DOCTORAL THESIS

Copper Metabolism in Health and Disease: Focus on Copper in Adipogenesis and α-Lipoic Acid in Wilson Disease

Ekaterina Kabin

TALLINNA TEHNIKAÜLIKOOL
TALLINN UNIVERSITY OF TECHNOLOGY
TALLINN 2023

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69/2023

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TAL TECH PRESS

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This dissertation was accepted for the defence of the degree of Doctor of Philosophy in Chemistry and Biotechnology on 06/11/2023

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Defence of the thesis: 15/12/2023, Tallinn

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Ekaterina Kabin

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ISSN 2585-6898 (publication)
ISBN 978-9916-80-083-6 (publication)
ISSN 2585-6901 (PDF)
ISBN 978-9916-80-084-3 (PDF)
Printed by EVG Print

TALLINNA TEHNIKAÜLIKOOL
DOKTORITÖÖ
69/2023

Vase ainevahetus tervise ja haiguse tingimustes: fookus vasele adipogeneesil ja α-lipoehappele Wilsoni tõve korral

EKATERINA KABIN

TAL TECH
KIRJASTUS

Dedicated to my PhD

I'm juggling words, a grand finesse,
Seven meters above, in the air I impress,
On the tightrope, 'midst the beasts' duress,
Embers glowing, my skills, they confess...

And the onlookers in silent awe,
Marvel at the sight they saw,
"What lies ahead, what more, what flaw?
This young lass's wit, it leaves us in awe!"

Like a blade, the questions dance,
One by one, in an eager trance,
"You're not at our level, this isn't chance,
But your performance, it's a vibrant expanse!"

Applause and cheers now resound,
I stand tall, on solid ground,
We, once students, now renowned,
Today, as doctors, our achievements unbound!

We'll keep juggling, our hearts set aglow,
For science beckons, its wonders to show,
To fathom life's secrets, let's continue to grow,
That's the passion within us, the vibrant, eternal glow!

Ekaterina Kabin, 2023

Contents

List of Publications

The list of author's publications, on the basis of which the thesis has been prepared:

I Smirnova J., **Kabin E**., Järving I., Bragina O., Tõugu V., Plitz T., Palumaa P.
Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent.
Sci Rep, 8(1):1463. doi: 10.1038/s41598-018-19873-2.

II **Kabin E.**, Dong Y., Roy S., Smirnova J., Smith J. W., Ralle M., Kelly Summers K., Yang H., Dev S., Wang Y., Devenney B., Cole R. N., Palumaa P., Lutsenko S.
α-lipoic acid ameliorates consequences of copper overload by up-regulating selenoproteins and decreasing redox misbalance.
Proc Natl Acad Sci, 120 (40), #e2305961120. DOI: 10.1073/pnas.2305961120.

III Yang H., **Kabin E.**, Dong Y., Zhang X., Ralle M., Lutsenko S.
Atp7a-dependent copper sequestration contributes to termination of β-catenin signalling during early adipogenesis. Manuscript.

Author's Contribution to the Publications

Contribution to the papers in this thesis are:

I The author contributed to the planning of experiments, performed experimental work, analysed the data and contributed in manuscript preparation.

II The author contributed in conceptualization of the study, planned and performed experiments, analysed the data, prepared and edited the manuscript.

III The author contributed to the planning of the experiments, performed experimental work, analysed the data and contributed in manuscript preparation and editing.

Introduction

Copper plays a crucial role as a protein cofactor, contributing significantly to essential biological processes like cellular respiration, redox signaling, and the scavenging of reactive oxygen species, among others. Studies of copper homeostasis have broadened our understanding of its functions. More and more new players involved in interaction with copper are emerging, filling gaps in our knowledge. It is now evident that copper holds particular interest, as its correct balance is critical in health and disease, including cancer, obesity, cardiovascular and neurodegenerative disorders. Despite extensive studies, many processes regulated by copper are still not fully understood, including cellular signalling, differentiation, and lipid metabolism, which are among the crucial topics.

Disturbances in copper homeostasis, particularly copper overload, can result in a range of harmful effects. Wilson disease (WD) illustrates a disruption in copper metabolism due to mutations in the gene responsible for encoding the copper-dependent ATPase, ATP7B. This condition manifests through the accumulation of copper in the body, primarily within the liver and brain. Wilson disease can be treated by Cu(I)-chelating therapy. Several chelating agents that bind copper and help to excrete it are in use nowadays, however, WD is in search of new treatment options.

In this thesis, we estimated dissociation constants of Cu(I)-binding agents currently used in WD therapy as well as several other copper-chelating agents, using an ESI-MS based approach previously established in our laboratory. Furthermore, we studied the potential WD-therapeutic properties of α-lipoic acid (LA), which has relatively high Cu(I)-binding affinity. In addition, we studied the effect of excess copper on differentiation of adipose cell line. Obtained results show the link between LA action and selenium metabolism and are of biomedical importance.

Abbreviations

AOC3	Amino oxidase copper-containing 3 (also known as SSAO)
ATOX1	Copper chaperone for P_{1B}-type ATPases
ATP7A	Cu(I) P_{1B}-type ATPase defective in Menkes disease
ATP7B	Cu(I) P_{1B}-type ATPase defective in Wilson disease
BAL	British anti-Lewisite (dimercaptopropanol)
BCS	Bathocuproine disulfonate
CCO	Cytochrome C oxidase
CCS	Copper chaperone for SOD1
COX17	Copper chaperone for cytochrome C oxidase
CTR1	High affinity copper uptake protein 1
DETC	Diethyl-dithiocarbamate
DHLA	Dihydrolipoic acid, reduced form of α-lipoic acid
DMS	Dimercaptosuccinate
DTT	Dithiothreitol
ESI-MS	Electrospray ionization mass spectrometry
GSH	Reduced glutathione
GSSG	Oxidized glutathione
IMS	Intermembrane space
LA	α-lipoic acid
MBD	Metal-binding domain
MD	Menkes disease
MT	Metallothionein
NAFLD	Non-alcoholic fatty liver disease
PA	D-penicillamine
PPARα	Peroxisome proliferator-activated receptor α
ROS	Reactive oxygen species
SOD1	Cu,Zn-superoxide dismutase
SSAO	Semicarbazide-sensitive amine oxidase (also known as AOC3)
TG	Triglyceride
TGN	*trans*-Golgi network
TR	Trientine
TTM	Tetrathiomolybdate
WD	Wilson disease

1 Literature Overview

1.1 Copper as an essential biometal

1.1.1 Copper metabolism

Copper is an essential biometal, which plays a role of a cofactor for numerous enzymes. Cu-dependent enzymes participate in cell respiration, scavenging of reactive oxygen species (ROS), cell signaling, differentiation, angiogenesis, lipid metabolism, synthesis of neuromediators and even cell death (the process is known as cuproptosis) (Boal and Rosenzweig 2009, Kaplan and Lutsenko 2009, Argüello, Raimunda et al. 2013, Yang, Ralle et al. 2018, Tsang, Davis et al. 2021, Tsvetkov, Coy et al. 2022).

Human organism contains approximately 75–100 mg of copper and average daily copper intake from the diet is 1–1.5 mg (Burkhead and Collins 2022, Chen, Song et al. 2023). Copper is absorbed in the small intestine, mostly in the duodenum and jejunum, by enterocytes (Kaplan and Lutsenko 2009, Hordyjewska, Popiołek et al. 2014, Lutsenko 2021). In the extracellular space, it exists in the form of Cu(II) ions and before the uptake into cell Cu can be reduced by a metalloreductase of the STEAP family (Ohgami, Campagna et al. 2006, Tsang, Davis et al. 2021). Cu enters cells in the form of Cu(I) ions mainly through a trimeric CTR1 transporter, which does not require ATP energy for Cu transport (De Feo, Aller et al. 2009, Kaplan and Maryon 2016). Alternatively, CTR2 and divalent metal transporter, DMT1, can transport Cu into the cell (Prohaska 2008, Kaplan and Lutsenko 2009, Kaplan and Maryon 2016, Lutsenko 2021). The intracellular environment is reducing, and within the cell Cu is present in Cu(I) form, which is highly redox-active (Iakovidis, Delimaris et al. 2011, Hordyjewska, Popiołek et al. 2014, Kaplan and Maryon 2016, Lutsenko 2021, Tsang, Davis et al. 2021). Cu ions may react with hydrogen peroxide and initiate ROS formation via Fenton reactions (equation 1 and 2). Therefore, intracellular transport and handling of Cu ions (Figure 1) needs to be strictly regulated to avoid oxidative stress (Iakovidis, Delimaris et al. 2011, Hordyjewska, Popiołek et al. 2014).

$$LCu(II) + H_2O_2 \rightarrow LCu(I) + {}^{\bullet}OOH + H^+ \qquad \text{(eq 1)}$$
$$LCu(I) + H_2O_2 \rightarrow LCu(II) + {}^{\bullet}OH + OH^- \qquad \text{(eq 2)}$$

Glutathione (GSH) is a major ligand, participating in formation of an exchangeable Cu pool (Maryon, Molloy et al. 2013, Kaplan and Maryon 2016). GSH readily binds Cu(I) as soon as it enters the cell, and exchanges it with other proteins which have higher Cu(I)-binding affinity (Banci, Bertini et al. 2010, Maryon, Molloy et al. 2013, Lutsenko 2021). Special small proteins called copper chaperones bring Cu(I) ions to the target proteins (Kaplan and Lutsenko 2009, Palumaa 2013). Copper chaperones have a higher affinity to Cu(I) ions compared to GSH but lower than their target cuproenzymes, which favors fast and effective Cu(I) delivery without causing harmful side reactions within the cell (Banci, Bertini et al. 2010). Copper chaperone for superoxide dismutase (CCS) brings Cu(I) to Zn,Cu-superoxide dismutase (SOD1) (Culotta, Klomp et al. 1997) (Figure 1), COX17 and SCO1 are copper chaperones for mitochondrial cytochrome c oxidase (CCO) (Glerum, Shtanko et al. 1996, Beers, Moira Glerum et al. 2002, Banci, Bertini et al. 2008), and antioxidant protein 1 (ATOX1) traffics Cu(I) to copper-ATPases, located in the membranes of the Golgi complex (Klomp, Lin et al. 1997, Hamza, Schaefer et al. 1999). ATOX1 is also present in the cell nucleus and functions there as a Cu-dependent transcription regulator

contributing to the cell cycle regulation (Itoh, Kim et al. 2008, Öhrvik and Wittung-Stafshede 2015, Matson Dzebo, Blockhuys et al. 2018).

Metallothioneins (MT-s) also contribute to the formation of the exchangeable cellular Cu pool (Coyle, Philcox et al. 2002). They have a strong affinity to Cu(I) ions and can play the role of cellular Cu depot (Freedman and Peisach 1989, Banci, Bertini et al. 2010). The synthesis of MT-s is increased in response to elevated Cu, while in low Cu conditions Cu-MT-s release Cu(I) ions to other proteins, such as copper chaperones or SOD1 (Freedman and Peisach 1989, Liu, Fabisiak et al. 2000, Coyle, Philcox et al. 2002).

Cu ATPases, ATP7A and ATP7B, which reside in the membranes of the trans-Golgi network (TGN) (Figure 1, (Camakaris, Voskoboinik et al. 1999, Lutsenko, Barnes et al. 2007, Lutsenko 2021), regulate cellular Cu content in several ways. ATP7A and ATP7B mediate the metallation of numerous cuproenzymes which are secreted from the cell by the vesicular transport (Lutsenko 2021). Additionally, in elevated Cu conditions, they can pump Cu(I) ions into vesicles for storage or distribution within the cell (Pierson, Muchenditsi et al. 2018, Lutsenko 2021). Finally, ATP7A and ATP7B can be localized in cellular membrane and excrete Cu from the cell: intestinal ATP7A releases dietary Cu into the bloodstream where it is bound by extracellular Cu(II) carriers like albumin, liver ATP7B facilitates excretion of excess Cu into bile, which leaves the organism with feces (Lutsenko, Gupta et al. 2008, Gupta and Lutsenko 2009, Hordyjewska, Popiołek et al. 2014). In addition to biliary Cu excretion, a small amount of copper is excreted also with urine and saliva (Hordyjewska, Popiołek et al. 2014).

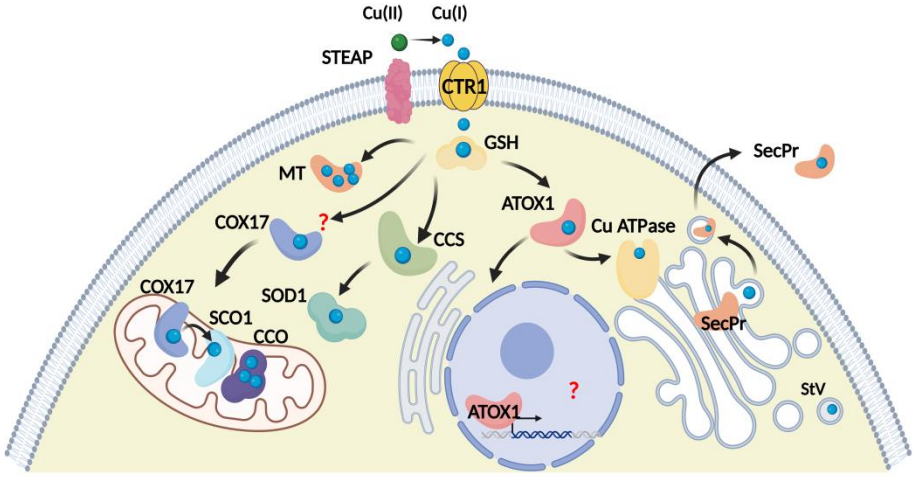

***Figure 1. A simplified representation of the intracellular Cu trafficking pathways**. Green sphere – Cu(II) ion; blue spheres – Cu(I) ions. STEAP – metalloreductase; Ctr1 – copper influx transporter; GSH – glutathione; MT – metallothioneins; Cox17 and Sco1 – copper chaperones for cytochrome C oxidase (CCO); CCS – copper chaperone for Cu,Zn-superoxide dismutase (SOD1); ATOX1 – copper chaperone for ATP7A and ATP7B Cu ATPases; SecPr – secretory protein; StV – storage vesicle. The figure was created with BioRender.*

The general mechanism described above is a current consensus; however, there are many details and more players that are involved in all steps of the cellular Cu handling.

1.1.2 Copper proteome

The copper proteome, also called cuproproteome (Robinson and Winge 2010), includes a wide range of proteins participating in various pathways of Cu metabolism in the extra- and intracellular locations. A recent study has stated that 54 proteins comprise the copper proteome in human cells (Blockhuys, Celauro et al. 2017). However, further studies of Cu-dependent processes, most of which are connected to various pathological conditions, can expand the list of Cu-binding proteins or their potential partners (Lutsenko 2021). Cys residues in proteins play an essential role in the binding of bioactive Cu(I) ions, which exist in the intracellular redox conditions. Some of the important intracellular copper proteins, copper chaperone COX17, MT-s, and Cu-ATPases ATP7A and ATP7B (Figure 1), are discussed below.

1.1.2.1 COX17

Copper chaperone COX17 is a member of the Cu-delivery system for CCO (Glerum, Shtanko et al. 1996, Beers, Moira Glerum et al. 2002, Banci, Bertini et al. 2008). Initially, Cox17 was identified and characterized in *Saccharomyces cerevisiae* (Glerum, Shtanko et al. 1996), and later it was found to be functionally highly conserved from yeasts to mammals (Takahashi, Kako et al. 2002). Human COX17 is composed of 63 amino acids, and its molecular mass is approximately 6.9 kDa (Robinson and Winge 2010). COX17 contains two conserved Cys-X_9-Cys motifs, which form two helical hairpins (Banci, Bertini et al. 2008). COX17 has 6 Cys residues which are all conserved, despite low sequence similarity (34%), between human and yeast Cox17 (Robinson and Winge 2010, Gladyck, Aras et al. 2021). Under physiological conditions, 2 of them (Cys 23 and Cys 24) are participating in Cu(I) binding (Figure 2). The rest of Cys form structurally important disulphide bridges: Cys36-Cys45 and Cys26-Cys55 (Arnesano, Balatri et al. 2005, Banci, Bertini et al. 2008, Banci, Bertini et al. 2011). Functional studies have demonstrated that when all Cys are oxidized and form disulphide bonds, Cu(I) coordination is not possible (Palumaa, Kangur et al. 2004, Banci, Bertini et al. 2008). However, when all Cys are reduced, COX17 can bind cooperatively 4 Cu(I) ions (Palumaa, Kangur et al. 2004, Arnesano, Balatri et al. 2005).

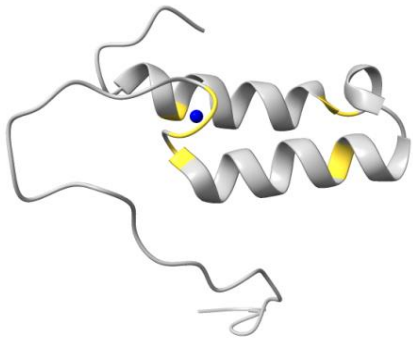

Figure 2. A crystal structure of human COX17*. PDB code: 2RNB. Cu is shown as a blue sphere, Cys residues are presented in yellow. The figure was generated with Chimera.*

COX17 is a substrate for Mia40 protein, which has oxidoreductase activity and participates in the formation of two disulfides in COX17, hence granting proper folding of COX17 (Banci, Bertini et al. 2010, Banci, Bertini et al. 2011).

COX17 was found in both the cytosol and mitochondria (Oswald, Krause-Buchholz et al. 2009). Therefore, it was thought to bind Cu(I) in the cytosol and transport it to the mitochondrial intermembrane space (IMS). However, later NMR studies showed that the functional protein resides in the IMS, leaving the true mechanism elusive. (Beers, Glerum et al. 1997, Banci, Bertini et al. 2008). Holo-form of COX17 transfers Cu(I) ions via protein-protein interactions to the Cu chaperones Sco1, Sco2 and Cox11 within the IMS. In turn, Sco1/Sco2 donate Cu(I) ions to Cu_A site of CCO, and Cox11 donates Cu(I) to the Cu_B site of CCO (Cobine, Pierrel et al. 2006, Banci, Bertini et al. 2010, Robinson and Winge 2010).

The absence of COX17 has different effects in different organisms. COX17 deficient yeast cannot propagate on nonfermentable food sources and have a respiratory deficiency; however, this phenotype can be rescued with the addition of exogenous copper salts (Glerum, Shtanko et al. 1996). *In vitro* experiments demonstrate that COX17 is essential for the formation and activation of CCO, as well as the organization of mitochondrial membranes (Oswald, Krause-Buchholz et al. 2009, Vanišová, Burská et al. 2019). The functioning of COX17 is even more critical for mammals. Mice lacking COX17 die in early prenatal age, which highlights the importance of COX17 and suggests that it plays a significant role in organismal development (Takahashi, Kako et al. 2002).

1.1.2.2 Metallothioneins

MT-s are a superfamily of small metal-binding proteins (approximately 7 kDa) which are highly conserved within living organisms (Klaassen, Liu et al. 1999, Coyle, Philcox et al. 2002, Vašák and Meloni 2011, Calvo, Jung et al. 2017). MT was initially isolated from the cortex of a horse kidney in 1957 (Margoshes and Vallee 1957) and got its name due to its high sulphur and metal content (Kägi, Vallee et al. 1961). MT-s are Cys-rich proteins, lacking aromatic amino acid residues, and His as well (Kissling and Kägi 1979, Kojima, Berger et al. 1979, Coyle, Philcox et al. 2002).

There are four classes of MT-s, MT-1 to MT-4, respectively. In humans, the family of MT-s encoding genes is located in 16q13 chromosome (Vašák and Meloni 2011). Among them, at least 10 are encoding functional isoforms of MT-1 while MT-2 to MT-4 are present with only one isoform (Karin, Eddy et al. 1984, Sadhu and Gedamu 1988, Stennard, Holloway et al. 1994, Coyle, Philcox et al. 2002). MT-1 and MT-2 are widely expressed in all human tissues and are most abundant in the liver, kidney, intestine and pancreas. MT-3 is specifically expressed in the nervous system, and MT-4 is expressed in epidermal cells (Bremner and Beattie 1990, Coyle, Philcox et al. 2002, Thirumoorthy, Shyam Sunder et al. 2011). Mice express one member of each family and yeast have only two MT-s (Cup1 and Crs5) (Culotta, Howard et al. 1994, Jensen, Howard et al. 1996, Miles, Hawksworth et al. 2000). Moreover, several bacterial MT-s are known: SmtA in cyanobacterium *Synechococcus elongates* (Olafson, Abel et al. 1979), MymT in pathogen *Mycobacterium tuberculosis* (Gold, Deng et al. 2008), and PsdMTs which was found in *Pseudomonas* group (Winsor, Griffiths et al. 2016, Habjanič, Mathew et al. 2020). Additionally, studies of cadmium detoxification by various bacteria including *Bacillus* genera may indicate that more bacterial MT-s could exist (Arce-Inga, González-Pérez et al. 2022).

Mammalian MT-s are composed of 61-68 amino acid residues including 20 highly conserved Cys residues, which are located in the β (N-terminal) and α (C-terminal) domains (Kojima, Berger et al. 1976, Kissling and Kägi 1979, Kojima, Berger et al. 1979). Cys are forming Cys-Cys, Cys-X-Cys or Cys-X-Y-Cys motifs where X and Y are non-Cys amino acid residues (Kojima, Berger et al. 1976, Robbins, McRee et al. 1991, Merrifield,

Huang et al. 2002). α-domain binds four and β-domain three divalent metal ions (e.g. Zn(II) or Cd (II)) with tetrahedral coordination geometry (Figure 3), whereas in case of monovalent ions like Cu(I) 4 to 6 metal ions can occupy each cluster with trigonal or digonal coordination (Merrifield, Huang et al. 2002). Yeast MT has a single cluster which can be filled with 8 Cu(I) ions (Figure 4) (Calderone, Dolderer et al. 2005). MT-s can bind different metal ions at the same time and fill domains with two different metal cations (e.g. rat Cd_5,Zn_2-MT-2, Figure 3). Also, forms containing a mix of metal ions are usually extracted from organ tissues: Cu and Zn containing MT-1 and MT-2 are isolated from the human brain, $Cu(I)_4Zn_{3-4}$MT-3 is isolated from human, bovine or equine brain, and mixed Cu and Zn form of MT-4 is isolated from tongue epithelium (Uchida, Takio et al. 1991, Quaife, Findley et al. 1994, Calvo, Jung et al. 2017).

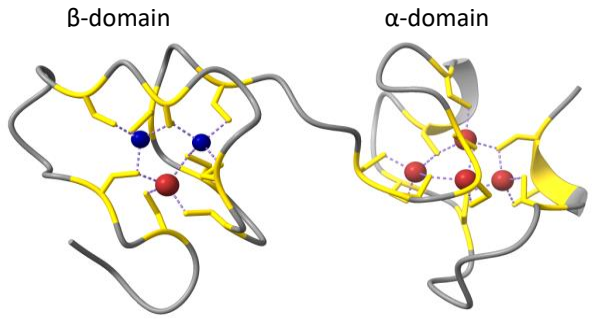

Figure 3. A crystal structure of the rat Cd_5,Zn_2-MT-2. *PDB code: 4MT2. Two Zn(II) and one Cd(II) are located in the N-terminal ß-domain, and the other four Cd(II) are in the C-terminal α-domain. Zn ions labelled with blue, Cd ions labelled with red, Cys residues are presented in yellow. The figure was generated with Chimera.*

MT-s have many different functions in the organism, which vary between the isoforms. For instance, MT-3 has neuronal growth inhibitory function, which is absent for MT-1 and MT-2 (Uchida, Takio et al. 1991). MT-s are involved in maintaining homeostasis and transport of essential biometals like Zn and Cu (Seagrave, Hanners et al. 1986, Quaife, Findley et al. 1994, Jacob, Maret et al. 1998, Suzuki, Someya et al. 2002, Banci, Bertini et al. 2010), detoxification of heavy metals (such as cadmium, led and mercury) (Piotrowski, Trojanowska et al. 1974, Kägi and Kojima 1987, Morcillo and Santamaría 1993, Jacob, Maret et al. 1998, Chen, Ma et al. 2014), protection against oxidative stress and control of cellular redox state (Andrews 2000, Uchida, Gomi et al. 2002, Meloni, Faller et al. 2007), anti-inflammatory response (Coyle, Philcox et al. 2002, Inoue, Takano et al. 2009), and cell cycle (Uchida, Takio et al. 1991). In addition, MT-s help to buffer excess Cu in conditions of Cu overload forming redox silent complexes and protecting cells against Cu toxicity (Bremner 1987, Krężel and Maret 2017, Krężel and Maret 2021).

Expression of MT-s is regulated in numerous ways. MT-s synthesis can be induced in response to cytosolic fluctuation of metals, antioxidants, inflammatory cytokines, ROS, and glucocorticoids (Piotrowski, Trojanowska et al. 1974, Durnam, Hoffman et al. 1984, De, McMaster et al. 1990, Andrews 2000, Zhang, Georgiev et al. 2003). Cellular content of MT-s is known to be elevated both in low and high Cu conditions including Wilson and Menkes disease pathologies (Leone, Pavlakis et al. 1985, Ogra, Aoyama et al. 2006) (Mulder, Janssens et al. 1992, Kelly and Palmiter 1996, Huster, Purnat et al. 2007, Bhattacharjee, Yang et al. 2016, Muchenditsi, Talbot et al. 2021, Rowan, Mangalaparthi et al. 2022).

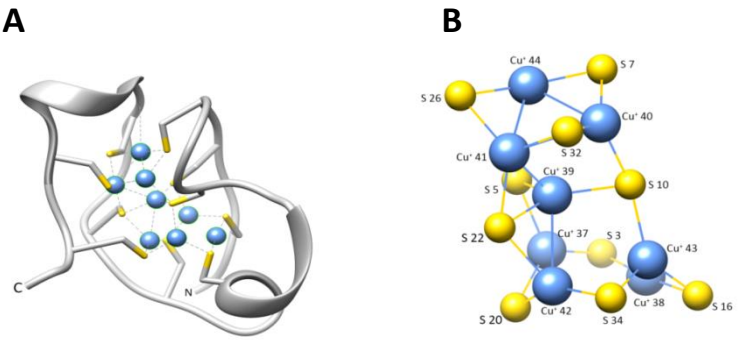

Figure 4. Cu(I) ions coordination in Cu(I)-loaded MT. *A) A crystal structure of S. cerevisiae $Cu(I)_8$-MT, PDB code: 1RJU, and B) the Cu(I)-binding geometry by Cys residues. Light-blue spheres represent Cu ions, sulphur atoms are presented in yellow. The figure was generated with Chimera.*

1.1.2.3 Copper-transporting P-type ATPases ATP7A and ATP7B

Copper-transporting P_{1B}-type ATPases, ATP7A and ATP7B, are highly homologous proteins encoded in humans by X and 13 chromosomes, respectively (Frydman 1990, Chelly, Tümer et al. 1993, Vulpe, Levinson et al. 1993). They are large transmembrane proteins (1500 amino acid residues in ATP7A and 1465 in ATP7B), which reside normally in membranes of Golgi network (Figure 1, (Lutsenko, Barnes et al. 2007)). Both ATP-ases have a long N-terminal cytosolic part with 6 metal-binding domains (MBD-s), 8 transmembrane (TM) helixes which form a pathway allowing Cu(I) transport into the Golgi lumen, and a cytosolic ATP-binding domain (Figure 5, (Lutsenko, Gupta et al. 2008, Banci, Bertini et al. 2010)). MBD-s receive Cu(I) ions from ATOX1, as they have higher affinity for Cu(I) ions (Banci, Bertini et al. 2010). ATP-binding domain has DKTG (DKTGT in case of ATP7B) motif (Figure 5) conserved in the entire family of P-type ATPases (Lutsenko, Gupta et al. 2008, Banci, Bertini et al. 2010). Autophosphorylation of Asp in ATP-binding domain facilitates series of conformational changes, which result in Cu(I) transport through the TM domain into the TGN lumen. After Cu translocation, the initial conformation of the transporter is restored (Lutsenko, Gupta et al. 2008, Banci, Bertini et al. 2010).

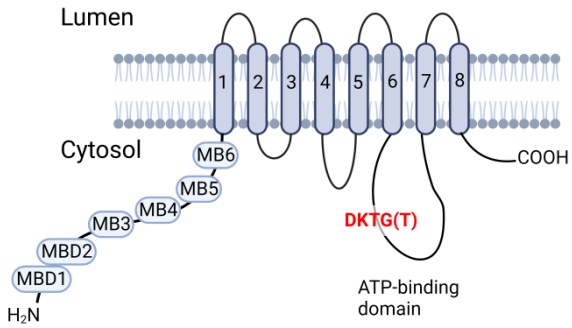

Figure 5. A schematic representation of the copper ATP-ase structure. *Numbers 1-8 represent each transmembrane helix. MBD1-6 – metal binding domains 1 to 6. DKTG(T) sequence – conserved ATP-binding motif in ATP-binding domain. The figure was created with BioRender.*

Expression pattern of ATP7A and ATP7B is different: ATP7A is abundant in most organs except for the liver, where ATP7B is the major Cu-ATPase (Lutsenko, Barnes et al. 2007, Horn, Møller et al. 2019). Within the secretory pathway, ATP7A and ATP7B mediate metallation of cuproenzymes such as peptidyl-α-monooxygenase (PAM), (El Meskini, Culotta et al. 2003), tyrosinase (Petris, Strausak et al. 2000), dopamine-β-hydroxylase (DBH), (Xiao, Ackerman et al. 2018), amino oxidase 3 (AOC3) also known as semicarbazide-sensitive amine oxidase (SSAO) (Yang, Ralle et al. 2018), extracellular superoxide dismutase 3 (Qin, Itoh et al. 2006) and lysyl-oxidase (LOX) (Shanbhag, Jasmer-McDonald et al. 2019) in case of ATP7A, and ceruloplasmin in case of ATP7B (Hellman, Kono et al. 2002).

Expression and subcellular localization of both ATP-ases depend on the cellular Cu level. Under basal Cu conditions, both proteins reside mostly in the TGN, under low Cu conditions they reside in TGN exclusively, and elevation of Cu in cytosol causes relocation of both ATP-ases to vesicular compartment (Petris, Mercer et al. 1996, Hung, Suzuki et al. 1997, Yang, Miura N Fau - Kawarada et al. 1997, Schaefer, Hopkins et al. 1999, Lutsenko, Gupta et al. 2008). The abundance of ATP7B in rat liver is highest before the onset of biliary Cu excretion (early prenatal age) and decreases after the birth, agreeing with cellular Cu content (Schaefer, Hopkins et al. 1999).

1.1.3 Copper-dependent pathogenesis and processes

1.1.3.1 Menkes disease

Loss-of-function mutations in either ATP7A or ATP7B lead to deleterious effects on Cu metabolism. Menkes disease (MD), also known as kinky hair syndrome, is caused by dysfunction of ATP7A. MD is characterized by accumulation of copper in enterocytes and kidneys, but copper deficiency in the brain and peripheral organs, which causes neurological retardation, decreased myelination, slow growth, anemia, arterial abnormalities, rigid muscles, hypopigmentation and other symptoms (Kodama, Murata et al. 1999, Shim and Harris 2003, Uriu-Adams and Keen 2005, Tümer and Møller 2010, Fujisawa, Kodama et al. 2022). When cultured, fibroblasts of MD patients accumulate Cu due to lack of Cu efflux ability (Kodama, Murata et al. 1999, Bhattacharjee, Yang et al. 2016). MD patients receive Cu supplementation intravenously in a form of Cu(II)-His

complex to promote Cu delivery to tissues (Sarkar, Lingertat-Walsh et al. 1993, Christodoulou, Danks et al. 1998). Other agents (e.g. disulfiram and dimercaptosuccinate) act as copper ionophores (Horn, Møller et al. 2019, Oliveri 2020). The recent study with Cu-ionophore elesclomol showed promising therapeutic results in the mouse model of MD (Guthrie, Soma et al. 2020). Most untreated MD patients die in early childhood, on average by the age of 3, and the therapy has to be started as early as possible (Kodama, Murata et al. 1999, Fujisawa, Kodama et al. 2022).

1.1.3.2 Wilson disease

a) Pathophysiology of Wilson disease

Wilson disease was first described in late 1880-s and later, in the beginning of 1900-s, named after a physician Samuel Alexander Kinnier Wilson (Tanner 2019). WD is a rare disorder with an occurrence of one out of 30-50 thousand births (Sandahl, Laursen et al. 2020). WD is an autosomal recessive disorder, which is caused by loss-of-function mutations in the ATP7B encoding gene (Frydman 1990). The main organ expressing ATP7B is the liver, but this protein is also abundant in the brain, kidney, placenta, mammary gland, and lungs (Lutsenko, Barnes et al. 2007, Lutsenko, Gupta et al. 2008, Horn, Møller et al. 2019). Clinically, symptoms of WD vary depending on the type of mutation and its location within the protein structure, as well as other factors such as genetic background and even diet (Schiefermeier, Kollegger et al. 2000, Einer, Leitzinger et al. 2019, Wooton-Kee, Robertson et al. 2020, Muchenditsi, Talbot et al. 2021, Dev, Kruse et al. 2022, Gottlieb, Dev et al. 2022). Despite large variability in the phenotypic manifestations of WD, the patients most commonly suffer from hepatic and neurological problems, which are caused by the accumulation of Cu in the liver and brain (Das and Ray 2006, Litwin, Gromadzka et al. 2013). Accumulation of excess Cu in the liver causes liver inflammation, morphological changes, and cirrhosis. In turn, copper overload in the CNS causes neurodegeneration in lenticular nuclei, cortex, thalamus and cerebellum (Das and Ray 2006, Litwin, Gromadzka et al. 2013). The most common consequences of disruptions in mentioned brain areas include dystonia, problems with speech, tremor and also psychiatric disorders like depression, paranoid psychosis, suicidal tendencies and others (Das and Ray 2006).

One of the main markers of WD is low level of ceruloplasmin (Kojimahara, Nakabayashi et al. 1995). Ceruloplasmin is a cuproenzyme, which is an important regulator of iron metabolism (Lee, Nacht et al. 1968, Hellman and Gitlin 2002) Metallation of ceruloplasmin occurs in the secretory pathway in hepatocytes and is dependent on the function of ATP7B (Hellman, Kono et al. 2002). Failure to incorporate Cu ions into apo-ceruloplasmin results in its quick degradation and leads to low blood ceruloplasmin levels, which is an important feature of WD (Kojimahara, Nakabayashi et al. 1995, Das and Ray 2006, Huster, Finegold et al. 2006). Another sign of WD is the appearance of brown Kayser-Fleischer rings in the cornea of the eyes (described independently by Kayser and Fleisher at the beginning of the 20th century) (Das and Ray 2006, Lutsenko, Barnes et al. 2007, Tanner 2019). Kayser-Fleischer rings are composed of visible deposits of sulfur-copper complexes which do not affect visual functions (Langwińska-Wośko, Litwin et al. 2016, Midena, Frizziero et al. 2019). Additionally, alanine aminotransferase (ALT), a marker of liver injury or inflammation, is known to be often increased in WD patients even at a young age (Huster, Purnat et al. 2007).

Cu overload also causes changes at the cellular level. Liver biopsies from WD patients and animal models demonstrate enlarged nuclei and abnormal mitochondria in

hepatocytes. These changes in cellular morphology do not depend on the species (Haywood, Loughran et al. 1985, Kumaratilake and Howell 1989, Yano, Sakamoto N Fau - Takikawa et al. 1993, Howell and Mercer 1994, Huster, Finegold et al. 2006). Moreover, increased Cu concentration in lysosomes, nuclei and increased DNA synthesis were reported (Huster, Finegold et al. 2006). In response to increased levels of Cu, synthesis of MT-1 and MT-2 is upregulated (Huster, Purnat et al. 2007, Burkhead, Ralle et al. 2011). Elevated hepatic Cu can decrease methylation of DNA and suppress activity of several nuclear factors including liver X receptor (LXR), retinoid X receptor (RXR) and peroxisome proliferator-activated receptor alpha (PPARα) which, in turn affects such processes as lipid metabolism, folate metabolism and inflammatory response ((Muchenditsi, Talbot et al. 2021, Dev, Muchenditsi et al. 2022) and reviewed in (Dev, Kruse et al. 2022)). Changed levels of other biologically significant metals (Zn, Fe) were also reported in WD patients and WD model organisms. However, these data are not consistent, possibly, because of differences in species and genetic background, as well as in the age of animals and sets of their organs used for such studies (Buiakova, Xu J Fau - Lutsenko et al. 1999, Litwin, Gromadzka et al. 2013, Meacham, Cortés et al. 2018, Washington-Hughes, Roy et al. 2023).

b) Treatment of Wilson disease

Without proper treatment patients with WD die (Das and Ray 2006). Prescription of copper-chelating agents combined with low-Cu diet and Zn(II) salts supplement comprise first-line treatment (Uriu-Adams and Keen 2005, Das and Ray 2006, Hordyjewska, Popiołek et al. 2014). Zn(II) ions induce the synthesis of MTs and prevent copper absorption from the gut (Uriu-Adams and Keen 2005, Das and Ray 2006). However, when it comes to acute liver failure, which cannot be treated with copper chelators, only liver transplantation can help (Das and Ray 2006, Catana and Medici 2012).

Copper-chelating agents used in the WD therapy include D-penicillamine (PA), triethylene tetramine dihydrochloride (trientine) (TR), ammonium tetrathiomolybdate (TTM), and dimercaptopropanol (2,3-propan-1-ol), which is also called British anti-Lewisite (BAL) (Uriu-Adams and Keen 2005, Das and Ray 2006, Hordyjewska, Popiołek et al. 2014).

Historically, BAL was the first drug which was tested on WD patients but it has adverse effects – the injections were painful and patients developed tachyphylaxis, i.e. a rapid and short-term onset of drug tolerance (Tanner 2019). Nowadays, treatment with BAL is more likely an exception than a rule (Vilensky and Redman 2003).

PA was introduced in 1956 (Walshe 1956), and it is the most frequently used agent for WD treatment (Horn, Møller et al. 2019, Litwin, Członkowska et al. 2019, Tanner 2019). PA chelates copper and, also, similarly to zinc, causes synthesis of MT-s and stimulates urinary Cu excretion (Das and Ray 2006). However, PA leads to numerous side effects, which include deterioration of neurologic symptoms, which can have even late-onset character (Brewer 1999, Das and Ray 2006). These side-effects could be explained with release of great amounts of copper to blood and its transport into the brain (Brewer 1999, Li, Chen et al. 2016).

TR is an effective WD drug in hepatic patients and can be successful both for initial therapy or for difficult-to-treat cases (Rupp and Weiss 2019). TR promotes urinary excretion of Cu but has side effects which are similar to PA (including mental worsening) but which occur more rarely (Kim, Chung et al. 2013, Rupp and Weiss 2019).

TR tends to generate toxic complexes with iron, especially when they are consumed at the same time (Li, Chen et al. 2016) (Das and Ray 2006).

Ammonium TTM is a better drug for WD treatment then PA and TR, as it has milder side effects (Brewer 1999, Das and Ray 2006). However, long-term safety and efficiency of TTM are elusive and there are also certain difficulties with storage of TTM, as it is very sensitive to oxidation. Bis-choline TTM is free from these complications and has recently completed the Phase II of clinical trials (Weiss, Askari et al. 2017).

In addition, Chinese medicine uses dimercaptosuccinic acid (DMS) for WD therapy besides the mentioned chemicals (Li, Chen et al. 2016).

Despite intensive studies and therapeutic use the comparative metal-binding properties of copper chelators are not known.

1.1.3.3 Cell differentiation

During differentiation, the cell takes the right place and functions in the organism according to its fate. The cell receives various signals from the environment and modulates its metabolism and synthesis of proteins correspondingly. For instance, it is known that the redox balance of the cell fluctuates during the cell cycle, and redox potential of GSH:GSSG couple increases from cell proliferation through differentiation, and the intracellular environment is the most oxidizing during apoptosis (Go and Jones 2013). Similarly, the cellular needs of Cu and the expression and localization of proteins involved in its transport change during differentiation (Lutsenko 2021).

In cultured neuroblastoma cells, Cu content increases upon differentiation, similar to the expression of genes that regulate Cu influx (*CTR1* and *CTR2*), mitochondrial transport and respiration (*COX17*, *COX1*), and secretory pathway (*ATOX1, ATP7A* and peptidyl-glycine-α-monooxygenase (*PAM*)). The significant changes in mitochondrial and secretory copper proteins indicate that differentiated cells have more actively respiring mitochondria on one hand and that the expression of secretory PAMs and copper delivery via the ATOX1 and ATP7A pathway is critical for neuronal cell functioning (Hatori, Yan et al. 2016). Similar events were observed in myocytes: during and after differentiation, the cells increased Cu influx and expression of ATP7A and ATP7B. Additionally, the assembly of myotubes in the early stages of myogenesis was shown to depend on the levels of cellular Cu, showing that copper has multiple roles in the maturation of muscle cells (Vest, Paskavitz et al. 2018). Similarly, Cu uptake is increased in maturated 3T3-L1 adipocytes in parallel with corresponding changes in Cu-influx proteins. Semicarbazide-sensitive amine oxidase (Ssao) expression is significantly increased in these cells, and Atp7a was demonstrated to be upregulated for delivery of Cu to Ssao (Yang, Ralle et al. 2018). Several studies have demonstrated that Cu is a critical factor in the differentiation of mesenchymal stem cells (MSCs), which can in dose-dependent manner boost or decrease the differentiation rate or shift the differentiation of MCSs towards osteoblasts or adipocytes lineages (Rodríguez, Ríos et al. 2002, Burghardt, Lüthen et al. 2015, Li, Xia et al. 2019, Noori, Hoseinpour et al. 2022). However, these studies did not target a detailed exploration of the cellular Cu-handling machinery in MSCs; therefore, additional investigations are needed.

1.1.3.4 Lipid metabolism

Cu and lipid metabolism are linked in a tight reciprocal interplay. More specifically, a Cu-deficient diet results in the development of blood cholesterolemia with significantly increased levels of low-density and high-density lipoproteins (LDL and HDL, respectively) in serum and plasma (Lei 1983, Klevay, Inman et al. 1984). These changes affect red

blood cells by changing the composition of the cellular membrane causing hemolysis and anemia (Jain and Williams 1988). In Cu-deficient diet, expression of apolipoprotein E (ApoE) and lecithin cholesterol acyltransferase (LCAT) is increased in the intestine, and expression and activity of several Cu-dependent enzymes including LOX, SOD1, CCO and DBH are decreased in other tissues (Allen and Klevay 1978, Cunnane, McAdoo et al. 1986, Burkhead and Lutsenko 2013). The activity of LOX is critical for collagen and elastin cross-linking and is essential for maintaining the structural integrity of blood vessels (Burkhead and Lutsenko 2013). Moreover, feeding mice dams with a low-Cu diet during weaning causes abnormal development of the pups reflecting in reduced brain weight, lower levels of phospholipids in the liver, hepatomegaly, and critically reduced activity of serum ceruloplasmin (Cunnane, McAdoo et al. 1986). Also, the recent study with *Atp7b*$^{-/-}$ mice showed that at a young age (4 weeks) these animals have significantly decreased Cu levels in the brain compared to age-matched wild-type mice, which was accompanied by dramatic changes in brain structures, and altered lipid metabolism (Washington-Hughes, Roy et al. 2023). Also the intestine of *Atp7b*$^{-/-}$ mice was found to be Cu-deficient, which causes mitochondrial dysfunction, increased synthesis of triglycerides (TG-s) and mislocalization of apolipoprotein B (ApoB). The mislocalization of ApoB results in the decreased assembly of chylomicrons, which facilitate the uptake of fat from the diet and its secretion from the enterocytes (Pierson, Muchenditsi et al. 2018).

Under conditions of hepatic Cu excess, the expression of proteins involved in the synthesis of cholesterol is decreased, resulting in decreased levels of TG-s and very-low density lipoproteins (VLDL) in serum (Huster, Purnat et al. 2007, Ralle, Huster et al. 2010, Krishnamoorthy, Cotruvo et al. 2016). A recent study showed that excess hepatic Cu affects the expression and activity of enzymes that participate in the synthesis of sterols, and, results in decreased levels of liver sterols. In turn, these changes inhibit the activity of the liver X nuclear receptor (LXR), which dysregulates lipid metabolism further (Hamilton, Koganti et al. 2016, Dev, Muchenditsi et al. 2022). Similar to NAFLD, liver steatosis is common in patients and animal models of Wilson disease despite high liver Cu content (Stättermayer, Traussnigg et al. 2015). However, data in the literature is inconsistent, which could be explained by genetic variability (Liggi, Murgia et al. 2013, Stättermayer, Traussnigg et al. 2015, Blades, Ayton et al. 2021, Muchenditsi, Talbot et al. 2021).

In adipocytes, Cu is essential for the activity of SSAO, which is highly expressed in white adipose tissue (WAT), and is involved in various processes (Morin, Lizcano et al. 2001, Mercier, El Hadri et al. 2007, Weston, Shepherd et al. 2015, Salmi and Jalkanen 2019, Blades, Ayton et al. 2021). Expression of SSAO is increased in WAT in case of obesity (Mészáros, Szombathy et al. 1999, Weiss, Klocker et al. 2003, Yang, Liu et al. 2019). The recent study with 3T3-L1 cells demonstrated that chelation of Cu or its limited transport to the secretory pathway by dysregulation of Atp7a, results in cellular hypertrophy, increased size of lipid droplets and levels of TG-s. A similar result was obtained also by the inactivation of SSAO and the authors concluded that SSAO is responsible for the choice of the energy source and its inactivation leads to utilization of lipid-dependent pathways in the cells (Yang, Ralle et al. 2018).

Because of the complicated balance between Cu and lipid metabolism, more studies are needed to untangle this enigmatic and essential interplay.

1.1.3.5 Cuproptosis

It is known that during cancerogenesis and tumor growth cell metabolic pathways undergo significant changes (reviewed in (Pruitt 2016)). Numerous studies have shown that Cu requirements are much higher in tumors compared to healthy tissues, as Cu is involved in such processes as angiogenesis, cell proliferation and formation of metastasis (Yoshida, Ikeda et al. 1993, Díez, Arroyo et al. 2009, Lavilla, Costas et al. 2009, Callejón-Leblic, Gómez-Ariza et al. 2018, Lelièvre, Sancey et al. 2020). Studies have demonstrated that additional Cu supplementation causes an increased rate of tumor progression (Ishida, Andreux et al. 2013, Brady, Crowe et al. 2014, Chen, Min et al. 2022). This could be explained by the copper-induced increase of the production of ROS which is linked with malignant cell transformation, and activation of down-stream signaling pathways such as mitogen-activated protein kinase (MAPK) cascade, ULK1/2 cascade and influences on the secretion of pro-angiogenic molecules and pro-inflammatory cytokines (fibroblast growth factor (FGF) and IL-1α, respectively) (Mandinov, Mandinova et al. 2003, Turski, Brady et al. 2012, Tsang, Posimo et al. 2020, Chen, Min et al. 2022). Moreover, alterations in the functioning of such members of the cellular Cu-handling machinery as CTR1, ATOX1, SOD1 or ATP7A can also contribute to tumorigenesis (reviewed in (Lelièvre, Sancey et al. 2020)). Therefore, cellular Cu balance has become an important target in cancer-related studies and cancer therapy. Currently, two opposite approaches are in use: Cu chelation to limit Cu bioavailability, and, increasing cellular Cu levels using Cu ionophores which facilitate Cu transport into the cell (Chen, Min et al. 2022, Tsvetkov, Coy et al. 2022).

One of the ionophores with a potent anti-cancer ability is elesclomol (Berkenblit, Eder et al. 2007, Kirshner, He et al. 2008, Gao, Huang et al. 2021), whose mechanism of action was elusive for a long time. Recently, studies by Tsvetkov and co-workers revealed the details underlying the mechanism of elesclomol action related to Cu toxicity (Tsvetkov, Coy et al. 2022). Briefly, in combination with elesclomol, Cu is transported into the cells where it causes cell death through loss of iron-sulfur clusters (Fe-S) containing proteins (in particular, FDX1) and through binding to lipoylated mitochondrial dehydrogenase complexes (in particular, dihydrolipoyl acetyltransferase (DLAT)) and causing their aggregation. This mechanism of Cu-dependent cell death was termed cuproptosis (Tsvetkov, Coy et al. 2022). Cuproptosis was triggered also by another Cu ionophore, disulfiram (Tsvetkov, Coy et al. 2022). These findings stimulated cancer research in elucidation of the molecular basis of Cu and ionophores interplay in the tumor cell and promoted Cu-focused clinical trials and therapy in the field of cancer.

1.2 Theoretical principles of protein-metal binding

1.2.1 Protein-Cu(I) binding affinities

Cu(I) ions are termed “soft” ions and they prefer to bind to S and N atoms which are represented in Cys, Met, and His amino acid residues in proteins (Permyakov 2009, Hatori, Inouye et al. 2017, Permyakov 2021). Cu (I) and (II) ions have different coordination numbers when they form coordination spheres with ligands. Cu(I) prefers coordination numbers 2, 3 or 4, while Cu(II) coordination numbers are 4, 5 or 6 (Permyakov 2009).

An essential thermodynamic parameter of protein-metal interaction is the metal-binding affinity, which is expressed by the dissociation constant of the protein-metal

complex, K_D (equation 3 and 4). The lower values of K_D mean a higher affinity for metal binding (Bisswanger 2008).

$$Cu^+ + P \leftrightarrow Cu^+\text{-}P \qquad \text{(eq 3)}$$

$$K_D = \frac{[Cu^+]\times[P]}{[Cu^+\text{-}P]} \qquad \text{(eq 4)}$$

Evaluation of metal-binding affinities is important for understanding the mechanisms of protein metallation *in vivo* and finding out factors, that affect this process (Xiao and Wedd 2010, Young and Xiao 2021). However, the experimental determination of K_D can be complicated, and careful planning of the *in vitro* experiments is needed to avoid discrepancies in the determined values (Young and Xiao 2021). *In vitro* experiments for determination of Cu(I)-binding affinities are difficult due to a number of reasons including instability of Cu(I) ions in mild oxidative conditions, lack of pH control, and possible oxidation of Cys residues, which are forming the coordination sphere (Xiao and Wedd 2010).

Almost all Cu(I)-binding proteins have extremely high binding affinities, making direct titration unsuitable for their determination (Xiao and Wedd 2010, Young and Xiao 2021). Several indirect (competitive) methods could be used for the determination of K_{Cu}, including spectrophotometric methods (detection of Cu(I)-ligand and Cu(I)-protein complexes at different wavelengths), spectroscopic methods (e.g. NMR where DTT is used as reducing agent and competing ligand), isothermal titration calorimetry (observation of changes in heat) and mass spectrometric methods (Palumaa, Kangur et al. 2004, Xiao and Wedd 2010, Kirsipuu, Zadorožnaja et al. 2020, Young and Xiao 2021, Smirnova, Gavrilova et al. 2022). A new method called microscale thermophoresis, which combines the sensitivity and variability of thermophoresis and the precision of fluorescent detection, was recently developed. It could, potentially, be used for the determination of Cu(I)-binding affinities, as it can be used for quantification of K_D-s in the low picomolar range (Jerabek-Willemsen, André et al. 2014).

1.2.2 Mass-spectrometric methods in the determination of Cu(I)-binding affinities

Mass spectrometry (MS) involves ionizing the analyte and sorting the ions based on their mass-to-charge ratio. Numerous ionization techniques have been developed, but electrospray ionization (ESI) has proven particularly effective for protein analysis. ESI-MS is known for its “soft ionization” approach, which can avoid the fragmentation of sample molecules and dissociation of molecular complexes by minimizing the applied energy (Raza, Engen et al. 2007). ESI-MS allows for rapid and reliable analyses of many non-covalent interactions, which are preserved during ionization and consumes a small amount of material (Banerjee and Mazumdar 2011). Therefore, the protein-ligand complexes can be monitored directly, as they will be transferred into the gas phase and detected in the ESI-MS spectrum (Ganem, Li et al. 1991, Katta, Chait et al. 1991, Smith, Light-Wahl et al. 1992, Craig, Veenstra et al. 1997).

ESI-MS-based approach can also be used for the determination of Cu(I)-binding affinities by measuring the competition of protein and Cu(I)-binding ligand with known Cu(I)-binding affinity for the metal ion. One competing ligand can be DTT, which application has many advantages: 1) DTT keeps Cu(I) ions stable and avoids their oxidation and recombination; 2) DTT is a reducing agent and mimics the cellular redox

conditions, and 3) being a non-ionic compound DTT does not reduce ionization efficiency in the ESI-MS spectra even at millimolar concentrations (Palumaa, Kangur et al. 2004, Banci, Bertini et al. 2010). In the ESI MS study by Banci and co-workers, K_{Cu} values for proteins were determined through the competition of proteins and DTT for Cu(I) ions and estimation of the concentration of DTT or the free Cu(I) ions, where 50% of the protein is in Cu(I)-loaded form (Banci, Bertini et al. 2010). By this scheme, apparent dissociation constants K_{Cu} were estimated for the main intracellular Cu(I)-binding proteins, including Cu chaperones, Cu_A site of CCO, SOD-1, metal-binding domains of ATP7A and MT-s (Banci, Bertini et al. 2010). In this study conditional K_{Cu}(DTT)= 7.94 x 10^{-12} M was used (Krężel, Leśniak et al. 2001), however, in 2011 the value of K_D(DTT) was corrected by four orders of magnitude (K_{Cu}(DTT)= 5.01 × 10^{-16} M, (Xiao, Brose et al. 2011), which should be taken into account in further studies. ESI-MS-based approach could be used also for determination of dissociation constants for Cu(I)-ligand complexes by determining their relative Cu(I)-binding affinities towards reference Cu(I)-binding protein, for which the Cu(I)-binding affinity is known.

1.3 α-Lipoic acid

1.3.1 α-Lipoic acid, its chemical properties, metabolism and *de novo* synthesis

α-lipoic acid (LA)(Figure 6), also known as thioctic acid, is a natural sulphur-containing fatty acid (Packer, Witt et al. 1995). Reed and co-workers reported LA isolation for the first time in 1951 (Reed, DeBusk et al. 1951, Reed 2001). LA has two optical isomers (Figure 6A, B), R-LA is naturally active and is synthesized in organisms and S-LA is believed to prevent R-LA from polymerization in racemic mixtures (Packer, Witt et al. 1995, Shay, Moreau et al. 2009).

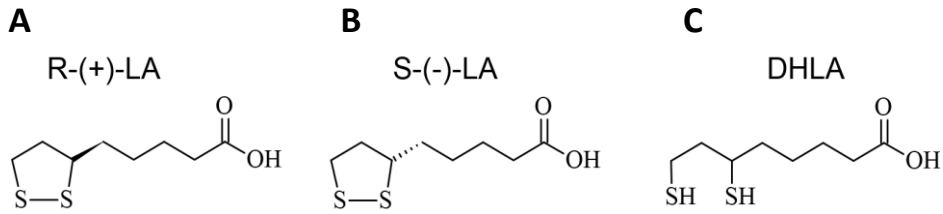

Figure 6. Chemical structure of LA. *(A) R-lipoic acid, (B) S-lipoic acid, and (C) dihydrolipoic acid. Structures are drawn in ChemSketch software (by ACD/Labs Inc).*

Reduced form of LA, dihydrolipoic acid (DHLA, Figure 6C) has two free thiol groups. Reduction potential of LA/DHLA redox couple (-320 mV, (Searls and Sanadi 1960)) is more potent even compared to GSH/GSSG couple (-240 mV for pH 7.0 (Rost and Rapoport 1964, Jones 2002)) and allows reduction of disulphide bonds in oxidized glutathione and in proteins such as thioredoxin, as well as regeneration of other antioxidants such as ascorbate and vitamin E (Packer, Witt et al. 1995, Bast and Haenen 2003).

Exogenous LA gets into the human organism from dietary sources or as a supplement. Spinach, broccoli, meat (mainly muscle meats, kidney and liver), eggs and tomatoes are among the most LA-rich products (Akiba, Matsugo et al. 1998, Kataoka 1998, Shay, Moreau et al. 2009, Durrani, Schwartz et al. 2010, Gorąca, Huk-Kolega et al. 2011, Saha,

Ahammad et al. 2018). LA gets absorbed in the gastrointestinal (GI) tract while the absorption rate can fluctuate depending on several factors such as presence of other nutrients, and LA formulation (liquid or solid, single pure isomer or racemic mixture) (Hermann, Niebch et al. 1996, Maglione, Marrese et al. 2015). At least two transporters participate in uptake of LA into the cell: monocarboxylate transporter (Takaishi, Yoshida et al. 2007) and Na^+-dependent multivitamin transporter (Grassl 1992, Prasad, Wang et al. 1998, Balamurugan, Vaziri et al. 2005). Additionally, LA can pass biological membranes thanks to its amphiphilic properties (Packer, Witt et al. 1995, Yi and Maeda 2005). From intestinal cells, LA is quickly transported into blood plasma and gets distributed throughout the whole body being detected in liver, heart, skeletal muscles and other organs (reviewed in (Shay, Moreau et al. 2009)). In numerous studies involving the administration of LA, researchers observed LA's presence in the brain, indicating its capability to traverse the blood-brain barrier (Panigrahi, Sadguna et al. 1996, Arivazhagan, Shila et al. 2002).

Once in the cell, LA is rapidly reduced to DHLA by NADH- and NADPH-dependent oxidoreductases (Handelman, Han et al. 1994, Constantinescu, Pick et al. 1995, Jones, Li et al. 2002, May, Qu et al. 2007) and is a substrate for further metabolic reactions including beta oxidation (Hermann, Niebch et al. 1996, Teichert and Preiss 2002, May, Qu et al. 2006, Gorąca, Huk-Kolega et al. 2011, Theodosis-Nobelos, Papagiouvannis et al. 2021). The most common LA metabolites found in animal studies are DHLA, bisnorlipoate, tetranorlipoate, β-hydroxy-bisnorlipoate, or their bis-S-methylated derivatives (Kataoka 1998, Shay, Moreau et al. 2009).

In organisms, LA is synthesized *de novo* in protein-bound form from octanoic acid and a sulfur source by the enzyme lipoic acid synthase (LIAS in humans) (Hiltunen, Autio et al. 2010, Mayr, Feichtinger et al. 2014, Solmonson and DeBerardinis 2018). LA synthesis was initially discovered in prokaryotes (Reed, Leach et al. 1958), later LA was demonstrated to be synthesized in mammalian mitochondria (Packer, Witt et al. 1995, Morikawa, Yasuno et al. 2001, Yi and Maeda 2005) and, additionally, in plant chloroplasts (Yasuno and Wada 2002). LA synthesis has been the most intensively studied in Saccharomyces cerevisiae (Schonauer, Kastaniotis et al. 2009, Solmonson and DeBerardinis 2018).

The importance of endogenous production of LA was shown in 2005 in a murine model by Yi and Maeda (Yi and Maeda 2005). The experiments revealed that mice carrying a single copy of the lipoic acid synthase encoding gene (*Lias*$^{+/-}$) exhibited decreased antioxidant capacities. In contrast, mice lacking both copies of the gene (*Lias*$^{-/-}$) did not survive beyond the early prenatal stage of development, and attempts to rescue them through maternal LA supplementation during pregnancy were unsuccessful (Yi and Maeda 2005).

1.3.2 The role of α-lipoic acid in enzymatic activity

Lipoic acid is a key cofactor to at least five mitochondrial enzyme complexes involved in energy and amino acid metabolism. They include glycine cleavage system (GCS) and the family of 2-ketoacid dehydrogenase complexes: pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (OGHD), branched-chain ketoacid dehydrogenase (BCKDH) and 2-oxoadipate dehydrogenase (OAHD) (Reed 2001, Cronan John 2014, Solmonson and DeBerardinis 2018).

Each of 2-ketoacid dehydrogenase complexes consist of three subunits (Figure 7A), E1-E3, and R-LA is covalently bound to Lys residue in the E2 subunits where it facilitates

coupled chemical reactions (Cronan John 2014, Mayr, Feichtinger et al. 2014, Solmonson and DeBerardinis 2018). The reactions are similar in their mechanism. Shortly, E1 subunit is a thiamine-dependent decarboxylase and it provides the reaction substrate, which is specific for each complex. E2 subunit has dihydrolipoamide S-acyltransferase activity and reacts with coenzyme A (CoA) producing acetyl-CoA. Finally, E3 subunit, dihydrolipoamide dehydrogenase (DLD), reacts with NAD^+ and dihydrolipoamide during oxidation of $FADH_2$ (Figure 7A, (Cronan John 2014, Solmonson and DeBerardinis 2018)).

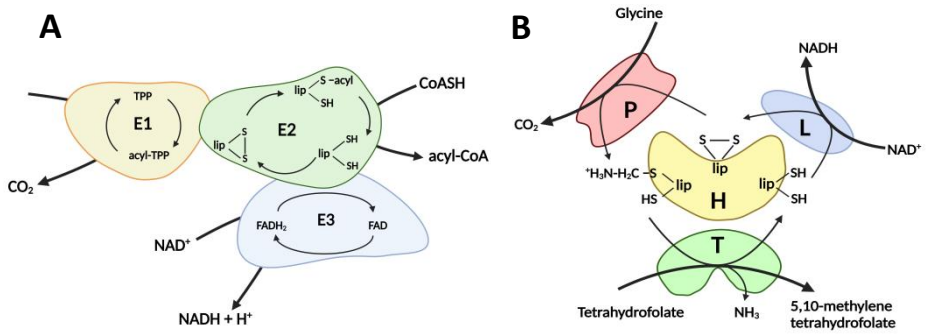

Figure 7. Schematic representation of lipoylated enzyme complexes. *(A) 2-ketoacid dehydrogenase complex; E1, E2 and E3 are the subunits of the complex. (B) A schematic representation of glycine cleavage system; P, L, H and T are the subunits of the complex. Bold arrows indicate the main enzymatic reactions of each complex. Figure was created with BioRender.*

In the GCS, LA is covalently bound to H protein (Figure 7B) and catalyses the decarboxylation of Gly assisting the methylation of tetrahydrofolate to form 5,10-methylene tetrahydrofolate, which is an important cofactor in the nucleic acids synthesis (Kumaran, Patel et al. 2013, Cronan John 2014, Theodosis-Nobelos, Papagiouvannis et al. 2021). In all complexes of 2-ketoacid dehydrogenases and in GCS lipoylated moiety interacts with all other subunits during the reactions (Figure 7A, B) (Cronan John 2014).

The activity of 2-ketoacid dehydrogenases plays a crucial role in aerobic organisms by serving as a link between glycolysis and the TCA cycle, providing the necessary carbon source. Moreover, acetyl-CoA formed by DLAT is also required in the TCA cycle and other metabolic pathways, including synthesis of amino acids and fatty acids (Guest and Russell 1992, Cronan John 2014, Solmonson and DeBerardinis 2018). Therefore, mutations in genes encoding subunits of dehydrogenase complexes can have deleterious effects (Solmonson and DeBerardinis 2018). Moreover, in cuproptosis (discussed above), aggregation of lipoylated DLAT leads to the cell death (Tsvetkov, Coy et al. 2022).

1.3.3 Therapeutic properties of α-lipoic acid

Since its discovery, there has been extensive research on α-lipoic acid. It has emerged as a valuable dietary supplement and a strong contender for the treatment or prevention of various diseases, including diabetes, inflammations, some forms of cancer, neurodegenerative diseases, heavy metal poisoning and others (Packer, Witt et al. 1995, Shay, Moreau et al. 2009, Theodosis-Nobelos, Papagiouvannis et al. 2021, Tripathi, Ray et al. 2023).

1.3.3.1 Safety profile

The safety and toxicity of LA have been the subject of intensive studies for years. Acute toxicity and subchronic toxicity experiments have been conducted on various species, including mice, rats, cats, and dogs (Grunert 1960, Packer, Witt et al. 1995, Hill, Werner et al. 2004, Cremer, Rabeler et al. 2006, Shay, Moreau et al. 2009). Surprisingly, cats are the least LA-tolerant species, with an LD_{50} of 30 mg/kg body weight in the case of acute oral administration, which comprises only 13 mg/kg body weight (bw) as the maximum tolerated dose (MTD) (Hill, Werner et al. 2004). In contrast, for rats, the acute toxicity LD_{50} is > 2000 mg/kg bw, and a long-term study (2 years of oral administration of LA) revealed a no-observed-adverse-effect-level (NOAEL) dose of 60 mg/kg/day (Cremer, Rabeler et al. 2006, Cremer, Rabeler et al. 2006).

Pharmacokinetic studies in humans were conducted in healthy volunteers by several research groups, and the influence of differences in LA administration (oral or intravenous) and dosages were revealed (Shay, Moreau et al. 2009). Numerous clinical trials were conducted for human diseases including diabetes (ALADIN (I–III), OPRIL, SYDNEY (I and II), DEKAN), multiple sclerosis and metabolic syndrome. The trials were multicenter, randomized, double-blinded and included placebo-groups (Shay, Moreau et al. 2009). Oral doses of LA ranged from 100 mg/day (intravenous infusion, 3 weeks (Ziegler, Hanefeld et al. 1995)) to 2400 mg/day (1200 mg twice a day, oral supplementation, 2 weeks, (Yadav, Marracci et al. 2005), and the duration of the studies varied from 2 weeks to 24 months (Reljanovic, Reichel et al. 1999, Yadav, Marracci et al. 2005, Shay, Moreau et al. 2009). In all studies, LA did not show significant adverse effects and showed improvements in patients compared to corresponding placebo groups (Ziegler, Hanefeld et al. 1995, Reljanovic, Reichel et al. 1999, Sola, Mir et al. 2005, Yadav, Marracci et al. 2005, Shay, Moreau et al. 2009).

1.3.3.2 Metal chelation

In addition to antioxidant properties of LA, it is capable of chelating various metal ions. It was found that both LA and DHLA can chelate Cd(II) and reduce Cd-induced toxicity in cultured hepatocytes. However, speculating on the details of the chelation mechanism, the authors hypothesized that LA facilitates extracellular metal chelation while DHLA has more prominent chelation properties within the cell (Müller and Menzel 1990). Later, Cd(II) scavenging by LA and its protective role against Cd toxicity were demonstrated *in vivo* (Sumathi, Baskaran et al. 1996, Saleh, El-Sayed et al. 2017). Another study described the ability of LA and its derivatives (bisnor- and tetralipoate) to bind Zn(II), Cu(II) and Mn(II) in aqueous solutions (Sigel, Prijs et al. 1978). Moreover, LA can chelate and increase biliary elimination of Hg in mercury poisoned rats (Gregus, Stein et al. 1992). Additionally, LA and DHLA, can form complexes with Fe(II) and Fe(III) *in vitro* and reduce Fe-induced toxicity and Fe accumulations *in vivo* (Bonomi, Werth et al. 1985, Bonomi, Pagani et al. 1992, Camiolo, Tibullo et al. 2019). Moreover, LA has strong protective abilities against Pb exposure despite its weak abilities to eliminate Pb accumulations from soft tissues compared to another chelator DMS (Gurer, Ozgunes et al. 1999, Pande and Flora 2002).

In vitro, LA and DHLA can also bind Cu(II) and Cu(I) ions, respectively (Kang and Shi 1994), decrease Cu(II)-dependent oxidation of ascorbate, and inhibit Cu(II)-induced lipid peroxidation (Ou, Tritschler et al. 1995). In daily doses as low as 100 mg/kg five times per week, LA was able to significantly decrease accumulation of Cu in the studies of Cu-nanoparticles toxicity study *in vivo* (Khalaf, Zaki et al. 2016), and reduce hepatitis as well as decrease signs of oxidative stress in Long-Evans Cinnamon (LEC) rats which are

one of the animal models of WD (Yamamoto, Watanabe et al. 2001). Despite extensive research efforts, there is a scarcity of quantitative data available concerning the interactions between LA and metal ions.

1.3.3.3 Antioxidant and anti-inflammatory properties

Some of the antioxidant and anti-inflammatory properties of LA are caused by its redox and metal-chelating abilities discussed above. Additionally, numerous studies LA demonstrates its potent radicals scavenging properties, being able to neutralize harmful reactive species including hydroxyl and singlet oxygen (reviewed in (Packer, Witt et al. 1995)). As referenced above, LA thiol groups can reduce oxidized disulphides of other proteins, but in addition to this LA can induce production of endogenous antioxidants. So, in some *in vivo* and *in vitro* studies, it was shown to facilitate more effective uptake of ascorbate (Xu and Wells 1996, Suh, Shigeno et al. 2001, Michels, Joisher et al. 2003). Moreover, DHLA reduces cystine (Cys-Cys) to Cys (2Cys) in the extracellular space and enhances Cys cellular uptake, which is necessary for synthesis of GSH (Han, Handelman et al. 1997). Further studies have demonstrated that LA supplementation induces *de novo* synthesis of GSH via nuclear factor erythroid 2–related factor 2 - Kelch-like ECH-associated protein 1 pathway (Nrf1-Keap1). So, LA can causes nuclear localization of Nrf1 acting as pro-oxidant or prevents Nrf2/Keap1 complex formation, and the consequent induction of synthesis of antioxidant proteins is facilitated by interaction of Nrf2 with antioxidant response elements (ARE) (reviewed in details in (Shay, Moreau et al. 2009)). γ-glutamyl cysteine ligase (GCL), which mediates the first step in GSH synthesis, is one of the proteins regulated by ARE (Meister and Anderson 1983, Ferguson and Bridge 2016).

LA has been studied for its potential as an antioxidant and nuclear factor-κB (NF-κB) inhibitor (Packer, Witt et al. 1995, Shay, Moreau et al. 2009, Tripathi, Ray et al. 2023). LA has demonstrated the ability to decrease the expression of adhesion molecules induced by tumor necrosis factor α (TNF-α) in cultured endothelial cells and inhibit NF-κB-dependent expression of metalloproteinase-9 in vascular smooth muscle cells (Kunt, Forst et al. 1999, Zhang and Frei 2001, Kim, Kim et al. 2007). Also, LA successfully inhibits lipopolysaccharides-induced (LPS) synthesis of other inflammatory cytokines including TNF-α, interleukin (IL) β and IL-6 in a cellular model of kidney sepsis (Li, Fu et al. 2015). In a recent animal study, LA significantly decreased gut inflammatory response to irinotecan, an anti-cancer drug, and, therefore, increased the survival rate (Costa, Costa et al. 2020). Moreover, LA was demonstrated to inhibit activation of T cells (Suzuki, Aggarwal et al. 1992) and prevent replication of human immunodeficiency virus (HIV) DNA in cultured cells (Baur, Harrer et al. 1991). However, LA was not able to reduce HIV RNA levels neither increase number of CD4 cells despite improved blood GSH and lymphocyte proliferation response in patients in a 6 months long phase II trial study (Jariwalla, Lalezari et al. 2008).

To sum up, LA multifaceted effect on various signalling pathways makes it a potential therapy candidate for numerous diseases (Packer, Witt et al. 1995, Shay, Moreau et al. 2009, Tripathi, Ray et al. 2023). LA role in prevention and treatment of diabetes and neurodegenerative diseases are discussed in the following chapters.

1.3.3.4 Obesity and diabetes

In addition to participating in the aforementioned signaling pathways, LA can interact with numerous protein kinases. Among them, direct or indirect LA signaling via adenosine monophosphate-activated protein kinase (AMPK) has been shown to regulate cellular glucose uptake (Shay, Moreau et al. 2009). In an animal study, LA inhibited AMPK activity in the hypothalamus and decreased food intake. Conversely, it increased AMPK activity in skeletal muscle, resulting in increased glucose uptake and fatty acid metabolism (Kim, Park et al. 2004). Additionally, activation of AMPK was shown to activate the sirtuin 1 (SIRT1) signaling pathway, which is involved in cellular metabolic adaptation (Chen, Kang et al. 2012). Several studies demonstrated that LA administration to insulin-resistant obese rats results in increased glucose uptake from the blood (Jacob, Streeper et al. 1996, Streeper, Henriksen et al. 1997, Peth, Kinnick et al. 2000), and that this was a result of an increased abundance of glucose transporter GLUT4 (Khamaisi, Potashnik et al. 1997). Further *in vitro* studies evidenced that LA causes rapid relocalization of GLUT4 and GLUT1, resulting in increased glucose uptake in both 3T3-L1 adipocytes and L6 myotubes. This effect depends on phosphatidylinositol 3-kinase (PI3K) and AKT1 serine/threonine-protein kinase activity, and it can further activate insulin receptor substrate-1 (IRS1) (Estrada, Ewart et al. 1996, Yaworsky, Somwar et al. 2000). LA was also shown to induce liposysis *in vivo* and *in vitro* (Hamano 2002, Hamano 2006, Fernández-Galilea, Pérez-Matute et al. 2012) and inhibit differentiation of 3T3-L1 preadipocytes (Cho, Moon et al. 2003, Hahm, Noh et al. 2014).

To sum up, LA has ability to to enhance metabolic rates in obese and insulin-resistant individuals by reducing blood glucose levels and inducing lipolysis. It also has the potential to decrease inflammatory responses and increase antioxidant levels within the organism without causing severe side effects. All these properties collectively position LA as a significant player in combating diabetes mellitus, and caused by it polyneuropathies and CVD-s. The clinical trials in obese and diabetic patients (ALADIN (I–III), OPRIL, SYDNEY (I and II), DEKAN and others) have demonstrated significant improvements in major affected markers of diabetis after LA administration (Shay, Moreau et al. 2009, Rahimlou, Asadi et al. 2019). Interestingly, in contrast to the US clinical research groups who are claiming need for more clinical trials (Shay, Moreau et al. 2009, Rahimlou, Asadi et al. 2019), in Germany, LA is used for treatment of diabetic polyneuropathy since 1950-s (Biewenga, Haenen et al. 1997).

1.3.3.5 Neurodegenerative diseases

Due to its strong metal-chelating, antioxidant, and anti-inflammatory properties, as well as its ability to improve a cell's energy status, LA is the focus of studies aiming to prevent or treat several neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS) (Shay, Moreau et al. 2009, Molz and Schröder 2017, Salehi, Berkay Yılmaz et al. 2019).

In AD, β-amyloid (Aβ) plaques, dyshomeostasis of metals (Zn, Cu and Fe) and redox misbalance and, hence, inflammation are playing the most crucial role in disease progression (Hardy and Higgins 1992, Praticò and Delanty 2000, Selkoe and Hardy 2016) (Squitti, Salustri et al. 2017, Bagheri, Squitti et al. 2018). *In vitro* studies demonstrated that LA inhibits Aβ fibrillization (Ono, Hirohata et al. 2006) and is able to protect neuronal cells from both Aβ and peroxide toxicity, partially via Akt/PI3-K signalling (Zhang, Xing et al. 2001). Moreover, synthesis of cytokines IL-1B and IL-6 was inhibited by LA in neuroblastoma cells (Dinicola, Proietti et al. 2017). In murine AD, chronic

administration of LA showed promising results by improving animals' memory and decreasing oxidative stress (Quinn, Bussiere et al. 2007, Farr, Price et al. 2012).

However, dysregulation of Cu is considered to be a much earlier event in the AD (Metsla, Kirss et al. 2022). Distribution of Cu is not even in AD patients: it is high in a blood and CSF and low in the brain (Schrag, Mueller et al. 2011, Ventriglia, Bucossi et al. 2012, Li, Zhang et al. 2017). The recent study showed that LA is able to increase intercellular Cu in concentration-dependent manner in human neuroblastoma cells (Metsla, Kirss et al. 2022). Additionally, LA improved the climbing score in AD model of *Drosophila melanogaster*, which have altered geotaxis compared to wild-type (Metsla, Kirss et al. 2022). These findings suggest that LA possesses potential Cu-ionophore properties. Further studies are needed to determine whether it can address Cu imbalance in the organism, potentially serving as a treatment not only for AD but also for the aforementioned WD and MD.

2 Aims of the Study

The aim of the current thesis was to broaden knowledge of copper functions and properties of copper chelating agents. More specifically, the aims were as follows:

- To investigate Cu(I) binding properties of copper-chelating agents used in the treatment of Wilson disease with the application of the ESI-MS technique and to determine their apparent dissociation constants;
- To study properties of α-lipoic acid as a candidate for WD treatment using 3T3-L1 *Atp7a*$^{-/-}$ mouse-derived preadipocytes, a model of copper overload;
- To study the role of copper in adipogenesis using mouse-derived 3T3-L1 and 3T3-L1 *Atp7a*$^{-/-}$ cells.

3 Materials and Methods

For the current study, the following methods were used, and are described in the respective publications:

- ESI-MS studies of Cu(I) binding to Cox17 and MT-2a (Publication I)
- ESI-MS studies of demetallation of Cox17 and MT-2a using Cu chelating agents (Publication I)
- Determination Cu(I)-ligand dissociation constants for selected Cu chelating agents (Publication I)
- Studies of the protective effect of LA against Cu toxicity in HuH7 hepatocellular carcinoma cells (Publication I)
- Morphometric studies of 3T3-L1 wt and *Atp7a*$^{-/-}$ cells (Publication II)
- Immunostaining and confocal microscopy (Publications II and III)
- Elemental analysis with ICP-MS and/or AAS of cells and mice organs (Publication II and III)
- Cell lysis and protein quantification (Publications II and III)
- Western blot analysis (Publications II and III)
- Tandem Mass Tag labeling mass spectrometry and bioinformatic data analysis using R-studio and QIAGEN Ingenuity Pathway Analysis (IPA) (Publications II and III)
- RNA extraction, cDNA synthesis, RT-qPCR (Publications II and III)
- Analysis of protein oxidation state using nLC-MS/MS (Publication II)
- Liquid chromatography coupled with ICP-MS (Publication II)
- Mice husbandry and tissue collection (Publications II and III)
- Adenoviral infection and transfection (Publications II and III)
- Determination of cellular redox conditions with live-cell imaging (Publication II)
- Seahorse XF Cell Mito Stress Test (Publication II)
- X-ray fluorescence microscopy (Publication III)
- Differentiation of adipocytes (Publication III)
- Triglyceride levels measurement (Publication III)
- Oil red O staining (Publication III)
- Generation of 3T3-L1 *Atp7a*-knockdown cell line using sh-RNA plasmids (Publication III)

4 Results

4.1 Publication I – Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-lipoic acid as a potential anti-copper agent

- Cu(I)-binding affinities for five major WD treatment drugs were determined: D-penicillamine (PA), trientine (TR), 2,3-dimercapto-1-propanol (BAL), meso-2,3-dimercaptosuccinate (DMS) and tetrathiomolybdate (TTM);
- Cu(I)-binding affinities positively correlate with the number of S atoms in the compound and negatively correlate with the number of atoms between thiol groups;
- The Cu(I)-binding affinity for the reduced form of α-lipoic acid (DHLA) was determined and found to be 2 and 3 times higher than that of TR and PA respectively;
- LA demonstrated strong protective abilities against Cu toxicity in HuH7 hepatocellular carcinoma cell culture.

4.2 Publication II – α-lipoic acid ameliorates consequences of copper overload by up-regulating selenoproteins and decreasing redox misbalance

- Mouse-derived 3T3-L1 preadipocytes accumulate copper after deletion of the *Atp7a* gene and exhibit impaired morphology characteristic for the WT line;
- 3T3-L1 *Atp7a*$^{-/-}$ cells have a decreased ratio of reduced and oxidized glutathione (GSH:GSSG) in the cytosol, mitochondria, and nuclei, and a significant increase in the oxidation of Cys and Met amino acid residues in proteins;
- Excess Cu does not cause cuproptosis in *Atp7a*$^{-/-}$ preadipocytes;
- 3T3-L1 *Atp7a*$^{-/-}$ cells have impaired respiratory function compared to WT;
- 3T3-L1 *Atp7a*$^{-/-}$ preadipocytes have a significantly reduced cellular selenium (Se) content;
- Extracellular Cu chelator bathocuproinedisulfonic acid (BCS) rescues impaired morphology of 3T3-L1 *Atp7a*$^{-/-}$ cells and lowers intracellular Cu content;
- BCS does not improve GSH:GSSG ratio in *Atp7a*$^{-/-}$ cells compartments;
- BCS does not have any effect on cellular Se content, neither in WT nor in *Atp7a*$^{-/-}$ cells;
- LA rescues impaired morphology of 3T3-L1 *Atp7a*$^{-/-}$ cells similarly to BCS at both lower and higher concentrations without causing visible cytotoxicity;
- LA does not have any effect on cellular Cu content, does not form intracellular small-molecular-weight complexes with Cu, nor causes Cu redistribution from the cytosol;
- In 3T3-L1 WT cells, LA causes relocalization of the Atp7a protein into the vesicles of the secretory pathway;

- LA increases cellular Se content in 3T3-L1 cells in a dose-dependent manner and enhances the synthesis of selenoproteins regardless of the cells' genotype;
- LA increases the abundance of reduced GSH and improves GSH:GSSG ratio in the cytosol, mitochondria and nuclei of *Atp7a*$^{-/-}$ cells;
- LA reverses the oxidation of Cys and Met residues in proteins of *Atp7a*$^{-/-}$ cells;
- LA and BCS improve the respiratory function of *Atp7a*$^{-/-}$ cells;
- Hepatic and cerebral Se content is significantly decreased in *Atp7b*$^{-/-}$ mice with advanced WD;
- Supplementation of *Atp7a*$^{-/-}$ cells with another antioxidant, n-acetylcysteine (NAC), decreases cell area but is less efficient than LA;
- Supplementation of *Atp7a*$^{-/-}$ cells with NAC does not improve the respiratory function of *Atp7a*$^{-/-}$ cells;
- Supplementation of *Atp7a*$^{-/-}$ cells with elemental Se decreases cell area but is less efficient than LA;
- Supplementation of *Atp7a*$^{-/-}$ cells with Se does not cause a decrease in cellular Cu content and does not improve cellular respiratory function.

4.3 Publication III – Atp7a-dependent copper sequestration contributes to termination of β-catenin signaling during early adipogenesis

- Differentiation of 3T3-L1 adipocytes requires changes in the expression of Cu-handling machinery proteins;
- Expression of Atp7a increases, and its localization pattern is changed in differentiating 3T3-L1 adipocytes in Cu-independent manner;
- Deletion of the Atp7a protein alters intracellular Cu redistribution in the process of adipogenesis and causes Cu accumulation in the cell nucleus;
- Deletion of the Atp7a protein results in the inhibition of adipogenesis in the early stage;
- The proteomes of 3T3-L1 WT and *Atp7a*$^{-/-}$ cells differ in D0-D3 adipogenic transition:
    - In WT cells, proteins associated with lipid metabolism and storage are up-regulated; RhoA and actin cytoskeleton signaling proteins are down-regulated; osteoclast differentiation is inhibited
    - In *Atp7a*$^{-/-}$ cells, proteins associated with the synthesis of triglycerides are down-regulated; proteins facilitating osteoclast differentiation are up-regulated;
- Atp7a protein deletion in 3T3-L1 cells causes the stabilization of β-catenin and an increased abundance of Wnt, which affects Wnt/β-catenin signaling and adipogenesis;
- In Atp7b$^{-/-}$ mice livers, Wnt/β-catenin signaling is affected similarly to *Atp7a*$^{-/-}$ adipocytes.

5 Discussion

5.1 Cu(I)-binding affinity is a critical characteristic of de-coppering drugs

Copper is an essential bioelement that has many "faces". It is necessary for the functions of enzymes involved in many processes including but not limited to cell respiration, scavenging of ROS, cell signalling, differentiation, angiogenesis, lipid metabolism and synthesis of neuromediators (Boal and Rosenzweig 2009, Kaplan and Lutsenko 2009, Argüello, Raimunda et al. 2013, Yang, Ralle et al. 2018, Tsang, Davis et al. 2021). Because of these significant Cu contributions to cell physiology, disturbances in Cu homeostasis can have deleterious effects. The most dramatic examples are Menkes disease (MD), characterized by an inborn copper deficit, and Wilson disease (WD) characterized by systemic copper overload (Shim and Harris 2003, Hordyjewska, Popiołek et al. 2014).

WD is an autosomal recessive disorder, which is caused by the loss-of-function mutations in the ATP7B encoding gene (Frydman 1990). WD patients experience copper build-up in various tissues, particularly the liver and the brain. This accumulation leads to oxidative stress, dysfunction in mitochondria and metabolism, and the development of hepatic and neurologic pathologies (Gitlin 2003, Shim and Harris 2003, Das and Ray 2006, Lutsenko, Barnes et al. 2007, Gupta and Lutsenko 2009).

In contrast to many other genetic disorders, WD is treatable. Prescription of copper-chelating agents combined with low-Cu diet and Zn(II) salts supplement comprise first-line treatment (Uriu-Adams and Keen 2005, Das and Ray 2006, Hordyjewska, Popiołek et al. 2014). Several de-coppering drugs have been used for the treatment of WD: D-penicillamine (PA), triethylene tetramine dihydrochloride (trientine) (TR), ammonium tetrathiomolybdate (TTM), and dimercaptopropanol (2,3-propan-1-ol), which is also called British anti-Lewisite (BAL) (Uriu-Adams and Keen 2005, Das and Ray 2006, Hordyjewska, Popiołek et al. 2014, Tanner 2019). FDA-approved, and the most frequently used Cu-chelators PA and TR are beneficial; however, they have very slow response time and many side effects (Brewer, Terry et al. 1987, Brewer 1999, Gitlin 2003, Chen, Feng et al. 2012, Li, Chen et al. 2016). TTM salts have a faster response time (30 days) and milder side effects (Brewer, Hedera et al. 2003).

Considering the distinct action of PA, TR and TTM, it is tempting to speculate that information on how drug interacts with other intracellular ligands in addition to Cu could enhance our understanding of the drug therapeutic potential. When it comes to copper-chelating agents, characterizing their copper-binding properties is of particular interest. Here (Publication I), we determined apparent dissociation constants K_D for PA, TR, BAL, DMS, DHLA, diethyl-dithiocarbamate (DETC) and TTM using ESI-MS-based approach elaborated in the previous studies. K_D for the chelators were determined by the demetallation of hCox17 and comparison with the K_D of DTT. Estimated apparent dissociation constants are summarized in the Table 1. As it is seen from the experiments with Cox17, DETC demonstrates higher affinity, than DTT, being approximately 587 times better chelating agent (Table 1). K_D(TTM) was not calculated from experiments with Cox17, as TTM demetallates Cu_1Cox17 at stoichiometric concentrations. K_D(TTM) were determined from the experiments with MT-2a (Table 1), as it can remove Cu(I) ions from Cu-loaded form of MT-2a similarly to DETC.

K_D-s for TR and PA are very similar; however, they are about four orders of magnitude higher than K_D for TTM. BAL and DMS are also characterized by three and two orders of magnitude lower K_D values respectively as compared to TR or PA. For DHLA K_D is equal to 8.05×10^{-17} M, which is about two orders of magnitude higher than that of DETC or DMS but is, at the same time, lower than the apparent dissociation constants for TR and PA.

Table 1. Apparent dissociation constants (±SD) for Cu-binding ligands.
** - taken from the reference (Xiao, Brose et al. 2011).*

Ligand	**C_{50} (mM) for Cu_1Cox17**	**K_D (M)**
DTT	3.10 ± 0.26	5.01×10^{-16} *
DETC	0.00528 ± 0.00032	8.53×10^{-19}
TTM	< 0.001	$< 1.6 \times 10^{-19}$
TR	1.08 ± 0.13	1.74×10^{-16}
DLA	0.498 ± 0.098	8.05×10^{-17}
PA	1.47 ± 0.33	2.38×10^{-16}
DMS	0.00322 ± 0.00041	5.17×10^{-19}
BAL	0.00938 ± 0.00148	1.52×10^{-18}
Ligand	**C_{50} (mM) for Cu_{10}MT-2a**	**K_D (M)**
DETC	0.772 ± 0.087	8.53×10^{-19}
TTM	0.021 ± 0.004	2.32×10^{-20}

To achieve the therapeutic outcome, a potential WD drug must satisfy several criteria. Firstly, it should effectively remove excess copper without interfering with vital Cu-binding enzymes. Secondly, it should have the ability to penetrate the blood-brain barrier, to neutralize the neurotoxic effects of excess Cu in the brain. While TTM exhibits the highest affinity among all chelators used in WD treatment, our study's K_D(TTM) value suggests that it can extract Cu(I) from crucial Cu-containing enzymes like SOD1, potentially causing harm. Conversely, given its K_D value close to those of the drugs in use, DHLA emerges as a promising candidate for treating Wilson disease.

5.2 Excess copper causes numerous alterations in 3T3-L1 cell functioning

Copper and lipid metabolism are involved in a complicated reciprocal interplay, which is not fully understood. A Cu deficient-diet, as well as Cu deficiency in general, are risk factors of cardiovascular diseases and NAFLD due to decreased synthesis and activity of Cu-dependent enzymes, and increased levels of HDL-s and LDL-s in blood (Allen and Klevay 1978, Lei 1983, Klevay, Inman et al. 1984, Cunnane, McAdoo et al. 1986, Burkhead and Lutsenko 2013). Excess Cu also alters lipid metabolism and causes

decreased levels of TG-s and VLDL in blood serum; however, in WD it causes liver steatosis and fatty-liver similar to NAFLD (Huster, Purnat et al. 2007, Ralle, Huster et al. 2010, Stättermayer, Traussnigg et al. 2015, Krishnamoorthy, Cotruvo et al. 2016).

Despite recent findings that Cu is needed for the activity of Ssao in adipocytes (Yang, Ralle et al. 2018), as well as for differentiation of numerous cell types (Rodríguez, Ríos et al. 2002, Burghardt, Lüthen et al. 2015, Hatori, Yan et al. 2016, Vest, Paskavitz et al. 2018, Li, Xia et al. 2019, Noori, Hoseinpour et al. 2022), the role of Cu in the development and functioning of the adipose tissue is not fully clear. Moreover, the *in vivo* studies of adipogenesis are complicated because pre-adipose cells are engaged in signaling with each other and other cell types (Ma, Li et al. 2020, Bagchi and MacDougald 2021). Therefore, to broaden our knowledge about functioning of Cu in the adipogenesis, we used 3T3-L1 mouse-derived cell line, which is a well-known model for the white adipose tissue.

3T3-L1 cells naturally do not express Cu-efflux transporter Atp7b, which enables the elimination of their Cu excreting ability by deleting the gene encoding another copper transporter Atp7a (Bhattacharjee, Yang et al. 2016, Yang, Ralle et al. 2018). Previous study with 3T3-L1 cells demonstrated that the synthesis of TG-s and the size of lipid droplets depend on the cellular Cu status. Moreover, Cu deficiency caused hypertrophy of adipocytes, and mature 3T3-L1-s heterozygously lacking *Atp7a* gene (*Atp7a*$^{+/-}$ cells) lose the ability to normally redistribute Cu into the secretory pathway, which also leads to the cellular hypertrophy (Yang, Ralle et al. 2018).

The most significant changes that occurred in 3T3-L1 preadipocytes upon the complete *Atp7a* deletion with CRISPR/Cas9 were increased intracellular Cu content and altered cell morphology. *Atp7a*$^{-/-}$ cells are significantly enlarged, flattened and contain several nuclei (Publications II and III). We demonstrated that these morphological changes are not an off-target effect of the CRISPR/Cas9 system as approximately 70% down-regulation of expression of Atp7a protein by shRNA causes also similar phenotype (Publication III). Cu chelation with an extracellular Cu-chelating agent BCS decreased intracellular Cu content and rescued the impaired morphology of 3T3-L1 *Atp7a*$^{-/-}$ preadipocytes proving that changes in the phenotype are caused by excess Cu (Publication II). X-ray fluorescence imaging demonstrated that in WT preadipocytes Cu is located in perinuclear area (Publication III), however, *Atp7a*$^{-/-}$ cells have significantly higher Cu levels throughout the cell and, additionally, their nuclei are overloaded with Cu (Publication III). In addition to elevated Cu, Atp7a-deficient cells also have significantly higher Zn (previously reported *in vivo* by (Meacham, Cortés et al. 2018)) and Mn content, whereas Fe cellular content is not altered (Publication II).

Excess Cu is known to cause significant redox stress (Das and Ray 2006, Bhattacharjee, Yang et al. 2016, Dev, Kruse et al. 2022, Dev, Muchenditsi et al. 2022). By using live-cell imaging we quantified the dynamic oxidation of GRX1-roGFP sensor which reflects the GSH:GSSG ratio (Publication II). We found that the content of GSSG is increased in cytosol, mitochondria and nuclei of *Atp7a*$^{-/-}$ cells compared to WT. The shift in redox homeostasis does not look large (Publication II, Figure 4A-C), but, it is sufficient to cause serious oxidation of Cys and Met residues in the proteome of *Atp7a*$^{-/-}$ cells (Publication II). Additionally, the respiratory function of *Atp7a*$^{-/-}$ preadipocytes was altered being consistent with the increased mitochondrial redox stress, which is not caused by cuproptosis (Publication II). Furthermore, we found that *Atp7a*$^{-/-}$ preadipocytes have significantly depleted content of Se and decreased expression of some selenoproteins which are involved in redox homeostasis (Publication II). This finding is

consistent with a recent study, which reported on reciprocal relations of Se and Cu, where excess Cu inhibited synthesis and activity of several selenoproteins by decreasing cellular Se content through a poorly understood mechanism (Schwarz, Lossow et al. 2020).

Atp7a$^{-/-}$ preadipocytes also did not respond to the adipogenic stimuli during the standard 8-day differentiation protocol (Publication III). Compared to WT, these cells contained a relatively smaller number of mature adipocytes, smaller lipid droplets and, correspondingly, significantly lowered TG content. We showed that the function of Atp7a in cellular Cu homeostasis is critical in the early stage of adipogenesis (Publication III), as the induction of the master regulators of adipogenesis *Pparγ* and *C/EBPα* (Tanaka, Yoshida et al. 1997) did not occur in *Atp7a*$^{-/-}$ cells on day 3 of differentiation (D3). Analysis of proteomes of WT cells, *Atp7a*$^{+/-}$ and *Atp7a*$^{-/-}$ cell line in D0-D3 transition (Publication III) identified that proteins of the Wnt/β-catenin signaling pathway are most significantly changed in *Atp7a*$^{-/-}$ cells. Wnt/β-catenin signaling is known to be involved in adipogenesis (de Winter and Nusse 2021). Normally, in the cells undergoing adipogenesis, the classical Wnt pathway is disabled, synthesis and secretion of endogenous Wnt is inhibited, and β-catenin is phosphorylated and rapidly degraded in the cytosol. These events allow activation of the master regulator C/EBPβ and consecutive expression of other adipogenic regulators, C/EPBα and PPARγ (de Winter and Nusse 2021). However, in conditions of Cu overload, we see that the expression of Wnt, and, especially, Wnt10 is enhanced while β-catenin is stabilized and relocated to the nuclei (Publication III), which extends the Wnt/β-catenin signaling and suppresses adipogenesis. Two potential molecular mechanisms that could explain the stabilization of β-catenin are as follows. Firstly, an excess of copper is known to induce oxidative stress, and 3T3-L1-*Atp7a*$^{-/-}$ cells have exposed a significant imbalance in redox homeostasis ((Bhattacharjee, Yang et al. 2016) and Publication II). Oxidative stress can stabilize β-catenin and augment the expression of Wnt target genes (Funato, Michiue et al. 2006). Additionally, increased levels of Cu have been observed to inhibit the activity of GSK-3β (Hickey, Crouch et al. 2011). GSK-3β-mediated phosphorylation is a critical step in the degradation of β-catenin and the termination of Wnt/β-catenin signaling.

Some of the *in vitro* results obtained in the current studies were further supported by results from *in vivo* studies with *Atp7b*$^{-/-}$ mice as models of Wilson disease. We found that in the liver of WD mice β-catenin is stabilized similarly to the 3T3-L1 *Atp7a*$^{-/-}$ cells on D3, which does not occur in wild-type mice (Publication III). Moreover, the elemental analysis of liver, brain, kidney and heart in two age groups demonstrated that in younger WD animals (4w) hepatic Cu levels were already high, whereas no changes occurred in other organs compared to WT, and levels of Se in all organs were similar despite the genotype. However, disease progression in 20 weeks old WD mice leads to significantly reduced levels of Se in the liver and the brain, accompanied with excessive Cu accumulation in these organs (Publication II). Considering that excess Cu affects β-catenin signaling and causes Se deficiency both *in vitro* and *in vivo*, our findings will open new perspectives in WD-related research. More studies are needed to find out, how exactly Cu causes these events – whether it is a direct influence of an impaired gene regulation, a consequence of the redox misbalance, or a combination of both.

5.3 α-Lipoic acid mitigates the effects of excess copper by increasing selenoprotein expression and reducing oxidative imbalance

WD can be treated with Cu chelation supported with low Cu-diet and Zn salts (Uriu-Adams and Keen 2005, Das and Ray 2006, Hordyjewska, Popiołek et al. 2014). The most frequently used Cu-chelating agents, PA and TR, may have serious side effects including neurological worsening caused by increased levels of Cu in blood after its excretion from the liver (Brewer 1999, Gitlin 2003, Chen, Feng et al. 2012, Li, Chen et al. 2016). Long-term safety of TTM salts is still being investigated (Li, Chen et al. 2016, Sabine, Stefanie et al. 2022, Ambi, Stanisavljevic et al. 2023). Our data has shown that low micromolar TTM can cause Cu(I) ion release from SOD1 (Publication I). Additionally, TTM has been shown to inhibit activity of SOD1 (Juarez, Betancourt et al. 2006). Therefore, the need for a chelating agent, which removes excess Cu effectively and safely, stimulates the search for new alternatives.

Our study (Publication I) demonstrated that LA, also known as thioctic acid, exhibits a stronger affinity to Cu(I) ions compared to PA and TR but weaker than TTM. LA is a naturally occurring, short fatty acid with two sulfhydryl groups capable of engaging in redox reactions (Searls and Sanadi 1960, Ou, Tritschler et al. 1995, Packer, Witt et al. 1995). LA endogenous nature (Reed, DeBusk et al. 1951, Packer, Witt et al. 1995), known safety profile (Ziegler, Hanefeld et al. 1995, Reljanovic, Reichel et al. 1999, Yadav, Marracci et al. 2005, Shay, Moreau et al. 2009), and ability to cross the blood-brain barrier (Panigrahi, Sadguna et al. 1996, Shay, Moreau et al. 2009) in synergy with our results of its Cu(I)-binding affinity (Publication I), suggest that LA could have beneficial effects in a cellular model of Cu overload. Therefore, we used LA in 3T3-L1 *Atp7a*$^{-/-}$ preadipocytes to investigate the mechanisms by which LA operates, with a focus on its relevance to WD.

We demonstrated that LA can reverse Cu-dependent morphological changes of uniformly enlarged and flattened *Atp7a*$^{-/-}$ preadipocytes with the kinetics similar to the external chelator BCS in concentrations lower, comparable and higher than that of BCS, without causing visible signs of cytotoxicity (Publication II). However, in contrast to BCS, LA did not decrease intracellular content of Cu, neither formed low-molecular weight complexes, neither caused Cu relocation from the cytosol. However, cytosolic trafficking of Atp7a in WT in response to LA (Publication II) shows its potential contribution into safe Cu storage in vesicles. Comparison of the proteomes of the cells treated with LA or BCS for 10 days compared to non-treated *Atp7a*$^{-/-}$ cells revealed a novel bioactivity of LA. As it would be expected, proteins involved in metals homeostasis were the most significantly changed in BCS-treated cells, including down-regulated Mt-1 and Slc30a1 Zn(II)-influx regulating transporter, and up-regulated chaperone for SOD1 (Ccs) (Publication II, Fig 3). Cells treated with LA had significantly up-regulated selenoproteins regardless of their genotype (abundance of selenoproteins was increased in both WT and *Atp7a*$^{-/-}$ cells). Further elemental analysis (Publication II) confirmed that LA, but not BCS, increases cellular Se content in a dose-dependent manner and rescues *Atp7a*$^{-/-}$ preadipocytes from Se-deficiency, which means that these processes are not dependent on the cellular Cu status. Furthermore, LA was able to normalize cellular Mn content in *Atp7a*$^{-/-}$ cells and did not cause alterations in levels of other biologically significant metals, Fe and Zn. Quantification of the dynamic oxidation of GRX1-roGFP sensor in live cells demonstrated that LA was able to increase the fraction of GSH in the cytosol, mitochondria and nuclei of *Atp7a*$^{-/-}$ cells, thus improving the impaired redox balance.

Analysis of the proteome further showed that LA can reverse oxidation of Cys and Met residues of the *Atp7a*$^{-/-}$ cells' proteins (Publication II), which are located in different cellular compartments and are involved in regulation of cytoskeleton assembly (Map6, Myo1c, Tagln2), chromosome maintenance and RNA processing (Eef2, Eif4g2, Mcm6), and mitochondria function (Idh3a, Glud1). Additionally, LA was able to improve the respiratory function of mitochondria, although less efficiently compared to BCS (Publication II).

LA is an antioxidant on its own (Searls and Sanadi 1960), therefore we needed to test whether its positive effect on *Atp7a*$^{-/-}$ cells is related to the improved Se uptake and selenoprotein synthesis or it is a Se-independent antioxidant activity. Treatment with another antioxidant N-acetylcysteine (NAC) (Publication II) showed some improvement in *Atp7a*$^{-/-}$ cell morphology suggesting an important role of the oxidative stress on the cytoskeletal proteins, but NAC did not rescue mitochondrial functions. Supplementation of *Atp7a*$^{-/-}$ preadipocytes with elemental Se also showed improvements on cell size and morphology similar to LA, however did not improve respiratory function of mitochondria (Publication II).

Here, we demonstrate that LA can restore cell morphology and improve redox balance regardless of the cellular Cu status. LA reverses oxidation of many significant proteins which are involved in cytoskeleton arrangement, RNA processing, and mitochondrial functioning. LA can enhance Se uptake and up-regulate synthesis of selenoproteins. A re-evaluation of the previously obtained proteomics and RNA sequencing of *Atp7b*$^{-/-}$ mice livers (Muchenditsi, Talbot et al. 2021, Dev, Muchenditsi et al. 2022) showed that the main changes of the selenoprotein levels happen post-translationally and not at the mRNA level (Publication II). We could hypothesize that it occurs through stabilization of the RNA-processing machinery caused by the LA-reversed oxidation of the proteins; however this potential mechanism requires further studies. Positive effect of LA on the redox balance (Publication II) of the Cu-overloaded nucleus of *Atp7a*$^{-/-}$ cells (Publication III) also deserves more attention. The mechanism behind the LA-induced vesicular trafficking of Atp7a in WT cells is not clear (Publication II), and opens a field for further research. Proteins of cytoskeleton and their dynamics in Cu-overloaded cells can be affected either by direct Cu(I) binding to Cys-X-X-Cys motifs (Böhm 2015, Perrin, Roudeau et al. 2017) or by the oxidative stress (Sakai, Li et al. 2012, Wilson and González-Billault 2015, Cao, Yu et al. 2019, Goldblum, McClellan et al. 2021). We propose that LA-mediated antioxidant protection reverses the changes in the cytoskeleton of *Atp7a*$^{-/-}$ cells, but more studies are required to clarify the detailed mechanism.

In individuals with WD (both in human patients and animal models), there is a reduction in antioxidant capacity, including a decline in glutathione peroxidase levels (Gromadzka, Przybyłkowski et al. 2023). Considering our discovery that *Atp7b*$^{-/-}$ mice have lower Se levels in tissues, notably the liver, it is tempting to speculate that administering LA as a supplementary treatment could safely alleviate oxidative stress, particularly in the later stages of the disease.

6 Conclusions

The principal findings of this study are:

- From all studied chelating agents, TTM demonstrated the highest affinity for Cu(I) ions, followed by DMS, DETC, BAL, DLA, TR and PA (in the descending order) (Publication I);
- LA possesses a potent ability to protect cells from Cu-induced cytotoxicity (Publication I);
- Excess Cu causes cellular morphological abnormalities, affects redox balance, causes oxidation of Cys and Met residues in proteins and decreases respiratory function of in 3T3-L1 *Atp7a*$^{-/-}$ cells (Publication II)
- Excess Cu is associated with Se deficiency in cell and murine models of WD (Publication II);
- LA rescues impaired morphology of *Atp7a*$^{-/-}$ cells, improves cellular redox status, reverses Cu-induced protein oxidation, increases intracellular Se content and up-regulates synthesis of selenoproteins without decreasing cellular Cu levels (Publication II);
- In *Atp7a*$^{-/-}$ cells, excess Cu is predominantly localized in cell nuclei (Publication III);
- Excess Cu prevents differentiation of adipose cells prolonging Wnt/β-catenin signaling in the early adipogenesis (Publication III);
- In the liver of murine model of WD, β-catenin is stabilized similarly to the cell model (Publication III).

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Acknowledgements

First and foremost, I express my deepest gratitude to professor Peep Palumaa and professor Svetlana Lutsenko – my supervisors and, furthermore, my scientific father and mother. Thank You for Your infinite wisdom and never-ending support, for Your enthusiasm and kindness, for Your healthy criticism, and for every piece of advice. You are the ones who inspired me to move forward, and even further.

My sincerest gratitude goes to Dr. Julia Smirnova, who welcomed me into the laboratory world, trained and guided me through many difficulties that awaited me on the Path of the Biochemist.

I extend my warmest thanks to Dr. Vello Tõugu for his priceless advice, constructive critics and inspiring discussions. I thank Merlin, Andra Noormägi and all other current and former members of the lab of Metalloproteomics. Special thanks go to Olga Bragina, who introduced me to the cell culture, and to Sigrid Kirss for wonderful technical support. All the people of Metalloproteomics have created an enjoyable environment and provided lots of support. Clearly, I would not be here without you.

Here are my special thanks for every member of Lutsenko's lab, current and former, who worked shoulder to shoulder with me on the research. I especially like to thank Yu Wang, Shubhrajit Roy, Kelly Summers, Som Dev, Xuechun Zhang, Cat McCann, Alexander Zak, Li Wang, Abigael Muchenditsi and Benjamin Devenney for the incredible opportunity to learn something new, enjoy the research and have fun on a daily basis. Special thanks go to Bruce (Yixuan) Dong for his contribution into the lipoic acid and β-catenin projects. Being equally passionate about science, we even survived through the COVID-19 lockdown, work on shifts, and JHMI mask policy together.

Also, I would like to thank co-authors of my publications and colleagues from the Department of Chemistry and Biotechnology in TalTech and from the department of Physiology in JHMI.

I thank ChatGPT-3.5 for the translation of my poem from my mother tongue and for the help with the spelling and grammar of my thesis' body.

I am saying thank you to all the funding agencies for their financial support: Estonian Research Council, the Archimedes Foundation and the Education and Youth Board of Estonia, National Institutes of Health, and the Graduate School of Biomedicine and Biotechnology. I thank the „TUT Institutional Development program for 2016 – 2022" Graduate School in Biomedicine and Biotechnology which received funding from the European Regional Development Fund under program ASTRA 2014-2020.4.01.16-0032 in Estonia.

From the depths of my heart, I express my gratitude to my family. Most likely, I was a difficult daughter and a somewhat invisible sister, and certainly, almost unbearable wife throughout these years. But I love all of you and your love was (and still is) truly supporting me in the darkest times.

I extend my gratitude to all my friends who live in many countries. My special thanks go over the ocean to Jenifer and Kyle, Beatrix and Daniel, Ziggy, David, Tina and Chuck (as well as to four-pawed Taz and Obi), Valerie and David, Molly and Chris, Bella and Lindsay, as well as to Natalia and Nikita who are family no matter what. All of you turned my time abroad into something special and unforgettable. Thank you for all the fun and all the travels together!

Abstract

Copper metabolism in health and disease: focus on copper in adipogenesis and α-lipoic acid in Wilson disease

Copper is an essential micronutrient which is required as a key cofactor for proteins and enzymes participating in numerous biological functions ranging from cell respiration to synthesis of neuromediators. Misbalance in copper homeostasis can result in deleterious effects. For example, loss-of-function mutations in ATP7A and ATP7B copper transporters cause Menkes disease (MD) and Wilson disease (WD), respectively. MD is characterized by accumulation of copper in enterocytes due to dysfunction of ATP7A, peripheral copper deficiency and lethality in early childhood. In contrast, WD patients suffer from accumulation of excess copper in tissues, especially in liver and brain. Lipid metabolism is also dysregulated in WD and WD patients frequently develop liver steatosis.

WD patients require chelation therapy, supportive treatment with zinc salts, and low-Cu diets. The most frequently used Cu-chelating agents are D-penicillamine (PA) and trientine (TR). However, they have a high probability of causing serious side effects. Namely, successful excretion of copper from liver to urine often improves hepatic symptoms but worsens neurological manifestations due to increased copper in the bloodstream. Tetrathiomolybdate salts (ammonium – and bis-choline tetrathiomolybdate) have milder side effects but their long-term safety and efficiency are still being investigated (bis-choline TTM is in clinical trials).

Copper-chelating medications need to compete with cellular Cu(I)-binding proteins for copper ions to decrease excess Cu burden in the cell. However, the crucial Cu(I)-binding affinities of the copper-depleting drugs, essential for comprehending their therapeutic mechanisms, remained unknown.

In the current research, we investigated Cu(I)-binding affinities for a row of commonly known Cu-chelating agents, including PA, TR and TTM, via the process of demetallation of Cox17 and MT-2 copper-loaded proteins, which was monitored by the ESI-MS technique. One of the Cu(I)-binding agent, dihydrolipoic acid which is a reduced form of α-lipoic acid (LA), which has not been previously tested in WD therapy, was estimated to have Cu(I)-binding affinity stronger than that of TR and PA but weaker than that of TTM.

The second part of this research was to test whether LA reduces excess copper in a cellular model of copper overload and to investigate possible mechanisms of action. We found that LA does not decrease cellular copper levels but ameliorates consequences of copper overload by up-regulating selenoproteins and decreasing redox misbalance.

Finally, to better understand the role of copper in adipogenesis of the fat tissue in health and disease, we used a mouse-derived cell model of white adipose tissue. It revealed that Atp7a-dependent copper sequestration contributes to termination of β-catenin signalling during early adipogenesis.

Lühikokkuvõte

Vase metabolism tervise ja haiguse korral: fookus vasele adipogeneesil ja α-lipoehappele Wilsoni tõve korral

Vask on oluline kofaktor paljudele valkudele ja ensüümidele, mis tagavad mitmeid elutähtsaid funktsioonide alates raku hingamisest kuni neuromediaatorite sünteesini. Vase homöostaasi häiretel on organismile kahjustav toime. Näiteks mutatsioonid vase transporterites ATP7A ja ATP7B põhjustavad vastavalt Menkesi tõbe (MT) ja Wilsoni tõbe (WT). MD iseloomustab vase kuhjumine enterotsüütides ATP7A düsfunktsiooni tõttu, vase puue perifeerseted keha piirkondades ja surm varajases lapsepõlves. WT all kannatavatel patsiendidel toimub kudedes, eriti maksas ja ajus, hoopis vase kuhjumine. WT-s on lisaks häiritud lipiidide metabolism ning patsientidel leitakse sageli maksasteatoosi.

WT patsientidele on vajalik kelaatravi, toetav ravi tsingisooladega ja madala vase sisaldusega dieet. Kõige sagedamini kasutatavad vasekelaatorid on D-penitsillamiin (PA) ja trientiin (TR), kuid need ained põhjustavad tõsiseid kõrvaltoimeid. Nimelt võib edukas vase eritumine maksast uriiniga parandada küll maksa seisundit, aga samas kutsuda esile neuroloogilisi tüsistusi, kuna vase sisaldus veres suureneb. Molübdaadi sooladel (ammoonium- ja bis-koliini tetratiomolübdaat) on leebemad kõrvaltoimed, kuid nende pikaajalist ohutust ja efektiivsust alles uuritakse (bis-koliini TTM on kliinilistes uuringutes).

Vase kelaatravimite eesmärk on konkureerida raku Cu(I)-siduvate valkudega vaskioonide pärast, et vähendada liigse vase kuhjumist rakus. Nende valkude Cu(I)-sidumise afiinsus on hästi dokumenteeritud, kuid vase kelaatoritel Cu(I)-sidumise afiinsused, mis on olulised nende terapeutiliste mehhanismide mõistmiseks,olid siiani teadmata. Käesolevas töös uurisime laialt kasutuses olevate vasekelaatorite, sealhulgas PA, TR ja TTM-i Cu(I)-sidumise afiinsusi, kasutades vasega seotud valkude Cox17 ja MT-2 demetallisatsiooni protsessi, mida jälgisime ESI-MS-tehnikaga. Leidsime, et ühel Cu(I)-siduvat ainel, dihüdrolipoehapel, mis on α-lipoehappe (LA) redutseeritud vorm ja mida ei ole varem WD ravis testitud, Cu(I)-sidumise afiinsus on tugevam kui TR ja PA, kuid nõrgem kui TTM.

Selle töö teise osa eesmärgiks oli testida, kas LA vähendab üleliigse vase kontsentratsiooni rakus vase kuhjumise puhul ning uurida võimalikke toimemehhanisme. Me leidsime, et LA ei vähenda rakusisese vase hulka kuid parandab vase kuhjumise tagajärgi selenoproteiinide sünteesi ülesreguleerides ja vähendades redoks tasakaalu häireid.

Lõpuks, et paremini mõista vase rolli rasvkoe arengus tervises ja haiguses, kasutasime hiirest pärinevat rasvkoe eellasrakkude mudelit. See näitas, et Atp7a-sõltuv vase sekvesteerimine panustab β-kateniini signaliseerimise terminatsiooni varajases adipogeneesis.

Appendix

Publication I

Smirnova J., **Kabin E.**, Järving I., Bragina O., Tõugu V., Plitz T., Palumaa P.
Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent.
Sci Rep, 8(1):1463. doi: 10.1038/s41598-018-19873-2.

SCIENTIFIC REPORTS

OPEN

Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent

Received: 27 October 2017
Accepted: 9 January 2018
Published online: 23 January 2018

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Wilson disease is an autosomal recessive genetic disorder caused by loss-of-function mutations in the P-type copper ATPase, *ATP7B*, which leads to toxic accumulation of copper mainly in the liver and brain. Wilson disease is treatable, primarily by copper-chelation therapy, which promotes copper excretion. Although several de-coppering drugs are currently available, their Cu(I)-binding affinities have not been quantitatively characterized. Here we determined the Cu(I)-binding affinities of five major de-coppering drugs – D-penicillamine, trientine, 2,3-dimercapto-1-propanol, *meso*-2,3-dimercaptosuccinate and tetrathiomolybdate – by exploring their ability to extract Cu(I) ions from two Cu(I)-binding proteins, the copper chaperone for cytochrome c oxidase, Cox17, and metallothionein. We report that the Cu(I)-binding affinity of these drugs varies by four orders of magnitude and correlates positively with the number of sulfur atoms in the drug molecule and negatively with the number of atoms separating two SH groups. Based on the analysis of structure-activity relationship and determined Cu(I)-binding affinity, we hypothesize that the endogenous biologically active substance, α-lipoic acid, may be suitable for the treatment of Wilson disease. Our hypothesis is supported by cell culture experiments where α-lipoic acid protected hepatic cells from copper toxicity. These results provide a basis for elaboration of new generation drugs that may provide better therapeutic outcomes.

Wilson disease is characterized by loss-of-function mutations in a P-type copper ATPase, ATP7B, which is expressed mostly in liver[1,2]. The WD protein has dual roles, it is functioning in the transport of copper into the trans-Golgi network, for incorporation into the plasma protein ceruloplasmin[3,4], and into the bile, for excretion of excess cellular copper[5]. Defective ATP7B functioning is causing reduced incorporation of copper into ceruloplasmin and copper accumulation primarily in liver and in brain, leading to liver disorders and/or neuropsychiatric symptoms[6].

Unlike many other genetic disorders, Wilson disease is treatable, primarily by copper-chelation therapy, which promotes copper excretion. Several de-coppering drugs have been used for the treatment of Wilson disease, two of which have also been approved by the FDA. D-penicillamine (PA), the first orally administered copper-chelating agent available, was approved for therapeutic use in 1956[7]. PA induces copper excretion into urine[8]; however, it also has many adverse effects[9]. The second oral copper chelating drug, trientine (TR), was approved in 1982. TR also acts by enhancing urinary excretion of copper, however, it is better tolerated than PA[9,10]. In addition to these two major drugs, two other copper-chelating compounds have been used for treatment of Wilson disease in the past: an injectable drug, British anti-Lewisite (BAL or dimercaptopropanol), which was used in the UK in 1951[11] and dimercaptosuccinate (DMS), which has been used in China for half a century already[12]. In Western medicine, BAL and DMS are used primarily for the treatment of arsenic, mercury, and lead poisoning[13,14]. The fifth de-coppering drug selected for our study is tetrathiomolybdate (TTM), which was introduced in 1984 and was used in a limited number of patients with Wilson disease. Initial studies with ammonium TTM[15] and a recently completed Phase II clinical study with bis-choline TTM[16], demonstrate that TTM acts

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rapidly, resulting in improvements in copper control and this is accompanied by improved neurologic outcomes and stabilization of liver function, with a favorable safety profile[17].

De-coppering drugs should compete for copper ions with cellular Cu(I)-binding proteins, for which the Cu(I)-binding affinities are known[18]. However, the Cu(I)-binding affinities of de-coppering drugs, which are of fundamental importance for understanding their therapeutic action, are currently not known. In the current study, Cu(I)-binding affinities of de-coppering drugs were determined from their competition with two cellular Cu(I)-binding proteins, Cox17 and metallothionein (MT), which have different Cu(I)-binding affinities[18]. These proteins were reconstituted to form Cu_1Cox17 and Cu_{10}MT metalloforms and incubated with increasing concentrations of the de-coppering drugs. Demetallation of the copper proteins was monitored by electrospray ionization mass spectrometry (ESI MS)[18] and the copper-binding affinities were determined from dose-dependent demetallation curves. The absolute values for dissociation constants (K_d) of Cu(I)-drug complexes were obtained by comparing demetallation potencies of de-coppering drugs with those of copper chelators with known copper-binding affinities such as dithiotreitol (DTT) and diethyl dithiocarbamate (DETC). Analysis of structure-activity relationships suggested that an endogenous biologically active substance, α-lipoic acid (LA), may serve as a potential de-coppering drug. LA was able to protect hepatic cells from copper toxicity *in vitro*, which further supports its potential as an anti-copper agent.

Methods

Reagents. Chemical reagents: PA, TR, BAL, DMS, LA, ammonium TTM, diethylammonium DETC were purchased from Sigma-Aldrich, DTT (ultrapure) from USB Corporation, DLA from Santa Cruz Biotechnology. Bischoline TTM (WTX101) was provided by Wilson Therapeutics AB. Rabbit apo-MT2A was purchased from Bestenbalt LLC and apo-$Cox17_{3S\text{-}S}$ was produced as described previously[19]. Recombinant human Cu,Zn-SOD was purchased from Biovision Inc. All solutions were prepared immediately before the experiments in 20 mM ammonium acetate, pH 7.3 buffer in the absence of organic solvents. To avoid oxidation of DTT and proteins, the buffer was saturated with argon.

Reconstitution of proteins with Cu(I) ions. Lyophilized apo-Cox17 was dissolved in 20 mM ammonium acetate, pH 7.3 containing 50 μM DTT and metallated at 1 μM concentration with one equivalent of Cu(I)–DTT complex in the presence of 50 μM DTT. Cu_{10}MT2 was reconstituted in the presence of 10 mM DTT from 3 μM apo-MT2 dissolved in 20 mM ammonium acetate, pH 7.3 by addition of 10-fold excess of Cu(I)–DTT. The stock solution of Cu(I)–DTT complex was prepared by dissolving Cu(II)-acetate at 1.3 mM concentration in argon-saturated 20 mM ammonium acetate containing 10 mM DTT at pH 7.3.

ESI-MS settings and determination of copper-binding affinities. DTT is suitable for applications in ESI MS as being a nonionic compound it does not suppress the ionization efficiency of proteins during ESI-MS substantially, which enables detection of protein peaks even if ligand is present in millimolar concentrations. The Cu(I)-binding affinity of DDT has been studied extensively[18,20], and was therefore used as a standard for determination of Cu(I)-binding affinities of DETC, PA, TR, TTM, BAL and DMS. It should be mentioned that DTT is air sensitive and its Cu(I)-binding affinity depends on pH of solution[20]. Therefore all experiments with DTT have been conducted in oxygen-free argon-saturated solutions at pH = 7.3, where the Cu(I)-binding affinity has reliably been determined ($K_d = 5.01 \times 10^{-16}$ M)[20]. Since DETC, PA, TR TTM and DMS are ionic compounds, they lead to ionic suppression of protein peaks in ESI MS spectra. However, ionic suppression is low at submillimolar concentrations and presumably similar for all protein peaks. In a standard experiment, the reconstituted protein was incubated with increasing concentrations of copper chelators for 1 min and samples were injected into the electrospray ion source of an Agilent Technology 6540 UHD Accurate-Mass Q-TOF MS instrument (Agilent) by a syringe pump at 7 μL/min. ESI-MS spectra were recorded for 10 min in the m/z region from 100 to 3,000 Da using the following instrument parameters: capillary voltage = 3500 V, drying gas = 4 l/min, drying gas temperature 100 °C, nebulizer 15 psig, skimmer voltage = 65 V. ESI MS spectral composition was stabilized after 5 min of monitoring time and spectra between 8–10 min were averaged for further analysis. Obtained ESI MS spectra were deconvoluted using the Mass Hunter software (Agilent). The fractional content of metallated protein forms was calculated from the integrated peak areas for metallated and nonmetallated protein forms. C_{50} values for compounds have been calculated by fitting the dose-dependent demetallation curve to the simple 1:1 binding isotherm (in case of Cu_1Cox17) or Hill equation (in case of Cu_{10}MT). K_d values for the Cu(I)-protein complexes at pH 7.3, 20 mM ammonium acetate, and 25 °C were determined using previously determined K_d values for protein-Cu(I) complexes[18] and the re-estimated apparent K_d for the Cu(I)–DTT complex of 5.01×10^{-16} M[20]. K_d values for PA, TR, BAL, DMS and TTM, were determined by the ability of these ligands to extract Cu(I) ions from Cu_1Cox17 or Cu_{10}MT in comparison with DTT (in case of Cu_1Cox17) or DETC (in case of Cu_{10}MT) respectively as described above.

Demetallation of Cu,Zn-SOD with TTM. Commercial lyophilized Cu,Zn-SOD was dissolved in argon-saturated 20 mM ammonium acetate pH 7.3 to the final concentration of 5 μM, and injected at different time points into an Agilent Technology 6540 UHD Accurate-Mass Q-TOF MS instrument by a syringe pump at 7 μL/min. ESI-MS spectra were recorded for 10 min in the m/z region from 500 to 3,000 Da using the instrument parameters presented above. ESI MS spectrum of the sample exposed two peaks: Cu,Zn-SOD and partially demetallated Zn-SOD (Fig. S9A). Following incubation of the sample with 10 μM ammonium TTM, there was a time-dependent shift in intensities of the peaks, whereas peak of Cu,Zn-SOD disappeared and peak of apo-SOD appeared in the spectrum (Fig. S9A). Spectral changes could be interpreted with extraction of Cu(I) and Zn(II) ions from Cu,Zn-SOD and Zn-SOD and formation of Cu-SOD and apo-SOD. The demetallation process occurred with a half-life of 23 ± 14 min (Fig. S9B).

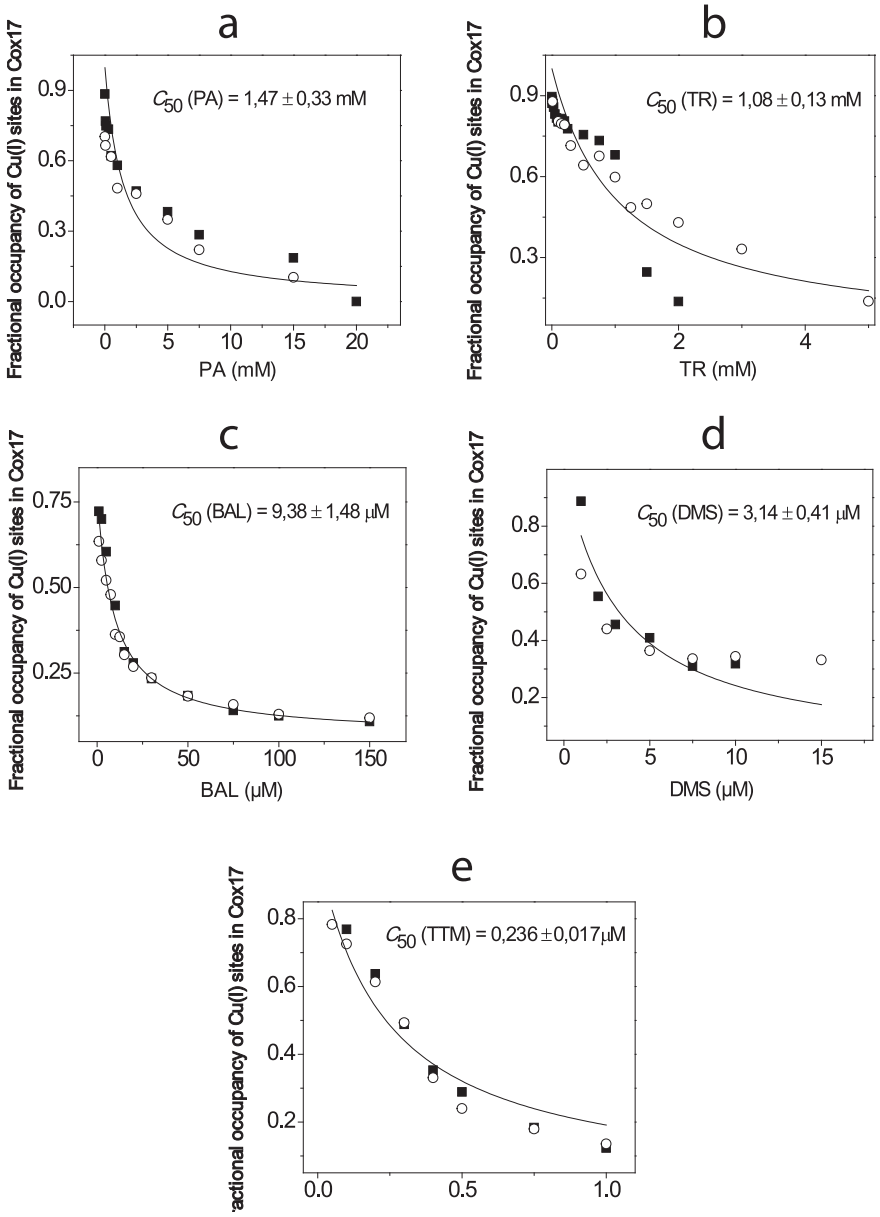

Figure 1. Determination of the relative Cu(I)-binding affinity of de-coppering drugs in competition with Cu_1Cox17. Fractional occupancy of Cu(I)-binding sites in Cox17 at different concentrations of PA (**a**), TR (**b**), BAL (**c**), DMS (**d**) and TTM (**e**) in a metal competition experiment. Conditions: Cox17 1 μM; 20 mM ammonium acetate, pH = 7.3, DTT 50 μM; T = 25 °C. Results of duplicate experiments are presented with different symbols. The solid line shows the fitting curve with hyperbolic equation (y = P1*(1 − [x/(P2 + x)]) + P3), where P2 = C_{50}.

Cell culture. Huh7 hepatocyte derived cellular carcinoma cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and 50 U/ml penicillin, 50 μg/ml streptomycin solution (PAA) in an incubator at 37 °C and 5% CO_2. The medium was changed every 2–3 days and cells were split using Trypsin-EDTA solution (Gibco).

Ligand	C_{50} (mM) for Cu_1Cox17	K_d (M)
DTT	3.10 ± 0.26	5.01×10^{-16}*
DETC	0.00528 ± 0.00032	8.53×10^{-19}
PA	1.47 ± 0.33	2.38×10^{-16}
TR	1.08 ± 0.13	1.74×10^{-16}
BAL	0.00938 ± 0.00148	1.52×10^{-18}
DMS	0.00314 ± 0.00041	5.07×10^{-19}
TTM	<0.001	$<1.6 \times 10^{-19}$
DLA	0.498 ± 0.098	8.05×10^{-17}
Ligand	**C_{50} (mM) for $Cu_{10}MT$**	**K_d (M)**
DETC	0.772 ± 0.087	8.53×10^{-19}
TTM	0.0211 ± 0.0014	2.32×10^{-20}

Table 1. Determination of K_d values for de-coppering drugs. The efficiency of de-coppering drugs to demetallate Cu_1Cox17 and/or $Cu_{10}MT$ (C_{50}) was in linear correlation with K_d values for Cu(I)-binding complexes (conditions: 20 mM ammonium acetate, pH 7.3, 25 °C). *Taken from reference[20].

Cell viability measured by WST-1. The effects of $CuCl_2$ on the cells were determined using the cell viability assay WST-1 (Roche). WST-1 assay allows colorimetric measurement of cell viability due to reduction of tetrazolium salts to water-soluble formazan by viable cells. 105 cells were seeded in triplicates in a 96 well plate and cultivated for 24 h. Various concentration (0–100 μM) of $CuCl_2$ were added and the measurements were performed 24 hours after cells treatment. The experiments with no $CuCl_2$ added were used as a negative control. 5 μl/well of WST-1 reagent was added to 100 μl of cell culture medium, incubated at 37 °C for 2 h and absorbance was measured at 450 nm using TECAN Genios Pro microplate reader. For LA testing, cells in triplicates were preincubated with various concentrations (0–100 μM) of LA (dissolved in ethanol) for 24 h and medium was changed to fresh medium, containing 50 μM of $CuCl_2$ and various concentrations (0–100 μM) of LA. Cell viability was determined after 24 h incubation with WST-1 test. Experiments were performed twice.

Results and Discussion

Demetallation of Cu_1Cox17 by the de-coppering drugs. All de-coppering drugs were able to demetallate Cu_1Cox17; however, it occurred at different concentrations with different drugs. Demetallation potency of compounds was defined as the concentration where 50% of Cu_1Cox17 was demetallated and was denoted as C_{50}. PA and TR extracted Cu(I) from Cu_1Cox17 at millimolar concentrations with the corresponding $C_{50}(PA) = 1.47$ mM (Figs 1A and S1) and $C_{50}(TR) = 1.08$ mM (Figs 1B and S1). BAL extracted Cu(I) from Cu_1Cox17 at micromolar concentrations with $C_{50}(BAL) = 9.38$ μM (Figs 1C and S3). DMS and TTM showed very low C_{50} values: $C_{50}(DMS) = 3.14$ μM (Figs 1E and S4) and the $C_{50}(TTM)$ was less than 1 μM (Figs 1F and S5). C_{50} values were determined also for reference compounds, DTT ($C_{50}[DTT] = 3.10$ mM) (Fig. S6) and DETC ($C_{50}[DETC] = 5.28$ μM) (Fig. S7). Based on the linear relationship between C_{50} and K_d values and the known K_d value for DTT[20], we calculated the K_d values for studied compounds and the results are presented in Table 1. As an example we present here the mathematics for calculation of K_d value for DETC. DETC was 587 times more effective Cu(I) chelator than DTT ($C_{50}[DTT] / C_{50}[DETC] = 587$), which is in line with earlier results obtained with a different ESI-MS setup[18]. Consequently the K_d for Cu(I)-DETC complex should also be 587 times lower as that for DTT and therefore $K_d(DETC) = 5.01 \times 10^{-16} / 587 = 8.53 \times 10^{-19}$ M.

Demetallation of $Cu_{10}MT$ by the de-coppering drugs. In the second series of experiments, we explored the ability of different de-coppering drugs to extract copper from $Cu_{10}MT$, which has 42 times higher Cu(I)-binding affinity as compared to Cu_1Cox17[18]. TTM demetallated $Cu_{10}MT$ at micromolar concentrations through a stepwise process. First, a complex $Cu_{10}MT$-TTM was formed with 1:1 stoichiometry (Fig. 2A). Multiple peaks appeared at higher concentrations of TTM, indicative of the binding of a second, third and fourth TTM molecule to the complex and the simultaneous build-up of apo-MT (Fig. 2A). The binding of multiple TTM ions appeared to lead to the opening of Cu(I)-thiolate clusters and dissociation of Cu(I) ions from the protein. As dissociation of Cu(I)-thiolate clusters of $Cu_{10}MT$ occurred cooperatively we did not observe hyperbolic demetallation curves like in case of Cu_1Cox17. By this reason fitting of the $Cu_{10}MT$ demetallation curves by the influence of TTM was performed by using the Hill equation[18]. A $C_{50}(TTM)$ value of 21.1 μM was obtained by taking into account all CuMT-TTM complexes (Fig. 2B). Both salts of TTM, ammonium and bis-choline TTM, behaved similarly in ESI-MS experiments. Earlier HPLC/ICP experiments have also demonstrated the binding of TTM to CuMT[21]; however, the exact composition of ternary complexes remained unclear.

DETC also extracted Cu(I) from $Cu_{10}MT$ (Fig. 2C) at millimolar concentrations and the corresponding $C_{50}(DETC)$ was equal to 0.77 mM (Fig. 2D). Other de-coppering drugs were unable to demetallate $Cu_{10}MT$ up to millimolar concentrations. Based on the linear relationship between C_{50} and K_d values and the known K_d value for DETC (see Table 1), the K_d value for TTM was calculated ($K_d = 2.32 \times 10^{-20}$ M) and inserted into Table 1. The absolute value of K_d for $Cu_{10}MT$ of 4.1×10^{-16} M was first determined in 2010[18] based on the absolute value of available K_d for the Cu(I)-DTT complex known at that time. Subsequently, the K_d for the Cu(I)-DTT complex has been corrected from 7.94×10^{-12} M[22] to 5.01×10^{-16} M (pH 7.3)[20]. Based on the corrected absolute dissociation constant for Cu(I)-DTT complex, the corrected K_d for $Cu_{10}MT$ was calculated to be 2.59×10^{-20} M.

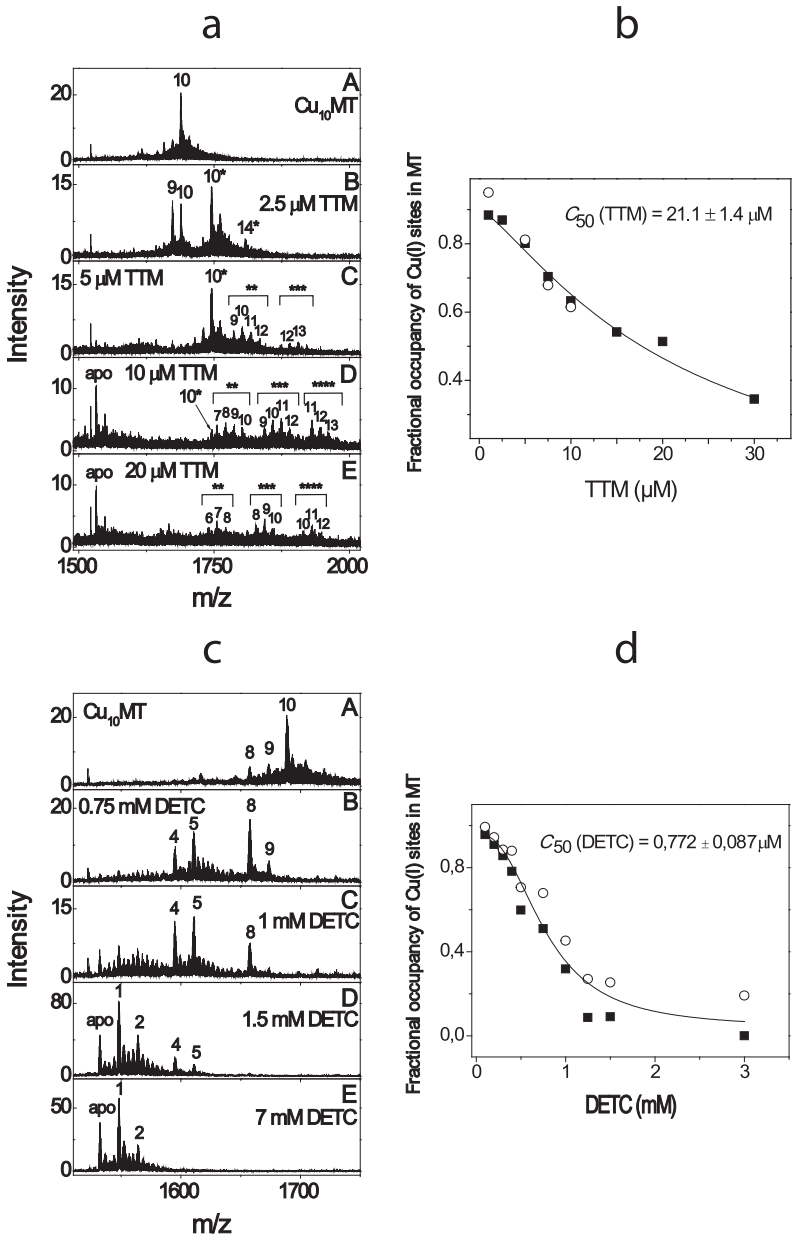

Figure 2. Determination of the relative Cu(I)-binding affinity of TTM and DETC in competition with $Cu_{10}MT$. ESI-MS spectra of $Cu_{10}MT$ in the presence of 1 μM–20 μM TTM (**a**) and 0.1–7 mM DETC (**c**). Conditions: MT 3 μM; 20 mM ammonium acetate, pH = 7.3, DTT 10 mM; T = 25 °C. Ions with a charge state + 5 are shown; numbers on the peaks denote the metal stoichiometry of the complex. Number of asterisks denotes number of TTM molecules in the complex. Fractional occupancy of Cu(I)-binding sites in MT at different concentrations of TTM (**b**) and DETC (**d**) in a metal competition experiment. Results of duplicate experiments are presented with different symbols. The solid line shows the fitting curve with Hill equation (y = START + (END − START) * x^n / (K^n + x^n)), where $K = C_{50}$.

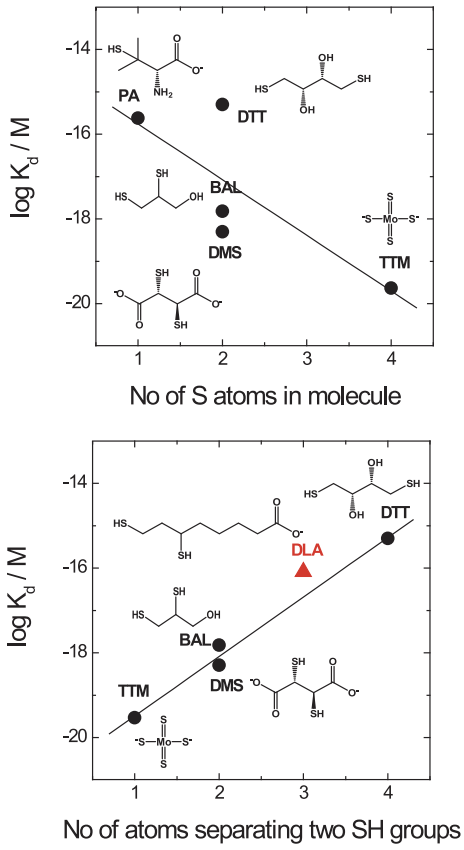

Figure 3. Structure-activity relationships. Correlation between the number of sulfur atoms in the molecule of copper chelators and their Cu(I)-binding affinity (**a**); correlation between the number of carbon atoms separating two SH groups in the molecule of copper chelators including DLA and their Cu(I)-binding affinity (**b**).

TTM had almost equal copper-binding affinity ($K_d = 2.32 \times 10^{-20}$ M) to that of MT. Indeed, TTM can form a 1:1 TTM-Cu_{10}MT complex at concentrations equimolar to MT, whereas at higher concentrations (from 5 to 30 μM) it can extract Cu(I) ions from Cu_{10}MT (Fig. 2A).

Clinical implications of demetallation by de-coppering drugs. The obtained information can be applied to the clinical context. For a sustainable treatment effect in Wilson disease, de-coppering drugs must extract copper from intracellular copper stores, which have, however, different Cu(I)-binding affinities. MT has a high affinity for Cu(I) ions and only one de-coppering drug studied, TTM, had the ability to demetallate Cu_{10}MT at low micromolar concentrations. For estimation of the physiological concentration of TTM, we used data from oncology patients where TTM 180 mg/day resulted in C_{max} of 5.7 μM[23]. At such concentrations, TTM can partially demetallate CuMT, which most probably forms the basis of its fast and efficient therapeutic action.

MT has one of the highest copper-binding affinities among cellular Cu(I) proteins[18] and TTM may therefore extract copper also from several other essential copper proteins. Other cellular copper chaperones and copper transporters have copper-binding affinities similar to that of Cox17[18] and most probably they can also be demetallated by TTM. Intracellular copper enzymes Cu,Zn-SOD (antioxidative defense) and mitochondrial cytochrome c oxidase (mitochondrial electron transfer) have copper-binding affinities comparable to that of MT[18]. However, the dissociation of metal ions from the active sites of these enzymes is very slow[18], which might protect them from fast demetallation by TTM. It has been shown that TTM does not inhibit mitochondrial cytochrome c oxidase activity in hepatic mitochondrial preparations at concentrations up to 100 μM even after 16-h incubation[24], strongly suggesting that TTM cannot demetallate cytochrome c oxidase. Nevertheless, in the same study, TTM inhibited Cu,Zn-SOD in a time-dependent manner[24]. Our data confirm that 10 μM TTM can extract copper from 5 μM Cu,Zn-SOD with a half-life of 23 min (Fig. S8). Thus, treatment with high doses of TTM may demetallate Cu,Zn-SOD, which should be taken into account in refinement of therapeutic doses for TTM.

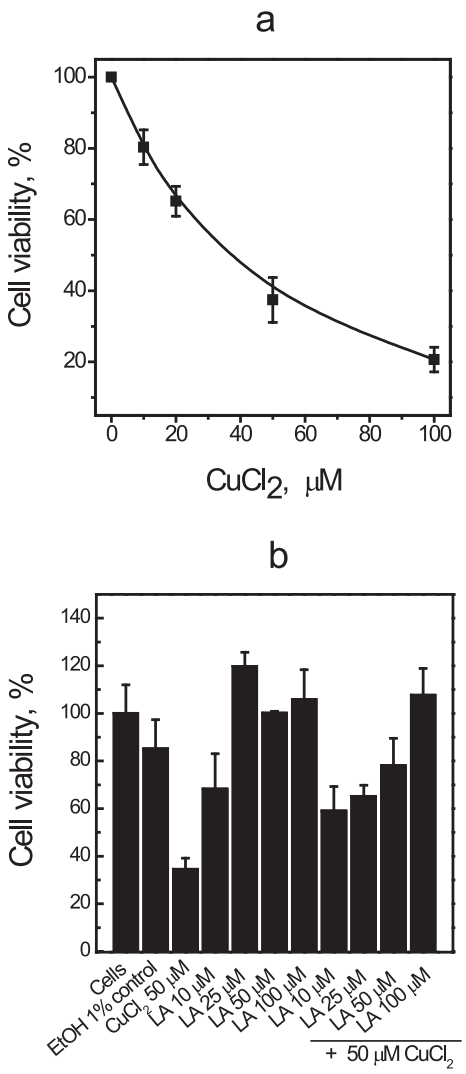

Figure 4. Effect of LA on cellular copper toxicity. Viability of Huh7 cells determined by WST-1 assay after 24 h of exposure to $CuCl_2$ (**a**). Viability of Huh7 cells after 24 h incubation with different concentrations of LA and additional treatment with 50 μM $CuCl_2$ for next 24 h, measured with WST-1 test. Data are shown as mean ± SD.

The K_d values for PA and TR were 2.38×10^{-16} M and 1.74×10^{-16} M, respectively. Therapeutic doses of PA and TR (750–1500 mg/day[17]) are substantially higher than that for TTM. The current study suggests that PA and TR cannot effectively compete with CuMT at therapeutic concentrations (approximately 50–100 μM); however, they can partially compete with copper chaperones. At therapeutic concentrations, BAL and DMS effectively demetallated copper chaperones and only partially CuMT; however, the action of DMS is restricted mainly to the extracellular space since it is unable to cross biological membranes.

Structure-affinity relationship analysis and identification of new copper-binding drugs. The Cu(I)-binding affinity of the de-coppering drugs studied varied by four orders of magnitude depending upon the molecular structure of the compounds. One can speculate that the number of sulfur-containing groups in the molecule, which are well adapted for the coordination of Cu(I) ions, is an important determinant of the binding affinity. Indeed, a positive correlation between the Cu(I)-binding affinities and the number of the S-atoms in the molecule (Fig. 3A) was observed: TTM, with four S-atoms, has the highest Cu(I)-binding affinity, and PA with only one S-atom has the lowest affinity. However, the Cu(I)-binding affinity of DTT is considerably different

from that of BAL and DMS, although all these compounds carry two SH groups. Thus, not only the number, but also the position of the sulphur-containing groups appears to affect the Cu(I)-binding affinity of compounds. Indeed, the Cu(I)-binding affinity decreased substantially with increasing number of atoms between the sulfhydryl groups: TTM > BAL, DMS > DTT (Fig. 3B). This structure-affinity relationship explains why TTM has the highest Cu(I) affinity of all de-coppering agents studied and opens new perspectives for the rational design of new copper-binding drugs.

In order to test the validity of this structure-activity relationship and its applicability for design of new copper-binding drugs, we looked for biomolecules with three intercalating atoms between two SH groups and found that dihydrolipoic acid (DLA) fits these criteria. The experimental Cu(I)-binding affinity of DLA was equal to $K_d = 8.05 \times 10^{-17}$ M (Fig. S9, Table 1), which fits well the predicted relationship between logK_d and the number of atoms between two SH groups, thus confirming the structure-activity relationship. The Cu(I)-binding affinity of DLA was 2 and 3 times higher than that of TR and PA respectively, however, the affinity was not high enough to directly decopper intracellular high-affinity Cu stores like Cu_{10}MT when tested experimentally (data not shown). *In vivo*, DLA is formed from α-lipoic acid (LA) by reduction in a cellular environment. LA is a well-tolerated food supplement that is able to cross biological membranes. Considering its higher Cu(I)-binding affinity than that of chelators approved for the treatment of Wilson disease (PA, TR), good membrane and blood brain barrier permeability[25] as well as very good tolerability[26], we suggest that LA might be a suitable de-coppering drug for treatment of Wilson disease. Some exploratory attempts to use LA in Wilson disease have been reported[27,28], however, the potency of LA in the treatment of Wilson disease has not been explored in cellular experiments.

Effect of LA on cellular copper toxicity. To further explore the potential of LA as a de-coppering drug we performed preliminary experiments with Huh7 hepatic cell lines to test the putative protective effect of LA against copper toxicity. Similar experimental setup has been used earlier to demonstrate the protective effect of PA on copper toxicity exerted on hepatic cell lines[29,30]. The results in Fig. 4 demonstrate that copper is toxic to Huh7 cells with LD_{50} of approximately 30 μM of $CuCl_2$ (Fig. 4a) and LA exerts a clear dose-dependent protective effect on toxicity of 50 μM $CuCl_2$ (Fig. 4b). The protective effect on cellular level supports the potential therapeutic role of LA in treatment of Wilson disease, however, the full spectrum of therapeutic activities of LA should be evaluated in further cellular and animal studies.

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Acknowledgements

This work was supported by the Estonian Ministry of Education and Research (grant IUT 19-8 and 19-9) and by a research grant from Wilson Therapeutics AB. The authors thank Richard Tamme and Emma Marshman for medical writing support.

Author Contributions

P.P. and T.P. conceived the study; J.S., E.K. I.J. and O.B. carried out the main experiments; J.S., E.K., V.T., O.B. and P.P. analyzed the data; P.P. wrote the main draft of the paper and all authors participated in revising the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-19873-2.

Competing Interests: The authors declare that they have no competing interests.

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Publication II

Kabin E., Dong Y., Roy S., Smirnova J., Smith J. W., Ralle M., Kelly Summers K., Yang H., Dev S., Wang Y., Devenney B., Cole R. N., Palumaa P., Lutsenko S.
α-lipoic acid ameliorates consequences of copper overload by up-regulating selenoproteins and decreasing redox misbalance.
Proc Natl Acad Sci, 120 (40), #e2305961120. DOI: 10.1073/pnas.2305961120.

PNAS RESEARCH ARTICLE | CELL BIOLOGY

α-lipoic acid ameliorates consequences of copper overload by up-regulating selenoproteins and decreasing redox misbalance

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Edited by Vadim Gladyshev, Harvard Medical School, Boston, Massachusetts; received April 13, 2023; accepted August 18, 2023

α-lipoic acid (LA) is an essential cofactor for mitochondrial dehydrogenases and is required for cell growth, metabolic fuel production, and antioxidant defense. In vitro, LA binds copper (Cu) with high affinity and as an endogenous membrane permeable metabolite could be advantageous in mitigating the consequences of Cu overload in human diseases. We tested this hypothesis in 3T3-L1 preadipocytes with inactivated Cu transporter Atp7a; these cells accumulate Cu and show morphologic changes and mitochondria impairment. Treatment with LA corrected the morphology of *Atp7a*$^{-/-}$ cells similar to the Cu chelator bathocuproinedisulfonate (BCS) and improved mitochondria function; however, the mechanisms of LA and BCS action were different. Unlike BCS, LA did not decrease intracellular Cu but instead increased selenium levels that were low in *Atp7a*$^{-/-}$ cells. Proteome analysis confirmed distinct cell responses to these compounds and identified upregulation of selenoproteins as the major effect of LA on preadipocytes. Upregulation of selenoproteins was associated with an improved GSH:GSSG ratio in cellular compartments, which was lowered by elevated Cu, and reversal of protein oxidation. Thus, LA diminishes toxic effects of elevated Cu by improving cellular redox environment. We also show that selenium levels are decreased in tissues of a Wilson disease animal model, especially in the liver, making LA an attractive candidate for supplemental treatment of this disease.

copper | α-lipoic acid | selenoprotein | oxidative stress | Wilson disease

Significance

Copper is an essential biometal; however, Cu overload causes oxidative stress and debilitating pathologies, such as Wilson disease (WD). Renal Cu toxicity is observed in Menkes disease, and changing copper levels are seen in other neurologic disorders and aging. Copper chelators provide health benefits but have shortcomings, including limited permeability through brain barriers. We demonstrate that a natural metabolite—α-lipoic acid (LA)—decreases Cu toxicity similarly to chelation but acts through a different mechanism. While Cu chelators limit available Cu, LA boosts antioxidant defense by increasing levels of selenium and selenoproteins. Our finding that selenium is low in tissues of the WD animal model suggests that LA may have therapeutic value for WD, especially given its favorable safety profile.

Cu is an essential cofactor of enzymes participating in numerous biological processes ranging from cellular respiration to biosynthesis of neuromediators (1–4). Cu deficiency, observed in Menkes disease (MNK), is associated with lower activity of Cu-dependent enzymes, numerous metabolic abnormalities, and early death (4–8). MNK is caused by genetic mutations in ATP-driven Cu(I) transporter ATP7A. Inactivation of ATP7A results in the entrapment of dietary Cu in enterocytes, impaired Cu export from the intestine to the rest of the body, and systemic Cu deficit. Despite Cu deficiency, MNK patients exhibit paradoxical Cu accumulation in kidneys (9, 10), which worsens with Cu supplementation therapy and complicates patients' treatment. In another disorder of Cu homeostasis, Wilson disease (WD), mutations in the Cu(I) transporter ATP7B disrupt Cu export from the liver, disabling the major route of Cu removal from the body. WD patients suffer from Cu accumulation in tissues, especially in the liver and the brain, which causes oxidative stress, mitochondrial and metabolic dysfunction, and hepatic, neurologic, and behavioral pathologies (8, 11–14). WD is managed using Cu chelation therapy, supportive treatment with zinc salts, and low-Cu diets (4, 5, 11). The most frequently used Cu-chelators D-penicillamine (PA) and trientine (TR) (12, 15), while beneficial, have very slow response times and side effects, including neurological worsening (12, 16–18). Thiomolybdate salts have milder side effects and a faster response time (30 d), but their long-term safety and efficiency are still being investigated (15, 19, 20).

The need for safe and fast means of diminishing Cu toxicity stimulates search for new alternatives. A recent in vitro study found that alpha-lipoic acid (LA) binds Cu(I) ions with higher affinity than PA and TR (21). Lipoic acid, also known as thioctic acid, is an endogenously produced short fatty acid. It contains two sulfhydryl groups that can participate in redox reactions and also bind Cu(I) ions (21–24). LA is synthesized de novo from octanoic acid in mitochondria and serves as a cofactor for at least five dehydrogenase complexes (24–26). LA is already used for the treatment of diabetic neuropathy and is available as a dietary supplement (24, 27). Significantly, LA can pass through the blood–brain barrier—in contrast to PA or TR (27, 28). Consequently, we tested whether LA has beneficial effects in a cellular model of Cu overload and investigated the mechanisms of LA action and relevance of our findings to WD.

Author contributions: E.K., J.S., J.W.S., K.S., R.N.C., P.P., and S.L. designed research; E.K., Y.D., S.R., J.S., J.W.S., M.R., K.S., and Y.W. performed research; H.Y., S.D., B.D., and R.N.C. contributed new reagents/analytic tools; E.K., Y.D., J.S., J.W.S., M.R., K.S., Y.W., P.P., and S.L. analyzed data; S.R., K.S., H.Y., S.D., and Y.W. read and commented on the manuscript; B.D. was involved in animal husbandry, read and commented on the manuscript; and E.K., Y.D., P.P., and S.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2305961120/-/DCSupplemental.

Published September 26, 2023.

Results

Elevated Cu Induces Morphological Changes in *Atp7a*$^{-/-}$ 3T3-L1 Cells. To determine whether LA ameliorates consequences of Cu overload, we used *Atp7a*$^{-/-}$-3T3-L1 cells (29), further referred to as KO. These cells lack Cu-transporter Atp7a and thus are unable to export Cu, which causes intracellular Cu accumulation (*SI Appendix*, Fig. S1 *A–F*). Nondifferentiated cells were used because LA inhibits differentiation of adipocytes (30–32). The obvious difference between control and KO cells is their morphology. Nondifferentiated 3T3-L1 preadipocytes can be either elongated or flattened (Fig. 1*A*), whereas KO cells are uniformly flattened and enlarged (Fig. 1*B*). Treatment with the Cu chelator BCS linked these morphological changes to cellular Cu content. Short-term treatment (24 to 48 h) did not significantly change cellular Cu and did not affect KO morphology (Fig. 1 *C–F* and *SI Appendix*, Fig. S2 and Table S1). After 10 d of treatment, Cu levels decreased significantly and were closer to that of WT (4.03 ± 0.86 fg Cu/cell and 3.14 ± 0.49 fg Cu/cell, respectively, Fig. 1 *C* and *G*) Similarly, the fraction of flattened KO cells decreased to 61.3 ± 4.0 % and was close to that of WT (51.9% ± 9%, Fig. 1*D* and *SI Appendix*, Fig. S2 and Table S1). By day 17, Cu levels in the BCS-treated KO cells returned to nontreated WT levels and, morphologically, KO cells were indistinguishable from WT (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S2). The majority of nontreated KO cells (87.1 ± 3.0%) remained enlarged and flattened (Fig. 1 *D–G* and *SI Appendix*, Fig. S2 and Table S1). The need for a long treatment time can be ascribed to BCS being membrane impermeable and only limiting Cu entry into cell, instead of sequestering intracellular Cu or facilitating Cu efflux.

Lipoic Acid Corrects *Atp7a*$^{-/-}$ Cell Morphology without Changing Cellular Cu Content. Treatment with LA, which is membrane permeable, did not improve cell morphology after 24- or 48-h treatment (Fig. 1 *D* and *F* and *SI Appendix*, Fig. S2 and Table S1). However, after 10 d, about 60% of cells treated with 5 μM LA were no longer enlarged (Fig. 1*D* and *SI Appendix*, Fig. S2 and Table S1) similarly to BCS-treated cells. After 17 d of treatment with either 5 μM or 25 μM LA, KO cells looked like WT (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S2 and Table S1). Morphometric analysis confirmed that the decrease in cell area accompanied changes in cell shape (Fig. 1 *F* and *G*) and occurred at all LA concentrations (*SI Appendix*, Fig. S2). As the major changes in KO cell phenotype require 10 d of treatment with either reagent, most of the further analyses were completed at this time point.

While LA corrects morphology of KO cells at concentrations and with kinetics similar to BCS, further studies found marked differences in the BCS and LA mechanisms of action. In contrast to BCS, LA did not decrease cellular Cu content at all tested LA concentrations (Fig. 2*A* and *SI Appendix*, Fig. S3 *A* and *B*). To determine whether LA chelates Cu intracellularly, we examined the effect of LA on localization of Atp7a in WT cells. Atp7a localization is sensitive to cytosolic Cu levels: under low Cu, Atp7a is targeted to the *trans*-Golgi network (TGN), whereas elevated Cu triggers Atp7a trafficking from the TGN to vesicles (33, 34). In BCS-treated 3T3-L1 cells, Atp7a had expected perinuclear localization, consistent with the TGN targeting (Fig. 2*B* and *SI Appendix*, Fig. S4). In contrast, in cells treated with LA, Atp7a localized to vesicles (Fig. 2*B* and *SI Appendix*, Fig. S4), resembling the Atp7a response to Cu elevation (*SI Appendix*, Fig. S4). This result argued against LA chelating Cu and limiting its availability. To verify this conclusion, we characterized Cu speciation in cells using size-exclusion chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS). Analysis of homogenates of *Atp7a*$^{-/-}$ cells treated with or without LA demonstrated that, as expected, LA did not decrease Cu content in *Atp7a*$^{-/-}$ cells and that the vast majority of excess copper was in a protein-containing fraction and not in the low-molecular-weight fraction (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S5).

We also considered the possibility that LA limits Cu availability in the cytosol by redistributing Cu to other compartments. To examine this possibility, we took advantage of the fact that the expression of cytosolic Cu(I)-binding proteins, metallothioneins MT-1 and MT-2, is up-regulated by elevated cytosolic Cu (34–38). Indeed, the *Mt-1* and *Mt-2* mRNA levels were higher in KO cells compared to WT (Fig. 2*E* and *SI Appendix*, Fig. S6). Treatment with BCS for 10 d, which lowered Cu content (Fig. 2*A*), decreased the *Mt-1* mRNA levels, whereas treatment with LA did not (Fig. 2 *F* and *G*). Upregulation of *Mt-2* mRNA was decreased by LA treatment but remained higher than the WT levels (*SI Appendix*, Fig. S6), providing evidence for differences in Mt-1 and Mt-2 regulation, previously reported for Zn (39). Taken together, the data provided compelling evidence that LA improves *Atp7a*$^{-/-}$ cells' phenotype by mechanisms other than limiting Cu availability. If LA does not chelate or redistribute Cu, then how does LA ameliorate Cu effects?

LA Increases Abundance of Selenoproteins. To better understand how LA affects KO cells, we compared changes in proteomes of KO cells treated with 10 μM BCS or 5 μM LA for 10 d using tandem mass tag (TMT) labeling and mass spectrometry. In nontreated KO cells, 167 proteins were up-regulated and 191 down-regulated (fold change $\log_2 > 0.5$, *P*-value 0.05; *SI Appendix*, Fig. S7*A*) compared to WT; these proteins participate in cellular metabolism, signaling, cell adhesion, and morphology (*SI Appendix*, Table S2). BCS treatment significantly changed proteins involved in metals metabolism and transport: Mt-1, Zn transporter Slc30a1, and Cu transporter Slc31a2 (Ctr2) were down-regulated, and Cu chaperone for superoxide dismutase (Ccs) was up-regulated (Fig. 3*A* and *SI Appendix*, Fig. S8 and Tables S3 and S4) in agreement with the decrease in cellular Cu content.

Cellular response to LA was completely different. The most significant change in the LA-treated KO cells (both fold change and p-value) was upregulation of selenoproteins. Out of 13 selenoproteins detected in our mass-spectrometry dataset, eight showed increased abundance after treatment with LA, including glutathione peroxidase 1 (Gpx1), phospholipid hydroperoxide glutathione peroxidase also known as glutathione peroxidase 4 (Gpx4), selenoprotein H (SelenoH), selenoprotein N (SelenoN), selenoprotein S (SelenoS), selenoprotein T (SelenoT), and selenoprotein W (SelenoW) (Fig. 3*B* and *SI Appendix*, Table S3). We verified the LA-dependent increase in selenoproteins by performing size-exclusion ICP-MS, which demonstrated not only a significant increase in the Se levels but also a predominant association of Se with the high-molecular-weight (protein) fraction (Fig. 3 *C* and *D*). In WT cells, treatment with LA also increased the abundance of selenoproteins Gpx1, SelenoH, SelenoW, and selenoprotein N (SelenoN) (*SI Appendix*, Fig. S7*B*).

LA Specifically Increases Cellular Selenium. To determine whether LA regulates expression of selenoproteins at the transcriptional level, we performed RT-qPCR analysis of mRNA levels and did not find a strong correlation between the levels of selenoproteins and their respective mRNA with or without LA treatment (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S9). For most selenoprotein transcripts, the increase after LA treatment was either small (1.1 to 1.3 fold) or not observed, suggesting that the LA-induced increase of selenoproteins abundance is largely posttranscriptional. Cellular selenium levels are critical for selenoprotein synthesis because selenium should

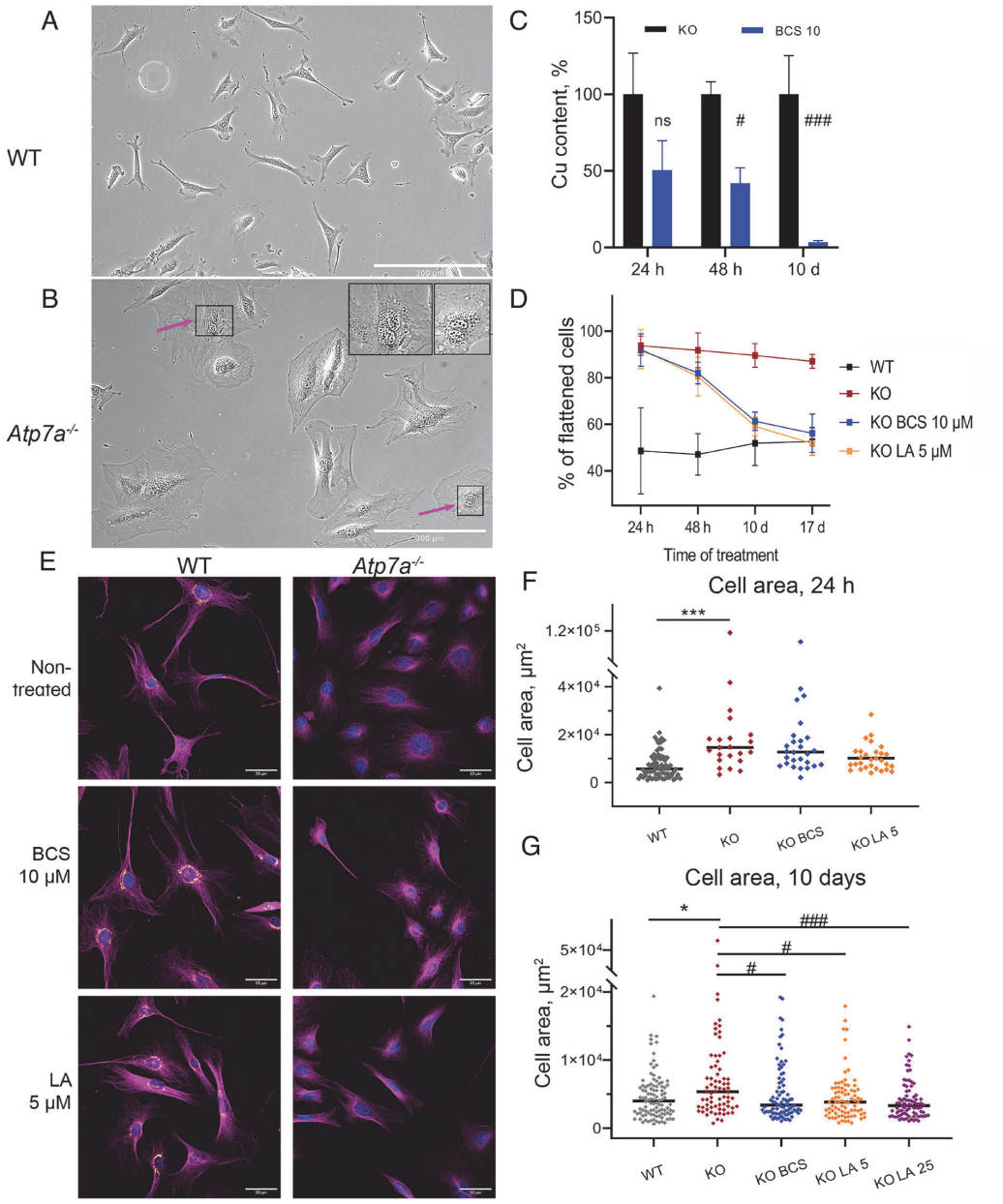

Fig. 1. α-lipoic acid reverses Cu-dependent changes in cell morphology of Atp7a-deficient preadipocytes. (*A*) Fibrillar morphology of WT preadipocytes and (*B*) flattened phenotype of KO cells; changes in nuclei size are indicated with arrows in the zoomed upper-right corner of the panel (scale bar, 300 μm). (*C*) Cellular Cu content in KO preadipocytes is decreasing during treatment with 10 μM BCS. (*D*) The number of flattened KO cells is decreasing during treatment with 10 μM BCS (blue) and 5 μM LA (orange) compared to nontreated conditions (dark-red). (*E*) Morphological changes in KO preadipocytes after 17 d of treatment with 10 μM BCS or 5 μM LA compared to the WT cell line visualized with immunostaining. Magenta α-tubulin, orange Atp7a (scale bar, 50 μm). Changes in cell area after 24 h (*F*) and 17 d (*G*) of treatment with BCS or LA. Each dot corresponds to an individual cell. The black line represents the median cell area. Cell areas were determined using ImageJ software. For panels (*C*, *F*, and *G*): comparison to nontreated WT cells designated with asterisks (*); comparison with the nontreated KO cells designated with sharp (#); *, # *P*-value < 0.05; ***, ### *P*-value < 0.001.

be available for incorporation into selenocysteine. As a result, abundance of selenoproteins often correlates with the cellular selenium content (40–43). We hypothesized that Se availability is low in the KO cells and that LA increases Se levels and selenoprotein synthesis. ICP-MS analysis confirmed that intracellular Se in KO cells was significantly lower than in WT cells (0.53 ± 0.03 fg/cell and 0.89 ± 0.09 fg/cell, respectively, Fig. 3*G*). BCS did not change Se content in either WT or in KO cells in agreement with the data on selenoprotein abundance (Fig. 3 *A* and *E*–*G* and *SI Appendix*, Fig. S9 and Table S3). In contrast, LA increased Se levels in both WT and KO cells in a dose-dependent manner. In KO cells treated with higher concentrations of LA (25 and 100 μM), Se content

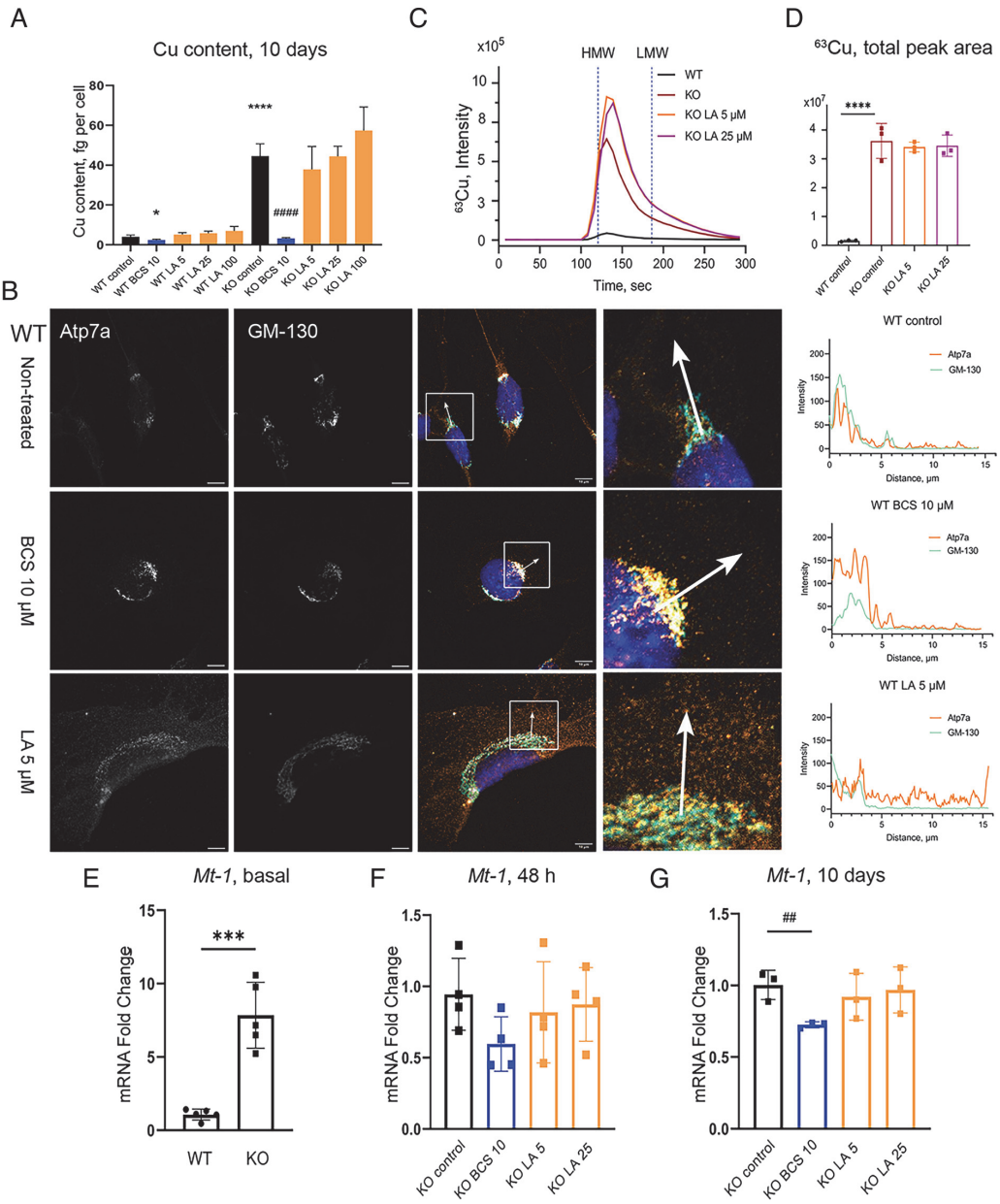

Fig. 2. LA does not alter cellular Cu content and causes relocalization of Atp7a in 3T3-L1 cells. (*A*) Cu content in WT and KO preadipocytes after 10 d of treatment with 10 µM BCS or different concentrations of LA. (*B*) Localization of Atp7a in 3T3-L1 WT cells after 10 d of treatment with 10 µM BCS or 5 µM LA. Orange Atp7a, cyan GM-130 (scale bar, 10 µm). Differences in the localization pattern of Atp7a and GM-130 are shown in white squares. Colocalization of Atp7a and GM-130 is quantified using ImageJ and visualized in GraphPad Prism. (*C*) Average chromatogram (n = 3 per group) of ^{63}Cu species in cell lysates of 3T3-L1 KO preadipocytes after 10 d of treatment with different concentrations of LA and (*D*) quantification of total ^{63}Cu peak areas normalized by ^{133}Cs signal and protein content (mean ± SD, n = 3). Nontreated WT and KO cells were used as respective controls. Vertical blue dashed lines (120.8 s and 186 s) indicate the retention time of high- and low-molecular-weight species (HMW and LMW, correspondingly) based on retention time of CuHSA (M ≈ 66 kDa) and Cu-EDTA (Mw 355.8 Da), respectively (*SI Appendix*, Fig. S5). (*E*) Relative expression of *Mt-1* in WT and KO preadipocytes on day 0 (n = 5), (*F*) after 48 h (n =4), and (*G*) after 10 d (n=3) of treatment with BCS or LA. For panels (*A*, *D*, *E*, and *G*): *P*-values for WT cells shown with asterisks (*) and for KO cells with sharp (#); * – *P*-value < 0.05; ## – *P*-value < 0.01; ***, ### – *P*-value < 0.001; **** – *P*-value < 0.0001.

(0.82 ± 0.04 fg/cell and 0.98 ± 0.05 fg/cell, Fig. 3*G*) became similar to that in nontreated WT cells (0.89 ± 0.09 fg/cell, Fig. 3*G*).

To test whether the effect of LA was specific to Se, we measured Fe, Zn, and Mn in control and KO cell lines after 24 h, 48 h, and 10 d of treatment. Fe levels were unchanged by Cu accumulation, and neither LA nor BCS altered Fe content in either WT or KO cells (*SI Appendix*, Figs. S10 *A* and *B* and S11*A*). Zn was approximately 25% higher in KO cells compared to WT (*SI Appendix*,

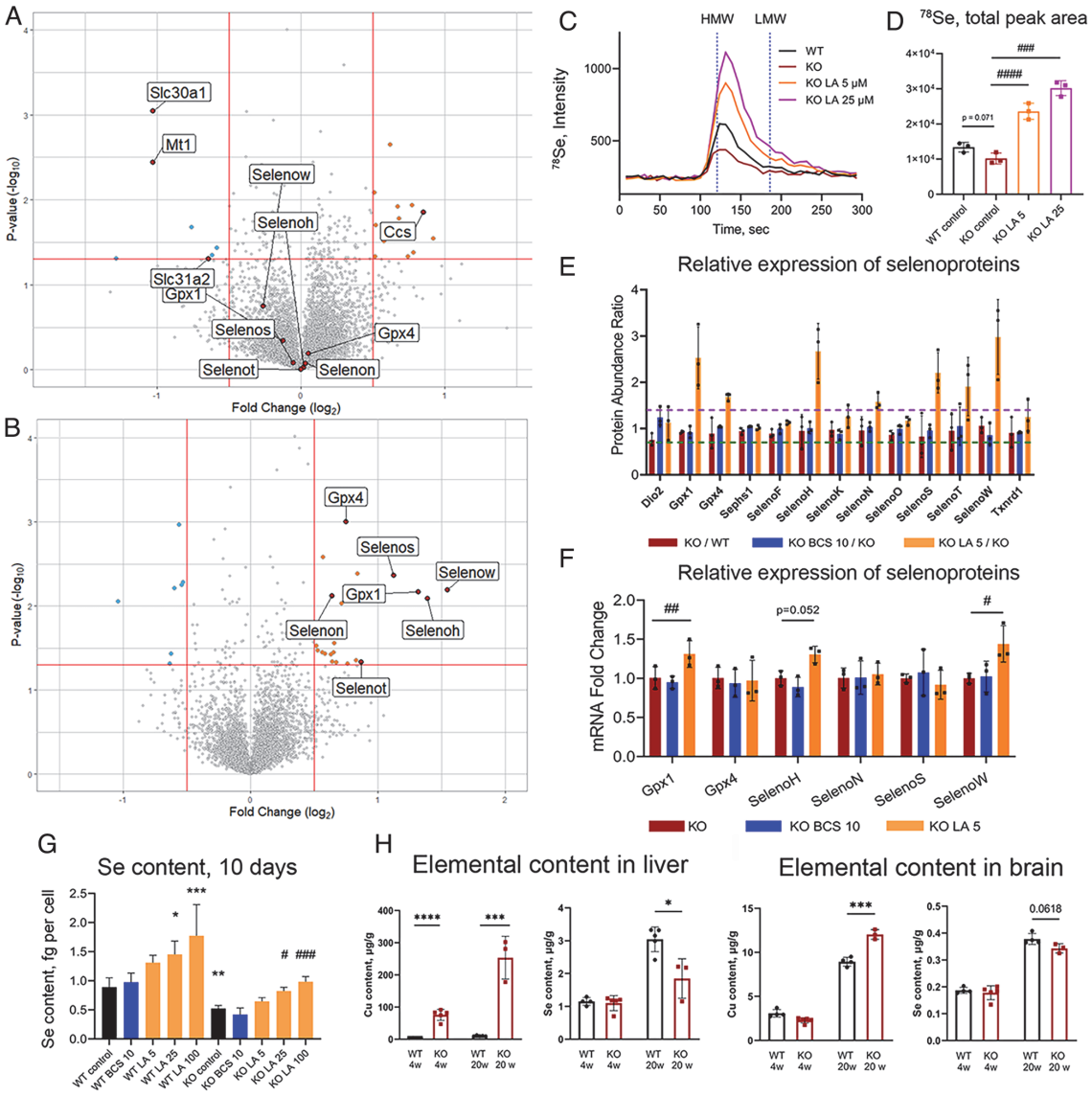

Fig. 3. LA up-regulates selenoproteins and increases cellular Se content. (*A*) Volcano plot comparing proteome of KO cells treated with 10 μM BCS for 10 d to nontreated KO cells and (*B*) KO cells treated with 5 μM LA for 10 d to nontreated KO cells. (*C*) Average chromatogram (n = 3 per group) of ^{78}Se species in cell lysates of KO preadipocytes after 10 d of treatment with different concentrations of LA and (*D*) quantification of total ^{78}Se peak areas normalized by ^{133}Cs signal and by protein content (data present as mean ± SD, n = 3). Nontreated WT and KO cells are used as controls. Vertical dashed lines (120.8 s and 186 s) demonstrate retention time of high- and low-molecular-weight species (HMW and LMW, correspondingly) based on retention time of CuHSA (Mw ≈ 66 kDa) and Cu-EDTA (Mw 355.8 Da), correspondingly (*SI Appendix*, Fig. S5). (*E*) Relative abundances of all selenoproteins identified by TMT labeling mass spectrometry in WT and KO preadipocytes after 10 d of treatment with 10 μM BCS or 5 μM LA (n = 3). The horizontal dashed lines at 1.4 (purple) and 0.7 (dark-green) indicate cutoff values for significant changes in protein abundance (upregulation and downregulation, correspondingly). (*F*) The mRNA for selenoproteins in KO cells relative to WT without treatment and after 10 d of treatment with 10 μM BCS or 5 μM LA (n = 3). For more details, see *SI Appendix*, Figs. S9 and S10. (*G*) Selenium content in WT and KO preadipocytes after 10 d of treatment with 10 μM BCS or different concentrations of LA. (*H*) Cu and Se content in the liver and brain tissue of 4- and 20-wk-old WT and *Atp7b*$^{-/-}$ mice; the two-tailed *t*-test was used for pairwise statistical analysis. For panels (*D*–*G*): comparison to nontreated WT cells is indicated with asterisks (*) and comparison with nontreated KO cells with sharp (#). For panels (*F*–*I*): *, # – *P*-value < 0.05; **, ## – *P*-value < 0.01; ***, ### – *P*-value < 0.001; ****, #### – *P*-value < 0.0001.

Figs. S10 *C* and *D* and S11*B*), similar to findings in the mouse model of Wilson disease (44). BCS returned Zn levels to that of WT after 10 d of treatment (*SI Appendix*, Fig. S11*B*), whereas LA did not (*SI Appendix*, Figs. S10*D* and S11*B*). Mn levels were significantly higher in KO cells compared to WT (*SI Appendix*, Figs. S10 *E* and *F* and S11*C*). After 10 d of treatment with either BCS or LA, Mn returned to the WT levels (*SI Appendix*, Figs. S10 *E* and *F* and S11*C*). Thus, increase in Se is specific to LA, whereas the effect on Mn is not.

***Atp7b*$^{-/-}$ Mice Have Decreased Selenium Content in The Liver and The Brain.** 3T3-L1 *Atp7a*$^{-/-}$ is a fibroblast model of Cu overload,

and whether Cu excess impacts Se content in vivo was unclear. To answer this question, we measured Se content in the liver and brain samples of *Atp7b*$^{-/-}$ mice, an animal model of Wilson disease (45), which accumulate Cu in tissues. In presymptomatic 4-wk-old *Atp7b*$^{-/-}$ mice, hepatic Se was not altered compared to the age-matched WT, even though Cu was elevated (Fig. 3*H*). At 20 wk, *Atp7b*$^{-/-}$ mice accumulated approximately three times more Cu in their livers compared to younger animals and had significantly decreased Se (Fig. 3*H*). A similar pattern was seen in the brain (Fig. 3*I*). In the heart, Se was markedly decreased even though Cu levels were comparable to WT (*SI Appendix*, Fig. S12*A*). Se content in the *Atp7b*$^{-/-}$ kidneys was not changed comparing to WT despite significantly elevated Cu (*SI Appendix*, Fig. S12*B*).

To determine whether low hepatic Se in *Atp7b*$^{-/-}$ mice reflects low levels of selenoproteins or their transcripts (or both), we took advantage of RNA sequencing and proteomics data for 20-wk-old *Atp7b*$^{-/-}$ mice that we generated previously (46, 47). These data demonstrated that out of 12 selenoproteins identified in the liver proteome, six showed significant downregulation (>1.5 fold, *P*-value < 0.05, *SI Appendix*, Table S5). This decrease was not due to the decreases in the selenoprotein transcripts: we detected mRNA for 20 out 25 selenoproteins, and most were either not changed or modestly up-regulated (*SI Appendix*, Table S5). Taken together, these results demonstrate that WD mice with a developed liver pathology, similarly to *Atp7a*$^{-/-}$ cells, have dysregulated selenium homeostasis, lower selenium, and diminished levels of several selenoproteins.

LA Protects Cu Overloaded Cells from Cu-Induced Redox Stress. Selenoproteins influence cellular redox balance by maintaining the ratio of reduced and oxidized glutathione (GSH:GSSG) and decreasing levels of peroxides (48, 49). We hypothesized that the LA-dependent increase in selenoproteins improves the antioxidant capacity of KO cells and diminishes oxidative stress caused by excess Cu. To test this hypothesis, we first used GRX1-roGFP2-based redox sensors targeted to different intracellular compartments (29, 50) and live cell measurements before and after treatments with LA for 10 d. Targeting of GRX1-roGFP2, MTS-GRX1-roGFP2, and NLLS-GRX1-roGFP2 to the cytosol, mitochondria, and nuclei, respectively, was verified by laser-scanning microscopy (*SI Appendix*, Fig. S13). As expected, nontreated KO cells show higher sensor oxidation in all three cellular compartments compared to nontreated WT cells (29) and (Fig. 4 *A*–*C*) treatment with LA significantly decreased sensor oxidation in the cytosol, mitochondria, and nuclei of KO cells, indicative of improved redox balance (Fig. 4 *A*–*C*).

To verify this conclusion, we examined protein oxidation in WT, KO, and KO cells treated with LA. We focused on the sulfur-containing amino acids, Cys and Met, because they are highly susceptible to oxidation, which can be prevented or reversed by other sulfur-containing molecules and/or by selenoproteins, such as methionine sulfoxide reductases MSRB1 (51). In total, there were 6,949 uniquely oxidized peptidoforms [i.e., unique peptides with specific modification(s)] across 2,271 proteins. To compare protein oxidation between the WT, KO, and KO+LA samples, we selected 2,400 peptidoforms that were found in all three groups and evaluated distribution of abundances of oxidized peptides normalized to total protein. This analysis demonstrated that the KO cells have significantly higher protein oxidation and that treatment with LA significantly decreases it (Fig. 4*D*). Initial evaluation of proteins undergoing LA-reversible oxidation found that these proteins are located in different cellular compartments and are involved in regulation of cytoskeleton assembly (Map6, Myo1c, Tagln2), chromosome maintenance and RNA processing (Eef2, Eif4g2, Mcm6), and mitochondria function (see examples in *SI Appendix*, Table S6).

To test whether the LA-dependent changes in redox balance and protein oxidation benefit cell functions, we measured mitochondria respiration using the Seahorse XF Cell Mito Stress Test (*SI Appendix*, Fig. S14*A*). The KO cells demonstrate a lower oxygen consumption rate (ref. 52 and *SI Appendix*, Fig. S14*B*) and lower spare respiratory capacity (Fig. 4*E*) than WT, consistent with increased mitochondria oxidation (Fig. 4*B*). After 48 h of treatment, both parameters were improved by either BCS or LA and became similar to WT cells (Fig. 4 *E* and *F* and *SI Appendix*, Fig. S14 *B* and *C*).

Finally, we examined whether the defects in mitochondria function in *Atp7a*$^{-/-}$ cells could be a result of a Cu-dependent loss of mitochondria protein lipoylation, as was observed in cancerous cells undergoing cuproptosis (53). However, western blot analysis of major lipoylated enzymes dihydrolipoamide S-acetyltransferase (Dlat) and dihydrolipoamide S-succinyltransferase (Dlst) found no difference in their abundance, arguing against LA altering this pathway in 3T3-L1 mouse fibroblasts (Fig. 4 *G*–*I*). Taken together, the data suggested that LA protects *Atp7a*$^{-/-}$ cells from Cu overload by diminishing Cu-induced oxidative stress.

Selenium alone Partially Replicates Effects of LA. Lipoic acid is an antioxidant on its own (54). Therefore, it was unclear whether the beneficial effects of LA are associated with LA increasing cellular Se content or via Se-independent antioxidant activity of LA. To distinguish between these possibilities, we compared the effects of a well-known antioxidant N-acetyl cysteine (NAC) and LA on *Atp7a*$^{-/-}$ cell morphology and mitochondria respiration after 10 d of treatment. NAC at 5 μM improved *Atp7a*$^{-/-}$ cells morphology, highlighting the importance of redox balance for cell shape and size. However, NAC was significantly less effective compared to LA and also had no effect on *Atp7a*$^{-/-}$ mitochondria respiration (Fig. 5 *A*–*C* and *SI Appendix*, Fig. S15). These data suggested that an increase of Se/selenoproteins abundance may be an important component of LA activity. To test this hypothesis, we treated cells with either 5 μM LA, or 1 μM Se in a form of selenite, or both 1 μM Se and 5 μM LA. Neither Se alone nor Se with LA decreased the cytosolic Cu as indicated by unchanged levels of *Mt-1* and *Ctr1* (Fig. 6 *A* and *B*). The *Mt-2* levels were decreased (*SI Appendix*, Fig. S6) but remained above the WT levels, suggesting that regulation of *Mt-2* was only partially dependent of Cu. Cell morphology was improved by treatment with either Se or LA (Fig. 6*C*), which produced similar results, and LA with Se treatment had strongest effect on cell morphology (Fig. 6 *C* and *D* and *SI Appendix*, Fig. S15). Thus, increased Se availability plays a significant role in improving morphology of *Atp7a*$^{-/-}$ cells. However, Se alone or in combination with LA did not improve mitochondrial respiratory function (Fig. 6*E* and *SI Appendix*, Fig. S16), pointing to an additional role of LA in mitochondria.

Discussion

Here, we show that the natural membrane permeable metabolite, α-lipoic acid, diminishes toxic effects of elevated Cu in a cellular model of Cu overload. The mechanism of LA action is distinct from that of Cu chelator BCS, which gradually reduces the intracellular Cu content in KO cells by limiting Cu availability. We show that LA does not prevent Cu entry into the cells, does not form intracellular small-molecular-weight complexes with Cu, and does not redistribute Cu away from the cytosol. Instead, LA improves cellular redox status and reverses protein oxidation while Cu is elevated. Using analysis of proteomes, treatments with either

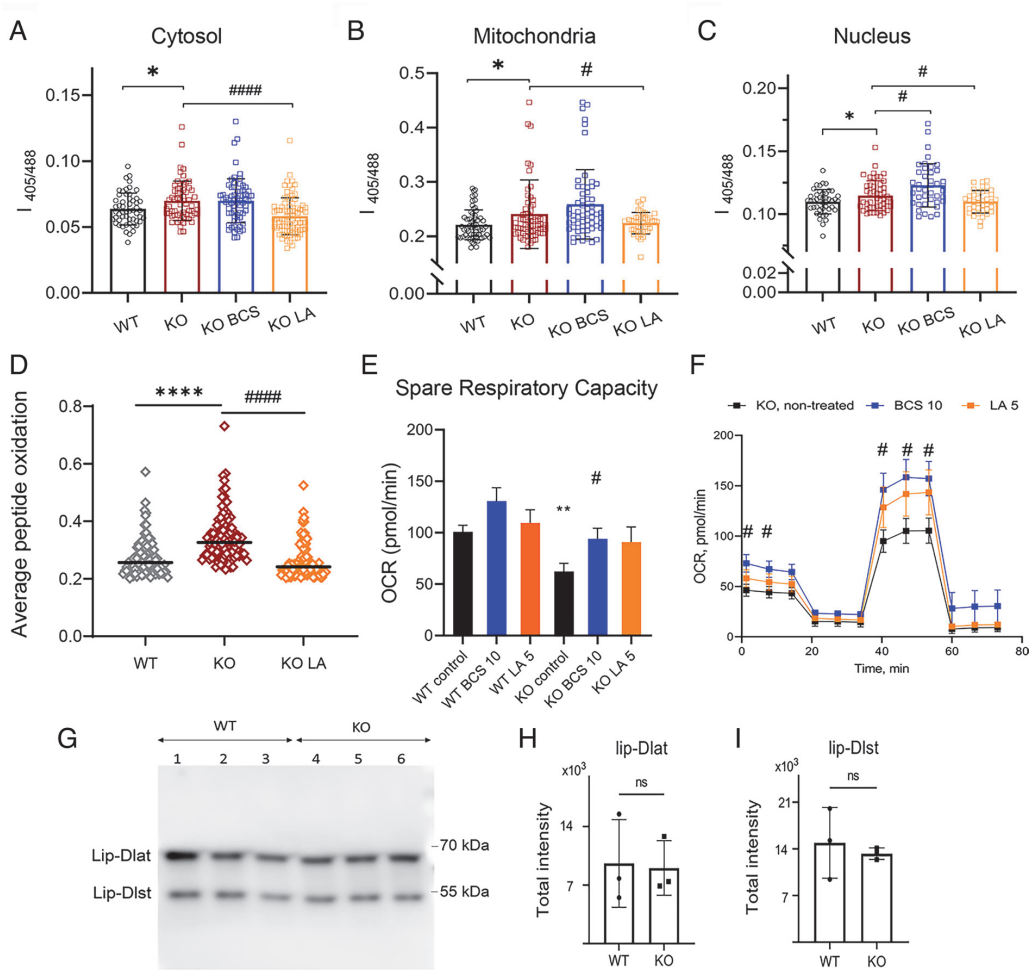

Fig. 4. Treatment with LA decreases redox misbalance in *Atp7a*$^{-/-}$ preadipocytes. Oxidation of the GRX-roGFP2 redox sensor in (*A*) cytosol, (*B*) mitochondria, and (*C*) nuclei of WT and KO preadipocytes after 10 d of treatment with 10 μM BCS or 5 μM LA (the number of cells in each group is > 40; n = 3). (*D*) Average oxidation of peptidoforms of WT and KO preadipocytes after 5 d of treatment with 5 μM LA estimated with quantitative redox proteomics. Peptides found in all treatment groups with a SD of mean less than 0.1 were selected for comparison. (*E*) Spare respiratory capacity (SRC) of WT and KO preadipocytes after 48 h treatment with 10 μM BCS or 5 μM LA. (*F*) Oxygen consumption rate (OCR) of KO preadipocytes after 48 h treatment with 10 μM BCS (blue) or 5 μM LA (orange) compared to nontreated KO cells (black). Data in *E* and (*F*) are mean ± SEM. (*G*) Representative western blot and (*H* and *I*) densitometric analysis of lipoylated proteins Dlat and Dlst in WT and KO cells. For panels (*A*–*F*): comparison with WT cells are shown with asterisks (*) and comparison with nontreated KO cells with sharps (#);*, # – *P*-value < 0.05; ** *P*-value < 0.01; ****, #### *P*-value < 0.0001.

Se or NAC and by evaluating Se species in the cells, we show that LA counteracts Cu-induced decrease of cells antioxidant capacity by increasing selenoprotein levels by increasing Se availability rather than altering transcription. Our data also suggest that in mitochondria, LA shows additional Se-independent activity.

BCS reverses changes in cellular Zn and Mn content and improves mitochondria respiratory function but does not increase Se, nor restore GSH:GSSG balance in either the cytosol or mitochondria, whereas LA does. This result suggests that oxidative stress in the KO cells is caused to a significant degree by a decrease in selenoproteins. In Wilson disease (in patients and animal models), antioxidant capacity is decreased, including a decrease in glutathione peroxidase (55). Given our finding that *Atp7b*$^{-/-}$ mice have lower Se levels in tissues, especially in the liver, it is tempting to speculate that treatment with LA could be a safe supplementary treatment that reduces oxidative stress, especially in the advanced disease. LA has already been tested in clinical trials for treatment of diabetic neuropathy (56) and neurodegenerative diseases (57), where it demonstrated a favorable safety profile at doses up to 1,800 mg/d (58). With administration of 600 mg of racemic LA, the maximum plasma concentrations of 6.86 ± 1.29 μg/mL (33 μM) could be reached (59). In our studies, we see improvements using 5 μM LA.

The LA-dependent increase in intracellular Se and upregulation of selenoproteins in WT and KO cells at concentrations as low as 5 μM is an unexpected effect of LA. Our data suggest that the most likely mechanism is facilitation of Se uptake, as we see more selenium with increasing LA concentration. How exactly LA increases Se uptake is still unknown. The apparent saturation of Se levels with higher LA concentrations and only small additive effect, when Se is combined with LA, point to a limiting step in this process, such as abundance/activity of a relevant transporter, which remains to be identified. Finally, by reversing protein oxidation of the RNA processing machinery, LA may facilitate

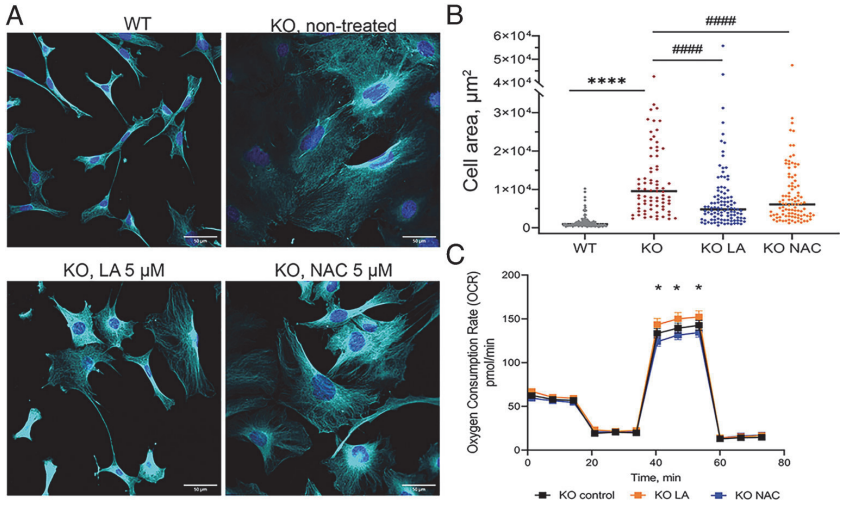

Fig. 5. NAC partially improves morphology but not mitochondrial respiration of *Atp7a*$^{-/-}$ preadipocytes. (*A*) Morphological changes in KO preadipocytes after 10 d of treatment with 5 μM LA or 5 μM NAC compared to nontreated KO and WT cell lines visualized with immunostaining. Cyan α-tubulin (scale bar, 50 μm). (*B*) Changes in cell area after 10 d of treatment with 5 μM LA or 5 μM NAC. Each dot corresponds to an individual cell. The black line represents the median cell area. Comparison to nontreated WT cells is designated with asterisks (*) and comparison with the nontreated KO cell line is designated with sharp (#). ****, #### – *P*-value < 0.0001. Cell areas were determined using ImageJ software. (*C*) Oxygen consumption rate (OCR) of KO cells treated with 5 μM LA (shown in orange) or 5 μM NAC (shown in blue) for 48 h compared to nontreated KO cells (shown in black). * – comparison of NAC-treated cells to LA-treated cells, * - *P*-value < 0.05.

protein synthesis of selenoproteins GPX4, SELENON, and SELENOS, which are less dependent on dietary Se (60, 61).

Cu negatively affects selenoprotein expression through a poorly understood mechanism not reversible by BCS (62), which is in agreement with our results. Selenoproteins play an essential role in protection of cells against oxidative stress. In WD mice, Se content is significantly lower in the liver, heart, and brain. In Se deficiency, the brain retains its Se content at the expense of other tissues (40). Therefore, diminished levels of Se and selenoproteins could be an important contributing factor to oxidative stress in WD, and LA may be beneficial as a supplementary treatment for WD. The ability of LA to improve cellular redox balance, decrease protein oxidation and restore cell morphology in the presence of elevated Cu supports this hypothesis. At the same time, our data do not exclude the possibility of minor changes in cellular Cu distribution contributing to improvement of cell phenotype. LA has relatively strong Cu(I)-binding affinity and some selenoproteins like SELENOH and SELENOW contain Cys-X-X-Sec motifs (63, 64), which are similar to Cys-X-X-Cys Cu-binding motifs. LA and selenoproteins can potentially bind excessive Cu(I) ions by these motifs into weak complexes and reduce Cu burden.

Lipoic acid improved mitochondrial respiratory function of *Atp7a*$^{-/-}$ cells, whereas neither Se alone nor NAC have beneficial effect on mitochondria. This result points to unique properties of LA that are necessary for this effect in mitochondria. Examination of protein lipoylation in mitochondria did not find significant effects on two major markers of this process, but more detailed evaluation is needed to better understand whether cuproptosis, ferroptosis, or other cell death mechanisms contribute to cells' response to Cu overload.

Previous studies found that Cu affects the structure of cytoskeletal proteins and their assembly into microtubules (65, 66). Oxidative stress affects the structure and dynamics of the cytoskeleton (67–70). Excess Cu can directly bind to Cys residues of cytoskeletal proteins or cause their oxidation, as we observed in our studies resulting in disrupted cytoskeleton arrangement and altered cellular morphology. We propose that BCS prevents unfavorable Cu binding to cytoskeletal proteins via Cu limitation and, therefore, restores the morphology of KO cells, and further estimation of Cu-binding affinities and protein competition experiments (21, 71) are needed to support this possibility. The LA-mediated protection of redox-sensitive Cys and Met residues of cytoskeletal proteins may have the same beneficial effect. Our study identified several cytoskeletal proteins protected from oxidation by LA treatment, and further studies will directly test the role of this reversible modification in the cytoskeletal proteins' function.

In summary, LA is a multifaceted player that diminishes the consequences of Cu overload and may serve as a supportive substance to chelation therapy, as it can restore cellular redox balance, protect proteome, and improve cellular functions.

Materials and Methods

Cell Morphology. 3T3-L1 preadipocytes were cultured in DMEM (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning Cellgro) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) and 1% Penstrep (Gibco). Cells were supplemented with fresh medium every 2 d and passaged after reaching 90% confluency. Both 3T3-L1 and 3T3-L1 *Atp7a*$^{-/-}$ preadipocytes were incubated with or without 5 μM, 25 μM, or 100 μM of lipoic acid (LA, Sigma-Aldrich, cat #62320-5G-F) for various periods of time as indicated in the text. Cells treated with 10 μM bathocuproinedisulfonic acid (BCS, Sigma-Aldrich) were used as a positive control for Cu chelation. Treatment with 1 μM sodium selenite (Na_2SeO_3, 214485, Sigma; referred to as Se or selenite), alone or in combination with 5 μM LA, or with 5 μM N-acetylcysteine (NAC, Sigma-Aldrich, A7250) for 10 d was used to assess the effect of alternative antioxidants on KO cell morphology. Freshly prepared chemical solutions were added to the cells every 2 d along with a fresh medium as described above. During treatment with LA and BCS, phase-contrast images (Olympus IX51) were taken every 24 h to monitor changes in cell morphology; phase-contrast cells for morphometric analysis after treatment with NAC, Se, or Se and LA combination were done on day 10 of treatment. Detailed cell morphology assessment is described in *SI Appendix, Supplementary Methods*.

Immunostaining. Control 3T3-L1 WT cell and *Atp7a*$^{-/-}$ cells were treated with or without one of the following: 5 μM, 25 μM LA, 10 μM BCS, or 20 μM $CuCl_2$ for several time points (24 h, 48 h, 10 d, or 17 d). At the end of each treatment, cells were transferred to poly-L-lysine (Sigma-Aldrich) or rat tail collagen (354236,

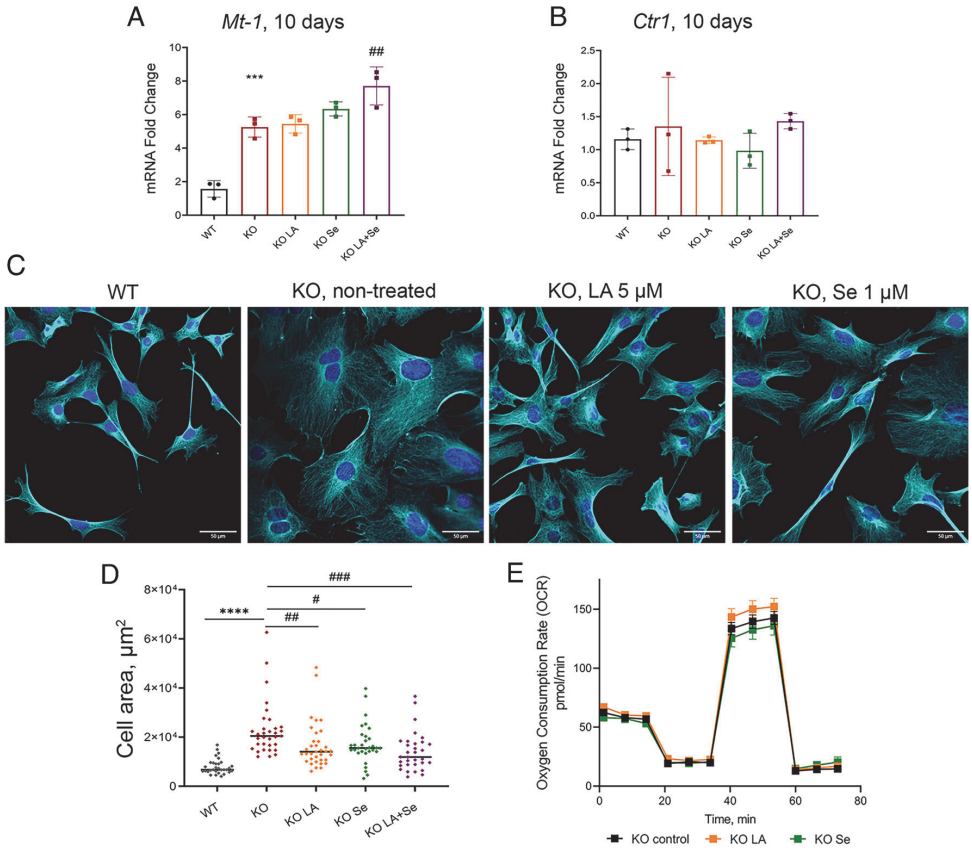

Fig. 6. Se improves morphology but not mitochondrial respiration of *Atp7a*$^{-/-}$ preadipocytes. (*A*) Relative expression of *Mt-1* and (*B*) *Ctr1* genes after 10 d of treatment with 5 μM LA or 1 μM Se, alone or in combination. (*C*) Morphological changes in KO preadipocytes after 10 d of treatment with 5 μM LA or 1 μM Se compared to nontreated KO and WT cell lines confirmed with immunostaining. Cyan α-tubulin (scale bar, 50 μm). (*D*) Changes in cell area after 10 d of treatment with 5 μM LA or 1 μM Se, alone or in combination. Each dot corresponds to an individual cell within the field of view. The black line represents the median cell area. Cell areas were determined using ImageJ software. (*E*) Oxygen consumption rate (OCR) of KO cells treated with 5 μM LA (shown in orange) or 1 μM Se (shown in dark-green) for 48 h compared to nontreated KO cells (shown in black). For panels (*A* and *D*): comparison to nontreated WT cells designated with asterisks (*); comparison with the nontreated KO cell line designated with sharp (#); # - *P*-value < 0.05; ## – *P*-value < 0.01; ***, ### – *P*-value < 0.001; **** – *P*-value < 0.0001.

Corning)-coated glass eight-chamber slides at a density of 0.7 × 10^4 cells/well. Further details are provided in *SI Appendix, Supplementary Methods*.

Immunoblot Analysis. Nontreated 3T3-L1 WT and *Atp7a*$^{-/-}$ cells were grown on 6-cm dishes. Details for the immunoblot experiment are provided in *SI Appendix, Supplementary Methods*.

TMT Labeling Mass Spectrometry, Peptide Identification, and Quantification. 3T3-L1 WT and *Atp7a*$^{-/-}$ preadipocytes were cultured in 60 mm dishes and treated with or without 5 μM LA or 10 μM BCS for 10 d. Cells were washed with ice-cold PBS, collected, and lysed with RIPA buffer with EDTA-free protease inhibitor cocktail on ice for 1 h and centrifuged at 3,000 *g* for 15 min. Protein concentration from the obtained lysate was estimated by the BCS assay. A mixture of 20 μg of total protein per well and 6× sample buffer were loaded to Laemmli SDS-PAGE gel and stained with Colloidal Blue staining (Thermo Fisher, LC6025) to confirm the quality of proteins. Protein lysate was further diluted to a concentration of 1 μg/μL. Then, proteins were reduced, alkylated, and precipitated in TCA/acetone mixture. Protein pellets were washed with ice-cold acetone, dried, and then reconstituted and proteolyzed. TMT labeling, peptide identification, quantification, and bioinformatics analysis are described in more details in *SI Appendix, Supplementary Methods*.

Biometals and Selenium Measurements with ICP-MS. 3T3-L1 WT and *Atp7a*$^{-/-}$ cells were cultured in 100-mm dishes and treated with or without 5 μM, 25 μM, or 100 μM LA or 10 μM BCS for 24 h, 48 h, or 10 d. Cells were washed, collected into 15-mL metal-free centrifuge tubes (VWR), suspended with PBS (Gibco, cat number 14190250), and counted using the Neubauer chamber for further normalization of metal (Cu, Zn, Fe, and Mn) and Se content. Cells were pelleted, and supernatant was removed. Cell pellets were stored in −80 °C until cell digestion. Further details of sample preparations for ICP-MS are described in *SI Appendix, Supplementary Methods*.

Liquid Chromatography Coupled with ICP-MS (LC-ICP MS). 3T3-L1 WT and *Atp7a*$^{-/-}$ preadipocytes were grown on 15-cm dishes as described above. KO cells were treated with 5 or 25 μM LA for 10 d; nontreated cells were used as a control. Cells were washed and collected into clean 1.7-mL tubes. Cells were pelleted, and supernatant was removed. Cell pellets were stored in −80 °C until required. Further details of sample preparations for LC-ICP MS are described in *SI Appendix, Supplementary Methods*.

RT-qPCR. 3T3-L1 WT and *Atp7a*$^{-/-}$ cells were grown on six-well plates and treated with either 5 μM, 25 μM LA, or 10 μM BCS for 48 h or 10 d. Cells were washed and collected into clean centrifuge tubes for RNA isolation. RNA isolation and further gene expression analysis details are described in *SI Appendix, Supplementary Methods*.

Mice Husbandry and Tissue Collection. Animals were housed at the Johns Hopkins University, School of Medicine (JHU SOM) animal care facility, and the studies followed the NIH guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee (protocol number M017M385).

Atp7b KO (*Atp7b*$^{-/-}$) and WT mice of C57BL/6x129S6/SvEv background (described in ref. 45) were fed with the standard pellet chow. At 4 wk or 20 wk after birth, mice were killed and perfused with PBS (Gibco, cat number 14190250). The liver, kidney, heart, and brain were used for Cu and Se analysis. Tissues were flash-frozen and stored at −80 °C until digestion. Details of sample preparations for ICP-MS are described in *SI Appendix, Supplementary Methods*.

Live Imaging. WT and *Atp7a*$^{-/-}$ cells treated with or without 5 μM LA or 10 μM BCS for 10 d transiently expressing GRX1-roGFP were imaged with the Zeiss LSM700 or Zeiss LSM800 confocal laser scanning microscope. The sensor was excited frame by frame with a 405-nm or 488-nm laser, and emission was detected with a 505 to 600 band-pass filter. Cells were treated with 2 mM H_2O_2 or 10 mM dithiothreitol to achieve complete sensor oxidation or reduction, respectively. $IR_{405/408}$ ratios were calculated using ImageJ software. Ratiometric ($IR_{405/408}$) images were prepared with ZEN and ImageJ software after background subtraction. Generation of adenoviral constructs, adenoviral infection, and transfection for compartmentalized expression of GRX1-roGFP sensors are described in more details in *SI Appendix, Supplementary Methods*.

Seahorse XF Cell Mito Stress Test. WT and *Atp7a*$^{-/-}$ cells were plated to the 96-well Agilent Seahorse XF Cell Culture Microplate and treated with or without 5 μM LA or 10 μM BCS for 48 h. Further details are provided in *SI Appendix, Supplementary Methods*.

Analysis of Protein Oxidation State. 3T3-L1 WT and *Atp7a*$^{-/-}$ preadipocytes were cultured in 60-mm dishes and treated with 5 μM LA 10 d. Cells were washed with ice-cold PBS and collected and lysed with RIPA buffer with EDTA-free protease inhibitor cocktail on ice for 1 h and centrifuged at 3,000 *g* for 15 min. Protein concentration from the obtained lysate was estimated by the BCS assay. A mixture of 10 μg of total protein per well and 4× sample buffer were loaded to Laemmli SDS-PAGE gel and stained with Colloidal Blue staining (Thermo Fisher, LC6025) to confirm the quality of proteins. Protein lysate was further diluted to a concentration of 1 μg/μL and further prepared for the analysis.

Analysis of protein oxidation state was done using modifications to the *Pan-Protein Adductomics* approach (72), which combines nanoflow-liquid and overlapping-window data-independent acquisition, high-resolution tandem mass spectrometry. Further details on sample preparations are provided in *SI Appendix, Supplementary Methods*.

Statistical Analysis. GraphPad Prism version 9.4.1 software (GraphPad Software) was used for all statistical analysis. One-way ANOVA was used unless otherwise mentioned. All data in the figures are shown as the mean ± SD unless specified. A *P* value of less than 0.05 was considered significant.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*. All data used for generation figures in the manuscript are included with the submission (supplement). We have submitted the mass-spectrometry datasets to ProteomeXchange in PRIDE database: http://www.ebi.ac.uk/pride under identifier PXD045233 (73). The data are currently private but will become available when manuscript is published, on 9 August 2023. The data are currently private but will become available when manuscript is published.

ACKNOWLEDGMENTS. This work was supported by grants from Dora Plus 1.2 and Kristjan Jaak Scholarship Programs awarded by Archimedes Foundation (Education and Youth Board, Estonia) to E.K., NIH grants R01 DK117396 and R01 DK071865 S.L. and by the Estonian Research Council no. 1289 to (P.P.). K.S. acknowledges a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada. We thank Dr. Tobias P. Dick [German Cancer Research Center (DKFZ), Heidelberg, Germany] for the GRX1-roGFP2 construct. TMT-MS/MS study was performed in JHU SOM Mass Spectrometry and Proteomics Facility. We thank Dr. Tatiana Boronina and Connie Talbot for help with the proteomics result analysis and Sean M. Burke, for assistance with the Pan-Protein Adductomics data processing. ICP-MS measurements were performed in the OHSU Elemental Analysis Core with partial support from NIH (S10RR025512 and S10OD028492). Cell live imaging was performed in the Johns Hopkins MicFac facility, which was funded by the NIH grant #S10 OD016374.

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Publication III

Yang H., **Kabin E.***, Dong Y.*, Zhang X., Ralle M., Lutsenko S.
Atp7a-dependent copper sequestration contributes to termination of β-catenin signalling during early adipogenesis.
Manuscript.

ATP7A-DEPENDENT COPPER SEQUESTRATION CONTRIBUTES TO TERMINATION OF β-CATENIN SIGNALING DURING EARLY ADIPOGENESIS

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Key words: copper, adipocytes, ATP7A, β-catenin

ABSTRACT

Adipocyte fate determination is tightly regulated by extrinsic signaling pathways and intrinsic metabolic and morphologic changes to maintain adipose tissue function. Copper (Cu) homeostasis is required for the normal metabolism of mature adipocytes, whereas the role of Cu in adipogenesis is unclear. We show that in differentiating 3T3-L1 cells, adipogenic stimuli trigger intracellular Cu re-distribution evident from the upregulation and trafficking of the Cu transporter Atp7a. Disrupting Cu homeostasis by the deletion of Atp7a causes Cu elevation, changes in cell morphology, and inhibition of adipogenesis. The initial step of differentiation, upregulation of C/EBPβ, is not affected in *Atp7a*$^{-/-}$ cells, whereas the subsequent upregulation of Pparγ is inhibited. To identify the underlying mechanism of Atp7a-dependent adipogenesis, we compared changes in *Atp7a*$^{-/-}$ and wild type 3T3-L1 proteomes during this period using unbiased quantitative mass-spectrometry. We found that loss of Atp7a specifically stabilized β-catenin, a negative regulator of adipogenesis. Importantly, pharmacological inhibition of Wnt/β-catenin signaling improves *Atp7a*$^{-/-}$ cells differentiation. Cu chelation, overexpression of Atp7a homologue, Atp7b, in *Atp7a*$^{-/-}$ cells rescued the downregulation of β-catenin in response to adipogenic stimuli, pointing to importance of Cu buffering in termination of β-catenin signaling. Abnormal upregulation of β-catenin was also observed *in vivo* in the livers of *Atp7b*$^{-/-}$ mice, which accumulate Cu, suggesting a tissue-independent crosstalk between Cu homeostasis and

the Wnt/β-catenin pathway. These results point to a new regulatory role of Cu in adipocytes and contribute to better understanding of the human disorders of Cu misbalance.

INTRODUCTION

Copper (Cu) has a multifaceted role in mammalian physiology and is essential for normal development and function of humans, animals, and many other species. Copper's best known function is serving as a cofactor of important metabolic enzymes, such as cytochrome c oxidase, superoxide dismutases SOD1 and SOD3, amino-oxidase AOC3 and many others[1]. It is now appreciated that Cu is also an important regulator of kinase-mediated signaling, autophagy, and even cell death[2-5]. Both Cu deficit and Cu excess are harmful to cells. Consequently, cells faithfully maintain Cu homeostasis using Cu transporting proteins, which ensure proper and timely Cu distribution to the intracellular compartments. Cu uptake is predominantly mediated by SLC31A1 (CTR1), whereas ATP-driven transporters ATP7A and ATP7B transport Cu into the secretory pathway to activate Cu-dependent enzymes and to lower cytosolic Cu by exporting excess Cu out of cells[6]. ATP7A and ATP7B are highly homologous, and both play critical roles in regulation of cellular Cu levels. Inactivation of either ATP7A or ATP7B is associated with debilitating and even fatal diseases[7].

Recently, the new role for ATP7A in cell differentiation was identified. Specifically, expression of ATP7A was found to change during neuronal differentiation[4,8]; and be associated with a transient decrease in the cellular Cu levels[9]. Loss of ATP7A function in neurons causes defects in their axon outgrowth and synaptogenesis[10]. Cu is required for myogenesis (during which ATP7A is significantly upregulated[11]), for spermatogenesis (where changes in the expression of ATP7A, ATP7B, and CTR1 occur at different steps of sperm maturation[12]), and for angiogenesis (neovascularization). Understanding of how Cu is used during cell differentiation remains very limited.

Cu homeostasis is required for normal functions of adipose tissue[13-15], however, the role of ATP7A in adipogenesis remains unclear. In mice, targeted deletion of *Atp7a* gene in adipocytes is associated with Cu elevation, age-dependent atrophy and diminished function of white adipose tissue.This is evident from the lower levels of serum leptin and adiponectin, elevated serum triacylglycerol, and hepatic steatosis[16]. It is not known whether these abnormalities reflect defects in adipocytes differentiation or the toxicity of elevated Cu in fully differentiated cells. Studies of adipocytes differentiation in animals are complicated by complex interactions with other cell types[17,18]. Consequently, to better understand the role of Cu homeostasis in

adipocytes, we used 3T3-L1 preadipocytes, an established model for adipocyte differentiation. We found that Atp7a-dependent Cu distribution is required for early steps of 3T3-L1 adipocytes differentiation and identified a new role for Cu in sustaining Wnt/β-catenin signaling, a conserved pathway regulating cell proliferation and differentiation. We demonstrate that the adipogenic stimuli alter the intracellular Cu distribution by regulation of Atp7a abundance and localization, and that without this regulation the Wnt/β-catenin is prolonged and adipogenesis is impaired. Moreover, we demonstrate that Cu accumulation leads to stabilization of β-catenin in an animal model of Wilson disease, which points to a new mechanism through which Cu can contribute to development of human pathologies.

RESULTS

Atp7a-dependent Cu distribution changes during adipogenesis.

To understand the role of Cu homeostasis in functional maturation of 3T3-L1 adipocytes, we first examined the expression status of major Cu transporters: Atp7a, Atp7b, and Slc31a1 (Ctr1) during a standard 8-day differentiation process[19]. Immunoblotting revealed that Atp7a was present in differentiated adipocytes, whereas Atp7b was not (**Fig. 1A**). Furthermore, expression of the Atp7a protein significantly increased upon induction of cell differentiation, when comparing fully differentiated adipocytes to pre-adipocytes (**Fig. 1B**). Quantitative PCR confirmed that *Atp7a* mRNA was upregulated right after the growth arrest, which occurs during the differentiation (**Fig. 1C**). By contrast, expression of *Ctr1* did not change at the early stage of differentiation, but it was upregulated at the later stage, on day 6 (**Fig. 1D**).

In adipocytes, Atp7a transfers Cu to Cu-dependent enzymes, such as Aoc3[20]. This process takes place in the *trans*-Golgi network, TGN. Atp7a also maintains cytosolic Cu levels by sequestering Cu into vesicles and exporting Cu via plasma membrane[6]. In either mature or non-differentiated adipocytes, we found that Cu limitation facilitated Atp7a retention in the TGN, whereas high Cu triggered Atp7a movement to the vesicles and plasma membrane (**Suppl. Fig. 1A, B**). Analysis of Atp7a localization during adipogenesis revealed that the balance between the two Atp7a functions (TGN delivery and Cu export) changes as adipocytes differentiate. In preadipocytes, Atp7a was found mainly in the TGN (**Fig. 1E**). On day 3 of differentiation (D3), Atp7a was upregulated and trafficked to vesicles, as well as to the plasma membrane (indicative of increased Cu sequestration and export), in addition to the TGN. On day 5, Atp7a mostly returned to the TGN, with only a small amount detected in vesicles. The fully mature adipocytes (day 8) had Atp7a in the TGN (**Fig. 1E**). Specifically, we found that the insulin in the adipocyte differentiation induction cocktail is sufficient to induce the trafficking of Atp7a in a Cu-

independent manner: we demonstrated that Atp7a relocalized to vesicles and plasma membrane in response to insulin in the presence of the Cu chelator TTM (**Suppl. Fig. 1C**).

Considering *Ctr1* levels increase only on day 6 of differentiation (**Fig. 1D**), the earlier upregulation and vesicular/plasma membrane targeting of Atp7a suggest that the initial steps of adipogenesis may require lowering of the cytosolic Cu. Taken together, the results show that the Atp7a-regulated Cu redistribution is an integral component of adipogenesis program.

Deletion of Atp7a alters the intracellular Cu redistribution and inhibits adipogenesis at the early stage.

To further examine the significance of Atp7a for the adipocyte differentiation program, we used 3T3-L1-*Atp7a*$^{-/-}$ cells, previously generated using CRISPR/Cas9. These cells lack the Atp7a-dependent Cu export and accumulate Cu ([21,22] and **Suppl. Fig. 2A, B**). We first examined the intracellular Cu distribution using X-ray fluorescence imaging (**Fig. 2A**), an analytical technique to quantitatively map multiple metals in cells and tissues with reasonable sensitivity, specificity, and resolution[23-25]. Our data demonstrate that abundant phosphate levels, as an integral component of nucleic acids, mainly accumulated in the nucleus and were comparable between control cells and *Atp7a*$^{-/-}$ cells. We observed that Cu was enriched in the perinuclear area of the wild type (WT) cells, and the levels of Cu in the nucleus were low (**Fig. 2A**). In contrast, in *Atp7a*$^{-/-}$ cells, Cu levels were increased throughout the cell and especially elevated in the nucleus (**Fig. 2A**). We also observed that *Atp7a*$^{-/-}$ cells show different morphology and often contained multiple nuclei (**Fig. 2B**, and reported in [22]**).** Analysis of cell differentiation in response to adipogenic stimuli found that maturation of 3T3-L1-*Atp7a*$^{-/-}$ cells into functional adipocytes was inhibited. At the end of the differentiation program, while there is no change of cell confluency, *Atp7a*$^{-/-}$ cells remained flat and contained less and smaller lipid droplets comparing to WT cells. The Oil red O staining, which stains lipids in red, also confirmed a paucity of lipid droplets in differentiated *Atp7a*$^{-/-}$ cells (**Fig. 2C**). In addition, when normalizing to the protein content, total triglyceride levels were significantly lower in 3T3-L1 *Atp7a*$^{-/-}$ cells compared to 3T3-L1 controls (**Fig. 2D**). To verify that these changes were caused by the loss of function of Atp7a and were not the off-target effects of CRISPR/Cas9, we generated an additional 3T3-L1 based cell line using shRNA, wherein Atp7a was down-regulated by approximately 70% (**Suppl. Fig. 2B**). These Atp7a knockdown cells accumulated Cu, had altered cell morphology (**Suppl. Fig. 2C, D**), and a diminished adipogenic differentiation (**Suppl. Fig. 2E**). Taken together, these

data suggest that Atp7a is required, in some capacity, for pre-adipocytes transitioning to mature adipocytes.

Adipogenesis is controlled by a balance of internal and external factors, which either stimulate or repress cells differentiation. During the early phase of the adipogenic differentiation, CCAAT/enhancer binding proteins C/EBPβ and C/EBPδ induce the expression of C/EBPα and PPARγ[26], which are the principal transcription factors that control early differentiation of preadipocytes into lipid-accumulating cells. The RT-qPCR analysis of *C/EBPβ* and *C/EBPδ* mRNA revealed that both genes were upregulated in WT cells 24h after induction of differentiation, as expected (**Fig. 2E, F**). In *Atp7a*$^{-/-}$ cells 24h after induction of differentiation, *C/EBPβ* mRNA levels were comparable to WT and *C/EBPδ* mRNA levels were even higher than control (**Fig. 2E, F**). Thus, this step of differentiation appears to be largely independent of Atp7a activity. In contrast, on D3, when abundance and localization of Atp7a changes significantly in control cells, the mRNA levels of *C/EBPα* and *Pparγ* in *Atp7a*$^{-/-}$ cells were markedly different compared to WT controls. The WT cells had *C/EBPα* and *Pparγ* upregulated by more than 50-fold relative to day 0, whereas *Atp7a*$^{-/-}$ cells had only modest upregulation of *C/EBPα* and very little upregulation of *Pparγ* (**Fig. 2G**). These effects on the expression of the differentiation markers suggested that the Atp7a activity is critical for the differentiation steps after the *C/EBPβ* and *C/EBPδ* induction, and that the loss of Atp7a inhibits the signaling leading to activation of *C/EBPα* and *Pparγ*.

Inactivation of Atp7a leads to the specific changes in the cell proteome.

To identify signaling pathways altered in *Atp7a*$^{-/-}$ cells, we compared changes in proteomes of WT 3T3-L1 cells, heterozygous *Atp7a*$^{+/-}$ cells (that differentiate similarly to WT [20]) and 2 clonal lines of *Atp7a*$^{-/-}$ cells during their day0-to-day3 (D0-D3) transition, using TMT-labelling mass-spectrometry (**Fig. 3A**). Based on changes in protein abundance, during D0-D3 period the WT cells increased fatty acid oxidation, fatty acid synthesis, and upregulated proteins involved in lipid storage (**Fig. 3B**). The RhoA and actin cytoskeleton signaling were decreased, and the processes leading to osteoclast differentiation were inhibited (**Fig. 3B**). When the proteome of *Atp7a*$^{-/-}$ cells was compared to WT on D3, main differences include an increased cell spreading with formation of protrusions (p-value 5.48×10^{-18}, z-core 4.184, **Fig. 3C**). Indeed, cell morphology and cytoskeleton of *Atp7a*$^{-/-}$ cells differs from WT 3T3-L1 cells, with *Atp7a*$^{-/-}$ cells being larger and often more flattened as showed by α-tubulin staining (**Fig. 3D**). Proteins associated with osteoclasts differentiation were upregulated in *Atp7a*$^{-/-}$ cells along with a

diminished metabolism of triglycerides (**Fig. 3C**). Taken together, these results pointed to early differentiation defects in *Atp7a*$^{-/-}$ cells compared to WT.

To identify the most affected adipogenic pathway in *Atp7a*$^{-/-}$, we focused on the highly changed genes during the D0-D3 transition in WT cells (**Fig. 3E**) using the cutoff of 4 fold-change. We then grouped those genes by applying the cluster function in Heatmap.2 R package, which uses euclidean measure to obtain distance matrix and complete agglomeration method for clustering, in the other words, the similarity of the expression change among those genes. By this way, we found the most changed genes are a group of genes failed to decrease in *Atp7a*$^{+/-}$ cells upon the induction of adipogenesis, including a well-known inhibitor of adipogenesis β-catenin. β-catenin is a key component of canonical Wnt/β-catenin signaling pathway and its translocation to nucleus induces the Wnt responsive genes. It is well studied that Wnt signaling regulates adipogenesis through the inhibition of C/EBPα and Pparγ expression [27]. The regulator of the early adipogenesis process C/EBPβ has been shown to negatively regulate Wnt pathway through inhibiting Wnt10b [28]. Interestingly, we found that while β-catenin was significantly down-regulated in WT and heterozygous *Atp7a*$^{+/-}$ cells, both of which could differentiate successfully, it was highly maintained in *Atp7a*$^{-/-}$ cell lines (Fig. 3E). Further, several other proteins involved in Wnt/β-catenin pathway were also decreased by more than 16-fold in WT and *Atp7a*$^{+/-}$ cells at D3 with only a mild decrease in *Atp7a*$^{-/-}$ cells (**Fig. 3F**). These differences in the proteomes suggest that *Atp7a*$^{-/-}$ cells do not properly down-regulate the Wnt/β-catenin signaling pathway, which in turn may block adipogenesis.

Elevated Cu stabilizes Wnt/β-catenin signaling *in vitro* and *in vivo*.

To test whether *Atp7a*$^{-/-}$ cells have sustained β-catenin signaling, we further investigated the expression of the Wnt/β-catenin pathway members. Wnt ligands, such as Wnt10b, Wnt10a and Wnt6, which activate the Wnt pathway and stabilize β-catenin protein levels,, have been shown to inhibit adipogenesis[29]. In *Atp7a*$^{-/-}$ cells on D3 during adipogenesis, the mRNA levels of *Wnt10*, and *Wnt6* were significantly higher compared to WT 3T3-L1 cells (**Fig. 4A**).Notably, *Wnt10b* showed the most significant increase, being upregulated by 2.5-fold (**Fig. 4A**). Furthermore, the Western blot analysis of β-catenin confirmed the proteomics findings, showing the decline of β-catenin' expression on D3 in WT cells (**Fig. 4B**). In contrast, levels of β-catenin remained high in *Atp7a*$^{-/-}$ cells, indicative of β-catenin stabilization (**Fig. 4B**). Such stabilization is expected to enhance β-catenin trafficking into the nucleus for the activation of its downstream targets. We tested this prediction using immunofluorescent staining, and found a strong nuclear localization

of β-catenin in *Atp7a*$^{-/-}$ cells but not in control cells on D3, consistent with a prolonged β-catenin signaling (**Fig. 4C**).

In order to examine whether this altered Wnt/β-catenin signaling contributes to defective adipogenesis, we treated *Atp7a*$^{-/-}$ cells with FH535, an inhibitor of Wnt/β-catenin signaling, during the D0-D3 differentiation step. Interestingly, FH535 increased the triglyceride content in *Atp7a*$^{-/-}$ cells (**Fig. 4D**) in agreement with an improved adipogenesis. Similarly, overexpression of Atp7b in *Atp7a*$^{-/-}$ cells partially rescued the adipogenesis defect, being reflected in the increased levels of triglycerides (**Fig. 4E**). Meanwhile, overexpression of Atp7b dramatically decreased β-catenin staining in the nuclei (**Fig.4F)**, as well as the total abundance of β-catenin (**Fig. 4G**)**.** Notably, overexpression of Atp7b-D1027A mutant, which cannot export Cu, had no effect on the β-catenin levels in *Atp7a*$^{-/-}$ cells on D3 (**Fig. 4G**), suggesting that cellular Cu content is the key factor, which induces accumulation of β-catenin. This was further supported by our observation that Cu chelator BCS down-regulates β-catenin levels in a dose-dependent way (**Fig. 4H**).

Finally, we tested whether the Cu-dependent stabilization of β-catenin is unique to *Atp7a*$^{-/-}$ adipocytes or copper elevation may have the similar effect in other cells or tissues. *Atp7b*$^{-/-}$ mice, a mouse model of Wilson disease, accumulate copper in the liver to high levels[30]. We found that the hepatic abundance of β-catenin is significantly increased in *Atp7b*$^{-/-}$ mice compared to WT (**Fig. 4I**). These results confirmed a stabilizing effect of elevated Cu on β-catenin pathway.

DISCUSSION

In this study, we investigated the role of Cu during 3T3-L1 adipocytes differentiation and identified a Cu-dependent step in the early adipogenesis program, and a new role for Cu in prolonging the Wnt/β-catenin signaling. It was previously demonstrated that the expression of Cu transporters, such as ATP7A, changes upon cell differentiation[31]. However, functional significance of this change has not always been apparent. In neuronal cells, upregulation of ATP7A is associated with higher flow of Cu to the secretory pathway and facilitated Cu delivery to Cu-dependent enzymes dopamine-β-hydroxylate (DBH) and peptidyl-α-monooxygenase (PAM), which are also upregulated during differentiation[32]. Similar events take place in adipocytes, where adipogenesis is associated with increased expression of Atp7a and Aoc3OC3, a Cu-dependent enzyme, which is highly abundant in mature adipocytes and which receives its Cu from Atp7a[25]. However, the timing of ATP7A upregulation in either adipocytes or

neuronal cells is not coordinated with the expression of Cu-dependent enzymes, i.e. Atp7a expression peaks much earlier than increases in Aoc3 and DBH/PAM occur in the respective cells [25]. These results indicate that the early adipogenesis (and neurogenesis) involves changes in Cu distribution, which may affect other processes that are modulated during this time. Previous studies have demonstrated a role for Cu signaling in the control of phosphodiesterase (PDE3) activity, whereby elevated Cu concentrations inhibit PDE3-dependent lipolysis[33].

In neuronal cells, early differentiation steps involve transient lowering of cellular Cu, which coincides with changes in cell morphology[9]. We demonstrate that in adipocytes, not only abundance of the Atp7a protein is increased in the early adipogenesis, but also Atp7a traffics to the vesicles/plasma membrane, thus lowering cytosolic Cu. These events occur when the transcriptional programs leading to lipid synthesis and cytoskeleton remodeling are being turned on, suggesting that these processes can be sensitive to the Cu levels. In fact, in mature adipocytes higher Cu stimulates lipolysis[33], whereas Cu depletion induces lipid accumulation[25]. We propose that Atp7a-mediated transient Cu deficit is beneficial for initiation of lipogenesis and lipid accumulation, and serves as an integral component of the entire differentiation program. The effects of Cu on cytoskeleton and cell motility were previously observed in 3T3-L1 cells[22], as well as in other cell types[34,35], however the role of Cu in adipocytes morphology should be studies furhter.

Inactivation of Atp7a results in the elevation of the cellular Cu and has dramatic consequences for adipocytes, manifested in inhibition of *Pparγ* expression and low lipid accumulation. Our studies of changes in the cell proteome during early steps of adipogenesis revealed that these effects are caused in part by a sustained Wnt/β-catenin signaling. In differentiating 3T3-L1 adipocytes, down-regulation of Wnt/β-catenin signaling, which is inhibitory to adipogenesis[36], coincides with the upregulation of Pparγ, a master regulator of adipogenesis. In 3T3-L1 *Atp7a*$^{-/-}$ cells, elevated Cu causes stabilization of β-catenin and elevated Wnt levels thus prolonging Wnt/b-catenin signaling and inhibiting adipogenesis. Two molecular mechanisms may explain β-catenin stabilization. Forst, excess Cu is known to cause oxidative stress, and 3T3-L1-*Atp7a*$^{-/-}$ cells were previously shown to have redox misbalance[22,37]. Oxidative stress can stabilize β-catenin and increase expression of Wnt target genes[38]. Moreover, elevated Cu was shown to inhibit activity of GSK-3β[39]. GSK-3β mediated phosphorylation is a central event for β-catenin degradation and termination of Wnt/β-catenin signaling. Further studies should determine whether Cu regulates β-catenin directly or through these indirect mechanisms

Finally, our finding that elevated Cu prolongs Wnt/β-catenin signaling not only in cultured adipocytes but also *in vivo* in *Atp7b*$^{-/-}$ liver offers a new mechanism for Cu-dependent pathologies. This discovery can contribute in better understanding of the human disorders associated with Cu misbalance.

FIGURES

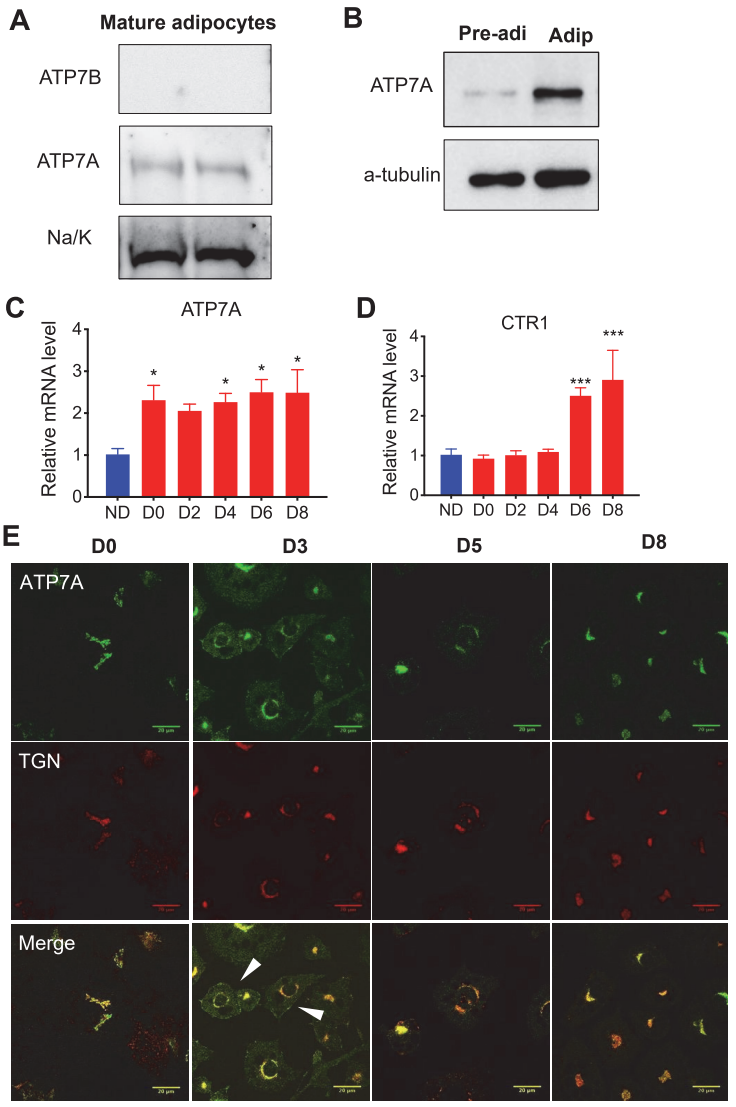

Figure 1. Cu transporter Atp7a is up-regulated and relocates during adipogenesis. (A) Protein levels of Atp7a and Atp7b in differentiated 3T3-L1 adipocytes. **(B)** Protein levels of Atp7a in pre-differentiated (pre-Adi) and differentiated (Adip) 3T3-L1 adipocytes. **(C)** mRNA levels of *Atp7a* (n=3) **(D)** Expression of *Ctr1* in pre-adipocytes, on day 0, day 2, day 4, day 6, and day 8 after adipogenic stimuli, normalized to the levels in preadipocytes (n=3). **(E)** Immunostaining of Atp7a (green) and TGN marker (syntaxin 6, red) in pre-adipocytes, on day 3, day 5, and day8 after adipogenic stimuli. All values present the mean ± SEM; one-way ANOVA was used to analyze the data in C and E. * $p<0.05$, *** $p<0.001$.

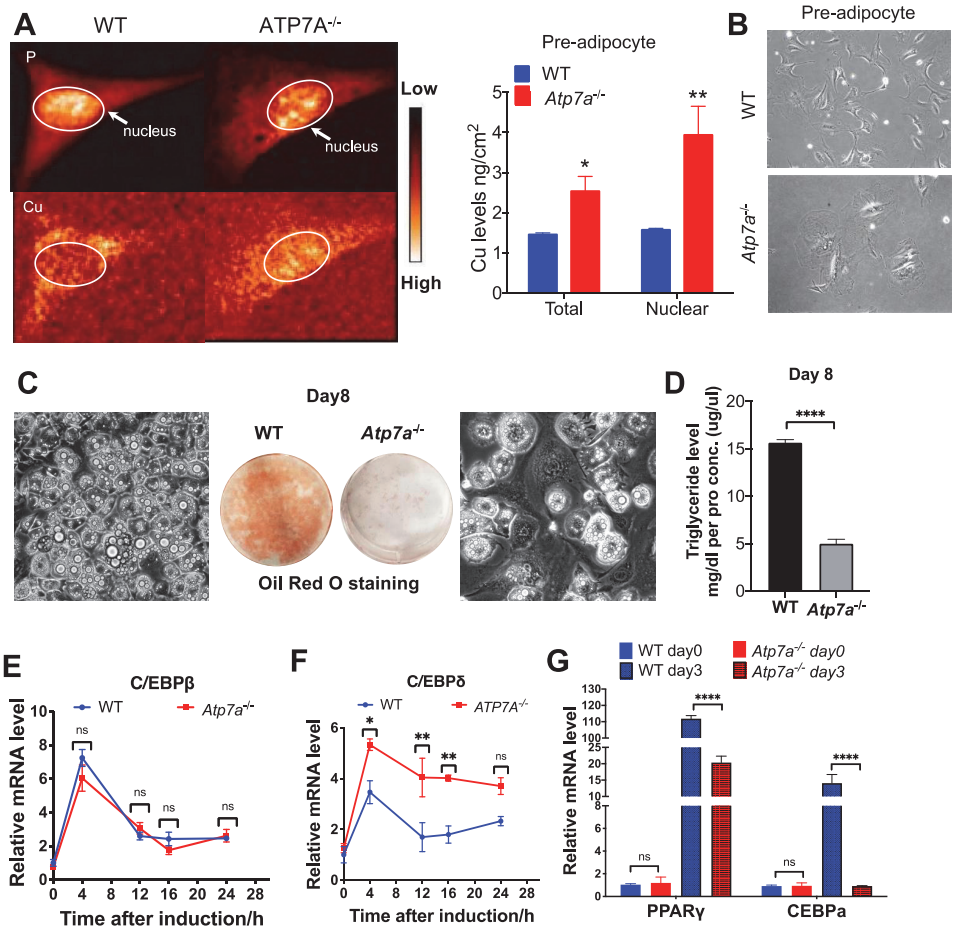

Figure 2. Atp7a inactivation is associated with changes in Cu distribution, cell morphology and inhibition of adipogenesis. (A) X-ray fluorescence imaging and quantification of Cu in control (WT) and *Atp7a*$^{-/-}$ preadipocytes. Signal of phosphate (P) serves as an abundant internal control and for identification of the location of cellular nuclei. **(B)** Differences in cell morphology between the WT and *Atp7a*$^{-/-}$ 3T3-L1 preadipocytes. **(C)** Differences in cell morphology and Oil red O staining in differentiated WT and *Atp7a*$^{-/-}$ 3T3-L1 adipocytes on day 8 (n=3). **(D)** Relative triglyceride content in differentiated WT and *Atp7a*$^{-/-}$ 3T3-L1 adipocytes (n=3). **(E)** The mRNA levels of *C/EBPβ* and **(F)** *C/EBPδ* in WT and *Atp7a*$^{-/-}$ cells during the first 24 hours following stimulation of adipogenesis, normalized to WT cells at time 0 (n=3). **(F)** The mRNA levels of *Pparγ* and *C/EBPα* in *Atp7a*$^{-/-}$ cells at day 0 and day 3, normalized to WT cells on day 0 (n=3). All values represent the mean ± SEM; mean ± SEM. Student t test was used for panel **C,** and two-way ANOVA was used for panels **A**, **E** and **F**. ns $p>0.05$, * $p<0.05$, ** $p<0.01$.

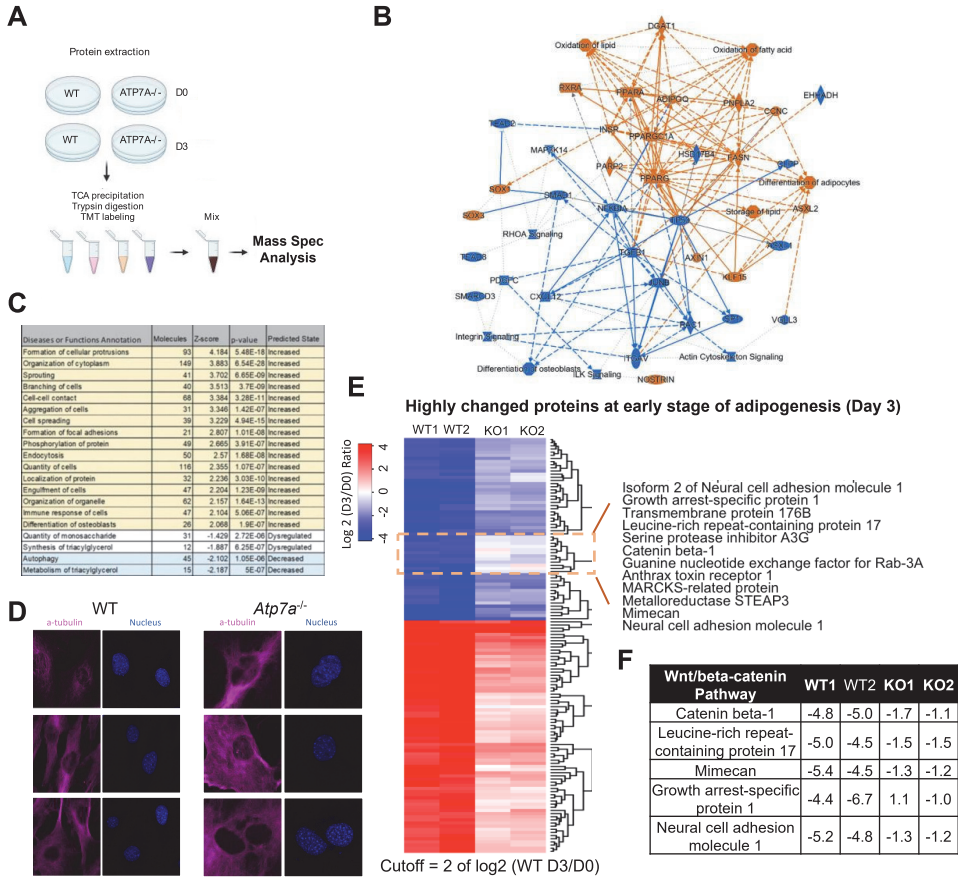

Figure 3. Analysis of proteomes of 3T3-L1 and *Atp7a*$^{-/-}$ cells during the early differentiation. (A) Schematic overview of TMT-labeling mass spectrometry. **(B)** Summary of processes primarily changed in 3T3-L1 cells during day0-day 3 transition: orange – up-regulated, blue down-regulated. **(C)** Major functional differences between the 3T3-L1 and *Atp7a*$^{-/-}$ cells on day 3. **(D)** Immunostaining of α-tubulin in WT and *Atp7a*$^{-/-}$ 3T3-L1 preadipocytes. **(E)** Heatmap of highly changed proteins in WT 3T3-L1 during the early adipogenesis stage, cut-off is 4 fold-change. Clustering function in Heatmap.2 package. The box area in the list indicates the least down-regulated genes in *Atp7a*$^{-/-}$ cells compared to the control adipocytes; β-catenin is among these least down-regulated protein. **(F)** Table of $\log_2$ fold change in abundance for the proteins involved in the Wnt/β-catenin pathway during D0-D3 transition.

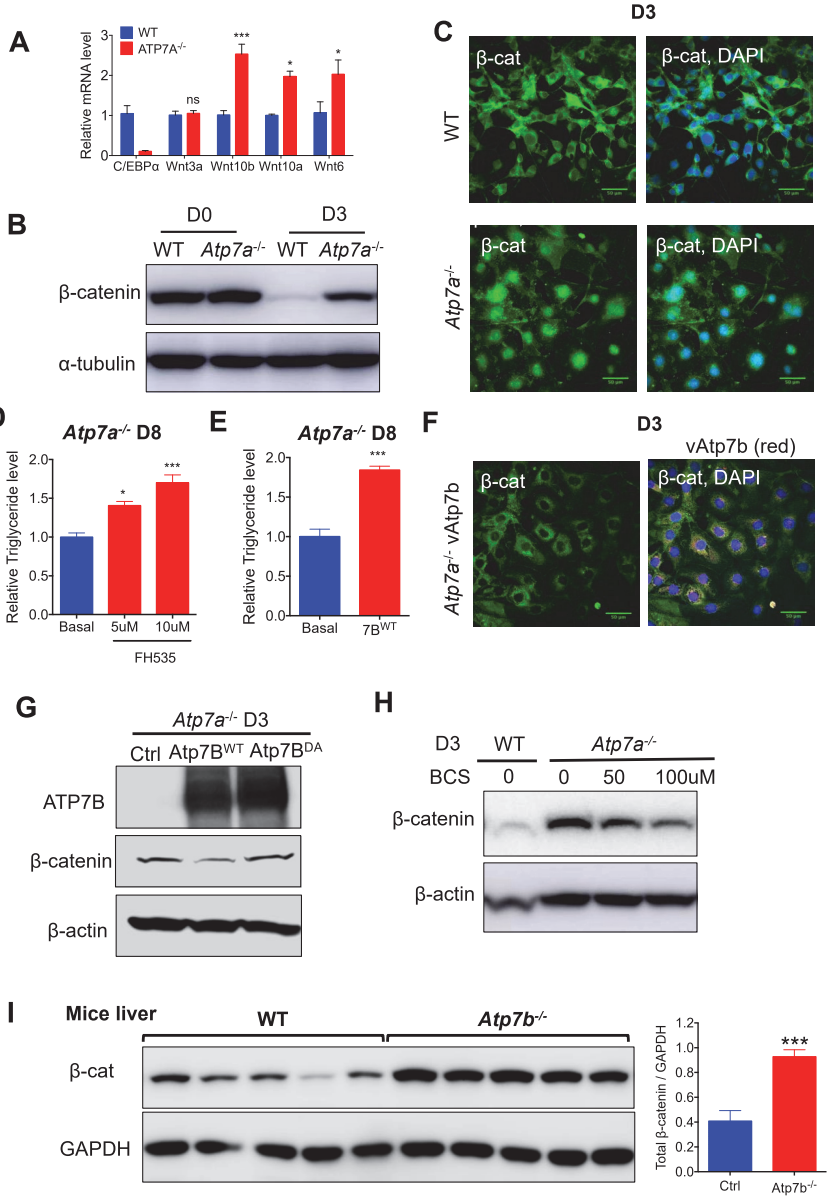

Figure 4. **Wnt/β-catenin signaling persists in Cu overloaded cells and livers.**
(A) Relative mRNA levels of *C/EBPα*, *Wnt10b*, *Wnt10a* and *Wnt6* in *Atp7a*$^{-/-}$ 3T3-L1 normalized to WT cells at day 3 (n=3). **(B)** Protein levels of β-catenin in WT and *Atp7a*$^{-/-}$ 3T3-L1 cells at day 0 and day 3, α-tubulin was used as internal control. **(C)** Immunostaining of β-catenin (green) and DAPI (blue) in WT and *Atp7a*$^{-/-}$ 3T3-L1 cells at day3. **(D)** Relative triglyceride levels in differentiated *Atp7a*$^{-/-}$ cells (day 8) in either basal medium or medium containing 5uM or 10uM of the β-catenin inhibitor FH535 from day0 to day 3 (n=3). **(E)** Triglyceride levels in differentiated *Atp7a*$^{-/-}$cells in basal medium following expression of WT

Atp7b. **(F)** Immunostaining of Atp7b-GFP (red) and β-catenin (green) in *Atp7a*$^{-/-}$ 3T3-L1 cells at day3. **(G)** Immunoblot of β-catenin in *Atp7a*$^{-/-}$ 3T3-L1 cells at D3 which infected with wild type Atp7b-GFP and DA mutant Atp7b-GFP. **(H)** Immunoblot of β-catenin in WT and *Atp7a*$^{-/-}$ 3T3-L1 cells at D3 with 50uM or 100uM Cu chelator BCS 2 days before and during differentiation. (**I**) Western blot (left) and densitometry (right) illustrate the elevation of β-catenin in copper-overloaded livers of *Atp7b*$^{-/-}$ mice (n=5) compared to WT livers. All values represent the mean ± SEM; mean ± SEM. Student t test was used for panels **E** and **G**; one-way ANOVA was used for the panel **D**; two-way ANOVA was used for the panel **A**. ns>0.05 * p<0.05, *** p<0.001

Materials and methods

Cell lines and culture conditions

3T3-L1 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Sigma Aldrich) and 1% Penstrep (Gibco). Differentiation of 3T3-L1 cells was done by sequential treatments with first basal medium (BM) for two days after cells reached confluence (day-2), second with basal medium containing IBMX, dexamethasone, insulin, and rosiglitazone (differentiation medium I, DMI) for 3 days (d0-d3), and last with basal medium containing insulin (differentiation medium II, DMII) for 2 days (d3-d5). On day 5, the medium was changed to basal medium for 3 to 4 days. Cells on day-2 were used as undifferentiated adipocytes, and cells on day 8 or day 9 were used as differentiated mature adipocytes.

Immunoblot analysis

Cells were cultured in 24-well plates, 6-well plates, or 100-mm dishes. Cells or tissue were lysed using 1xRiPA buffer (with EDTA free protease inhibitor cocktail) on ice for 1 h, and debris as well as nuclei were removed by centrifugation at 10000g for 15 min. Protein concentration in the resulting cleared lysate was determined by BCA assay. Before electrophoretic separation of proteins, each sample was combined with an equal volume of 2x Laemmlli sample containing 5% β-mercaptoethanol. Proteins were then resolved on 8% Laemmlli SDS-PAGE and transferred to PVDF membrane at 90 V for 90 min using CAPS buffer. Primary antibodies used in immunoblotting were: rabbit anti-ATP7A CT77 (Hycult biotech), mouse monoclonal anti-α-tubulin (Sigma, T8203), mouse anti-β-catenin (BD Biosciences, catalog #610153), rabbit anti-ATP7B (Abcam ab124973), mouse anti-Na/K ATPase (millipore 05-369). Secondary antibodies were: goat polyclonal anti-mouse IgG HRP-conjugate (Santa Cruz, SC-2005), goat polyclonal anti-rabbit IgG HRP-conjugate (Santa Cruz, SC-2004). All antibodies were used at a dilution of 1:1000.

Immunostaining of cultured cells

Immunostaining was performed as previously described. Cells were cultured on cover slips in a 12-well plate, fixed with 1:1 Acetone and Methanol solution for 30 s followed by 5 s washing in phosphate buffered saline (PBS), and blocked with 1% (w/v) BSA/1% (w/v) gelatin in PBS. Primary antibodies used in immunoblotting were: mouse monoclonal anti-rabbit anti-ATP7A CT77 (Hycult biotech), mouse anti-Syntaxin-6 (BD Transduction Laboratories, 610636), rabbit polyclonal anti-beta catenin (ab16051). Secondary antibodies were: goat polyclonal anti-rabbit IgG Alexa488-conjugate (Thermo, A11034), goat polyclonal anti-mouse IgG Alexa555-conjugate (Thermo, A31570). All antibodies were used at a dilution of 1:100. Cells were imaged with a Zeiss LSM 710. Images were processed with ZEN and ImageJ software.

Quantitative real-time PCR

Total RNA was isolated from cells with RNEasy kit (Qiagen), and corresponding cDNA pools were synthesized using Fast-Strand cDNA synthesis kit (Roche). RT–PCR was performed with SYBR green (Applied Biosystems) on an ABI 7500 Sequence Detection System (Applied Biosystems). For ddCt analysis, 18S or NoNo levels were used for normalization. Primers used in this study are listed in supplementary table 1.

CRISPR/Cas9 genomic edited cell line

Atp7a$^{-/-}$ 3T3-L1 cell line was generated by using CRISPR/Cas9 genomic editing and vector (pEF6/V5-His TOPO TA) expressing Cas9 were as previously described[37]. Briefly, two sgRNA oligos were synthesized for *Atp7a*: *Atp7a* target 1: 5'-GTTTTTCTGTATCCCTGTAATGG-3'*;* *Atp7a* target 2: 5'-CCTATGCTGTTTGTGTTTATTGC-3'). 3T3-L1 cells were transfected with 2 µg Cas9-sgRNA plasmid using Lipofectamine LTX with Plus Reagent (Life Technologies). Transfected cells were selected by the addition of 3µg/ml blasticidin in the cell culture medium for two weeks and then diluted into 96-well plates for cell cloning. *Atp7a*-downregulated cell clones were determined by Western blot assay. Mutations in the *Atp7a* gene were further identified through Sanger sequencing.

X-ray fluorescence microscopy (XFM)

Cultured cells were grown directly onto 4 x 4 mm silicon nitride membranes (SiN, Silson Ltd, Northhampton, England). SiN membranes were sterilized with UV radiation and incubated with 10 µL sterile 0.01% POLY-L-Lysine solution (Sigma-Aldrich, St Louis, MO) at 37ºC. After 30 min, POLY-L-Lysine was removed and 10 µl of cell containing media was added to the

membrane. Basal medium was added to the cultures after 15 min at 37ºC. After completion of the experiments, the membranes were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min at 37ºC, rinsed with PBS/isotonic 100 mM ammonium acetate/water, and then air-dried. XFM data were collected on beamline 2-ID-E at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL). SiN membranes were mounted onto kinematic sample holders and target cells were selected using a light microscope (Leica, Buffolo Grove, IL) equipped with a high precision, motorized x, y-stage (Ludl Electronic Products, Hawthorne, NY). Coordinates of target cells were recorded before mounting the sample onto the microprobe stage at the beamline. The microscope coordinates were translated into microprobe coordinates and the cell raster scanned in the x-y plane. The incident X-ray energy was tuned to 10 keV using a Si-monochromator, and the monochromatic beam was focused to 750 x 750 nm using a Fresnel zone plate. The sample was placed at 19º to the incident X-ray beam, and the resulting X-ray fluorescence was collected at 90º using an energy dispersive 4-element detector (Vortex ME-4, SII Nanotechnology, Northridge, CA). Elemental maps were created by extracting, background subtracting, and fitting the fluorescence counts for each element at each point using the program MAPS. The fluorescent photon counts were translated into µg/cm2 using calibrated X-ray standards (AXO products, Dresden, Germany).

Triglyceride levels measurement.

Cells monolayers cultured in 24 well-plate were washed two times with PBS and lysed with 80 µl 1 x RIPA on ice for 30 min. Cell lysates were vortexed for 30 s, and then triglycerides were quantified calorimetrically as glycerol by use of a commercial enzymatic assay (Infinity Triglycerides, Fisher Diagnostics). Triglyceride levels were expressed as total triglyceride per well. To compare triglycerides in different cell lines, triglyceride levels were normalized to protein concentration. Cell lysates were centrifuged at 3000*g* for 15 min at 4°C. Supernatants were used to measure protein concentration using BCA assay.

Oil red O staining

Cell monolayers cultured in 6 well-plates were washed two times with PBS and then fixed for 2 min with 4% formaldehyde. Cells were then washed two times with water followed by a wash with 60% isopropanol for 5 min. 60% isopropanol was removed completely and cells were left to dry at room temperature. 0.5% Stock Oil Red O (Sigma, O1391) was diluted with water (3:2), filtered through a 0.45 µm filter, and incubated with the fixed dried cells for 2 h at room

temperature. Cells were then washed extensively with distilled water, dried, and photographed using a camera.

Adenoviral Infection

Atp7a$^{-/-}$ 3T3-L1 preadipocytes were transduced with AdenoExpress ATP7B-GFP in pAdLOX for adenovirus-mediated protein expression. Briefly, 1% polylysine was added to the serum-free medium prior to the addition of AdenoExpress ATP7B-GFP virus, and then incubated at room temperature for 100 min prior to adding it to the PBS-washed cells. After incubation of the culture being transduced for OptiMEM media (Invitrogen Life Technologies) and then the media were replaced with complete media for 48 h.

Generation of ATP7A knock-down 3T3-L1 cells

The shRNA pLKO.1-puro plasmids expressed in DH5α *E.coli* strain were purchased from SigmaAldrich (MISSION® shRNA Bacterial Glycerol Stocks, clones nr TRCN0000101810, TRCN0000101812 and TRCN0000101813, products referred as shRNA-10, shRNA-12 and shRNA-13, correspondingly). Lentiviral pMD2.G envelope and psPAX2 packaging plasmids were purchased from Addgene (cat 12259 and 12260). HEK293T cell were used as a packaging cell line. Briefly, HEK293T cells were grown to reach 70-80% confluency and were co-transfected with pLKO.1-puro, pMD2.G and psPAX2 plasmids using Lipofectamine® LTX and Plus™ reagent (15338-100, Invitrogen) following the protocol of the manufacturer. Transfection medium was replaced with fresh medium 6 hours after transfection. Virus packaging was confirmed by the presence of virus plaques. Lentivirus particles containing medium was harvested 48 h after transfection and stored in -80˚C until transduction of 3T3-L1 cells. Before transduction, virus containing medium was slowly thawed on ice, diluted to obtain proper MOI and added to 3T3-L1 preadipocytes ($1x10^9$ particles/ml). Transduction medium was replaced with complete growth medium 24 h after infection. pLKO.1-puro plasmids expressing 3T3-L1 cells were selected using puromycin.

shRNA constructs sequences were:

shRNA-10:

5’CCGGCGTGCAAGGATCTTATGTCAACTCGAGTTGACATAAGATCCTTGCACGTTTTTG3’;

shRNA-12:

5’CCGGCGGACCATTGAACAGCAGATTCTCGAGAATCTGCTGTTCAATGGTCCGTTTTTG3’;

shRNA-13:

5’CCGGCCCGAGTGATAGCAGAGTTTACTCGAGTAAACTCTGCTATCACTCGGGTTTTTG3’.

458
459 **Mass spectrometry analysis**
460 Samples were prepared as described previously[20]. Briefly, Wild type, *Atp7a*$^{+/-}$, *Atp7a*$^{-/-}$, BSC
461 treated *Atp7a*$^{-/-}$ 3T3-L1 cells at confluent stage (day 0) or 3 days stimulated with differentiation
462 medium I, DMI (IBMX, dexamethasone, insulin, and rosiglitazone) day 3 were cultured and
463 washed three times with cold phosphate-buffered saline (PBS). Extraction solution consisted of
464 55 mM iodoacetamide in buffer (100 mM pH 8.5 Tris-HCl buffer, 4% SDS) was added to cells to
465 alkylate free cysteine thiols upon lysis per each condition. Cells were scraped immediately on
466 ice and sonicated. All samples were centrifuged at 16,000g for 5 min at 4 °C. Supernatants were
467 then incubated in the dark at a shaker with 1400 rpm for 1 h at room temperature. Protein
468 concentration was determined by BCA assay. 80 µg proteins from each sample were treated
469 with 70 mM DTT and incubated at room temperature (24 °C) for 45 min to reversibly reduce
470 their oxidise thiols. Samples were added one volume of 50 mM ammonium bicarbonate solution
471 (pH 7.0). Newly generated free thiols were subsequently alkylated using 80 mM NEM. Finally
472 proteins were then precipitated using trichloroacetic acid (TCA) and used to perform samples for
473 mass spectrum.
474 Approximately 1 µg of each fraction, (calculated based on the original amount of total protein)
475 was analyzed by liquid chromatography interfaced with tandem mass spectrometry (LC/MS-MS)
476 using a Thermo Easy-LC interfaced with a Fusion (www.thermofisher.com), and isotopically
477 resolved masses in precursor (MS) and fragmentation (MS/MS) spectra were extracted from
478 raw MS data without deconvolution and with deconvolution using Xtract or MS2 Processor in
479 Proteome Discoverer (PD) software (v1.4, Thermo Scientific) as described before[25]. Protein
480 quantification is based on the normalized median ratio of all spectra of tagged peptides from the
481 same protein[40].
482
483 **Bioinformatic Analysis**
484 Multivariate statistics and associated graphics were performed in R version 3.4.1. Fold change
485 of protein levels were analyzed using limma package. Heat maps were drawn using the
486 heatmap.2 function found in the gplots package. The associations between altered proteins and
487 pathways were evaluated using the Ingenuity Pathways Analysis software (Ingenuity Systems,
488 www.ingenuity.com). Differentially expressed proteins and their corresponding expression
489 values were loaded into the software and mapped to associated pathway functions that were
490 generated from existing literature from the Ingenuity Systems Knowledge Base.
491

Mice husbandry and tissue collection

Animals were housed at the Johns Hopkins University, School of Medicine (JHU SOM) animal care facility, and the studies followed the National Institutes of Health guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee (protocol number M017M385). Atp7b KO (*Atp7b*$^{-/-}$) and litter mate WT mice of C57BL/6x129S6/SvEv background (described in [41]) were fed with the standard pellet chow. At 20 weeks after birth, mice were euthanized and dissected to take different tissues. Liver samples were used for immunoblot analysis. Tissues were flash-frozen and stored at −80°C until protein extraction.

Statistical Analysis

Statistical analysis was performed using Prism software version 6.0b (GraphPad Software). Two-tailed Student's t tests were used when appropriate. All data in the figures are shown as the mean ± SEM. A p value of less than 0.05 was considered significant, and ns stands for p >0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Acknowledgements: This work was supported by the NIDDK and OD grant **2 R01 DK071865-14A1.** We thank Dr. S. Jayakanthan for providing the adenovirus encoding ATP7B and Dr. W.G. Wong for help and advice with the initial stages of this project. The analysis of proteomes was done at the Johns Hopkins Mass Spectrometry and Proteomics Core supported by the Johns Hopkins Institute for Clinical and Translational Research/Translational Science Cores (**NCATS center grant UL1 TR003098**); the JHU Sidney Kimmel Comprehensive Cancer Center (**NCI center grant P30 CA006973**) and the Hopkins Digestive Diseases Basic and Translational Research Core Center (**NIDDK center grant P30 DK089502**). Grants from Dora Plus 1.2 and Kristjan Jaak Scholarship Programmes were awarded by **Archimedes Foundation** (Education and Youth Board, Estonia) to E.K.

Author contributions: HY designed and performed experiments, analyzed the data, and wrote the manuscript; EK performed experiments, analyzed the data and edited the manuscript; YD, XZ and MR performed experiments and analyzed the data; SL provided financial support, analyzed the data, and edited the manuscript.

Competing interests: All authors declare no competing interests.

Data and materials availability: All data is available within manuscript or the supplementary materials.

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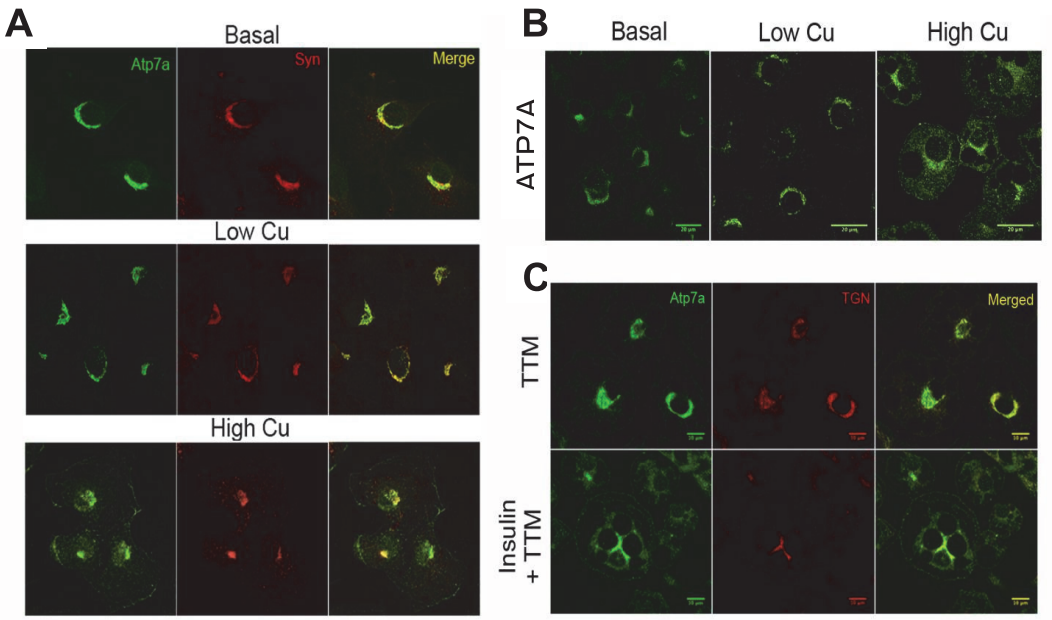

673

674 **Supplementary Figure 1. Trafficking of Atp7a in non-differentiated and differentiated 3T3-L1**
675 **adipocytes.**
676 (A) 3T3-L1 preadipocytes or (B) differentiated adipocytes were maintained in the regular growth medium
677 (basal) and treated with either 25 μM Cu chelator TTM (low Cu) or 50 μM $CuCl_2$ for 2 hours (High Cu).
678 Syn is TGN marker, syntaxin 6. (C) Atp7a localization in low Cu (25 μM TTM) or in cells treated with 1
679 μg/ml insulin for 2 hours in the presence of 25 μM TTM. All experiments have been repeated at least
680 three times.
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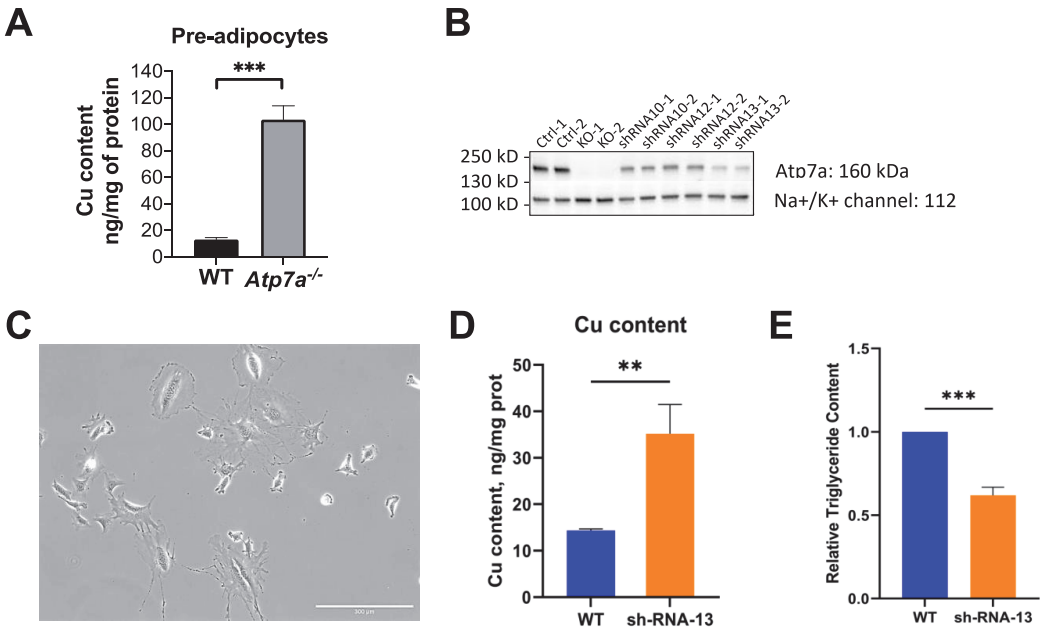
683
684



Supplementary Figure 2. Deletion of Atp7a alters the intracellular Cu redistribution and inhibits adipogenesis.

(A) Cu concentration in WT and *Atp7a*$^{-/-}$ 3T3-L1 cells before (preadipocytes) and after (adipocytes differentiation (n=3). (B) Immunoblotting of Atp7a and Na/K ATPase in WT and *Atp7a*$^{-/-}$ 3T3-L1 cells and Atp7a shRNA infected cells. (C) Representative image of Atp7a shRNA infected 3T3-L1 cells. (D) Cu content in control and Atp7a shRNA-13 infected WT 3T3-L1 cells (n=3). (E) Relative triglyceride levels of control and Atp7a shRNA-13 infected WT 3T3-L1 cells after differentiation (n=3). All values represent the mean ± SEM; mean ± SEM. Student t test was used to analyze the data and ** p<0.01, *** p<0.001.

Supplementary Table 1. Primers used for RT-qPCR analyses.

Wnt3a_f	CTCCTCTCGGATACCTCTTAGTG
Wnt3a_r	CCAAGGACCACCAGATCGG
Wnt4_f	AGACGTGCGAGAAACTCAAAG
Wnt4_r	GGAACTGGTATTGGCACTCCT
Wnt10a_f	GCTCAACGCCAACACAGTG
Wnt10a_r	CGAAAACCTCGGCTGAAGATG
Wnt6_f	GCAAGACTGGGGGTTCGAG
Wnt6_r	CCTGACAACCACACTGTAGGAG
beta-actin_f	ATGGAGCCGGACAGAAAAGC
beta-actin_r	TGGGAGGTGTCAACATCTTCTT
C/EBP delta_f	CGACTTCAGCGCCTACATTGA
C/EBP delta_r	CTAGCGACAGACCCCACAC
Atp7a_f	AGGATCGGTCAGCAAGTCAC
Atp7a_r	TACCAGCCTCCGAAAAACTGT
Ctr1_f	TCTCACCATCACCCAACCAC
Ctr1_r	AAAGCTCCAGCCATTTCTCCAG
CEBP alpha_f	CAAGAACAGCAACGAGTACCG
CEBP alpha_r	GTCACTGGTCAACTCCAGCAC
C/EBP beta_f	GTTTCGGGACTTGATGCAATC
C/EBP beta_r	AACAACCCCGCAGGAACAT
PPAR delta_f	TCCATCGTCAACAAAGACGGG
PPAR delta_r	ACTTGGGCTCAATGATGTCAC

Curriculum vitae

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2014–2017	Tallinn University of Technology, MSc (Gene technology)
2011–2014	Tallinn University of Technology, BSC (Gene technology)
2008–2011	Narva Kesklinna Gymnasium, with honours

Language competence

Russian	Native
Estonian	Fluent
English	Fluent

Professional employment

December 2022 – …	Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology
September 2019 – September 2022	Johns Hopkins University, School of Medicine, Department of Physiology, Baltimore, MD, USA, exchange trainee
September 2017 – December2017	Tallinn University of Technology, School of Science Department of Chemistry and Biotechnology Early Stage Researcher
June 2015 – October 2016	Tallinn Children Hospital, laboratory assistant

Academic Honors and Awards

2023	Top Poster Award at Physiology in Focus 2023 in Tallinn, Estonia, September 14–16, 2023
2022	Top Poster Award at FASEB's Trace Elements in Biology and Medicine, Conference in Asheville, North Carolina, USA, June 12–16, 2022
2020	Kristjan Jaak national stipendium for exchange studies
2019	Dora Pluss 1.2 action PhD student mobility scholarship
2019	Dora Pluss 1.1 action Short study visits scholarship

Professional Societies

2018 – …	Estonian Biochemical Society

Presentation at Conferences

September 2023	Physiology in Focus 2023, Tallinn, Estonia, poster presentation "α-Lipoic acid ameliorates consequences of copper overload by upregulating selenoproteins and decreasing redox misbalance"

May 2023	Spring School of Estonian Biochemical society, Nelijärve, Estonia, oral presentation "α-Lipoic acid ameliorates consequences of copper overload by upregulating selenoproteins and decreasing redox misbalance"
June 2022	Participant at the Johns Hopkins Mass Spectrometry Day, Baltimore, MD, USA
June 2022	FASEB's Trace Elements in Biology and Medicine Conference, Asheville, North Carolina, USA, poster presentation "α-lipoic acid reverses copper dependent changes in cell morphology of ATP7A-deficient preadipocytes"
June 2022	FASEB's Trace Elements in Biology and Medicine Conference, Asheville, North Carolina, USA, Flash-talk "α-lipoic acid reverses copper dependent changes in cell morphology of ATP7A-deficient preadipocytes"
November 2021	JHMI Physiology department retreat, Baltimore MD, USA, poster presentation "α-lipoic acid reverses copper dependent changes in cell morphology of ATP7A-deficient preadipocytes"
November 2019	Symposium "Frontiers in Metals in Medicine", Baltimore, MD, USA, participant
July 2019	11th international congress on Liver and Pancreatic diseases, Lisbon, Portugal, poster presentation "Protective effect of copper chelating ligands on copper toxicity in cell culture"
February 2019	Participant in winter school for PhD students "Writing Process Reengineering", Rakvere, Estonia
November 2018	Participant at EstEHG 20 annual conference, Viljandi, Estonia
August 2018	Participant at summer school for PhD students "Self-management and coping with stress", Saka manor, Estonia
February 2018	Participant in winter school for PhD students "Life after PhD"
May 2014	Spring School of the Estonian Biochemical society, oral presentation "Investigation of the redox properties of the Cys_4-type zinc finger by ESI-MS"

Publications

Yang H., **Kabin E.***, Dong Y.*, Zhang X., Ralle M., Lutsenko S.
Atp7a-dependent copper sequestration contributes to termination of β-catenin signalling during early adipogenesis. Manuscript.

Kabin, E.; Dong, Y.; Roy, S.; Smirnova, J.; Smith, J.W.; Ralle, M.; Summers, K.; Yang, H.; Dev, S.; Wang, Y.; Devenney, B.; Cole, R. N.; Palumaa, P.; Lutsenko, S. (2013). α-Lipoic acid ameliorates consequences of copper overload by upregulating selenoproteins and decreasing redox misbalance. *Proc Natl Acad Sci*, 120 (40), #e2305961120. DOI: 10.1073/pnas.2305961120.

Smirnova J., **Kabin E.**, Järving I., Bragina O., Tõugu V., Plitz T., Palumaa P. (2018). Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent. *Sci Rep*, 8(1):1463. doi: 10.1038/s41598-018-19873-2.

Smirnova, J.; **Kabin, E**.; Tougu, V.; Palumaa, P. (2018). Redox properties of Cys_2His_2 and Cys_4 zinc fingers determined by electrospray ionization mass spectrometry. *FEBS Open Bio*, 8 (6), 923−931. DOI: 10.1002/2211-5463.12422.

* - equal contribution

Elulookirjeldus

Isikuandmed

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Hariduskäik

2017–2023	Tallinna Tehnikaülikool, PhD
2014–2017	Tallinna Tehnikaülikool, MSc (Geenitehnoloogia)
2011–2014	Tallinna Tehnikaülikool, BSc (Geenitehnoloogia)
2008–2011	Narva Kesklinna Gümnaasium, kuldmedal

Keelteoskus

Vene keel	emakeel
Eesti keel	kõrgtase
Inglise keel	kõrgtase

Teenistuskäik

Detsember 2022 – ...	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, insener
September 2019 – September 2022	Johns Hopkinsi Ülikool, Meditsiinikool, Füsioloogia instituut, Baltimore, MD, USA, vahetusüliõpilane
September 2017 – Detsember2017	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, doktorant-nooremteadur
Juuni 2015 – Oktoober 2016	Tallinna lastehaigla, laborant-bioanalüütik

Stipendiumid ja tunnustused

2023	Parima postri auhind konverentsil “Physiology in Focus 2023” Tallinn, Eesti
2022	Parima postri auhind konverentsil FASEB’i Trace Elements in Biology and Medicine, Asheville, Põhja-Carolina, USA
2020	Kristjan Jaagu pikaajaliste vahetusõpingute stipendium
2019	Dora Pluss 1.2 pikaajaliste vahetusõpingute stipendium doktorantidele
2019	Dora Pluss 1.1 lühiajalise õpirände stipendium

Teadusorganisatsiooniline tegevus

2018 – …	Eesti Biokeemia seltsi liige

Konverentsidel osalemine

September 2023	Osalemine “Physiology in Focus 2023”, Tallinn, Eesti, poster-ettekanne “α-Lipoic acid ameliorates consequences of copper overload by upregulating selenoproteins and decreasing redox misbalance”

Mai 2023	Osalemine Eesti Biokeemia seltsi kevadkoolis, Nelijärve, Eesti, suuline ettekanne "α-Lipoic acid ameliorates consequences of copper overload by upregulating selenoproteins and decreasing redox misbalance"
Juuni 2022	Osalemine konverentsil "Johns Hopkins Mass Spectrometry Day", Baltimore, MD, USA
Juuni 2022	Osalemine konverentsil FASEB'i "Trace Elements in Biology and Medicine Conference", Asheville, Põhja-Carolina, USA, poster-ettekanne ja 90-sekundiline lühiettekanne "α-lipoic acid reverses copper dependent changes in cell morphology of ATP7A-deficient preadipocytes"
November 2021	Osalemine JHMI Füsioloogia instituudi konverentsil, Baltimore MD, USA, poster-ettekanne "α-lipoic acid reverses copper dependent changes in cell morphology of ATP7A-deficient preadipocytes"
November 2019	Osalemine sümpoosiumil "Frontiers in Metals in Medicine", Baltimore, MD, USA
Juuli 2019	Osalemine konverentsil "11th international congress on Liver and Pancreatic diseases", Lissabon, Portugal, poster-ettekanne "Protective effect of copper chelating ligands on copper toxicity in cell culture"
Veebruar 2019	Osalemine doktorantide talvekoolis "Writing Process Reengineering", Rakvere, Eesti
November 2018	Osalemine Eesti Inimesegeneetika ühingu 20. aastakonverentsil, Viljandi, Eesti
August 2018	Osalemine doktorantide suvekoolis "Enesejuhtimine ja stressiga toimetulek", Saka mõis, Eesti
Veebruar 2018	Osalemine doktorantide talvekoolis "Elu pärast PhD-d", Pärnu, Eesti
Mai 2014	Osalemine Eesti Biokeemia seltsi kevadkoolis, suuline ettekanne "Investigation of the redox properties of the Cys_4-type zinc finger by ESI-MS"

Publikatsioonid

Yang H., **Kabin E.***, Dong Y.*, Zhang X., Ralle M., Lutsenko S.
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Smirnova, J.; **Kabin, E**.; Tougu, V.; Palumaa, P. (2018). Redox properties of Cys_2His_2 and Cys_4 zinc fingers determined by electrospray ionization mass spectrometry. *FEBS Open Bio*, 8 (6), 923−931. DOI: 10.1002/2211-5463.12422.

* - võrdne panus

ISSN 2585-6901 (PDF)
ISBN 978-9916-80-084-3 (PDF)