins were identified (all proteins identified are detailed in Supplementary Table 1). Initial verification of the quality of data was made by plotting normalized spectra of WT vs. *Atp7b*^{−/−} mean spectral counts for each identified protein. The mean counts fell symmetrically on a 45° line, indicating that the data is suitable for further analysis (Fig. 1A). The identified Uniprot accession numbers were mapped to Entrez gene IDs and an ontology analysis for cellular component was performed with PIPE 2.0 (pipe2.systemsbio.net). Spectral counts for each cellular component were summed and the distribution plotted (Fig. 1B).

The distribution of proteins identified by weighted spectral count number indicates that over 86% of the peptide spectra were from proteins associated with nuclear or nuclear/cytoplasmic localization, confirming purity of our preparations. The next highest category was “uncategorized” at 5.22%. Non-nuclear proteins that consistently copurify with nuclei represent most other cellular compartments. All identified proteins were included in the analysis. Several proteins that are not typically characterized as nucleus-localized passed thresholds for being changed in abundance in the *Atp7b*^{−/−} mouse. Since they must be consistently copurified and pass both fold-change and T-test thresholds to be considered “changed,” they were included in subsequent analysis.

Copper accumulation alters the abundance of a limited number of proteins

Mean abundance for each protein was compared between WT and *Atp7b*^{−/−}. Those that passed T-test and Z-score thresholds were considered candidate proteins with altered abundance. There were 890 proteins having an average spectral count across all 7 samples exceeding 2.5. Another 50 proteins had fewer than 33% unique peptides and were excluded from quantitative analysis. A T-test p-value threshold of 0.10 and a Z-score threshold of ± 1.645 were used and 40 of the 840 proteins passed both criteria. There were two additional proteins that appeared to be present in one set of samples but not the other for a total of 42 differentially expressed protein candidates (Fig. 1A). Based on this analysis, roughly 5% of proteins had significantly altered abundance. P-value histograms were used to estimate that about 30 of the proteins with p-values less than 0.10 were likely to be true differential candidates.

Generation of an expression-based heatmap and dendrogram indicates that changes in abundance in the nuclear proteome provided clear separation of WT and *Atp7b*^{−/−} genotypes (Fig. 2). Although some variation amongst samples of the same genotype is detected, differences between genotypes are largely consistent across samples. Interestingly, proteins XRCC1, LIG3 and PNKP that had increased abundance in the *Atp7b*^{−/−} samples and function together in DNA repair had highly similar changes in abundance and were close together in the heatmap. Using the Z-score and T-test criteria (or manually added as described) 23 proteins had increased abundance (Table 1) and 19 proteins were decreased in abundance (Table 2).

Correlating the nuclear proteome with the transcriptome

Previous work analyzing the transcriptome and target nuclear proteins indicated that Selenoprotein H (SelH) was upregulated at the transcript and protein level in *Atp7b*^{-/-} liver at six weeks of age^{13, 14}.

The observation that SelH was also more abundant in the current shotgun proteomics analysis of hepatic nuclei was the first validation of an individual protein in the experiment. In all, five of the proteins identified as changed in this work also had altered transcript abundance as reported in¹³: SelH (C11orf31), CYP2C19, DBP, POR, and VRK1. These changes that we see across analytical methods indicate that the MuDPIT and spectral counting quantitation reveals protein-level molecular responses to Cu accumulation that are consistent with changes in the transcriptome.

Network analysis indicates targets in lipid metabolism and response to DNA damage

Previous microarray analysis of the *Atp7b*^{-/-} liver transcriptome identified attenuation of key cholesterol synthesis pathways through altered transcript abundance. Further studies revealed that signalling via SREBP2, a key transcriptional regulator of cholesterol synthesis, was intact¹³. Thus, which transcriptional activators of cholesterol synthesis were altered in *Atp7b*^{-/-} mice remained unclear. Our analysis of protein-level changes has yielded new information that may explain dysregulation of cholesterol biosynthesis. Specifically, changes in protein abundance indicate that two key nuclear receptors with function in lipid metabolism are less abundant in nuclei of *Atp7b*^{-/-} hepatocytes: NR3C1/Glucocorticoid receptor (GR) and NR1H4/Farnesoid X receptor (FXR) (Table 2). We used network analysis of the changed proteins to place functionally related proteins into both targeted metabolic pathways and identify key molecules in the pathways. The top network is populated predominantly by proteins with lower abundance in *Atp7b*^{-/-}, and is characterized by ontologies in lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism and includes both GR and FXR (Fig. 3). Both of these transcription factors have regulatory roles in lipid metabolism and carbohydrate metabolism²⁴⁻²⁷. Promyelocytic leukemia (PML) was also identified due to interaction with GR²⁸ and interestingly is also known to function as a heterodimer with retinoic acid receptor (RAR) as well as retinoid-x-receptor (RXR) to influence retinoic acid-dependent transcription²⁹. Thus, abundance of several key transcription factors is attenuated by Cu accumulation. Therefore, although SREBP2 signalling is intact, transcripts involved in cholesterol synthesis or lipid synthesis could be downregulated due to attenuation of these nuclear receptors. We confirmed the decrease in FXR abundance in nuclei by western blot (Fig. 4A), which showed a decrease consistent with that indicated by mean spectral count (Fig. 4B). Interestingly, attenuation of the nuclear receptor HNF4α was observed in Cu-treated HepG2 cells³⁰. However, we found no significant change in abundance of HNF4α in our analysis (Supp. Table 1), indicating that Cu accumulation may only target specific transcription factors, but not the entire nuclear receptor family.

Glucocorticoid receptor and serum glucocorticoid are downregulated

GR, which is also a ligand-activated nuclear receptor, was significantly less abundant in the proteomic analysis (Fig. 5a). GR effectors TRIM28/TIF1B and PML were also less abundant. The GR transcriptional activator is activated and translocated to the nucleus upon binding glucocorticoids including cortisol and corticosterone in response to inflammation. Reduced GR in the nucleus of *Atp7b*^{-/-} liver compared to wild type may indicate aberrant signalling or suppression of glucocorticoid signalling, mediating changes in both glucose and lipid metabolism: specifically, mobilizing fewer lipids into circulation³¹. In order to determine if the suppression of GR was a systemic physiological response, we tested levels of serum corticosterone, the main circulating glucocorticoid in mice (Fig. 5B). Although a

reduction in mean corticosterone is indicated, the change is relatively small compared to the change in GR in the nucleus. This result may indicate a functional disconnect between circulating glucocorticoid levels and liver GR signalling. Such a response would be consistent with reduced cholesterol levels, as a GR polymorphism in humans that renders GR less responsive to corticosteroids is associated with lower levels of cholesterol and insulin³².

DNA repair and RNA processing are targeted by Cu accumulation

The second top network in our analysis is populated primarily by proteins with increased abundance (Fig. 6). The ontologies associated with this network are cell death, DNA replication, replication and are consistent with upregulation of cell cycle machinery at the transcript level¹³. Novel in the proteomic analysis is increased abundance in *Atp7b*^{-/-} liver nuclei of three proteins that directly function in DNA (Fig. 7). XRCC1, LIG3, and PNKP function together in repair of DNA strand breaks³³⁻³⁵. This indicates an early response to DNA injury that may in the end affect genome stability. Though it is expected that a sustained inflammatory response like that observed later in disease may be associated with DNA damage and increased risk of tumour development, this observation indicates that the damage may indeed begin early in disease.

Consistent with 2D gel-based proteomics, we observed changes in proteins involved in RNA processing including ribosomal RNA processing proteins RRP1 and RRP12, serinearginine rich splicing factor 13A (FUSIP2), S1 RNA binding domain 1 (SRBD1), KHSRP/FUBP2^{36, 37} and ZCCHC8^{38, 39}. KHSRP may provide a mechanistic link between changes in RNA processing machinery reported in previous 2D gel proteomics study¹⁴ and cholesterol metabolism, in that the KHSRP is a modulator of low-density lipoprotein receptor (LDLR) mRNA in liver cells⁴⁰. Interestingly, ZCCHC8 is a 'zinc knuckle' RNA binding protein that coordinates Zn(II) through a binding site that includes a CXXC motif consistent with Cu(I) coordinating proteins⁴¹⁻⁴³.

Changes in metabolites indicate oxidative stress response and potential osmotic stress

Cu-associated targeting of nuclear receptors, particularly GR, taken with a reduction in serum glucocorticoids indicates that the liver metabolism in *Atp7b*^{-/-} may be significantly altered. We therefore analysed liver cell metabolites to determine if cell physiology may be significantly altered. Of 213 metabolites analysed, we found 10 were significantly changed with a fold change of ± 1.5 and P-value < 0.05 (Table 3). Among these, we observed a significant reduction in citrate levels as well as glycerophosphate isoforms and an increase in xylitol and sorbitol. A minor peak of glucose-6-phosphate was lower, but the major peak was not, thus the change in this molecule is unclear. The reduction in citrate may indicate that it is consumed to synthesize NADPH for GSH synthesis and is characterized as a response to oxidative stress in cells. Recent work indicates that the activity of aconitase (isocitrate synthase), which catalyzes conversion of citrate to isocitrate, is inhibited in the brains of *Atp7b*^{-/-} mice¹⁵.

Glycerol-3-phosphate levels were also decreased, as were glycerol-2-phosphate levels. SREBF1 regulates glycerol-3-phosphate synthesis, which also requires sorbitol, can be metabolized to make glycerol-3-phosphate⁴⁴. Increases in sorbitol, xylitol and xylose were unexpected, as consumption in the pentose phosphate pathway as a response to oxidative stress was expected.

The increase in sorbitol and other polyols indicates induction of the polyol pathway, which is observed in diabetes due to excess glucose. Fructose would be expected to accumulate as a result of sorbitol dehydrogenase activity, and we observe a trend toward increase (2-fold)

but not significant P-value (0.111). No transcript level change in sorbitol dehydrogenase or aldose reductase, key enzymes in the pathway were indicated in the transcriptome¹³.

Discussion

In this study, we have used the *Atp7b*^{-/-} mouse model of Wilson's Disease to gain insight into the molecular mechanisms of WD development. This work incorporates proteomics and metabolomics to build upon previous transcript and protein-level analysis. We identify key transcription factor targets in the hepatic nuclear proteome that are attenuated and directly link changes in cell physiology to reported changes in the transcriptome at an early stage of disease. We report an early response to DNA damage that is not apparent in other analyses and a selective change in liver metabolites including those involved in lipid metabolism, glutathione biosynthesis, and polyol metabolism.

We conclude that dysregulation of lipid and cholesterol metabolism observed in the liver of the *Atp7b*^{-/-} mouse can be explained in part by targeting of specific nuclear receptor transcription factors including FXR and GR in the nucleus and downstream responses. Although some indication of oxidative stress is indicated at all stages of disease in these animals¹⁵, no pathology or significant damage due to oxidative stress is indicated at early stages^{8, 13}. Although hepatocyte nuclei specifically retain Cu in these animals at early stages of disease^{8, 11, 14}, we conclude that hepatocytes respond through increasing abundance of DNA repair machinery and nuclear-localized GSH synthesis machinery.

Lipid metabolism and glucocorticoid levels may be targeted through Cu-induced destabilization of specific transcription factors including those in the ligand-activated nuclear receptor family. Nuclear receptors (NR) are characterized by a ligand-binding domain and two DNA-binding "zinc finger" domains, which each coordinate one Zn(II) through two CXXC motifs⁴⁵. Interestingly, the CXXC motif is characteristic of Cu(I) coordination in both Cu transporters and Cu chaperones⁴¹⁻⁴³.

It is possible that the high levels of reduced Cu in *Atp7b*^{-/-} hepatocytes will compete for the CXXC motif during or after protein folding and destabilizes specific NRs. Although it is not clear whether Cu can compete with Zn for incorporation in the DNA binding domain of all NRs, it has been shown that Cu effectively competes with Zn in binding to the apo-DNA binding domain of the estrogen receptor (ER), and this replacement inactivates DNA binding to the estrogen receptor element (ERE)⁴⁶. Although the oxidation state of Cu is not reported, the conditions (1 mM DTT and 10 μ M Cu) indicate that Cu(I) may be present. Further, electrospray ionization-mass spectrometry studies of CuCl₂ or CuSO₄ binding to a synthetic ER-DNA binding domain indicate two Cu atoms per 'zinc finger' that would necessarily disrupt tertiary structure and likely inhibit DNA binding⁴⁷.

Whether Cu specifically displaces Zn in the structure of GR and FXR is beyond the scope of this study, though it is tempting to speculate that this is a mechanism for NR attenuation in *Atp7b*^{-/-}. Cu-mediated destabilization of the HNF4 α NR has been reported in a Cu-treated hepatoma cell line, thus indicating Cu-responsive transcription targets of HNF4 α ³⁰. We identified HNF4 α in both wild type and *Atp7b*^{-/-} nuclei, though the levels were essentially the same (Supp. Table 1), suggesting the response may be different in liver tissue vs. a hepatoma cell line.

Low levels of GR in the nucleus may indicate a lower level of circulating corticosteroids, destabilization of GR or both, though our data indicate that circulating corticosterone is only mildly lower in *Atp7b*^{-/-} animals. However, suppression of GR signalling in the liver through destabilization of the protein or inhibition of DNA binding would indicate lower transcriptional levels of GR targets including cholesterol-7-hydroxylase (CYP7A1), which is

significantly downregulated at the transcript level in the liver of these animals¹³. Furthermore, reanalysis of the transcriptome of 6-week-old wild type and *Atp7b*^{-/-} livers by Gene Set Enrichment Analysis (GSEA) indicates that the PPAR signaling pathway is in the copper-downregulated gene set⁴⁸. Peroxisome proliferator-activated receptor (PPAR) is activated by GR in response to glucocorticoid signalling in the liver and this relationship is likely involved in suppression of inflammatory response⁴⁹. Thus, GR and other NR suppression may contribute to dysregulation of lipid metabolism as well as changes in inflammatory response, eventually contributing to a pathologic condition.

More broadly, suppression of FXR and GR may also inhibit biliary secretion, leading to toxic effects on liver cells and potentially influencing the development of colangiopathies⁵⁰. Interestingly, UDP-glucuronosyl transferase (UGT1A1), a key enzyme in bile acid synthesis was one of the non-nuclear proteins that were consistently identified in our analysis with less abundance in *Atp7b*^{-/-}, implying attenuation of bilirubin and steroid conjugation with UDP-glucuronate and attenuation of bile secretion across the canalicular membrane⁵¹. It was also reported that mutation of FXR, characterized as a bile acid receptor, in mouse predisposes the animals to hepatocellular carcinoma⁵².

The attenuation of key NR signalling provides mechanisms for changes in both lipid metabolism and cancer development in the *Atp7b*^{-/-} mouse model of WD. Our proteomics data also indicate an increase in the abundance of key DNA repair molecules XRCC1, LIG3, and PNKP, suggesting that DNA damage occurs early in disease. RNA processing proteins are also identified, consistent with 2D gel studies. KHSRP/FUBP2 protein regulates LDLR message stability and also associates with hnRNP A2 in oligodendrocytes, where hnRNP A2 critical in mRNA trafficking of myelin basic protein message⁵³. Curiously, we also found an increase in myelin expression factor 2 (MYEF2) in hepatocyte nuclei, providing another link between changes in liver and brain due to Cu accumulation.

Changes in metabolite levels including phosphoethanolamine and glycerol phosphates are consistent with altered lipid metabolism. Curiously, polyol sugars sorbitol and xylitol are at higher levels in the *Atp7b*^{-/-} mouse liver. Though the mechanism for this change is not clear, it may be linked a change in glucose metabolism use due to attenuated GR signalling (inhibition of gluconeogenesis and increase in glucose levels), whereby excess glucose may enter the polyol pathway and induce sorbitol accumulation as observed in diabetes⁵⁴⁻⁵⁶. It is possible that continued accumulation of sorbitol creates osmotic stress that leads to the ballooning hepatocytes observed later in disease⁸.

In utilizing a systems biology approach integrating observations of change in multiple types of biological molecules, we can begin to create a multifaceted mechanistic model for Cu toxicity in the mouse model of Wilson's disease. Our proteomics data focusing on the nucleus and metabolomics data analyzed in the context of transcriptome, metallome and previous proteomic data^{11, 13, 14, 48} indicates a copper-induced response to oxidative stress and DNA damage that is sufficient to forestall WD pathology at early stages of disease. Targeting of specific nuclear receptors mechanistically connects changes in lipid metabolism to specific transcriptional regulators and may influence other potentially hepatotoxic changes such as bile secretion and sorbitol metabolism. Accumulation of polyols may also influence osmotic potential and induce further hepatocyte damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Lawrence Gray and Abigael Muchen for assistance with animal handling and sample preparation. This work is supported by the National Institutes of Health P01 GM 067166 to SL and NIH 5P20RR016466-11 (7979) to JLB.

Bibliography

1. Lutsenko S, Barnes NL, Bartee MY, Dmitriev OY. *Physiol Rev.* 2007; 87:1011–1046. [PubMed: 17615395]
2. Lutsenko S, Gupta A, Burkhead JL, Zuzel V. *Arch Biochem Biophys.* 2008; 476:22–32. [PubMed: 18534184]
3. Bartee MY, Lutsenko S. *Biometals.* 2007
4. Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW. *Nat Genet.* 1993; 5:327–337. [PubMed: 8298639]
5. Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, Romano DM, Parano E, Pavone L, Brzustowicz LM, et al. *Nat Genet.* 1993; 5:344–350. [PubMed: 8298641]
6. Walshe JM. *Q J Med.* 1989; 70:253–263. [PubMed: 2602537]
7. Ferenci P, Czlonkowska A, Merle U, Ferenc S, Gromadzka G, Yurdaydin C, Vogel W, Bruha R, Schmidt HT, Stremmel W. *Gastroenterology.* 2007; 132:1294–1298. [PubMed: 17433323]
8. Huster D, Finegold MJ, Morgan CT, Burkhead JL, Nixon R, Vanderwerf SM, Gilliam CT, Lutsenko S. *Am J Pathol.* 2006; 168:423–434. [PubMed: 16436657]
9. Burkhead JL, Ralle M, Wilmarth P, David L, Lutsenko S. *Journal of Molecular Biology.* 2010 In press. 10.1016/j.jmb.2010.12.001
10. Klein D, Lichtmannegger J, Finckh M, Summer KH. *Arch Toxicol.* 2003; 77:568–575. [PubMed: 14574444]
11. Ralle M, Huster D, Vogt S, Schirrmeister W, Burkhead JL, Capps TR, Gray L, Lai B, Maryon E, Lutsenko S. *J Biol Chem.* 2010; 285:30875–30883. [PubMed: 20647314]
12. Burkhead JL, Gray LW, Lutsenko S. *Biometals.* 2011
13. Huster D, Purnat TD, Burkhead JL, Ralle M, Fiehn O, Stuckert F, Olson NE, Teupser D, Lutsenko S. *J Biol Chem.* 2007; 282:8343–8355. [PubMed: 17205981]
14. Burkhead JL, Ralle M, Wilmarth P, David L, Lutsenko S. *J Mol Biol.* 2011; 406:44–58. [PubMed: 21146535]
15. Sauer SW, Merle U, Opp S, Haas D, Hoffmann GF, Stremmel W, Okun JG. *Biochim Biophys Acta.* 2011; 1812:1607–1615. [PubMed: 21920437]
16. Buiakova OI, Xu J, Lutsenko S, Zeitlin S, Das K, Das S, Ross BM, Mekios C, Scheinberg IH, Gilliam TC. *Hum Mol Genet.* 1999; 8:1665–1671. [PubMed: 10441329]
17. Council, NR. Washington, DC: National academy of sciences. 1996.
18. Fei SS, Wilmarth PA, Hitzemann RJ, McWeeney SK, Belknap JK, David LL. *J Proteome Res.* 2011; 10:2905–2912. [PubMed: 21553863]
19. Wilmarth PA, Riviere MA, David LL. *J Ocul Biol Dis Infor.* 2009; 2:223–234. [PubMed: 20157357]
20. Liu H, Sadygov RG, Yates JRr. *Anal Chem.* 2004; 76:4193–4201. [PubMed: 15253663]
21. Zhang Y, Wen Z, Washburn MP, Florens L. *Anal Chem.* 2010; 82:2272–2281. [PubMed: 20166708]
22. Bolstad BM, Irizarry RA, Astrand M, Speed TP. *Bioinformatics.* 2003; 19:185–193. [PubMed: 12538238]
23. Ramos H, Shannon P, Brusniak MY, Kusebauch U, Moritz RL, Aebersold R. *Proteomics.* 2011; 11:154–158. [PubMed: 21182202]
24. Stayrook KR, Bramlett KS, Savkur RS, Ficorilli J, Cook T, Christie ME, Michael LF, Burris TP. *Endocrinology.* 2005; 146:984–991. [PubMed: 15564327]
25. Trauner M, Claudel T, Fickert P, Moustafa T, Wagner M. *Dig Dis.* 2010; 28:220–224. [PubMed: 20460915]

26. Watts LM, Mancham VP, Leedom TA, Rivard AL, McKay RA, Bao D, Neroladakis T, Monia BP, Bodenmiller DM, Cao JX, Zhang HY, Cox AL, Jacobs SJ, Michael MD, Sloop KW, Bhanot S. *Diabetes*. 2005; 54:1846–1853. [PubMed: 15919808]
27. Yang ZX, Shen W, Sun H. *Hepatology*. 2010; 4:741–748. [PubMed: 21286345]
28. Lin DY, Lai MZ, Ann DK, Shih HM. *J Biol Chem*. 2003; 278:15958–15965. [PubMed: 12595526]
29. Perez A, Kastner P, Sethi S, Lutz Y, Reibel C, Chambon P. *EMBO J*. 1993; 12:3171–3182. [PubMed: 8393784]
30. Song MO, Freedman JH. *Biochim Biophys Acta*. 2011; 1813:102–108. [PubMed: 20875833]
31. Macfarlane DP, Forbes S, Walker BR. *J Endocrinol*. 2008; 197:189–204. [PubMed: 18434349]
32. van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, Grobbee DE, de Jong FH, van Duyn CM, Pols HA, Lamberts SW. *Diabetes*. 2002; 51:3128–3134. [PubMed: 12351458]
33. Lu M, Mani RS, Karimi-Busheri F, Fanta M, Wang H, Litchfield DW, Weinfeld M. *Nucleic Acids Res*. 2010; 38:510–521. [PubMed: 19910369]
34. Moser J, Kool H, Giakzidis I, Caldecott K, Mullenders LH, Foustier MI. *Mol Cell*. 2007; 27:311–323. [PubMed: 17643379]
35. Weinfeld M, Mani RS, Abdou I, Aceytuno RD, Glover JN. *Trends Biochem Sci*. 2011; 36:262–271. [PubMed: 21353781]
36. Gherzi R, Lee KY, Briata P, Wegmuller D, Moroni C, Karin M, Chen CY. *Mol Cell*. 2004; 14:571–583. [PubMed: 15175153]
37. Min H, Turck CW, Nikolic JM, Black DL. *Genes Dev*. 1997; 11:1023–1036. [PubMed: 9136930]
38. Gustafson MP, Welcker M, Hwang HC, Clurman BE. *Biochem Biophys Res Commun*. 2005; 338:1359–1367. [PubMed: 16263084]
39. Lubas M, Christensen MS, Kristiansen MS, Domanski M, Falkenby LG, Lykke-Andersen S, Andersen JS, Dziembowski A, Jensen TH. *Mol Cell*. 2011; 43:624–637. [PubMed: 21855801]
40. Li H, Chen W, Zhou Y, Abidi P, Sharpe O, Robinson WH, Kraemer FB, Liu J. *J Lipid Res*. 2009; 50:820–831. [PubMed: 19141871]
41. Ralle M, Lutsenko S, Blackburn NJ. *J Biol Chem*. 2003; 278:23163–23170. [PubMed: 12686548]
42. Ralle M, Lutsenko S, Blackburn NJ. *J Inorg Biochem*. 2004; 98:765–774. [PubMed: 15134922]
43. Walker JM, Huster D, Ralle M, Morgan CT, Blackburn NJ, Lutsenko S. *J Biol Chem*. 2004; 279:15376–15384. [PubMed: 14754885]
44. Berry MN, Kun E, Werner HV. *Eur J Biochem*. 1973; 33:407–417. [PubMed: 4348396]
45. Laudet V. *J Mol Endocrinol*. 1997; 19:207–226. [PubMed: 9460643]
46. Predki PF, Sarkar B. *Environ Health Perspect*. 1994; 102(Suppl 3):195–198. [PubMed: 7843097]
47. Hutchens TW, Allen MH, Li CM, Yip TT. *FEBS Lett*. 1992; 309:170–174. [PubMed: 1505681]
48. He K, Chen Z, Ma Y, Pan Y. *Mammalian Genome*. 2011; 1–11. [PubMed: 21184083]
49. Chinetti G, Fruchart JC, Staels B. *Inflamm Res*. 2000; 49:497–505. [PubMed: 11089900]
50. Firrincieli D, Zuniga S, Poupon R, Housset C, Chignard N. *Dig Dis*. 2011; 29:52–57. [PubMed: 21691105]
51. Kadakol A, Ghosh SS, Sappal BS, Sharma G, Chowdhury JR, Chowdhury NR. *Hum Mutat*. 2000; 16:297–306. [PubMed: 11013440]
52. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. *Cancer Res*. 2007; 67:863–867. [PubMed: 17283114]
53. Snee M, Kidd GJ, Munro TP, Smith R. *J Cell Sci*. 2002; 115:4661–4669. [PubMed: 12415010]
54. Eriksson UJ, Naeser P, Brolin SE. *Diabetes*. 1986; 35:1356–1363. [PubMed: 3770312]
55. Gaynes BI, Watkins JBr. *Comp Biochem Physiol B*. 1989; 92:685–690. [PubMed: 2498032]
56. Poulsom R, Heath H. *Biochem Pharmacol*. 1983; 32:1495–1499. [PubMed: 6407486]

Fig. 1. A. Differential correlation plot of spectral counts for proteins identified in wild type and *Atp7b*^{-/-} liver nuclei by MuDPIT. B. Weighted distribution of cellular location gene ontologies for identified proteins.

Fig. 2. Heatmap and dendrogram based on normalized spectral counts for proteins with altered abundance in *Atp7b*^{-/-} compared to wild type liver nuclei.

Fig. 3. Highest scoring network indicating molecular relationships between changed proteins. The identified network has a highly significant score of 47. Genes or gene products are represented as nodes, and the connection between genes are supported by information in the Ingenuity Pathways Knowledge Base. Color indicates up- (red) or down- (green) regulation. Shapes of nodes represent functional classes of gene products (*rhomboid*, enzymes; *ovals*, transcription factors; *triangles*, kinases; *trapeziums*, transport and carrier proteins; and *circles*, others).

Fig. 4.

A. Spectral counts for FXRα. B. top: Densitometry quantitation of Western blot signal intensity for FXRα; bottom: representative Western blot and non-specific Coomassie bands.

Fig. 5.
A. Spectral counts for glucocorticoid receptor (GR). B. Serum corticosterone level mean, range and 95% CI.

Fig. 6. Highest scoring network indicating molecular relationships between changed proteins. The identified network has a score of 46. Genes or gene products are represented as nodes, and the connection between genes are supported by information in the Ingenuity Pathways Knowledge Base. Color and shape of nodes is defined in Fig. 3.

Fig. 7.

Average spectral counts for DNA repair proteins polynucleotide kinase-phosphatase (PNKP), DNA Ligase 3 (LIG3) and XRCC1 in wild type and *Atp7b*^{-/-} liver nuclei.

Table 1

Proteins with increased abundance.

Accession	Coverage (%)	MW (Da)	Description	Log ₂ Ratio	Z score	T-test
SELH MOUSE	37.1	12993	Selenoprotein H	3.61	-2.85	0.072
RRP1 MOUSE	11.3	54778	Ribosomal RNA processing protein 1 homolog A	2.61	-1.82	0.060
SRBD1 MOUSE	26.8	114093	S1 RNA-binding domain-containing protein 1	2.59	-2.49	0.039
GSTP1 MOUSE	20.5	23610	Glutathione S-transferase P 1	2.17	-2.60	0.030
MYEF2 MOUSE	37.9	63296	Myelin expression factor 2	2.16	-2.02	0.001
XRCC1 MOUSE	24.7	68972	DNA repair protein XRCC1	1.98	-1.91	0.001
CP4CA MOUSE	33.1	58332	Cytochrome P450 4A12A	1.69	-1.58	0.088
VRK1 MOUSE	35.9	49742	Serine/threonine-protein kinase VRK1	1.69	-1.74	0.001
DNL13 MOUSE	26	113019	DNA ligase 3	1.65	-1.89	0.0002
NOC4L MOUSE	21.3	58677	Nucleolar complex protein 4 homolog	1.58	-1.69	0.052
WDR74 MOUSE	15.9	42637	WD repeat-containing protein 74	1.57	-1.73	0.021
HMGB1 MOUSE	31.2	24895	High mobility group protein B1	1.45	-1.52	0.085
EPIPL MOUSE	15.4	723311	Epiplakin	1.40	-1.66	0.026
ABCF1 MOUSE	20.5	94946	ATP-binding cassette sub-family F member 1	1.30	-1.44	0.054
IMA2 MOUSE	25.9	57929	Importin subunit alpha-2	1.29	-1.87	0.042
ARI1A MOUSE	15.2	242093	AT-rich interactive domain-containing protein 1A	1.28	-1.16	0.043
UTP20 MOUSE	13.8	317745	Small subunit processome component 20 homolog	1.21	-2.01	0.010
PNKP MOUSE	47.5	57224	Bifunctional polynucleotide phosphatase/kinase	1.17	-1.45	0.008
RRP12 MOUSE	24	143132	RRP12-like protein	1.16	-1.62	0.074
RRP5 MOUSE	29.9	207780	Protein RRP5 homolog	0.80	-1.42	0.068
FBRL MOUSE	45.9	34308	rRNA 2'-O-methyltransferase fibrillar	0.73	-1.18	0.003
SFI3A MOUSE	26	31302	Splicing factor, arginine/serine-rich 13A	0.73	-1.44	0.069
NPM MOUSE	47.9	32561	Nucleophosmin	0.72	-1.47	0.00001

Table 2

Proteins with decreased abundance.

Accession	Coverage (%)	MW (Da)	Description	Log ₂ Ratio	Z score	T-test
FM03 MOUSE	36.3	60517	Dimethylalanine monooxygenase [N-oxide-forming] 3	-7.54	4.30	0.117
DBP MOUSE	19.7	34381	D site-binding protein	-4.43	4.17	0.044
GCR MOUSE	23.8	86054	Glucocorticoid receptor	-4.29	4.24	0.032
FGFR2 MOUSE	1.9	91985	Fibroblast growth factor receptor 2	-3.63	3.48	0.257
AL3A2 MOUSE	19.4	53944	Fatty aldehyde dehydrogenase	-3.01	4.12	0.001
CML01 MOUSE	8.1	25015	Probable N-acetyltransferase CML1	-2.79	4.18	0.041
NR1H4 MOUSE	21.9	56037	Bile acid receptor	-2.40	2.86	0.030
UD11 MOUSE	27.7	60124	UDP-glucuronosyl transferase 1-1	-2.35	2.78	0.022
DHE3 MOUSE	31.4	61338	Glutamate dehydrogenase 1, mitochondrial	-2.32	3.60	0.007
RFC4 MOUSE	21.2	39868	Replication factor C subunit 4	-2.23	3.59	0.0002
ZCHC8 MOUSE	29.2	78027	Zinc finger CCHC domain-containing protein 8	-1.95	1.89	0.008
NCPR MOUSE	19.6	77045	NADPH--cytochrome P450 reductase	-1.79	1.59	0.001
BHMT1 MOUSE	28.3	45022	Betaine--homocysteine S-methyltransferase 1	-1.70	2.50	0.060
PML MOUSE	27.8	98243	Probable transcription factor PML	-1.66	2.06	0.077
RS3 MOUSE	29.2	26675	40S ribosomal protein S3	-1.48	3.21	0.068
DH11 MOUSE	20.9	32365	Corticosteroid 11 -beta-dehydrogenase isozyme 1	-1.06	2.54	0.069
NF1B MOUSE	38.6	63508	Nuclear factor 1 B-type	-0.81	0.90	0.013
FUBP2 MOUSE	49.2	76811	Far upstream element-binding protein 2	-0.74	1.49	0.032
TIF1B MOUSE	48	88848	Transcription intermediary factor 1 -beta	-0.66	1.15	0.001

Table 3

Metabolites with altered abundance.

	Fold change	P- value
glycerol-3-phosphate	−1.91	0.032
citrate	−1.89	0.006
phosphoethanolamine	−1.86	0.008
glycerol -2 -phosphate	−1.67	0.004
glucose-6-phosphate (minor)	−1.59	0.001
xylitol	1.99	0.030
sorbitol	2.23	0.006
xylose	2.73	0.039
isoleucine	3.58	0.002
serine	3.87	0.002