THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Engineering Yeast for Functional Analysis of Human Copper Transport Proteins

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ABSTRACT

Copper (Cu) is an important trace element that plays a vital role in several biological processes. Many proteins that are involved in various biochemical pathways require Cu as a cofactor. In human cells, Cu uptake is mediated by the high-affinity Cu uptake protein Ctr1, followed by the cytoplasmic Cu chaperone Atox1, which shuttles Cu from the plasma membrane to the Wilson disease protein ATP7B, a P_{1B}-type ATPase located at the Golgi compartment. ATP7B incorporates Cu to Cu-dependent enzymes in the secretory pathway and also export excess Cu from the cells. Genetic defects in ATP7B may lead to a nonfunctional protein where Cu distribution is impaired, resulting in Wilson disease (WD). ATP7B is a multi-domain membrane-spanning protein with several protruding cytoplasmic domains. In contrast to its bacterial or yeast homologs, which have one or two metal-binding domains (MBD) respectively, ATP7B has six cytosolic MBDs. Many mechanistic questions around ATP7B function remain unanswered: *e.g.*, the reason for as many as six MBDs and how Cu is released in the Golgi lumen.

In this thesis, the development of a new yeast model system for investigating Cu transport by human proteins is described. The system probes Cu shuttling from Atox1 to ATP7B, and within ATP7B, with yeast growth as the readout. Using this system, combined with biophysical methods such as NMR and MD simulations in some studies, we investigated the roles of the six MBDs in ATP7B (Paper I and III), examined Cu release by ATP7B in the Golgi (Paper II), and studied the effects on Cu transport due to MBD mutations causing WD (Paper IV). Our results provide new mechanistic information on how the six MBDs cooperate to move Cu along them. We also identified residues important for Cu release from ATP7B to the Golgi lumen. The collected results highlight the yeast model as a useful system for studying human Cu transport proteins, in particular when combined with other experimental methods. The yeast model system may be utilized to gain further understanding of various human Cu transport proteins as well as of disorders related to Cu mismetabolism, such as cancer and neurodegeneration.

Keywords: ATP7B, Wilson disease protein, metal-binding domain, yeast model, Cu transport, Atox1, complementation assay, genetic engineering, yeast growth, mechanisms.

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers:

Paper I: Probing functional roles of Wilson disease protein (ATP7B) copper-binding domains in yeast.

Kumaravel P Shanmugavel, Dina Petranovic and Pernilla Wittung-Stafshede
Metallomics, 2017, 9(7):981-988. doi: 10.1039/c7mt00101k

Paper II: A Luminal loop of Wilson disease protein binds copper and is required for protein activity.

Birgit Köhn, Kumaravel P Shanmugavel, Min Wu, Michael Kovermann and Pernilla Wittung-Stafshede
Biophysical Journal, 2018, 115, 1-12. doi: 10.1016/j.bpj.2018.07.040

Paper III: Copper relay path through N-terminus of Wilson disease protein, ATP7B.
Kumaravel P Shanmugavel & Pernilla Wittung-Stafshede.
Metallomics, 2019, 11, 1472-1480. doi: 10.1039/C9MT00147F

Paper IV: Wilson disease missense mutations in ATP7B affect metal-binding domain structural dynamics.
Kumaravel P. Shanmugavel, Ranjeet Kumar, Yaozong Li & Pernilla Wittung-Stafshede
BioMetals, 2019. doi: 10.1007/s10534-019-00219-y

CONTRIBUTION REPORT

Paper I. I conceived the project together with PWS. Planned and performed all experiments and data analysis. I helped with writing the paper together with PWS.

Paper II. Planned and performed the yeast complementation and localization experiments, and analyzed the growth data. I helped with writing the paper together with P.W.S.

Paper III. I conceived the project together with P.W.S. Planned and performed all the experiments and data analysis. I helped with writing the paper together with P.W.S.

Paper IV. I planned and performed the yeast complementation, and localization, experiments. I analyzed the data and wrote the paper together with P.W.S.

Preface

This dissertation is submitted as partial fulfillment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering at the Chalmers University of Technology. The Ph.D. studies were carried out between September 2015 and October 2019 at the division of Chemical Biology, under the supervision of Prof. Pernilla Wittung-Stafshede. The research was funded by the Swedish research council, the Wallenberg foundation, and the Chalmers Foundation.

Kumaravel Ponnandai Shanmugavel

October 2019

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LIST OF ABBREVIATIONS

Ace1	Angiotensin converting enzymes-1
AD	Alzheimer's disease
ALS	Amyloid lateral sclerosis
Atox1	Antioxidant protein -1
ATP7A	Copper transporting ATPase-1
ATP7B	Copper transporting ATPase-2
BSA	Bovine serum albumin
Ccc2	Copper transporting ATPase
CCS	Copper chaperone for superoxide dismutase
CJD	Creutzfeldt-Jakob disease
CopY	Copper metalloregulatory repressor
COX	Cytochrome c oxidase
CP	Ceruloplasmin
Cryo-EM	Cryogenic electron microscopy
CSM	Complete supplement mixture
Ctr1	Copper transport protein-1
Ctr3	Copper transport protein-3
Cu	Copper
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
ER	Endoplasmic reticulum
Fe	Iron
Mac1	Metal-binding activator-1
MBD	Metal-binding domain
MD	Molecular dynamics
MES	2-(n-morpholino)ethanesulfonic acid
MNK	Menke's disease
MT	Metallothionein
Mtf1	Metal-regulatory transcription factor-1
NBD	Nucleotide binding domain
NMR	Nuclear magnetic resonance
PAM	Peptidylglycine alpha-amidating monooxygenase
PD	Parkinson's disease
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
Sco1	Scaffold protein for cytochrome C oxidase
Sco2	Scaffold protein for cytochrome C oxidase homolog 2
SD	Synthetic defined media
SDS	Sodium dodecyl sulfate
SOD1	Superoxide dismutase-1
TGN	Trans-golgi network
TMD	Transmembrane domain
UV-Vis	Ultraviolet-visible
WD	Wilson disease
α-syn	Alpha-synuclein
Aβ	Amyloid beta peptide

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1 INTRODUCTION

Metal ions play an important role in biological systems where they act as catalytic or structural cofactors of around one-third of all enzymes (Dean, Qin, and Palmer 2012). Transition metals, notably Mn, Fe, Co, Cu, Zn, Mo, V and Ni are particularly important to maintain the structural integrity of the associated proteins (Crichton 2019). Cu is involved in several key metabolic processes and is essential for life. In biochemical reactions, Cu has an important catalytic (i.e. oxidoreductases such as ascorbate oxidase, dopamine monooxygenase, galactose oxidase, and amine oxidase) and regulatory functions (proteins such as hephaestin, laccase, and zyklopen) that are needed for normal cellular growth, development, and physiological functions. These roles require tight regulation of Cu uptake and distribution in human cells to prevent Cu imbalance (Guengerich 2018). To regulate and avoid toxicity of free Cu ions, the intracellular concentration of Cu is regulated via dedicated proteins that facilitate its uptake, efflux, as well as distribution to target Cu-dependent proteins and enzymes (O'Halloran and Culotta 2000; Festa and Thiele 2011).

Malfunction of Cu regulation can lead to Menke's disease (Cu deficiency) and Wilson disease (Cu accumulation) (Stern et al. 2007). Wilson disease (WD) is an autosomal recessive disorder of Cu metabolism due to the mutations in the ATP7B gene. ATP7B is P_{1B}-type ATPase that is essential for the transport of Cu into the bile and loading of Cu to Cu dependent enzymes in the secretory pathway (Rodriguez-Castro, Hevia-Urrutia, and Sturniolo 2015). However, mutations in ATP7B can lead to a dysfunctional protein where Cu excretion by ATP7B into the bile is partly or completely hindered. WD is characterized by abnormal accumulation of Cu primarily in the liver cells. The frequency of the WD occurrence is 1 in 30000 individuals (Park et al. 1991). So far, more than 500 missense mutations have been identified in ATP7B (Nagano et al. 1998). Without treatment, WD tends to be fatal due to serious liver failures and/or severe neurological disorders observed in most WD patients (Rodriguez-Castro, Hevia-Urrutia, and Sturniolo 2015).

Intracellular Cu accumulation in cells can lead to many disturbances such as oxidative damage via ROS generation, mitochondrial damage, dysregulation of lipid metabolism and apoptosis (Stern et al. 2007). WD is mainly diagnosed using the knowledge based on clinical signs, histopathological findings, biochemical features and mutation analysis of ATP7B gene (Chappuis et al. 2005). Until now direct genotype–phenotype correlations in WD have been difficult to identify because of the phenotypic variability in WD patients and animal models (Rodriguez-Castro, Hevia-Urrutia, and Sturniolo 2015), which is one of the reasons why molecular mechanistic characterization is desired.

In humans, Cu^{2+} is reduced to Cu^{1+} prior to cell uptake by cell surface metalloreductases and taken up by high-affinity Cu^{1+} transporter. Uptake of Cu is mainly performed by Cu transport protein Ctr1 located in the cell membrane (Eisses and Kaplan 2005). After uptake, Cu is coordinated by the Cu chaperone Atox1, which shuttles the Cu to ATP7B (and its homolog ATP7A) in the Golgi apparatus. ATP7B then incorporates Cu to Cu-dependent enzymes such as ceruloplasmin in the secretory pathway (Huster 2010). At elevated intracellular Cu levels, ATP7B moves to vesicles that go to the plasma membrane and act as a Cu-exporter (Prohaska 2008). ATP7A is expressed in intestine and all tissues except liver and delivers Cu to cuproenzymes such as lysyl oxidase and tyrosinase (Caicedo-Herrera et al. 2018).

ATP7B is a multi-domain protein that consists of eight transmembrane domains (TMD) and three cytosolic domains namely: nucleotide-binding domain (N-domain), phosphorylation domain (P-domain), and actuator/dephosphorylation domain (A-domain). A unique feature of ATP7B (and ATP7A) is the presence of six cytosolic MBDs in the N-terminal region. The six MBDs are connected by peptide linkers of various lengths (Schushan et al. 2012). Similar Cu ATPase homologs in yeast and bacteria have only one or two MBDs (Morin et al. 2009). Cu transport by ATP7B is mediated via ATP hydrolysis and a subsequent phosphorylation-dephosphorylation cascade where ATP7B is thought to undergo significant conformational changes through domain-domain interactions while Cu is moved across the protein (Huster et al. 2012). The organization of the MBDs during the catalytic cycle is unknown due to limited structural information on the arrangement of MBDs within full-length ATP7B (Arioz, Li, and Wittung-Stafshede 2017). It has been hypothesized that Atox1 mediated Cu delivery triggers conformation changes within the MBDs initiating the catalytic cycle (Gonzalez-Guerrero and Arguello 2008). Although most WD mutations are found in the transmembrane domains, a considerable fraction of mutations are also observed in the MBDs (Huster et al. 2012; Schushan et al. 2012). Notably, there has been little focus on the luminal side of ATP7B and Cu release. It is speculated that Cu uptake by Cu-dependent enzymes may happen via direct binding of Cu ions in the lumen, after Cu release from ATP7B. In ATP7A, a protruding loop in the lumen has residues that bind Cu and was suggested as a Cu exit site (Barry et al. 2011). However, this has not been explored in ATP7B. In addition to this, how disease-causing mutations in MBDs contribute to ATP7B dysfunction is not known.

The aim of this thesis is to describe the development of an engineered yeast system for investigating the roles of MBDs, specific residues in ATP7B, and effects of WD mutations in MBDs in ATP7B Cu transport. Previous yeast models used to study Cu transport by ATP7B (Yuan et al. 1995) do not include Atox1 but rely on the yeast homolog Atx1. The model presented in this thesis includes human Atox1. For this purpose, we deleted yeast intracellular Cu transport proteins Ccc2 and Atx1 and complemented these deletions with human Cu transport proteins ATP7B and Atox1, which restored the wildtype phenotype. The system was used to assess the functional roles of the six MBDs in ATP7B (Paper I & III) and to study the effect on Cu transport by putative Cu-binding residues in the luminal loop of ATP7B. Complementary *in vitro* NMR spectroscopy was carried out to identify Cu-binding residues in the ATP7B luminal loop (Paper II). The yeast system was also used (together with *in silico* molecular dynamics) to assess the effect of WD mutations in MBDs (Paper IV).

Structure of the thesis:

Following this brief introduction, **Chapter 2** describes a theoretical background on the main research areas related to my work. The original work of this thesis is summarized and discussed, along with a description of the methodologies, in **Chapter 3.** Finally, a brief summary of the work is presented in **Chapter 4,** and with a conclusion and outlook about future directions of the research in **Chapter 5**.

2 BACKGROUND

This chapter highlights an overview of the key topics relevant to the work presented in the thesis. First, the biochemical properties and functions of Cu are described, followed by Cu homeostasis in eukaryotes. Further, the intracellular Cu transport proteins in the human cell are explained with a primary focus on ATP7B and Atox1 proteins. Then, a detailed introduction to the metal-binding domains of ATP7B is presented. Finally, a brief introduction on the Cu homeostasis disorders.

2.1 Function of copper (Cu) in biological system

Cu is an element located in the group 11 in the periodic table of elements whose name derived from latin: *cuprum*, with atomic number 29, atomic weight 63.54, and electronic configuration: $3d^{10}4s^{1}$. Cu is a naturally occurring metal, which in an aqueous environment has the preferred oxidation state Cu^{2+}, and in a highly reducing environment (such as the cytosol) is reduced to Cu^{1+} (Brose et al. 2014). Cu^{1+} readily coordinates with softer ligands, sulfur ligands such as cysteine, methionine or soft nitrogen ligands such as in histidine. On the contrary, Cu^{2+} binds harder ligands, nitrogen such as in lysine and oxygen ligands such as in glutamate. Cu is usually redox active, and is required to undergo a functional valence change ($3d^{10}4s^{0}$ (Cu^{1+}) to $3d^{9}4s^{0}$ (Cu^{2+}), in aqueous environment) every turnover (Syed Khalid Mustafa 2018). To facilitate this, some Cu-binding sites provide a ligand environment also known as entatic, meaning that Cu can be accommodated in a coordination between both preferred environments (Vallee and Williams 1968), thus allowing for an easy valence switch between Cu^{1+} and Cu^{2+}. The Cu-binding sites are classified based on absorption and EPR (Electron paramagnetic resonance) properties as Type I (blue Cu, mononuclear, electron transfer, mainly cupredoxins, and Cu binds two imidazole nitrogens from histidine and one sulfur from cysteine), Type II (mononuclear, non-blue Cu, and Cu is mainly bound to imidazole nitrogens from histidines) and Type III (dinuclear, EPR silent, and Cu center comprises two spin-coupled Cu ions, bound to imidazole nitrogens). Type II and Type III Cu-binding sites bind oxygen, and are mainly found in enzymes that function as oxidases or oxygenases (Malkin and Malmstrom 1970).

Cu is involved in numerous biological processes such as energy production, proper conformation of protein (*e.g.* collagen) in connective tissue, neuronal cell preservation, iron transport, phospholipid synthesis of cell membranes, antioxidant activity regulation of Cu-superoxide dismutase (Cu-SOD), immune response elicitation against infection, contraction of heart muscle, cholesterol metabolism and development of blood vessels, skins and joints (Josko and Natasa 2013). These numerous functions of Cu in biological systems render it as an essential component for human life. After iron and zinc, Cu is the third

most desired transition metal ion (in terms of amount) for various organisms (Bost et al. 2016). In addition to this, Cu^{2+} forms the most stable complexes in accordance with the Irving-Williams series (relative stabilities of complexes formed by transition metals based on crystal field stabilization energy and ionic radius) (Irving and Williams 1948). Proteins that use Cu as a cofactor play diverse and vital roles in maintaining the metabolic and catalytic activity in the cell (Festa and Thiele 2011). Different Cu-binding proteins play distinct roles in this maintenance where amine oxidase is responsible for oxidizing primary amines; ceruloplasmin (multi-Cu oxidase in plasma) is essential for iron transport; cytochrome c oxidase (oxidase enzyme in the mitochondrial respiratory chain) is involved in electron transport; dopamine β-hydroxylase catalyzes the conversion of dopamine to norepinephrine which is involved in catecholamine metabolism; hephaestin (multi-Cu ferroxidase) is responsible for iron transport across intestinal mucosa into portal circulation; lysyl oxidase directs the cross-linking of collagen and elastin; Cu-SOD acts as defence against reactive oxygen species; multi-functional enzyme peptidylglycine alpha-amidating mono-oxygenase (PAM) is involved in maturation and modification of neuropeptides (i.e., neurotransmitters, neuroendocrine peptides); and tyrosinase catalyzes melanin and other pigment production (Stern et al. 2007; Guengerich 2018). The examples of Cu dependent proteins are presented in Table 1.

Like all essential trace elements and nutrients, deficiency or overdose in the ingestion of Cu leads to decreased or increased Cu concentrations in the cells, each of which has its own unique adverse health effects. Humans have a complex homeostatic mechanism that maintains a constant supply of Cu and eliminates excess Cu (Bost et al. 2016). In all enzymes indicated above, Cu is being utilized as a redox active metal to catalyze reactions (Syed Khalid Mustafa 2018). However, Cu is also a potent cellular toxin (in free form) that is capable of catalyzing reduction or oxidation of other cellular proteins, membrane lipids and also DNA (Guengerich 2018). Through Haber-Weiss reaction ($Cu^{1+}+H_2O_2+O_2^- \rightarrow Cu^{2+}+O_2+OH^-+OH^{\bullet}$) (Kehrer 2000), Cu is known to produce hydroxyl radicals (**.**OH) from hydrogen peroxide (H_2O_2) and superoxide (O_2^-) *in vitro. In vivo* production of these radicals can induce cellular damage by lipid peroxidation, DNA damage (strand breakage and base oxidation), protein damage and mitochondrial damage (reduced respiratory activity) (Festa and Thiele 2011). The benefit of Cu as a cofactor and the potential toxicity of Cu make maintenance of Cu homeostasis a biological prerequisite for all living organisms (Bremner 1998); therefore any disturbance in this balance, especially in mammals leads to a set of metabolic diseases. A general overview of mammalian Cu homeostasis and disorders related to perturbation in this process will be discussed in the following section.

Table 1: List of Cu-dependent proteins and enzymes (Festa and Thiele 2011).

No	Protein	Function
1	Transcriptional Regulators Ace1 (angiotensin-converting enzymes), CopY (Cu metalloregulatory repressor), CsoR (Cu-sensing transcriptional repressor), Mac1 (metal-binding activator), CueR (DNA binding transcription regulator of Cu), Mtf1 (metal regulatory transcription factor), Spl7 (squamosa promoter binding protein 7)	Transcriptional activation in high or low Cu levels, metalloregulatory repressor
2	Chaperones and storage Atox1 (antioxidant 1), CCS (Cu chaperone for superoxide dismutase), CopZ (Cu chaperone Z), metallothionein	Cu delivery to P-type ATPase/Cu-SOD, cysteine rich metal binding and detoxification
3	Cell Surface/Secretory Compartment Transporters and Receptors (P_{1B}-type ATPases, Ctr, ethylene receptor)	Cu exporting/importing proteins
4	Oxidoreductases (Ascorbate oxidase, Dopamine-monooxygenase, Galactose oxidase, Amine Oxidase)	Involved in reduction of metabolites, oxidation of amines
5	Electron transfer/Energy production/Blue Cu proteins (Cytochrome c oxidase COX, Plastocyanin, NADH Dehydrogenase, Nitrite Reductase, Amicyanin)	Key enzymes in respiration and photosynthesis
6	Free Radical Scavenging (Cu-SOD)	Free radical scavenging
7	Oxidase (Laccase, Lysyl oxidase, Ceruloplasmin, Hephaestin)	Regulation of iron uptake, transport of iron
8	Monooxygenase (Methane monooxygenase, Phenylalanine hydrolase, Tyrosinase)	Enzymes involved in catalyzing the oxidation of phenols, melanin synthesis, hydroxylation of the aromatic side chain

2.6 Cu homeostasis and transport in humans

To ensure an optimal supply of Cu, the human body has a fine-tuned homeostatic system in which the Cu is absorbed, transported, distributed, stored and excreted by specific proteins. Several aspects of Cu homeostasis are known at the molecular level. An average of 70-80 mg of Cu is present in an adult human body. Each day around 0.5-2.5 mg of Cu is absorbed, and an approximately the same amount is excreted; thus a steady balance is maintained between dietary Cu absorption and its excretion (Hasan and Lutsenko 2012).

Only about 50 % of Cu is absorbed from the ingested Cu which enters the portal circulation after being absorbed from the small intestine. The portal blood contains several Cu binding molecules such as transcuprein, albumin and small peptides helping Cu transport primarily to the liver where the newly absorbed Cu is accumulated in the liver presumably via transcuprein or albumin within the first 2-6 hours (Syed Khalid Mustafa 2018; Festa and Thiele 2011). In the liver, Cu is then loaded to Cu-dependent proteins which are secreted into the blood such as ceruloplasmin, transcuprein, albumin, macroglobulin (Cabrera et al. 2008). One of the major blood Cu-binding protein is ceruloplasmin that carries around 65 – 95% Cu excreted from the liver (Figure 1), and therefore most of the changes in blood plasma Cu levels are associated with the changes in serum ceruloplasmin concentration (Linder et al. 1998). The main role of the

ceruloplasmin in the serum arises from its ferroxidase activity and its ability to carry Cu in the blood. The ferroxidase reaction (oxidation of Fe^{2+} to Fe^{3+}, reduction of Cu^{2+}) involves the oxidation of iron (Fe) before its incorporation into plasma transferrin (a protein that transports Fe to red blood cells to carry oxygen). This makes Cu-carrying ceruloplasmin essential for iron efflux from cells (Harris 2003).

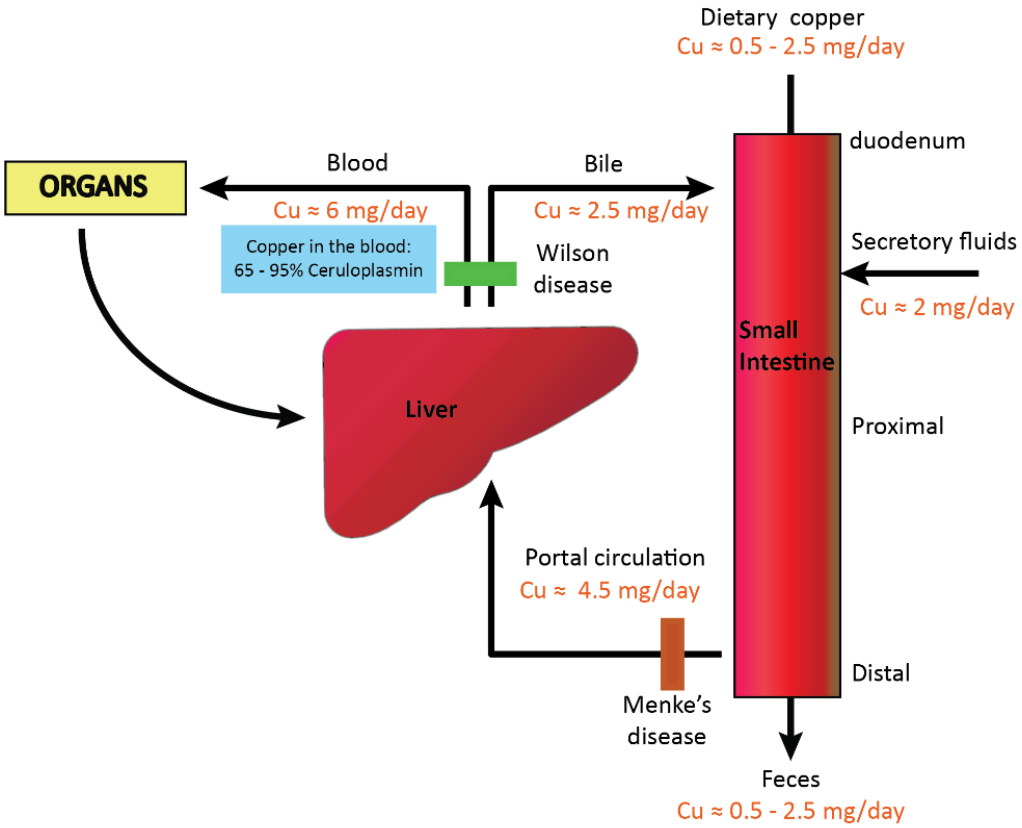


Figure 1: Overview of human copper homeostasis (Guengerich 2018).

The bile is the main route of Cu excretion and is important in the regulation of cellular Cu levels, and once Cu is excreted to the bile, it cannot be reabsorbed (one-way route). Although major excretion occurs in the bile, relatively low amounts of Cu are delivered to the urinary tract. This makes liver the main homeostatic controller maintaining Cu levels in the body (Fanni et al. 2005). Within few hours of intestinal absorption, Cu levels in other organs such as kidneys and the brain tend to rise with the decrease in liver Cu levels (Linder et al. 1998). Ceruloplasmin acts as a carrier for delivering Cu to these organs and also to other tissues(Ferapontova et al. 2005). Each cell has a set of proteins that deal with the uptake and distribution of Cu.

The high-affinity copper uptake protein Ctr1 (SLC31A1) is responsible for the import of extracellular Cu across the plasma membrane in human cells (Petris 2004). In humans, Cu induced endocytosis and degradation of the Ctr1 protein is used as a means to regulate steady-state levels of Ctr1 at the plasma membrane (Balamurugan and Schaffner 2006). Until now, transcriptional control specifically depending on

Cu, has not been identified in mammalian cells (Labbe, Zhu, and Thiele 1997; Cankorur-Cetinkaya, Eraslan, and Kirdar 2016). The Cu chaperones expressed in mammals are Atox1 (Atx1 yeast homolog), Cox17 (cytochrome C oxidase Cu chaperone) and CCS (Cu chaperone for SOD1) that delivers Cu to ATP7A/B (yeast Ccc2 homolog), and Cox11 (another protein involved in mitochondrial Cu delivery). Metallothioneins are also present in mammalian cells (Festa and Thiele 2011; Zhang et al. 2003). After Ctr1-mediated Cu uptake in cells, Atox1 shuttles Cu to ATP7A/B present in trans-Golgi network (TGN) which loads Cu to Cu dependent enzymes in the secretory pathway and expels Cu from the cell membrane during increased intracellular Cu levels. ATP7A/B shuttles between two localization in the cell, the Golgi apparatus and the cell membrane, to maintain Cu homeostasis and provides Cu to Cu dependent enzymes present in the Golgi apparatus (Ralle et al. 2010; Hardman et al. 2004).

The major Cu storage organ is the liver which takes up Cu from the portal circulation. ATP7A is expressed in many tissues except the liver, where its expression diminishes during maturation (Caicedo-Herrera et al. 2018). In liver and other tissues, the main Cu transporter is ATP7B which delivers Cu to cuproenzymes such as ceruloplasmin in Golgi apparatus, in a similar manner like ATP7A. ATP7B mobilizes excess Cu into the bile to prevent Cu overload (Figure 2) (Hasan and Lutsenko 2012).

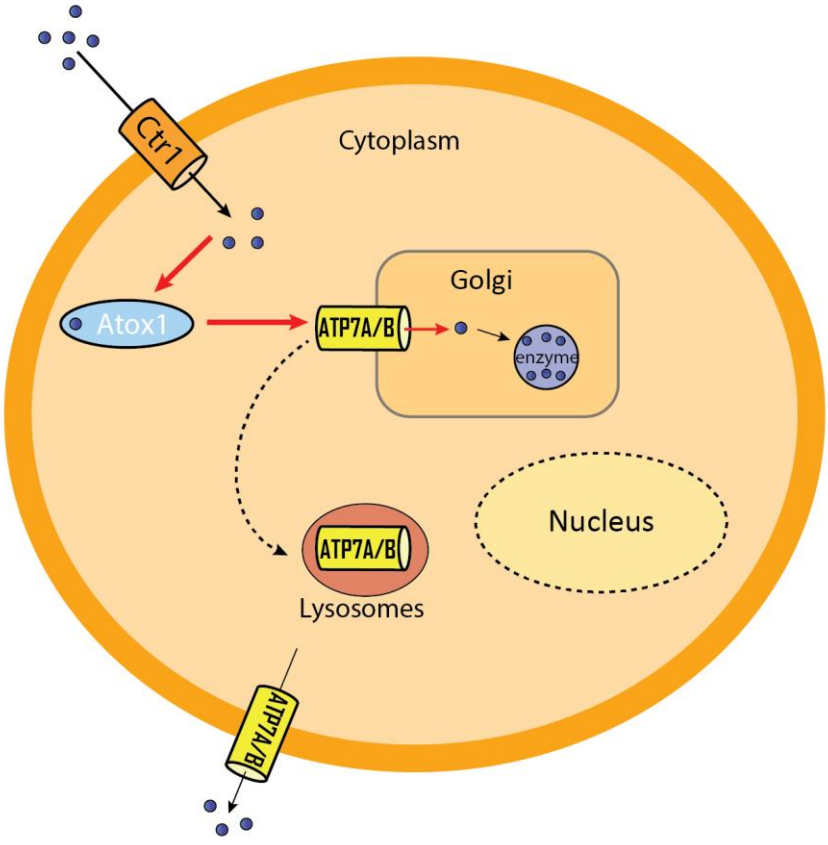


Figure 2: Schematic representation of proteins involved in copper transport in mammalian cells.

2.3 Cu transport in yeast and connection to iron uptake

The Cu homeostatic mechanism was first described in *Saccharomyces cerevisiae* (*S. cerevisiae*). The Cu transport proteins in human and yeast are homologous. The Cu import in *S. cerevisiae* is dependent on two functionally redundant high-affinity membrane Cu transporters; Ctr1 and Ctr3 (similar function to human Ctr1) (Pena, Puig, and Thiele 2000) and a low-affinity transporter Fet4, in which Cu is transported subsequently to the reduction from Cu^{2+} to Cu^{1+} by Fre1, a Cu-reductase located on the plasma membrane (Balamurugan and Schaffner 2006). However, the majority of Cu imported is via Ctr1 under low Cu conditions where Ctr1, Ctr3, and Fre1 are activated via the translational regulator MAC1 (Labbe, Zhu, and Thiele 1997). At elevated Cu levels, endocytosis and degradation of Ctr1 are promoted (Festa and Thiele 2011; Labbe, Zhu, and Thiele 1997).

Under basal conditions, cytoplasmic Cu chaperones Atx1 and CCS receive Cu from Ctr1 or Ctr3 and shuttle it to other Cu dependent proteins. Briefly, Atx1 delivers Cu to the P_{1B} type ATPase Ccc2 localized at the TGN, which then loads Cu to a Cu-dependent multi-Cu oxidase Fet3p (homologous to ceruloplasmin) (Festa and Thiele 2011). Another route for Cu is to Cu superoxide dismutase SOD1 that localizes both in cytoplasm and mitochondria, and receives Cu from CCS. SOD1 is crucial for detoxification of highly toxic superoxide radicals ($\cdot O_2^-$) and hence a great protection from oxidative stress (Rees and Thiele 2007). The mitochondria also need Cu, mostly for COX, which uses Cu for activity. For this, several proteins such as Cox17, Sco1, and Sco2 (Sco 1and 2 COX assembly protein) are involved in the delivery of Cu to COX (Balamurugan and Schaffner 2006). A pictorial illustration of Cu transport process in single cell eukaryotes is shown in Figure 3.

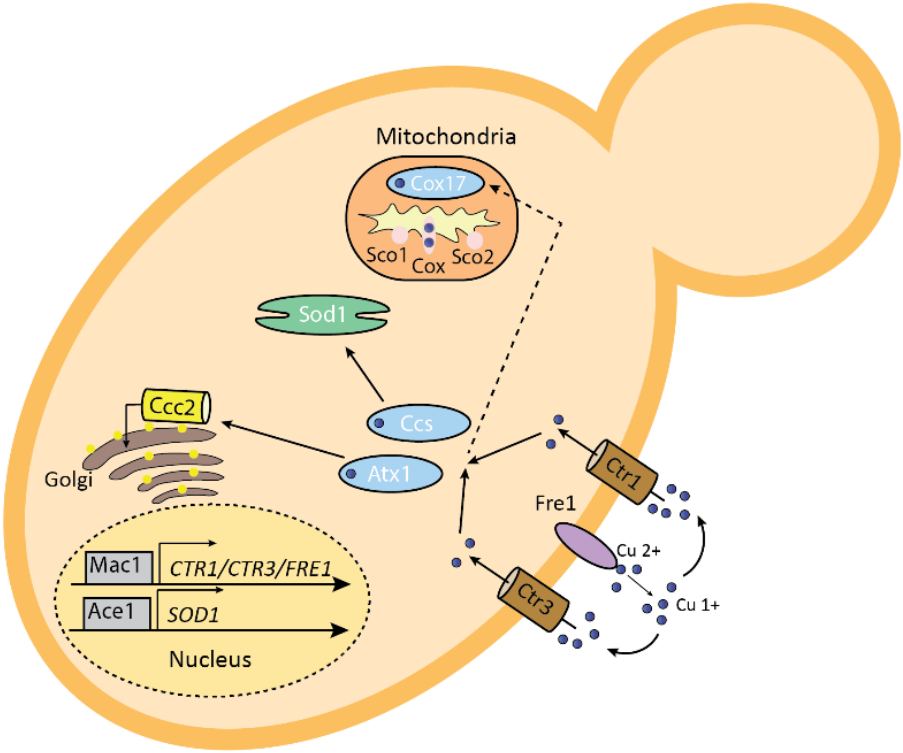


Figure 3: Illustration of copper transport process in the yeast.

Fet3p is an extracellular protein that is responsible for high-affinity iron uptake in yeast. The main function of Fet3p is to catalyze the oxidation of Fe^{2+} to Fe^{3+} (ferroxidase reaction) using oxygen as substrate (Hassett, Romeo, and Kosman 1998). Binding of Cu to Fet3p allows oxidase activity, as no oxidase activity is present in apo-Fet3p (Forbes and Cox 2000). Cell surface-localized Fet3p together with iron permease Ftr1 mediates high-affinity iron uptake. The oxidase function of Fet3p is required at the plasma membrane for iron uptake. The Fe^{3+} generated by Fet3p is a ligand for the iron permease Ftr1, which uptake extracellular iron (Stearman et al. 1996). Thus, Cu transport is connected to iron uptake in yeast (Figure 4).

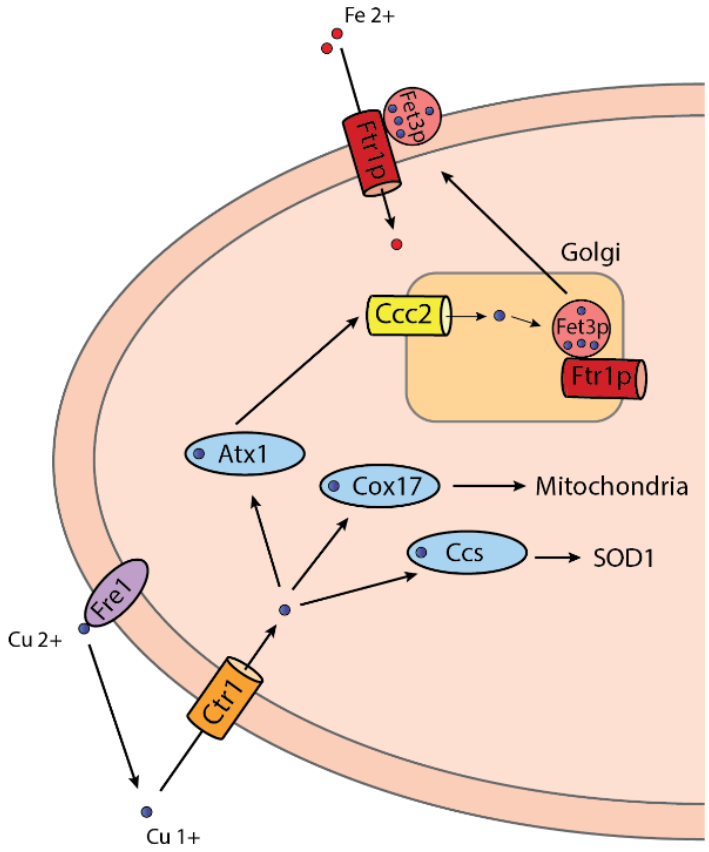


Figure 4: Yeast copper and iron homeostasis. Cu transport in yeast cells is closely linked to the iron transport. Ccc2 delivers Cu to Fet3p enzyme in the Golgi apparatus which is also coupled to iron import by Ftr1. Fre1 is a membrane-bound metalloreductase responsible for reduction of Cu.

2.4 Key Intracellular human Cu transport proteins

2.4.1 Cu chaperone Atox1

The first Cu chaperone Atx1 was originally discovered in *S.cerevisiae* yeast (1995), as a multi-copy suppressor of oxidative damage in yeast cells lacking SOD1 (Lin and Culotta 1995). The main role of Atx1 is to facilitate the delivery of Cu to Ccc2 present in the TGN (Pufahl et al. 1997). Antioxidant protein -1 (Atox1 or Hah1 cytosolic human Cu chaperone) was identified as a chaperone due to its similarity with Atx1 (the yeast Cu chaperone). Atox1 has a ferredoxin-like fold (βαββαβ) (Figure 6) and Cu-binding motif

MXCXXC (Met-X_1-cys-X_2-X_3-cys, where X_1 is mostly threonine, X_2 is majorly alanine, X_3 is serine) similar to Cu-binding motif of metal-binding domain (MBD) in ATP7B (Itoh et al. 2008; Huffman and O'Halloran 2001). The Cu-binding motif is located within the loop that connects the first β-strand and the following first α-helix. This motif binds Cu(I) (Hussain and Wittung-Stafshede 2007). Atox1 shuttles Cu to MBDs of ATP7A and ATP7B. This delivery of Cu to MBDs is confirmed by *in vitro* studies (Niemiec, Weise, and Wittung-Stafshede 2012). Direct protein–protein interactions between Atox1 and MBDs are essential for the delivery of Cu to the MBDs (Yu et al. 2017; Hamza et al. 1999). Atox1 can deliver Cu to all MBDs *in vitro*, but not all MBDs are equivalent in receiving Cu. The MBD1, MBD2, and MBD4 in ATP7B form Cu-bridged adducts with Atox1, whereas the adduct formation was not observed for MBD3, MBD5 and MBD6 in ATP7B but there is still Atox1-mediated Cu transfer (Arioz, Li, and Wittung-Stafshede 2017). The Cu affinities of MBDs are similar to Cu-binding affinity of Atox1, suggesting that Cu-exchange between Atox1 and MBDs is under kinetic rather than thermodynamic control (Wernimont, Yatsunyk, and Rosenzweig 2004).

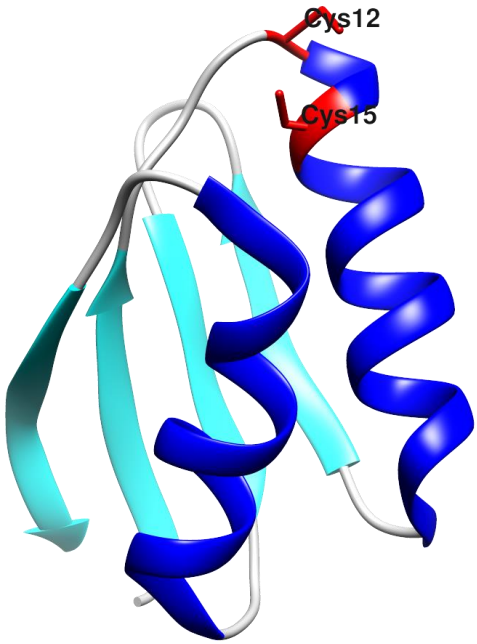


Figure 5: Secondary Structure of Atox1 (Protein database id: 1TL4, Cu-binding cysteine residues are indicated in red).

Apart from Cu transport, Atox1 is thought to have additional functions in the cell that have yet to be elucidated (Harrison et al. 2000; Blockhuys and Wittung-Stafshede 2017). The Lys60 residue is also conserved and was shown to be important for Cu delivery (Hussain, Rodriguez-Granillo, and Wittung-Stafshede 2009). In Atx1, the Lys60 was shown to be essential for antioxidant activity and Cu delivery to Ccc2 (Portnoy et al. 1999). Atox1 knockout cells displayed reduced Cu ATPase activity, and Atox1 deficient animals exhibited growth failure (Hamza et al. 2001).

2.4.2 P-type ATPases

The P-type ATPases (or E1-E2 ATPases) are a group of proteins that function as ion pumps that are found in bacteria, archaea, and eukaryotes. They vary depending upon the physiological process in humans that they are involved in (Palmgren and Nissen 2011). The sodium–potassium, proton-potassium, calcium, and plasma membrane proton pumps are prominent examples of P-type ATPases (heavy metal ATPases such as ATP7A, ATP7B. and non-heavy metal ATPases such as Ca^{2+}-ATPase of sarcoplasmic reticulum, Na^{+}/K^{+}-ATPase.) (Palmgren and Nissen 2011). The heavy metal ATPases form a distinct subgroup of P-type ATPase termed as CPx type ATPase (Solioz and Vulpe 1996).

The P-type ATPase transporter family usually functions as a cation uptake or efflux transporter, but certain proteins in this family are also involved in flipping phospholipids to maintain the asymmetric nature of the biomembrane (Sebastian et al. 2012). The unique feature of P-type ATPases is the auto-phosphorylation of a conserved aspartate (D) residue by ATP hydrolysis (energy source) for driving the ion transport. During the transport process, the transporter has a tendency to interconvert between at least two different conformations (open, E1 and closed, E2 states, (Huster et al. 2012)) after being bound with the inorganic phosphate (Pi) and the ion to be transported. P-type ATPases have a single catalytic subunit that hydrolyzes ATP (via N-domain and A-domain), contain an aspartyl phosphorylation site (P-domain), the binding site for the metal ion and catalyzes ion transport. The catalytic subunit is composed of a cytoplasmic section that consists of a phosphorylation domain (P-domain), nucleotide-binding domain (N-domain), actuator domain (A-domain) and a transmembrane segment (TMDs) with the binding site for the metal ion. The cytoplasmic section contains over half the mass of the protein. Some family of P-type ATPases have additional subunits for their function or accommodate additional regulatory domains (Stokes and Green 2003). P_1-type ATPases consist of the transition/heavy metal ATPases. P_{1A}-type ATPase is an atypical P-type ATPase, as the metal transport is mediated by the sub component of the complex such as K+ import pump of prokaryotes (KdpFABC). The P_{1B}-type ATPases have several N- and C-terminal MBDs which have a role in the catalytic activity regulation of the protein (Palmgren and Nissen 2011). P_{1B}-ATPases are involved in the transport of Cu+, Ag+, Cu2+, Zn2+, Cd2+, Pb2+ and Co2+ ions. The P_2-ATPases are non-heavy metal ATPases such as Na+, K+, H+, Ca2+, Mg2+ pumps and phospholipids, predominantly in eukaryotes. The P_3-ATPases consist of non-heavy metal ATPases of prokaryotes, plants and fungi; P_4-ATPases are flippases involved in the transport of phospholipids such as phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine; and P_5-ATPases are involved in regulation of homeostasis in the endoplasmic reticulum (ER) (Palmgren and Nissen 2011).

CPx type ATPases

CPx type ATPases belong to a subgroup of P-type ATPases (P_{1B}-ATPases), which have the unique feature of a conserved intramembranous cysteine-proline-cysteine or cysteine-proline-histidine motif (Solioz and Vulpe 1996). The novel features of CPx type ATPases are 1) putative metal-binding sites in the polar amino-terminal regions, 2) a conserved intramembranous CPC, CPH or CPS motif, 3) a conserved histidine –proline dipeptide (HP locus in N-domain) 34 to 43 amino acids C-terminal to the CPx motif, and 4) a unique number and topology of the membrane-spanning domains. The CPx motif is generally present in the 6th transmembrane domain, and this region is termed as ion transduction domain or ion channel. The

amino acids surrounding the proline residue vary with ion specificity of the transporter. The intramembranous CPC or CPx motif is a distinguishable feature of heavy metal pumping ATPases. Mutation of histidine present in the HP locus of ATP7B (H1069Q) makes the protein non-functional, thus causing abnormalities in Cu homeostasis (Solioz and Vulpe 1996).

ATP7B

Human ATP7A and ATP7B are highly homologous proteins which belong to the P1B-type subfamily of ATPases (Palmgren and Nissen 2011). They use the energy released from ATP hydrolysis to transport Cu across Golgi membranes. ATP7B is a multi-domain protein with a cytosolic region composed of A-domain, P- domain, N-domain, six MBDs, and has eight membrane-spanning TMDs (Figure 7). The cytoplasmic N-terminal part of the protein has six MBDs that are connected together in a linear chain by short flexible linkers (Banci et al. 2008). *In vitro* studies on MBDs of ATP7B have demonstrated that MBDs receive Cu from Cu chaperone Atox1 (Niemiec, Weise, and Wittung-Stafshede 2012). The MBDs consist of the consensus motif of MXCXXC that displays the Cu-binding site, similar to that in Atox1 (Niemiec, Weise, and Wittung-Stafshede 2012). A TGN retention signal is present in the amino terminus of ATP7B. The eight transmembrane segments are anchored in the Golgi membrane and have an appearance of a helical bundle. Adjacent transmembrane domains are connected by a loop segment (Andersson et al. 2014). The CPC motif in the transmembrane segment is a Cu-binding site. The protein uses ATP hydrolysis to move Cu. ATP binding to the N- domain initiates the Cu transport and Cu binds to the transmembrane region. Further, phosphorylation occurs at the aspartic residue in the DKTGT motif in the P- domain coupled to Cu release in the lumen. After this, the dephosphorylation of the aspartic acid residue converts the protein back to the initial conformation for the next cycle (Lutsenko et al. 2007). Studies on ATP7A have suggested that the luminal loop segment connecting the transmembrane domain 1 and 2 is important for Cu release. Mutation in the loop does not affect the ATP hydrolysis and phosphorylation but inhibits subsequent dephosphorylation (Barry et al. 2011).

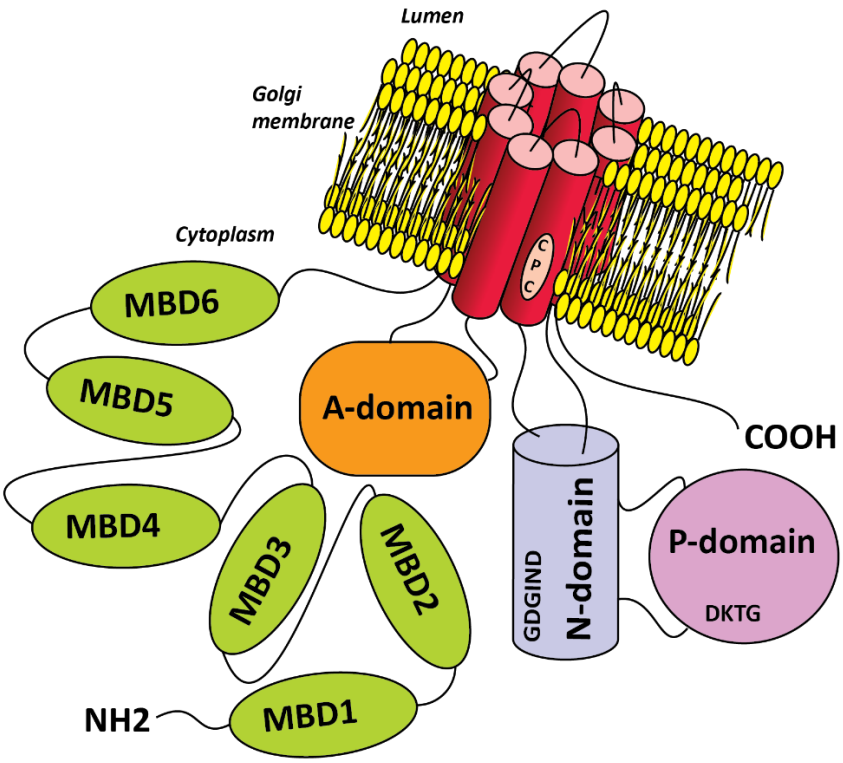


Figure 6: Structural organization of ATP7B. Domains of a Cu-P_{1B} type ATPase is illustrated for ATP7B; TMDs (red), six MBDs (green), A-domain (orange), P-domain (pink) and N-domain (blue, HP locus is present in this region). The linker that connects MBD4 and MBD5 consists of 76 residues which is the longest among other linkers. The linker between MBD3-MBD4 is 29 residues long, between MBD2-MBD3 is 24 residues in length, between MBD1-MBD2 is 13 residues and 6 residues between MBD5-MBD6 in ATP7B

In both ATP7B and ATP7A, there are four luminal protruding loops and each loop links two transmembrane domains. The luminal loop region that connects TMD1 and TMD2 in ATP7A and ATP7B contains numerous histidine and methionine residues in comparison to other luminal loop segments (Barry et al. 2011). Notably, the proposed luminal segment is longer in ATP7A than ATP7B and Cu binding to this region in ATP7A involves His and Met residues (Barry et al. 2011). Two distinct Cu-binding sites (3-coordinated site (His-(Met)2 site), and 2-coordinate site His-His or His-Met site) have been reported in this luminal loop segment and the segment is proposed as final Cu release site in ATP7A (Barry et al. 2011). In ATP7B, the luminal loop has many methionine and histidine residues, but less than ATP7A. There is limited knowledge on Cu release in ATP7B.

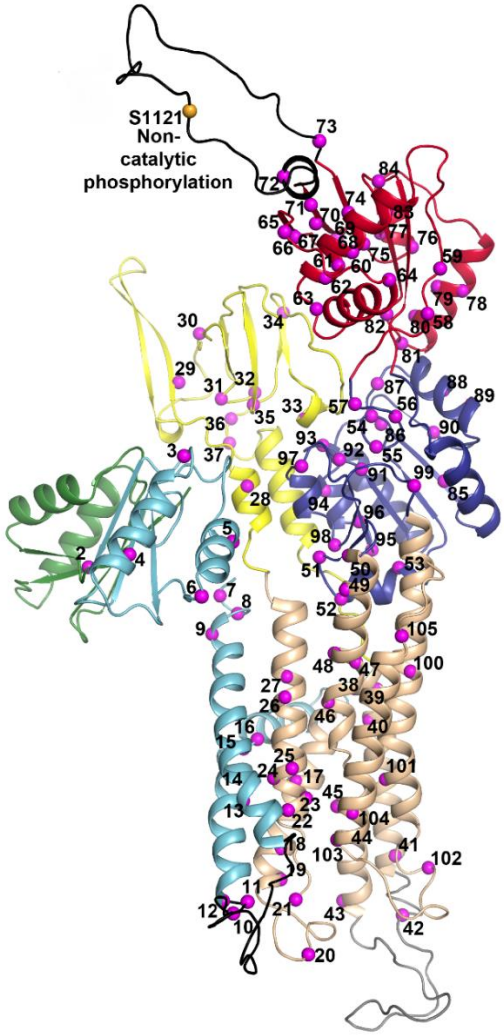


Figure 7: Three dimensional model of ATP7B.Homology model of ATP7B (1-4Δ MBD). The TMD (helices) are connected to MBD6 (cyan). MBD5 (green), A-domain (yellow), P-domain (blue), and N-domain (red). Magenta spheres indicate missense WD mutations (Gourdon et al. 2012)). *Used by permission of De gruyter, from Gourdon (2012).*

A cryo-EM structure, recently established, shows that ATP7B forms dimers (Figure 8). The contact between P- and N- domain and transmembrane contacts are responsible for ATP7B dimerization (Figure 8). Deletion of MBD1-4 in ATP7B did not disrupt the dimerization (Jayakanthan et al. 2017).

Localization of ATP7B changes with respect to intracellular Cu levels. In high intracellular Cu conditions, the ATP7B exits the Golgi and enters cytoplasmic membrane vesicles (Huang et al. 2014). Later, the vesicle containing ATP7B is translocated to the apical membrane, for exporting Cu in the process known as apical trafficking (Roelofsen et al. 2000). Changes in ATP7B localization help to maintain Cu homeostasis (Roelofsen, Balgobind, and Vonk 2004).

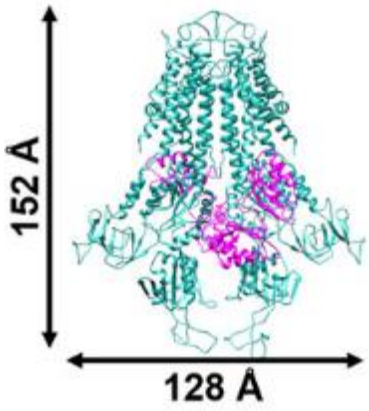


Figure 8: Dimer model of ATP7B (deletion of MBD1-4 did not disrupt dimer formation, P-N domains are proximal). MBD5 and MBD 6 is shown in magenta (Jayakanthan et al. 2017). *Used by permission of JBC, from Jayakanthan (2017).*

2.5 Metal binding domains of ATP7B

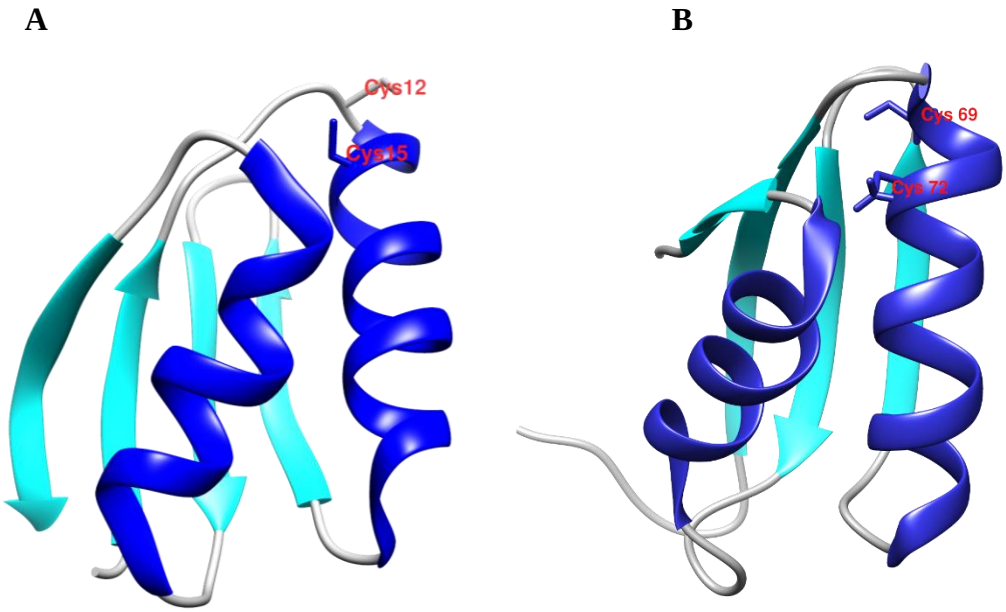
The MBDs in ATP7B are numbered starting from the first MBD in the N-terminal region, with MBD6 being the nearest MBD to the membrane-spanning part of ATP7B. The N-terminal MBDs consist of 630 residues that are nearly half the size of the full-length protein. Among 630 residues, 200 residues belong to the inter-domain linkers (Banci et al. 2008; Arioz, Li, and Wittung-Stafshede 2017). The structure of each MBD is similar to each other and to that of Atox1 (Fatemi et al. 2010; Banci et al. 2008). All MBDs have a ferredoxin-like fold (Figure 9) where the Cu-binding site is situated in a solvent-exposed loop between the β_1-strand and α_1-helix (Braiterman et al. 2015; LeShane et al. 2010). Numerous *in vitro* and *in silico* studies have been carried out with different constructs of MBDs (i.e., individual MBDs or as entire MBD1-6) (Rodriguez-Granillo, Crespo, and Wittung-Stafshede 2009; DiDonato et al. 2002).

To date, the exact roles of all six MBDs in ATP7B has not completely understood. Previous *in-vitro* studies have suggested that domain-domain interactions occur among MBDs. Also, interactions between MBDs and the core (transmembrane segment) of ATP7B have been described (Yatsunyk and Rosenzweig 2007). The function of the intramolecular interactions *in vivo* is not yet known. Localization studies on ATP7B have reported that the Golgi retention signal is present in the N-terminal MBDs (Huster et al. 2003).

There is no clear evidence suggesting MBDs interaction with other domains *in vivo*, but it has been shown that removal of MBDs in ATP7B has effects in phosphorylation and Cu transport activity. However, *in vitro* studies have shown MBD interaction with N- and A- domains (Tsivkovskii, MacArthur, and Lutsenko 2001). The MBDs was co-purified with the N-domain, and the interaction was found to be present in absence of Cu in the MBDs. This interaction was proposed to keep the ATP7B in inactive state at low Cu levels (Tsivkovskii, MacArthur, and Lutsenko 2001). Cu binding to the MBDs induced structural rearrangement resulting in disruption of MBD and N-domain interaction, and allowing ATP to bind N-domain (Bartee, Ralle, and Lutsenko 2009). However, NMR studies could not detect the interaction between

MBDs and N-domain in solution (Mondol, Aden, and Wittung-Stafshede 2016). The above data infer that the interaction between MBDs and N-domain appears to be transient or the interaction observed in the co-purification experiments comprised a small fraction of the sample (Mondol, Aden, and Wittung-Stafshede 2016). Similar to MBD-N-domain interaction studies, the interaction between N-terminal MBDs and A-domain, was observed by co-purification experiment (Gupta et al. 2011). Cu binding to the N-terminal MBDs was reported to enhance the interaction between MBDs and the A-domain and the interaction was found to stabilize the protein during ER to Golgi cargo experiment (Gupta et al. 2011).

Some studies have revealed that there is a strong interaction between MBDs that help to mobilize Cu in ATP7B (Yatsunyk and Rosenzweig 2007). A study on nanobodies binding to 1-6 MBDs in ATP7B showed that the interaction between MBDs is transient and modulating these interactions resulted in relocalization of ATP7B toward plasma membrane (Huang et al. 2014). It was hypothesized that Cu binding to MBDs of ATP7B induces conformational changes along the N-terminal region of the protein. However, *in vitro* observation of ATP7B's MBDs has provided evidence that the Cu binding to MBD causes some localized changes in the Cu binding loop but does not affect the overall fold of individual domains (Banci et al. 2008; Banci, Bertini, Cantini, Massagni, et al. 2009; Banci, Bertini, Cantini, Migliardi, et al. 2009). There is still no information on the structural orientation of MBDs in ATP7B.



Atox1 **MBD1**

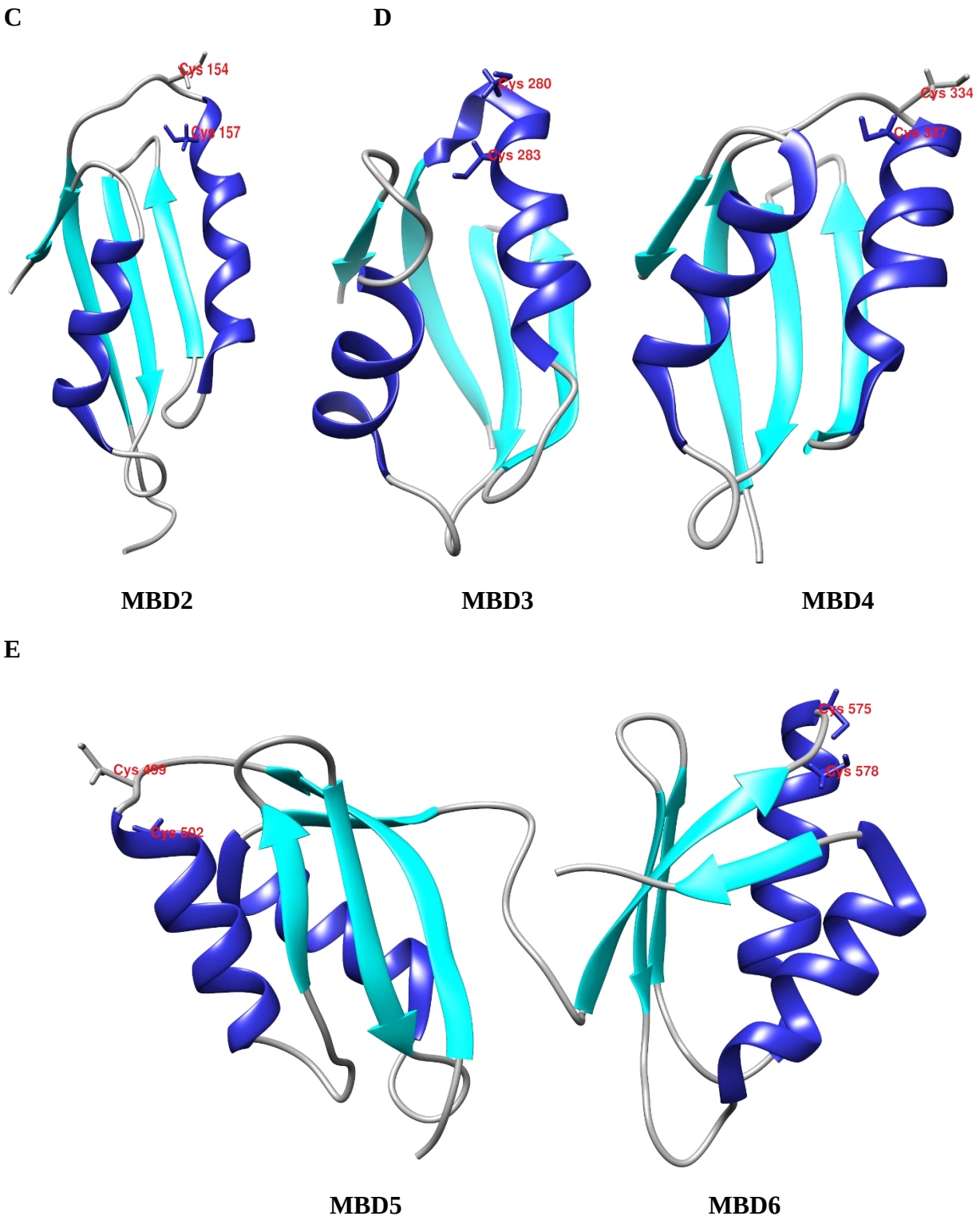


Figure 9: Secondary Structure of MBD. Secondary Structure of MBDs of ATP7B and Atox1 (Solution structure of A) Atox1, PdB: 1TL4 ; B) MBD1, Pdb: 2N7Y; C) MBD2, Pdb: 2LQB; D) MBD3-4, Pdb: 2ROP; and E) MBD5-6, Pdb: 2EW9). The Cu-binding cysteines are labeled in red.

2.6 Human diseases involving Cu mismetabolism

Cu uptake, distribution, and export processes are essential to maintain Cu homeostasis in cells. Any perturbation to these processes leads to Cu overload or Cu deficiency which have distinct critical effects on cellular function. Excess Cu intake, in turn, induces toxicity by interacting with other nutrients, and Cu deficiency disturbs the iron transport and metabolism, which results in anemia (Lutsenko et al. 2007) due to decreased iron concentration in the blood. On the other hand, Cu toxicity induces oxidative damage in the cells which results in DNA damage, peroxidation of lipids and mitochondrial damage (Prohaska 2008). Cu-related metabolic diseases are discussed in this section:

Wilson Disease (WD)

Wilson disease (WD) is a rare autosomal recessive genetic disorder of Cu transport and is caused by abnormal accumulation of Cu in the liver due to inactive ATP7B. Mutations in the ATP7B gene lead to dysfunction of Cu transport and hence the poor incorporation of Cu into ceruloplasmin as well as an impaired biliary Cu excretion leading to toxicosis (Cu accumulation) in the liver, brain, kidneys, eyes and other organs (Terada et al. 1998). Approximately 1 in every 30 000 individuals is affected by WD, and the onset is in the range of 3-50 years of age. Its widespread symptoms include hepatic disorder, neurological problems, skeletal and rarely renal or endocrine problems(Rodriguez-Castro, Hevia-Urrutia, and Sturniolo 2015). If untreated, the progression of this disease has adverse effects such as the development of encephalopathy and kayser-fleischer rings (discoloration at the outer rim of iris due to Cu accumulation). Early diagnosis and treatment can allow a patient to live a long and healthy life. The treatment for this disorder is Cu chelators (D-pencillamine, Zinc sulfate/acetate) which bind Cu and enables patients to excrete excess Cu (Chandhok et al. 2016).

Disease-causing mutations of ATP7B are missense, frameshift, non-sense or splice-site mutations (Harada et al. 2001). These mutations can hinder every step of the catalytic cycle of ATP7B, and the impact on the protein can range from mild to severe depending on which residues are affected (Huster et al. 2012). The most common WD mutations are observed in the conserved regions of the ATPase core (transmembrane domain) (Forbes, Hsi, and Cox 1999), but some mutations are also observed in other parts of ATP7B (i.e. A-domain, MBDs). The most common WD mutations are R778L, H1069Q, and R778W. The R778L (58% of all WD mutations) mutation is located in the TMD4 (lies close to the A-domain) and this specific mutation is observed in patients of southeastern Asian countries (Korean and Chinese patients) (Forbes and Cox 2000; Yoo 2002). The H1069Q (around 35-45% of all WD mutations) mutation is found in the N-domain and most prevalent among European descent (European and North America) (Parisi et al. 2018; Stapelbroek et al. 2004). The R778W mutation is common among Indian population (Hedera 2017; Inesi, Pilankatta, and Tadini-Buoninsegni 2014; Jang et al. 2017). ATP7B with R778L and H1069Q mutations are localized in the endoplasmic reticulum, and D765N /L776V mutation affects the localization of ATP7B on the TGN. Certain mutations in ATP7B do not affect the TGN localization, but disrupts Cu transport (Forbes and Cox 2000). Certain ATP7B mutations and domain deletions appear to destabilize the protein; as a result, the protein localizes to the ER. These localization problems were overcome by treatment with pharmacological chaperones such as phenylbutyrate (4-BPA) or curcumin that rescue the protein variants back to TGN (van den Berghe et al. 2009).

Until now, more than 800 mutations have been reported to cause WD in patients (Arioz, Li, and Wittung-Stafshede 2017). The wide spectrum of phenotypic variations of these mutations often makes it hard to get an accurate and early clinical diagnose for WD (Jang et al. 2017; Dong et al. 2016; Braiterman et al. 2014). Due to a wide variety of symptoms and age of onset, knowledge of how mutations in ATP7B link to symptoms in patients is still inconclusive. Numerous biochemical and biophysical studies on the properties of disease-causing mutations reported that the WD mutations have various effects on ATP7B function (Braiterman et al. 2014; Chen et al. 2015). Noticeably, the widely known effects are protein misfolding, impaired protein-protein interactions, decreased/increased phosphorylation activity, altered binding affinities for ATP or Cu, vesicle trafficking, aberrant localization due to protein conformational changes (Concilli et al. 2016; Chen et al. 2015; Huster et al. 2012). Among this, the most commonly observed effect is protein misfolding whereby the protein is retained in ER, which results in the abolished Cu transport in cells (Concilli et al. 2016; Payne, Kelly, and Gitlin 1998; Tsivkovskii, Efremov, and Lutsenko 2003).

Over 500 missense mutations have been identified in ATP7B (Stenson et al. 2017) that results in WD in patients, and nearly 33 (less than 10%) of these mutations are located in the MBDs (excluding linker regions). 10 of the 33 missense mutations are at residues in the first four MBDs, i.e., MBD1-4 and the remaining 23 mutations are found in MBD56. These numbers make the latter two domains, MBD56, a hotspot for WD mutations among the domains. The location of MBD56 is close to the Cu entry site in the membrane part of ATP7B and hints the importance of these domains in ATP7B function. In fact, some of these residues, i.e., positions 515, 532, 591, and 616 in MBD56, each have two different point-mutations resulting in WD (Figure 10). Only two of the 33 mutations are localized in the GMXCXXC Cu-binding motifs. The majority of the mutated positions are part of α-helices (R198G, I390V, E541K, L549P, T587M and R616Q), linker regions (M67V, R136G, G515V and G591S), whereas a few residues in β-strands (V519M and A595T) are affected (Kumar et al. 2017). Most of the WD mutations reported lack information about the impact on the protein function.

Menke's Disease (MNK)

Menke's disease (MNK) is a genetic disease that is mainly caused by a non-functional ATP7A and hence results in Cu deficiency. It is a rare X-linked disorder that affects 1 in 200 000 people worldwide. Mutational defects in ATP7A cause Cu to remain trapped within the lining of the small intestine and hence all Cu transport to the liver, blood and other organs are disrupted. Symptoms associated with this disease are usually observed with coarse, brittle, depigmented hair, mental retardation, skeletal defects, abnormal connective tissue growth and inability to control body temperature (Tümer and Møller 2009). Patients with MNK suffer from severe neurological abnormalities owing to the lack of cuproenzymes required for brain function and development as well as with a decrease in the COX activity. The cause of hypopigmentation of hair and Defective collagen, elastin polymerization and connective tissue abnormalities (loose skin, fragile bones, aortic aneurisms) are connected with reduced lysyl oxidase activity (Ojha and Prasad 2016). This disease is usually fatal even with the early diagnosis, and its treatment is carried out by both intraperitoneal and intrathecal Cu histidine administration to the central nervous system to prevent severe neurological problems.

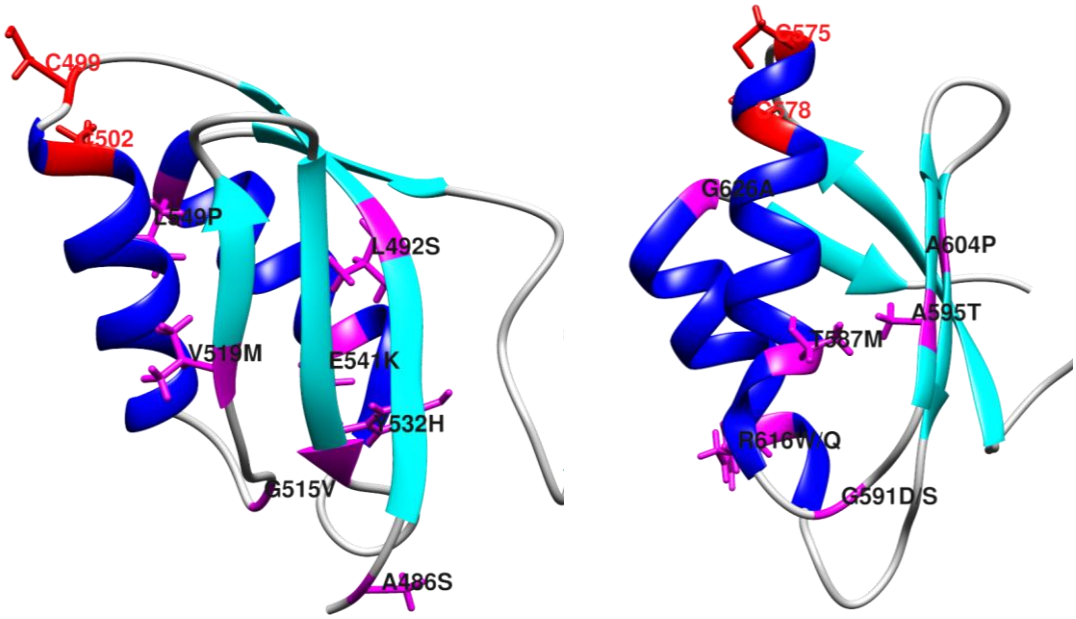


Figure 10: Secondary Structure of MBD 5 and 6 of ATP7B with WD-causing mutations highlighted.

Mutations in ceruloplasmin cause Aceruloplasminemia (Roberti Mdo et al. 2011), and mutations in SOD1 may lead to Amyloid Lateral Sclerosis (ALS or Lou Gehrig disease) (Waggoner, Bartnikas, and Gitlin 1999). Cu level misregulation may be involved in many diseases. Cu also binds amyloid-β and α-synuclein and is proposed to affect Alzheimer's (AD) (Atwood et al. 1998; Multhaup et al. 1998) and Parkinson's (PD) (Horvath, Werner, and Wittung-Stafshede 2018; Villar-Pique et al. 2017) disease progression. Also, prion protein binds Cu (Human Prion disease or Creutzfeldt–Jakob disease CJD) (Stockel et al. 1998). Finally, cancer progression seems to depend on high Cu levels (Khan and Merajver 2009).

3 RESEARCH CHAPTERS

This chapter presents the experimental methods used in the thesis and an overview of the results. All the results summarized here are the compilation of experimental work from papers I-IV. For more details about the presented research, the reader is referred to the appended papers.

3.1 Development of Yeast model system

OBJECTIVES

This study aimed to develop a novel yeast model system that probes Cu transport by human Atox1 and ATP7B.

MOTIVATION

The Atox1-ATP7B interaction is essential for maintaining Cu homeostasis in cells, and this interaction is important to understand the ATP7B mediated Cu transport function in human. There have been studies of ATP7B mediated Cu transport and localization in various cell types, including HepG2, neuronal cells, and cancerous cells, (Michalczyk et al. 2008; Huster et al. 2003; Chandhok et al. 2016), as well as model systems such as LEC rats, ATP7B deficient mice, and drosophila (Huster et al. 2007; Cater et al. 2004). ATP7B Cu transport activity is most extensively studied in yeast (Papur, Terzioglu, and Koc 2015), while ATP phosphorylation activity has been primarily observed *in vitro* experiments (Huster et al. 2012). Due to functional conservation, yeast can be used as a model to understand mammalian Cu homeostasis at the cellular and molecular level (De Freitas et al. 2003).

Previous studies using Δ*ccc2* yeast have provided information about the disease-causing mutations in ATP7B, the importance of MBDs, and Cu binding regions of ATP7B (Forbes, Hsi, and Cox 1999). All of these studies have been carried out in Δ*ccc2* yeast strain in absence of Atox1. The interaction site between Atox1-ATP7B is unknown. Previous yeast systems Δ*ccc2* and Δatx1 have not been successful in determining the Atox1-ATP7B interactions as of now. In order to acquire detailed information about Atox1-ATP7B interactions and also to determine which MBD of ATP7B receives Cu from Atox1, we have developed an advanced yeast model system that probes Cu flow from Atox1 to ATP7B (Paper I, (Ponnandai Shanmugavel, Petranovic, and Wittung-Stafshede 2017)).

METHODS

First, the yeast strain construction is explained, followed by the description of the working principle of yeast complementation assay. Further, the experimental setup of the yeast complementation assay is explained.

Construction of *Δccc2Δatx1* yeast strain

Yeast *Saccharomyces cerevisiae* CEN.PK 113-11C (*MATa SUC2 MAL2-8 URA3-52 HIS3-Δ1*) was used as reference strain. The yeast homologs of human *ATP7B* and *ATOX1* genes, *CCC2* and *ATX1*, are located on chromosomes IV and XIV of *S. cerevisiae*, respectively. The *Δccc2Δatx1 S. cerevisiae* strains were constructed by deletion of *CCC2* and *ATX1* genes in combination. Deletions of *CCC2* and *ATX1* genes were performed by replacing the gene with a KanMX deletion cassette which carries the kanamycin selectable marker (for detailed construction, refer paper I). The *Δccc2Δatx1* yeast strain were constructed by standard PCR techniques and homologous recombination.

Yeast Complementation assay

The copper transport pathway and iron transport pathway are interlinked in all eukaryotes. In yeast, the intracellular copper transport is responsible for the iron uptake and it is well coupled to each other. Fet3p is a multi-copper oxidase (like human ceruloplasmin, type 1 membrane protein with structure consisting of three cupredoxin-like fold) enzyme localized in the plasma membrane also traffics to vesicular compartments. The yeast cytosolic Cu chaperone Atx1 delivers Cu to Ccc2 localized in the Golgi, a Cu-binding ATPase that incorporates Cu into Fet3p (Forbes, Hsi, and Cox 1999). The main function of Fet3p is to catalyze the oxidation of Fe^{2+} to Fe^{3+} (ferroxidase reaction, $4Fe^{2+}+4Cu^{2+}\leftrightarrow 4Fe^{3+}+4Cu^{1+}$, $4Cu^{1+}+O_2+4H^{+} \leftrightarrow 4Cu^{2+}+2H_2O$) using oxygen as a substrate (Cankorur-Cetinkaya, Eraslan, and Kirdar 2016; Hassett, Romeo, and Kosman 1998). Binding of Cu to Fet3p initiates the oxidase activity, as no oxidase activity is present in apo-Fet3p and Cu bound Fet3p complexes with iron permease Ftr1 which mediates high-affinity iron uptake (De Freitas et al. 2003). The oxidase function of Fet3p is required at the plasma membrane for iron uptake and the Fe^{3+} generated by Fet3p is a ligand for the iron permease Ftr1 which uptake extracellular iron (Stearman et al. 1996). Taken all together, Ccc2 protein connects the Cu uptake pathway to iron uptake pathway in yeast and therefore, high-affinity iron uptake pathway of yeast cells is strictly dependent on Cu transport (Collins, Prohaska, and Knutson 2010).

Deletion of yeast copper transport proteins perturb the Cu transport. Disrupted intracellular Cu transport renders yeast deficient in high-affinity iron uptake due to inability to load Cu to Fet3p (De Freitas et al. 2003). The yeast grown in iron-limited medium requir completely functional copper transport for its survival. Any perturbation in Cu transport results in inactivation of Fet3p enzyme thereby ceased iron uptake in yeast which eventually leads to yeast growth limitations. The Δ*ccc2* yeast model (iron uptake deficient) was initially used to test whether mammalian Cu-ATPase can complement the function of Ccc2. The complementation function of the protein is assessed via growth (growth rate) in iron-limited conditions (Yuan et al. 1995). The growth rate of the Δ*ccc2* yeast strains in iron-limited conditions is associated with the protein function of Cu-ATPase. The growth rate of the Δ*ccc2* yeast strains was measured during the exponential growth phase since the exponential phase is considered to be the period of balanced uniform

multiplication of cells (also nutrients consumption and metabolic activities are known to be higher during exponential phase which involves the highest protein function) (Cooper 1991).

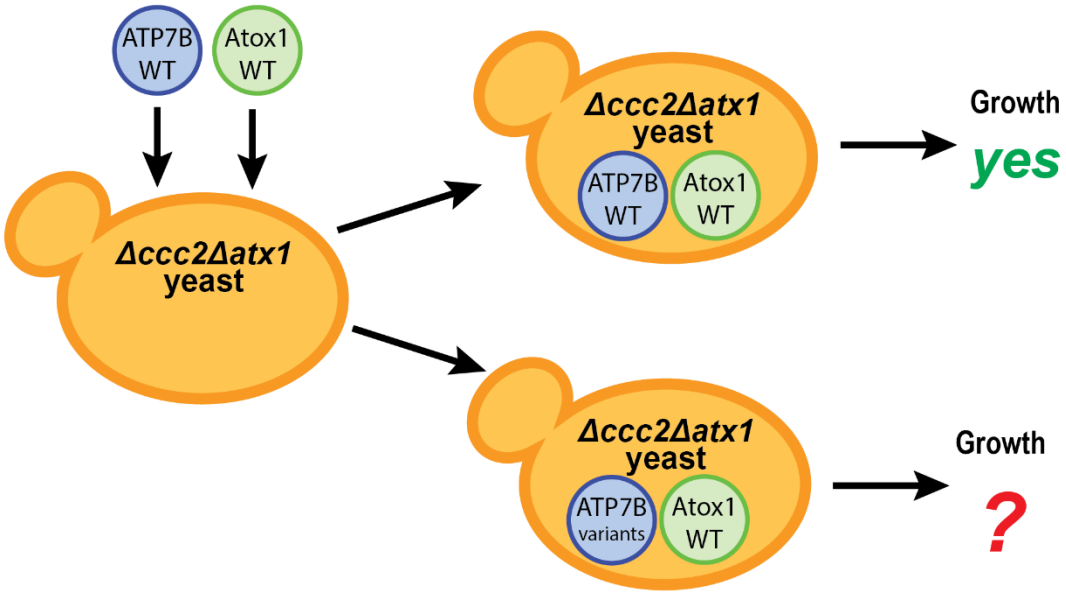


Figure 11: Yeast complementation system. (**A**) Schematic representation of the double deletion yeast system designed for human Cu transport studies. Plasmids with human ATP7B and ATOX1 were used to complement the yeast strains in which yeast endogenous CCC2 and ATX1 genes were deleted individually or in combination. In this system, we have investigated the consequences of wild type, mutated and truncated ATP7B variants by probing cell growth on Fe-limited media which requires functional Cu transport.

were subjected to yeast complementation assay. The growth rate of the yeast strains in iron-limited conditions was calculated. Measuring yeast growth rates in iron-limited medium is a sensitive method that can detect functional differences between mutant ATP7B variants. Numerous studies have been carried out to understand the mutational effect of ATP7B in Cu transport function (Papur, Terzioglu, and Koc 2015).

Experimental setup

The Fe-limited medium was prepared from the SD medium with the addition of 1 mM ferrozine (Fe chelator), 1 µM $CuSO_4$ and 100 µM $FeSO_4$. Fe-supplemented medium was prepared without ferrozine and 350 µM $FeSO_4$ and 500 µM $CuSO_4$. For the plating assay, yeast cells from pre-grown overnight cultures (30 °C, 200 rpm) were diluted to an optical density at 600 nm of 0.1, and then serial dilutions with sterile water were performed followed by plating in Fe-limited and Fe-supplemented medium and incubation at 30 °C for 3 days. For the determination of growth rate, yeast cells from pre-grown overnight culture were washed with ice cold deionized water and cultivated in fresh iron limited medium at an initial cell density of OD_{600} = 0.1. The growth of the cells was monitored spectroscopically (OD_{600}) for 30 hrs. All yeast growth experiments were carried out at identical conditions. Growth rates were calculated from the linear exponential growth phase ($\Delta\ln(OD_{600})$/hr) (Morin et al. 2009).

RESULTS AND DISCUSSION

Probing Cu transport in Yeast System

The constructed yeast strain Δ*ccc2*Δ*atx1* is high-affinity iron uptake deficient due to the lack of Cu delivery to Fet3p. Under iron limited conditions, the high-affinity iron uptake is vital for yeast survival. Therefore, the Δ*ccc2*Δ*atx1* strain was unable to thrive on iron limited medium because of the absence of functional Fet3p (high-affinity iron uptake deficient) (Figure 12). The growth of Δ*ccc2*Δ*atx1* strain was rescued in the presence of high iron or high Cu concentrations. The reason for the growth in high iron conditions (iron supplemented medium) is due to the fact that iron enters the yeast cell via low-affinity iron uptake pathways. Expression of human ATP7B protein in Δ*ccc2* background and human chaperone Atox1 in Δ*atx1* strains rescued the viability in an iron limited medium, as previously reported (Forbes, Hsi, and Cox 1999; Portnoy et al. 1999). The viability of the Δ*ccc2*Δ*atx1* yeast was not restored by the expression of either ATP7B or Atox1 protein whereas complementation of both proteins enabled the yeast viability under iron limited conditions (Figure 12).

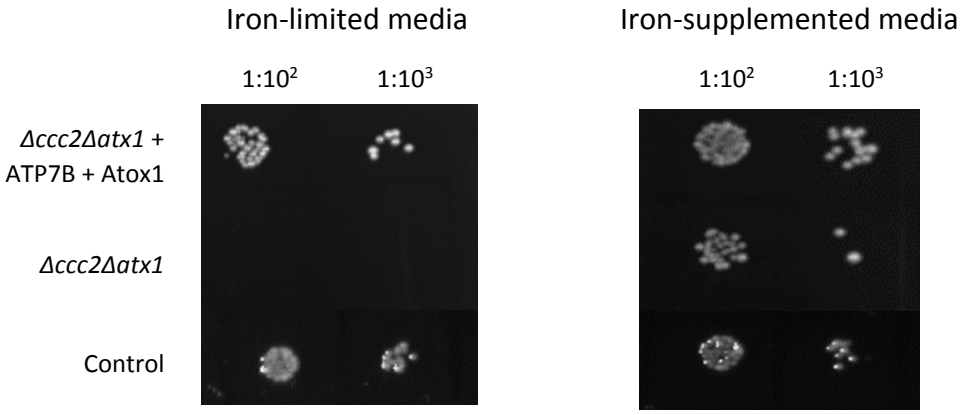


Figure 12: Yeast complementation assay. Plating assay showing serial dilutions of *Δccc2Δatx1* yeast cells, without and with added Atox1 and ATP7B plasmids (high-copy plasmids), as well as control (wild-type) yeast, spotted onto a agar plate prepared with Fe-limited and Fe-supplemented media.

Exponential growth rate of the yeast in iron-limited conditions relates to the Cu transport efficiency. The exponential growth rates of the yeast strains grown in iron limited conditions were determined. As expected the Δ*ccc2*Δ*atx1* strain had a poor growth rates in iron-limited conditions. However, the growth of Δ*ccc2* and Δ*atx1* yeast were restored when complemented with corresponding human Cu transport proteins ATP7B and Atox1. The growth data revealed that the deletion of one yeast genes and complementing it with a corresponding human gene restored the wildtype phenotype. Like in the plating assay, Δ*ccc2*Δ*atx1* yeast growth was recovered by expression of both ATP7B and Atox1 proteins and growth was restored similar to reference strain in iron-limited conditions (Figure 13). This suggests that both ATP7B and Atox1 are pivotal for the Cu transport in yeast in the absence of yeast Cu transport proteins Ccc2 and Atx1.

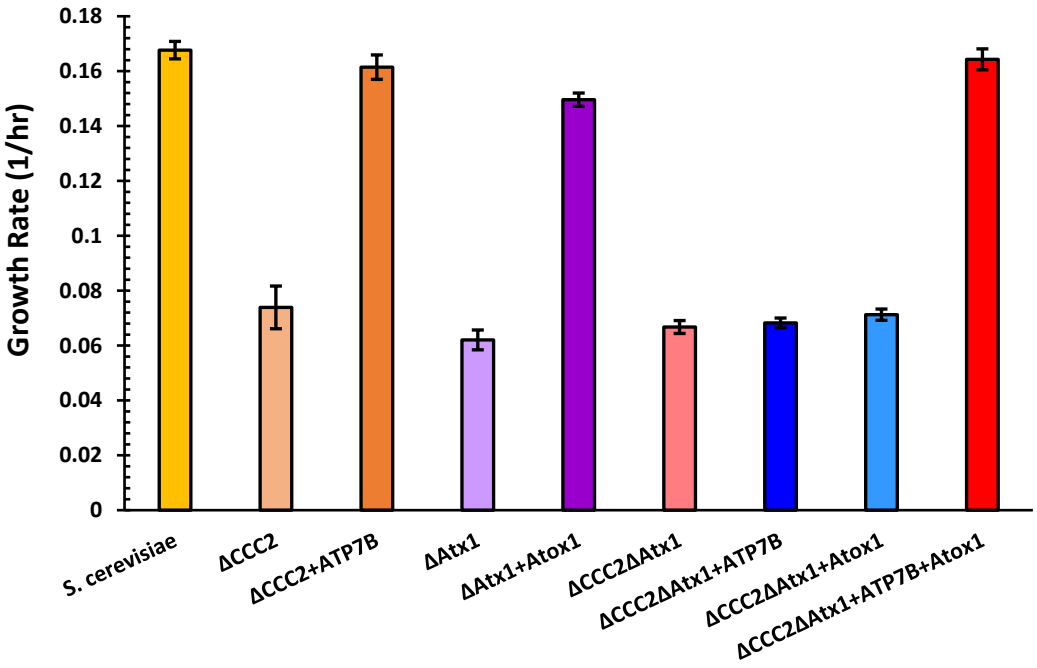


Figure 13: Growth rates for *Δccc2, Δatx1, Δccc2Δatx1* yeast strains complemented with ATP7B and Atox1 plasmids, under Fe-limited conditions. Error bars are based on the standard deviation for duplicate experiments.

The results here show that, ATP7B and Atox1 proteins can substitute Ccc2 and Atx1. ATP7B expression alone did not recover the growth in Δ*ccc2*Δ*atx1* which implies that both chaperone and transporter are required. Previous studies have used Δ*ccc2* yeast model in the complementation assay for functional analysis of ATP7B Cu transport and in thus relied on yeast Atx1 to shuttle Cu to ATP7B. The yeast model we established here *(*Δ*ccc2*Δ*atx1*) better mimics the real human cellular Cu transport.

3.2 Role of Metal-binding domains in ATP7B Cu transport

OBJECTIVES

This study aimed

- To assess the roles of the different MBDs in ATP7B mediated Cu transport
- To investigate the transport of Cu among the MBDs in ATP7B and establish the mechanistic model of Cu transport in MBDs.
- To investigate if cytoplasmic individual MBDs could act as chaperones.

MOTIVATION

A unique feature of ATP7B protein is the presence of six MBDs in the N-terminus. The functional role of MBDs in ATP7B is not completely known. It is not clear, whether Cu is transported through MBDs to the transmembrane site or Atox1 delivers Cu directly to the transmembrane Cu-binding sites like bacterial Cu-transport involving CopZ and CopA. There is no information on which MBDs in ATP7B receive Cu from Atox1 in cells. Until now, it is unclear how the Cu is transported along the MBDs in ATP7B during Cu transport, which MBDs delivers Cu to the transmembrane site, and what are the essential MBDs in ATP7B mediated Cu transport. Moreover, the MBDs that are involved in the regulatory interactions are not known. To date, many unresolved questions with regards to the role of the MBD still need to be answered.

The *Δccc2Δatx1* yeast system (described in Chapter 3.1) have shown to be valuable tool to probe the Cu transport between Atox1 and ATP7B. The yeast system can be applied to understand the effect of WD or non-disease causing mutation in Cu transport of ATP7B, and the effect of mutation in Atox1 in Cu transport. The advantage of using *Δccc2Δatx1* system, is the fact that the Cu is delivered to ATP7B by Atox1, unlike *Δccc2* yeast model. The information about how these MBDs are arranged in ATP7B is lacking due to limited structural information. Hence, there is lack of insights on the roles of MBDs in ATP7B. The *Δccc2Δatx1* yeast system can be used to unravel the roles of MBDs in ATP7B. In this chapter, using the established yeast model system, we assessed the roles of MBDs in Cu movement in ATP7B (Paper I (Ponnandai Shanmugavel, Petranovic, and Wittung-Stafshede 2017) and Paper III (Shanmugavel and Wittung-Stafshede 2019)), and explored the ability of individual MBDs to act as chaperones (Paper III).

METHODS

Domain Truncated ATP7B variants: The human *ATP7B* and *ATOX1* genes were cloned in separate plasmids as reported in methods part in Chapter 3.1. The MBDs truncated or mutated variants were amplified from p426GPD-ATP7B. Created domain truncated ATP7B constructs were as follows: domain deletions of one MBD to all six MBDs in the *ATP7B* gene (i.e., ATP7B 1DEL, ATP7B 1-2DEL, ATP7B 1-3DEL, ATP7B 1-4DEL, ATP7B 1-5DEL, and ATP7B 1-6DEL) and point-mutations in the *ATP7B* gene (i.e., ATP7B CPC-to-SPS, with two Cys replaced with Ser in transmembrane domain 6, and D1027A, with the transiently phosphorylated Asp1027 replaced by Ala) (Figure 14) (Ponnandai Shanmugavel, Petranovic, and Wittung-Stafshede 2017).

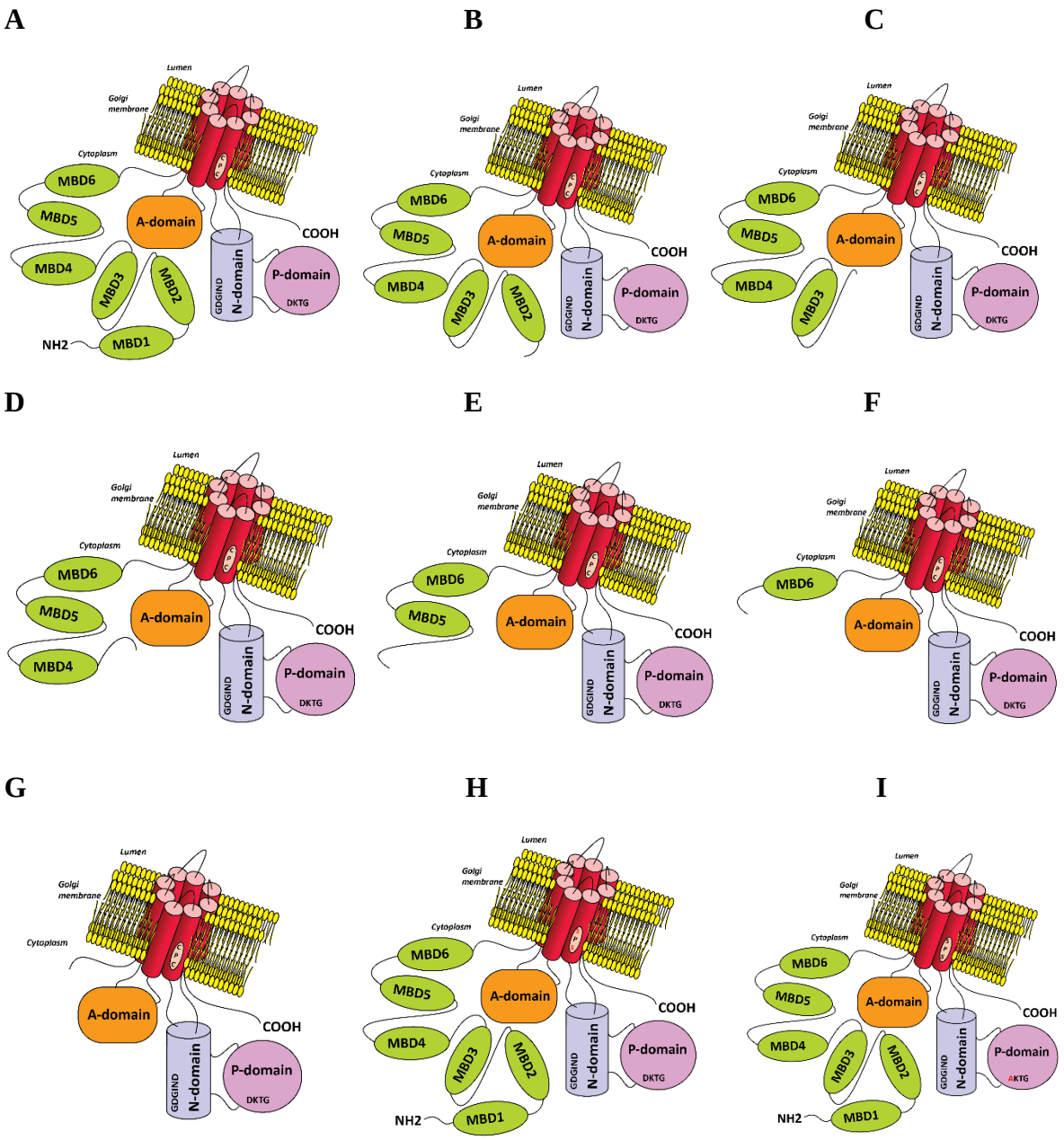


Figure 14: Schematic representation of MBD truncated ATP7B variants. MBD truncated ATP7B variants that were introduced in the engineered yeast strains. A) ATP7B; B) ATP7B 1 DEL; C) ATP7B 1-2DEL; D) ATP7B 1-3DEL; E) ATP7B 1-4DEL; F) ATP7B 1-5DEL; G) ATP7B 1-6DEL; H) ATP7B CPC-to-SPS; I) ATP7B D1027A.

Cu-site Mutated ATP7B mutants: The copper binding cysteines (CXXC) in the MBDs of ATP7B were mutated to serines (SXXS). Several combination of copper site mutations (CS) up to three MBDs were constructed (Figure 15). The mutations were introduced into the full-length ATP7B by quick change site-directed mutagenesis kit using the previously constructed p426GPD-ATP7B plasmid as a template (Shanmugavel and Wittung-Stafshede 2019).

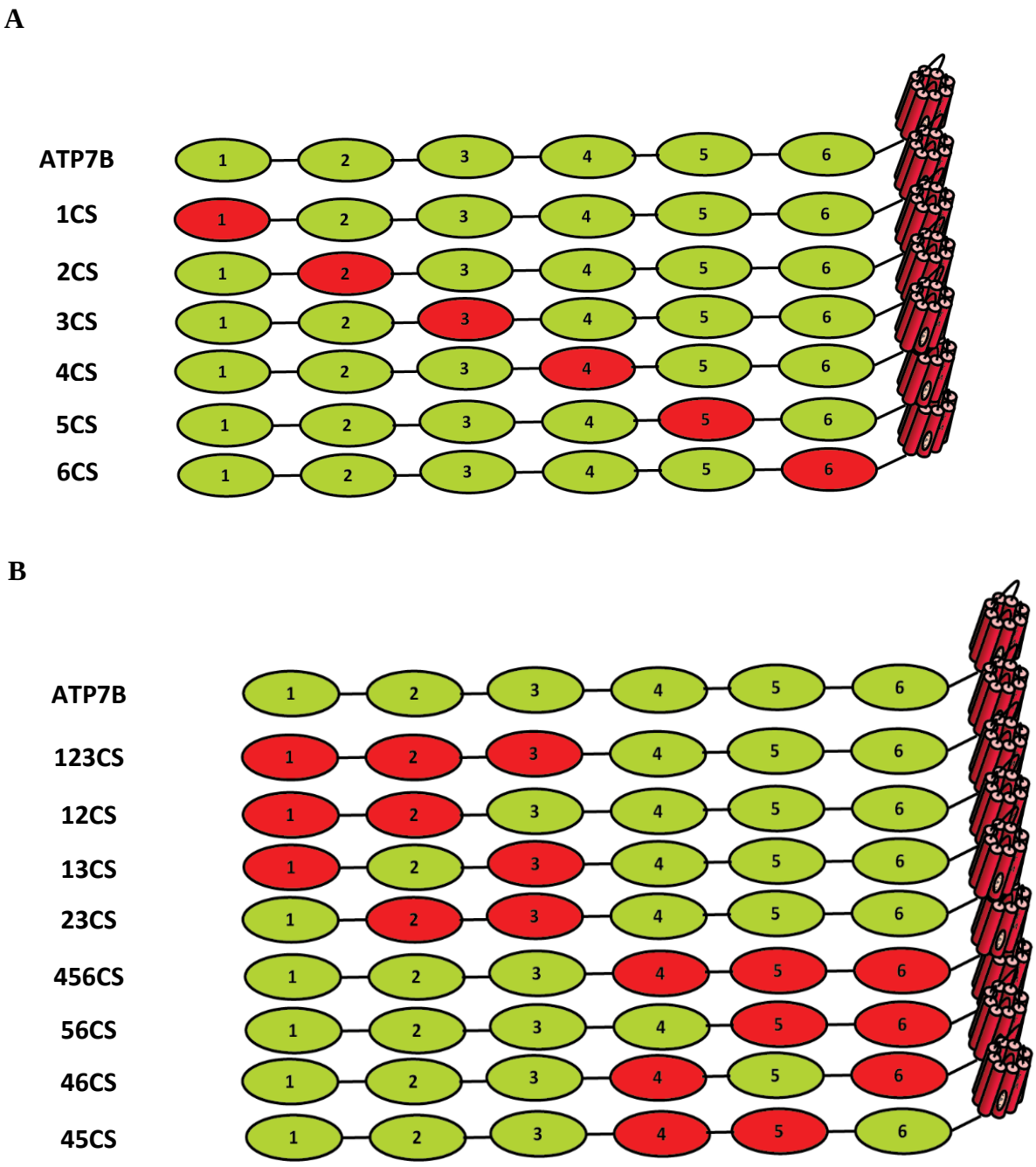


Figure 15: Schematic representation of ATP7B variants with Cu-sites in MBDs. ATP7B variants with Cu-sites in MBDs changed from CXXC to SXXS (as CS); red colour MBDs denotes mutated Cu-site; green colour MBDs denotes active Cu-site. A) Cu-site mutation in individual MBDs; B) Cu-sites mutation in two or three MBDs. All these variants were used with Atox1 as the Cu chaperone.

Individual MBDs: The sequences of the six ATP7B MBDs were amplified individually from the p426GPD-ATP7B plasmid. The amplified MBD genes were inserted into the p423GPD vector, similar to ATOX1 gene. The yeast complementation assay and growth analysis were carried out as described in methods part in Chapter 3.1.

RESULTS AND DISCUSSION

Metal binding domains in ATP7B mediated Cu transport

Different domain truncated ATP7B variants were constructed in order to assess the role of MBDs of ATP7B in Cu transport. Truncated ATP7B variants are created in which all N-terminal MBDs are deleted in succession (Figure 14). Apart from this, CPC to SPS ATP7B and D1027A ATP7B variants were also constructed in this work (no Cu transport activity (Iida et al. 1998); used as negative controls). The constructed ATP7B variants and ATOX1 gene on individual high-copy plasmid were introduced into our developed yeast strain (presented in Chapter 3.1) for their constitutive expression. The newly established yeast strains were grown in the iron-limited conditions, and the growth rate in the exponential phase was determined. The constructed ATP7B mutants were evaluated on their ability to complement the high-affinity iron uptake deficiency phenotype of Δ*ccc2*Δ*atx1* yeast. Western blot analysis confirmed that the expression amounts of ATP7B variants were very similar to that of wildtype ATP7B. The molecular sizes of truncated ATP7B variants matched the theoretical molecular sizes.

Deleting the first N-terminal MBD of ATP7B (ATP7B 1DEL) decreased the growth rate to 70% which indicates that the MBD1 is important for making Cu transport efficient. Removal of first and second MBD (ATP7B 1-2DEL) had also similar growth rate to that of ATP7B 1DEL. However, removal of third MBD (ATP7B 1-3DEL) restored the growth behavior like wildtype ATP7B which can be interpreted as ATP7B mediated Cu-transport is restricted by MBD3. Removal of MBD 1-4 (ATP7B 1-4DEL) and MBD 1-5 (ATP7B 1-5DEL) had similar growth like wildtype ATP7B. However, deletion of all six MBDs (ATP7B 1-6DEL) in ATP7B resulted in a drastic reduction of growth rate, similar to that of negative controls (Figure 16) which means that the Cu-transport is diminished when the N-terminal MBDs are removed. This result suggests that some of the MBDs are pivotal for ATP7B activity and further, indicates that Atox1 delivers Cu to one of the six MBDs of ATP7B.

Taken together, it appears that the presence of MBD1 promotes ATP7B mediated Cu transport whereas MBD3 presence limits Cu transport such that these negative effects might be cancelled out by interdomain interactions (MBD1-MBD3 interactions) in full-length ATP7B. As ATP7B 1-3DEL, ATP7B 1-4DEL and ATP7B 1-5DEL had similar growth, it appears that MBD 4 and 5 do not regulate ATP7B mediated Cu transport individually.

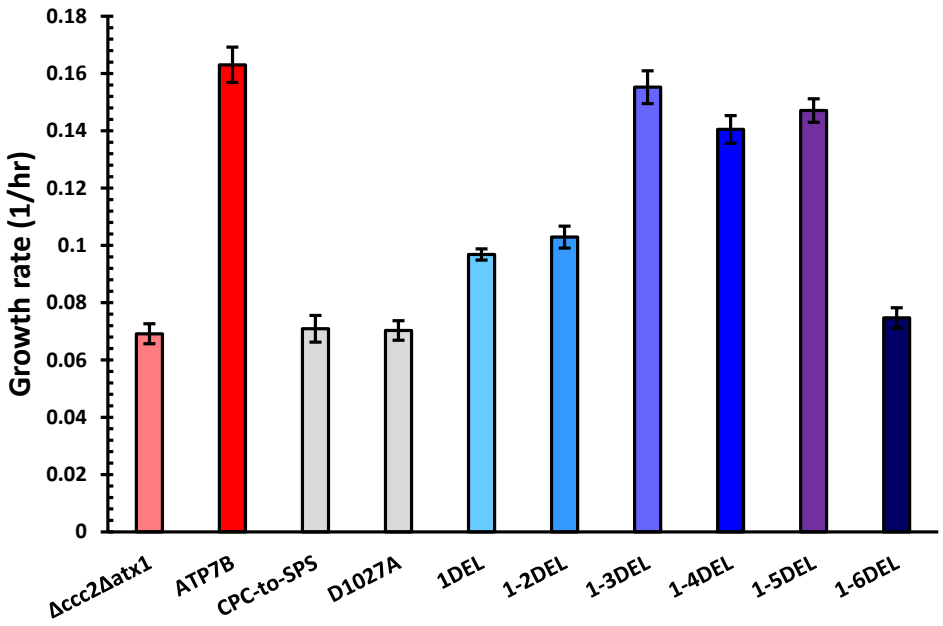


Figure 16: Growth rate of domain-deleted ATP7B. Growth rates of the *Δccc2Δatx1* yeast strain complemented with wildtype Atox1 and domain truncated ATP7B variants in high-copy plasmids under Fe-limited conditions. Error bars are based on standard deviations calculated for duplicate experiments.

Cu relay path in MBDs of ATP7B

Different combinations of Cu-binding site mutations pertaining to the three MBDs (one, two and three Cu-site) were constructed in ATP7B in order to better understand roles of MBDs in Cu transport through MBDs. Blocking the Cu-site in each MBD individually (with the presence of Cu-sites in other MBDs) did not have a substantial decrease in Cu transport (Figure 17). To unravel the more specific roles of MBDs in ATP7B mediated Cu transport, the six MBDs are divided into two sets, MBD1-3 and MBD4-6, and introduced all combinations of pairs of Cu-site mutated domains within the two sets while retaining the Cu-sites in the other three MBDs.

Role of MBD1-3in ATP7B Cu-site transport

Mutating the Cu-sites in MBD 1-3 resulted in normal Cu transport (Figure 18), which signifies that MBD1-3 are not required for the Cu-transport activity, but the domain appear to be involved in the regulatory process. Blocking the Cu-sites in MBD 12, the Cu transport is efficient as with all functional Cu-sites in MBD1-3. Blocking Cu-sites in MBD 13 or MBD 23, the Cu transport is much reduced, with MBD23 having a large effect in Cu transport (Figure 18). Taken together, it indicates that when Cu can bind to MBD3, the Cu-sites in MBD12 are not essential for efficient Cu transport. However, the domains themselves are needed as truncations of MBD1, or MBD12 resulted in much slower Cu transport (Figure 16). Overall, it implies that MBD3 is directly involved in inhibitory interactions with other ATP7B domains and that MBD12 must be present to release such interactions upon Cu loading (agrees with the data from NMR studies (Yu et al. 2017)).

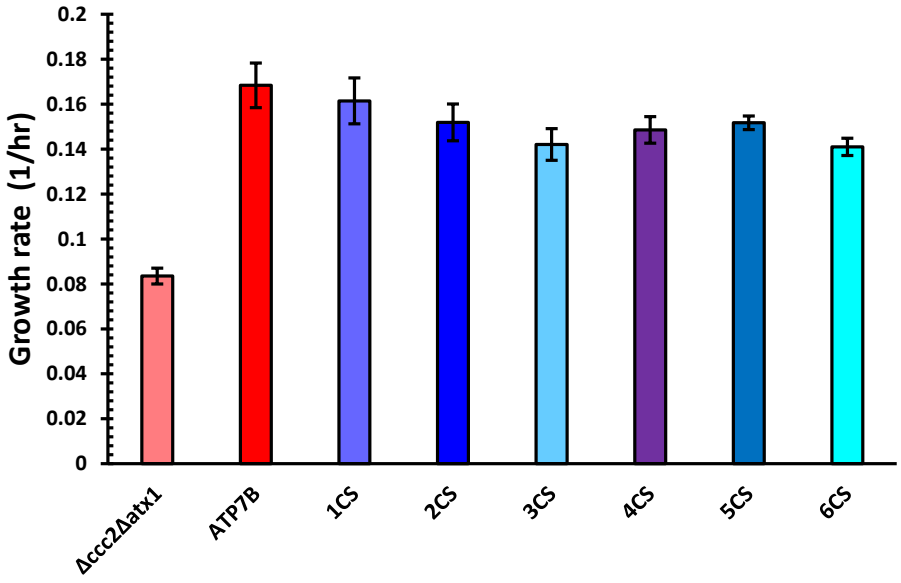


Figure 17: Growth rate of single MBD mutated Cu-site ATP7B variants. Growth rates of yeast expressing ATP7B variants with individual Cu-site mutated, in the *Δccc2Δatx1* yeast strain complemented with Atox1 plasmid at Fe-limited conditions. Error bars are based on standard deviations calculated for quadruplicate experiments.

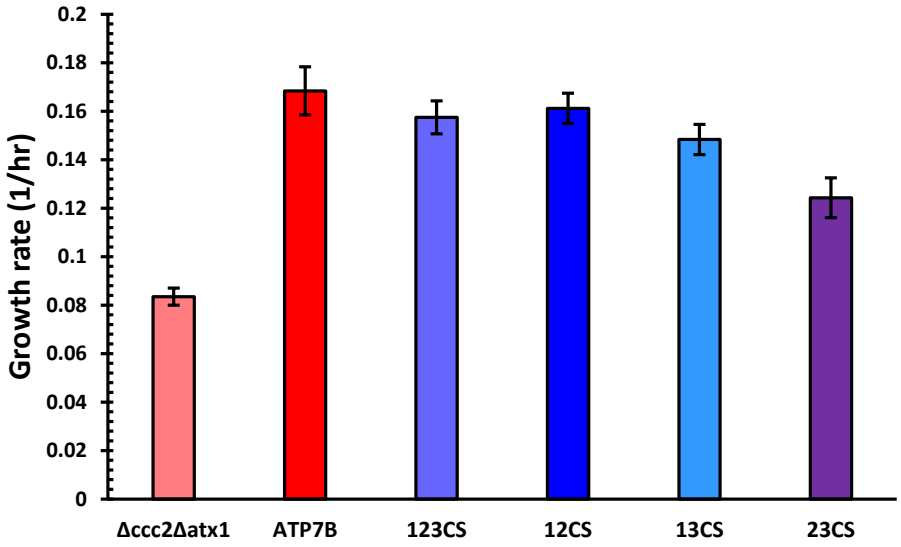


Figure 18: Growth rate of mutated Cu-sites in MBD1-3. Growth rates of yeast expressing ATP7B variants with two or three Cu-sites mutated among MBD1-3, in the *Δccc2Δatx1* yeast strain complemented with Atox1 plasmid at Fe-limited conditions. Error bars are based on standard deviations calculated for quadruplicate experiments.

Role of MBD4-6 in ATP7B Cu-site transport

Blocking all Cu-sites in MBD4-6 resulted in complete loss of Cu transport (Figure 19) which show that MBDs close to the membrane part are essential for Cu transport. Blocking the Cu-site in MBD4 did not possess a large reduction in Cu transport like MBD5 or 6 alone (Figure 17). This result suggests that MBD4 (likely structural role) appears to be involved in Cu relay from MBD3 to MBD56 via conformational rearrangement, as previously proposed [22].

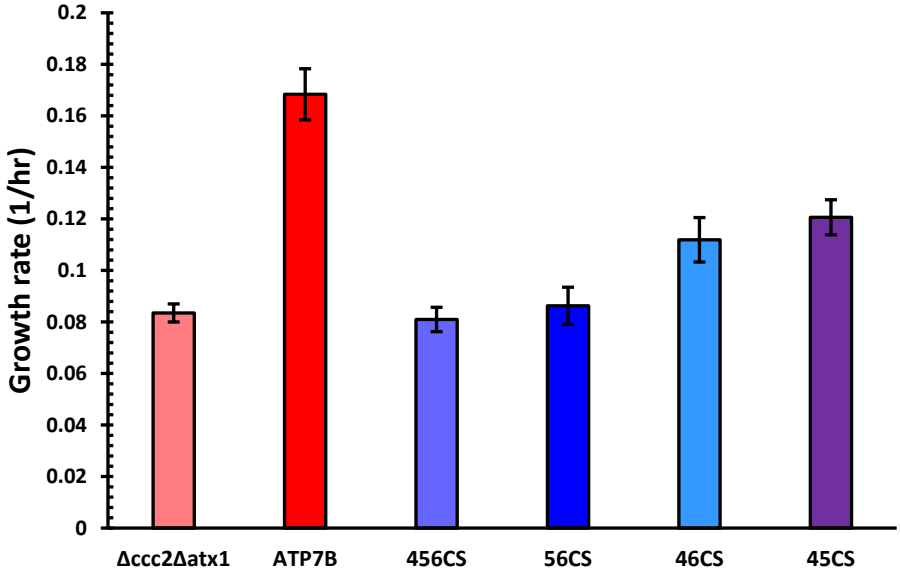


Figure 19: Growth rate of mutated Cu-sites in MBD4-6. Growth rates of yeast expressing ATP7B variants with two or three Cu-sites mutated among MBD4-6, in the *Δccc2Δatx1* yeast strain complemented with Atox1 plasmid at Fe-limited conditions. Error bars are based on standard deviations calculated for quadruplicate experiments.

Lack of Cu transport was observed when the Cu-sites in MBD56 are blocked. Blocking of Cu binding in combination with MBD5 or MBD6 displayed some Cu transport. These data signify that the Cu-site in either MBD5 or MBD6 is necessary for Cu transport. Presence of Cu-site in MBD6 (without Cu-site in MBD45) resulted in higher Cu transport than Cu-site in MBD5 (without Cu-site in MBD46), which implies path through MBD 6 dominates over MBD5 for reaching transmembrane Cu-site.

MBDs as cytoplasmic Cu chaperones

In yeast, the MBDs of Ccc2 when expressed individually complement the function of Cu chaperone Atx1 in absence of MBDs in Ccc2 (Morin et al. 2009). To understand the functional roles of cytoplasmic individual MBDs of ATP7B, we expressed the MBDs individually as a cytoplasmic single-domain protein instead of Atox1. All MBDs can replace Atox1, in presence of full-length ATP7B in our yeast system (Figure 20A). MBD1 and MBD3 as chaperones resulted in slightly lower Cu transfer activity when compared to MBD2, MBD4, MBD5 and MBD6 which displayed Atox1-like activity. These results show that all MBDs are well expressed as individual domains and adopt folded states capable of binding Cu.

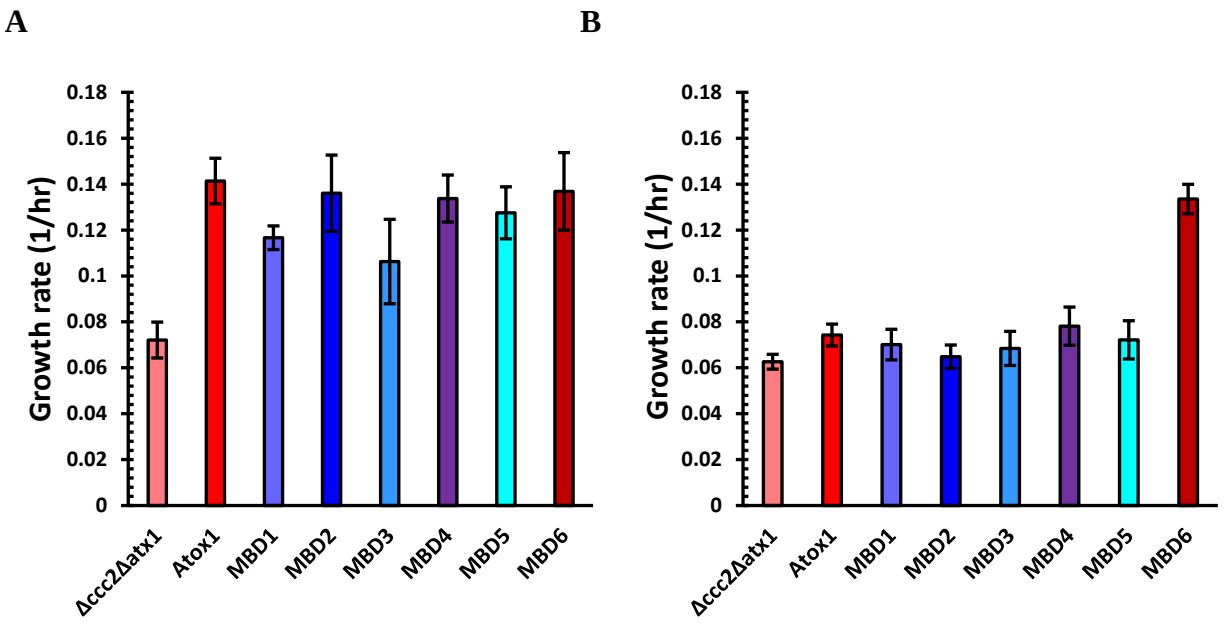


Figure 20: Growth rates of yeast expressing individual MBDs instead of Atox1, along with domain-truncated ATP7B variants in the *Δccc2Δatx1* yeast strain at Fe-limited conditions. **A.** Individual MBDs and full-length ATP7B. **B.** Individual MBDs and ATP7B DEL1-6. Error bars are based on standard deviations calculated for quadruplicate experiments.

Atox1 cannot deliver Cu to all six MBDs deleted ATP7B (Figure 16). However, with MBD1-6 deleted in ATP7B, we found that MBD6 as a cytoplasmic domain was able to deliver Cu to ATP7B whereas all other MBDs were non-functional (Figure 20B). This result implies that the MBD6 structural unit makes specific protein-protein interactions near the Cu entry site in the ATP7B membrane part.

Previous studies had shown the presence of functional Cu-site in MBD6, with the absence of Cu-site in other MBDs, or vice versa (Figure 17), and presence of functional Cu-site in MBD5, with the absence of Cu-site in other MBDs, restored Cu transport. This implies that one of the Cu-sites in MBD56 is required for Cu transport. Our important finding is that MBD1-3 forms a regulatory unit and one of the Cu-sites in MBD56 is essential for Cu-transport.

Our present results, together with the results from previous studies, unravelled the Cu relay path through MBDs (represented in Figure 21). It appears that MBD1-3 forms a regulatory unit that controls Cu transport. The cys residues in the Cu-sites of MBD1-3 appears to be involved in the internal interactions within the MBD1-3 unit (via transient disulfide bond formation). Cu binding (transferred from MBD2) to MBD3 releases internal MBD1-3 interactions which in turn will release inhibitory interactions with other ATP7B cytoplasmic domains, or Atox1 mediated Cu delivery to MBD4 will also result in the release of inhibitory interactions with other ATP7B cytoplasmic domains. From MBD4, the Cu is transferred to either of the MBD 5 or MBD6, or MBD 4 act as a linker that allows MBD3 to reach MBD5 or MBD6. MBD5 or MBD6 provide parallel routes for Cu entry into the transmembrane Cu-site and one of the Cu-site in MBD5

or MBD6 is essential for Cu transport. MBD6 (Figure 20B) makes possible interaction with the transmembrane domains, thus allowing Cu ion into the transmembrane Cu entry site.

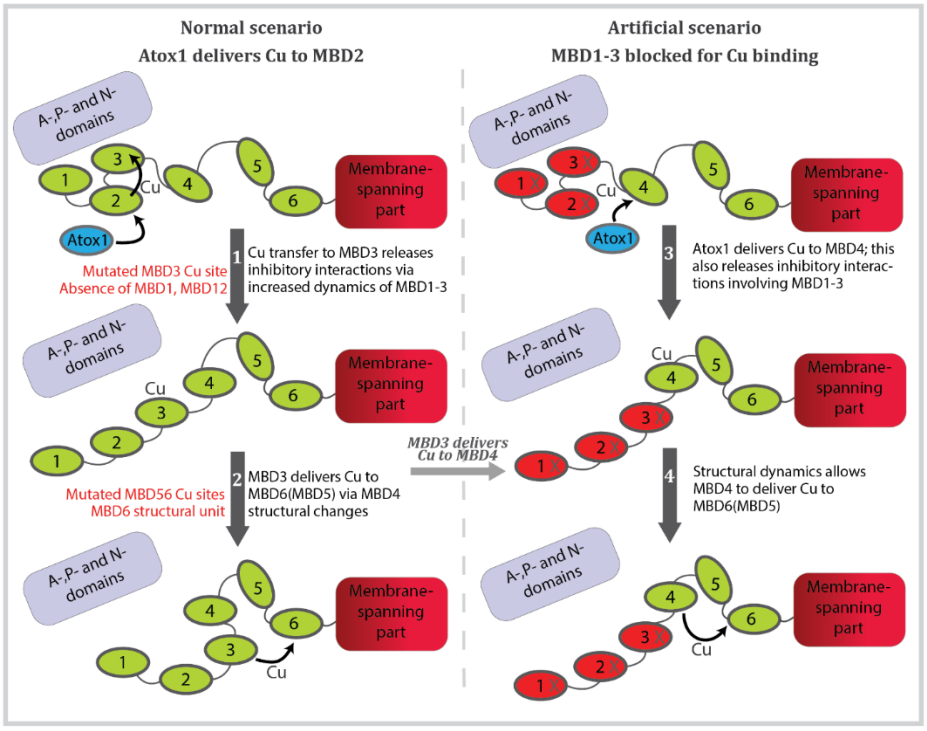


Figure 21: Schematic representation of Cu transport in the MBDs of ATP7B. Left, normal scenario. Right, for the case where MBD1-3 have mutated Cu-sites. The protein starts in an inhibitory form where MBD1-3 without Cu makes interactions with other (likely A-, P-, or N-) domains. Cu delivery by Atox1 to one of MBD1-3 Cu-sites, most likely the one in MBD2, results in further Cu transfer to MBD3. With Cu in MBD3, the domain interactions among MBD1-3 are released due to increased conformational dynamics; this relieves (inhibitory) interactions with other domains (1). This step can be blocked by mutation of Cu-binding residues in MBD3 or by truncation of MBD1 or MBD12 domains. Next, conformational changes, possibly mediated by MBD4 as a spacer, allows MBD3 to deliver Cu to MBD5 or, more likely, MBD6, for Cu delivery to the membrane-spanning part (2). Mutation of metal sites in MBD56 abolishes this step, and the MBD6 structural unit is required for correct docking at the membrane. In our artificial case, where Cu-sites in MBD1-3 are mutated, we propose that Atox1 initially delivers Cu to MBD4, and Cu in MBD4 results in increased structural dynamics providing a pulling force on the MBD34 linker such that the inhibitory interactions between MBD1-3 and other domains are released (3). MBD4 would then deliver Cu directly to MBD5 or MBD6, for delivery to the Cu-site in the membrane-spanning channel (4).

3.3 Role of luminal loop of ATP7B in Cu transport

OBJECTIVES

This study aimed to assess putative Cu-binding residues in the ATP7B luminal loop representing an "exit" Cu-site.

MOTIVATION

Human Cu-dependent enzymes such as blood-clotting factors, tyrosinase, lysyl oxidase, and ceruloplasmin go via the secretory pathway and depend on either ATP7A or ATP7B for Cu delivery (La Fontaine and Mercer 2007). From our previous study (described in Chapter 3.2), we know that Cu is received by MBDs from Atox1, as truncation of MBDs resulted in absence of Cu transport activity. The Cu release from ATP7B is not known and how the Cu is loaded to the Cu-dependent enzymes in the secretory pathway. It is important to determine the Cu release from ATP7B, in order to understand how the mutation in Cu release site or in the vicinity affect the Cu transport. Moreover, most studies on ATP7B focused on cytoplasmic domains, and less attention has been given to the luminal side of ATP7B and how Cu is loaded to the target Cu-dependent proteins in the TGN. The luminal loop connecting the transmembrane domains 1 and 2 contains putative Cu-binding residues in the form of methionine and histidine residues. Similar loop in ATP7A has been reported to bind Cu (Barry et al. 2011).

Our yeast system has proven to be a productive tool to unravel the roles of MBDs and Cu transport through MBDs. Using the same yeast system, we assessed the functional role of putative Cu binding luminal loop residues in Cu transport. In addition, we employed *in vitro* NMR to probe the Cu binding in the luminal loop. Apart from our yeast system, the NMR and MD simulation studies were required for the confirmation of Cu binding in the luminal loop and for determination of Cu-binding sites (Paper II (Kohn et al. 2018)). In our previous study (Chapter 3.1 and 3.2), NMR was not performed as the research questions concerned full-length ATP7B, which is not possible to study by NMR.

METHODS

Luminal loop ATP7B mutants: The ATP7B luminal loop mutants with all of M668, M671, H679, M682, and H686 mutated to Ala, or only all His mutated to Ala, or only all Met mutated to Ala, or the single mutation M682A were constructed. Mutations were introduced into the full length ATP7B by Quick-change site directed mutagenesis kit using the constructed p426GPD-ATP7B plasmid as a template. The yeast complementation assay and growth analysis were carried out as mentioned in methods part in Chapter 3.1. Immunofluorescence method was used to visualize the localization of ATP7B in yeast cells (refer Paper II & IV).

Nuclear Magnetic Resonance (NMR)

NMR spectroscopy is an analytical technique used to study the structure of molecules, the interaction of molecules, the kinetics or dynamics of molecules and the composition of mixtures of biological or synthetic solutions or composites. NMR's advantage is the unique ability of a nuclear spectrometer to allow

both the non-destructive and the quantitative study of molecules in solution and in solid state, as well as to enable the study of biological fluids. NMR can be used to determine molecular conformation in solution as well as studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion. Advancement in NMR spectroscopy makes it a reliable method to determine the protein structure (Louro 2013).

The nucleus of an atom is electrically charged and has the tendency to spin in the magnetic field. If an external magnetic field is applied, an energy transfer occurs between the lower energy level to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its lower energy level, energy is emitted at the same frequency. The signal that matches this transfer is measured and processed in order to yield an NMR spectrum for the nucleus. The precise resonant frequency of the energy transition is dependent on the effective magnetic field at the nucleus. The information about the nucleus's chemical environment can be derived from its resonant frequency (Marion 2013).

NOESY (Nuclear Overhauser Effect Spectroscopy) is an experiment in protein NMR that allows to study for large or complex proteins. NOESY spectra consist of protons on the both axis ($_1H^1$- $_1H^1$), the signals arise from protons that are close to each other in space even if they are not bonded (Louro 2013) . The most popular 2D-NMR are COSY (homonuclear correlation spectroscopy) and TOCSY (Total correlation spectroscopy). COSY is used to identify spins that are coupled together whereas in TOCSY the cross peaks of coupled protons are observed. In TOCSY, apart from directly coupled nuclei cross peaks, the cross peaks between nuclei which are connected by chain of couplings are also observed. Like NOESY, the TOCSY is widely used for large molecules with many separated coupling networks such as peptides, proteins and oligosaccharides (Fan and Lane 2016; Barbet-Massin and Pintacuda 2012).

NMR spectroscopy has shown great potential in determining the protein-protein interactions, ligand-protein interactions and metal-protein interaction. Thus, employed NMR in our work to identify the Cu-binding residues in the ATP7B luminal loop. In this study, two-dimensional homo-NOESY (^1H-^1H NOESY) and TOCSY (^1H-^1H TOCSY) experiments were performed for the assignment of chemical shifts for the ATP7B luminal loop peptides (synthesized). The assignment of proton resonance signals in luminal loop peptides was supported by the acquisition of a two-dimensional heteronuclear ^1H-^{13}C HSQC spectrum obtaining ^{13}C chemical shifts increasing the spectral resolution and also assisted in identifying the type of amino acid whose chemical shifts have been monitored by the ^1H-^1H NOESY and ^1H-^1H TOCSY spectra For the study of Cu(I) interaction with luminal peptides, the CuCl containing samples including stock solutions (CuCl in HCl) were prepared under reducing atmosphere (95% N_2, 5% H_2) in airtight NMR tubes (for experimental setup refer Paper II).

RESULTS AND DISCUSSION

Role of the luminal loop in ATP7B

To understand the functional role of the luminal loop (Paper II) that connects transmembrane domain 1 and 2 in ATP7B, four different ATP7B variants were constructed in which all putative Cu-binding residues in the luminal segment (histidine and methionine residues) are mutated into alanine (A). The proposed luminal loop contains three methionine (668, 671 and 682 site in full length ATP7B or M2, M5 and M16 site in the peptide) and two histidine residues (679 and 686 site in full length ATP7B or H13 and H20 site in the peptide) which are anticipated to be Cu(I) binding sites (Figure 22). The constructed ATP7B variants are denoted as i) H2A (mutation of both histidine residues in luminal loop to alanine), ii) M3A (mutation of all methionine residues in luminal loop to alanine), iii) M682A (Mutation of methionine residue at 682 site to alanine) and iv) M3H2A (mutation of all histidine and methionine residues in luminal loop).

Human **ATP7B**	654	FLCSLVFGIPV**M**A**L**M**I**Y**M**LIPS----------NEP**H**QS**M**VLD**H**NIIPGLSILNLIFFILCTFVQ 707
Human **ATP7A**	654	FLVSLFFCIPV**M**GL**M**IY**MM**V**M**D**HH**FATL**HH**NQN**M**SKEE**M**INL**H**SS**M**FLERQILPGLSV**M**NLLSFLLCVPVQ 724
L.Pnemophila **CopA**	85	FWIAL**M**LTIPVVILE--**M**GG----------**H**GLK**H**----FISG-NGSSWIQLLLATPVV 126
S.Cerevisiae **CCC2**	262	----TLLAIIC**M**LL--Y**M**IVP**MM**-WPTIVQ----------DRIFPYKETSFVRGLFYRDILGVILASYIQ 326

Figure 22: Alignment of luminal loop and nearby transmembrane sequences. Met and His residues are indicated with bold letters and the peptide segment (24 aa) that was used for NMR measurements are in red.

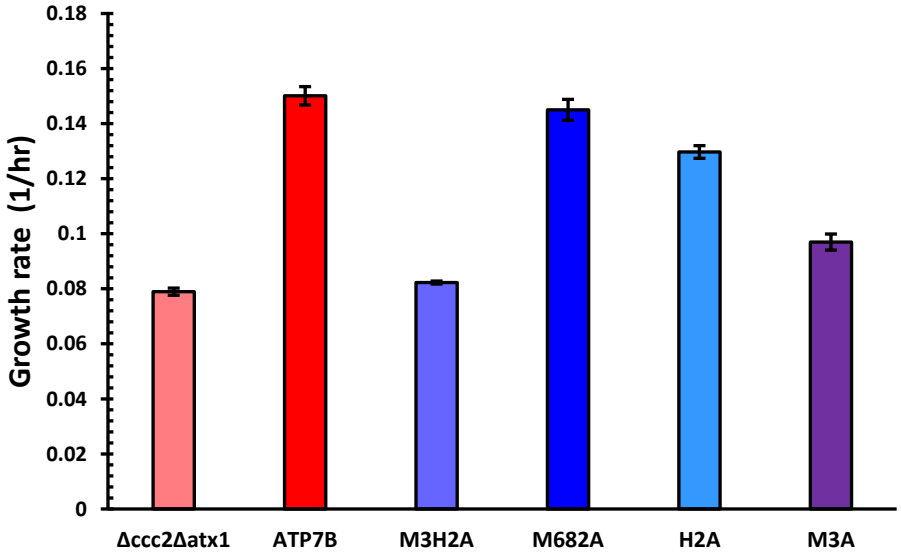


Figure 23: Luminal loop mutants in Δ*ccc2*Δ*atx1* yeast. Growth rate of the Δ*ccc2*Δ*atx1* yeast strain complemented with wildtype Atox1 and luminal loop ATP7B variants in Fe-limited conditions. Error bars are based on the calculation of weighted errors from six biological replicates.

The growth rate of M3H2A yeast matches the yeast strain without ATP7B (Figure 23). This demonstrates lack of Cu transport in M3H2A variant. Reduced growth rates were observed for H2A or M3A yeast. This shows that Cu transport in H2A or M3A variant is less functional but not completely inhibited. This result clearly indicates that both methionine and histidine residues are responsible for Cu transport. Replacing only one methionine (M682 site) had similar growth to wildtype ATP7B, indicating that M682 variant was able to transport Cu. Notably, replacing all methionine residues had more reduction in growth rate when compared to replacing all histidine in the loop. This implies methionine residues in the loop are more important than histidine residues. Another interesting observation is that when the growth rate differences between M3A and H2A compared to that of yeast without ATP7B *(*Δ*ccc2*Δ*atx1* yeast) are summed up, it equals the decreased growth effect of M3H2A variant. This suggests that the reduced growth rate observed in the M3A and H2A variant is the effect of loss of histidine or methionine residues in ATP7B mediated Cu transport. The western blotting analysis confirmed that the expression levels of these variants in yeast were similar to that of ATP7B wildtype. From the results, it can be concluded that most of the methionine and histidine residues present in the luminal loop in ATP7B are required for Cu transport.

Localization of ATP7B and its variants

Localization of all ATP7B variants were similar to those observed for cells with wildtype ATP7B (Figure 24). Moreover, all expressed proteins were distributed throughout the yeast cells in a dispersed pattern. This signifies that all ATP7B variant proteins are expressed efficiently in yeast cells and do not agglomerate due to protein misfolding in any specific compartment i.e. in ER. Thus, the introduced mutations in the luminal loop did not affect the biosynthesis (expression, folding and distribution) of full-length ATP7B. Taken together, western blot and immunostaining results clearly demonstrate that changes in yeast growth rates originate from the defects of ATP7B and not from the low expression levels or localization defects.

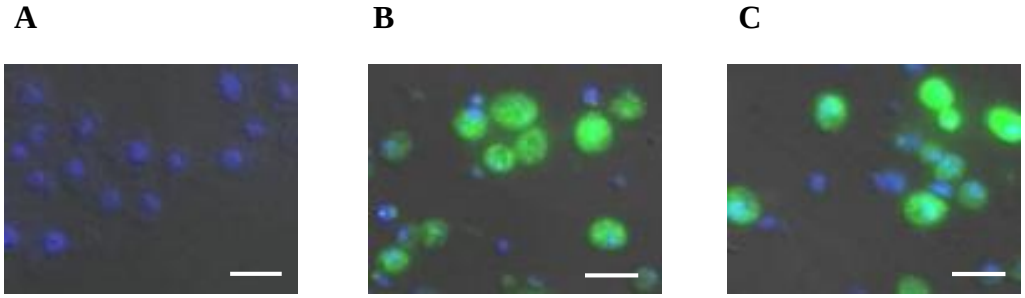


Figure 24: Cellular localization of ATP7B. Immunostaining of ATP7B variants in yeast cells using a ATP7B-specific antibody. All nuclei were visualized by DAPI staining. Δ*ccc2*Δ*atx1* yeast **A**. without any ATP7B plasmid as control, **B**. with wild type ATP7B, or **C**. with M3H2A ATP7B variant high-copy plasmid. (Scale bars 5 µm).

The luminal loop peptide (synthesized, 26 residues long) contains three methionine and two histidine sites which are referred as M2, M5, M16 and H13, H20. The peptides are denoted as L-WT (Luminal Wildtype), L-M3A (substitution of all three methionines (M668, M671 & M682) to alanines), L-H2A (substitution of two histidines (H679 & H686) to Ala) and L-M3H2A (comprising of all histidine and methionine mutations) (Figure 25).

```
L-WT     LMIYMLIPSNEPHQSMVLDHNIIP
L-M3A    LAIYALIPSNEPHQSAVLDHNIIP
L-H2A    LMIYMLIPSNEPAQSMVLDANIIP
L-M3H2A  LAIYALIPSNEPAQSAVLDANIIP
```

Figure 25: Luminal loop peptides. Primary sequences used in NMR experiments (with KK added to the N-terminus of all constructs to increase solubility, making each peptide 26 residues long).

Luminal loop peptide is unstructured in solution

The one-dimensional proton NMR spectroscopic data shows that the luminal loop peptide (L-WT) is unstructured (also supported by far-UV CD data) (Figure 26A). The chemical shifts arising from aromatic acids and several side chains resonances are shown in Figure 26B. The detected signals in this spectral range are notably derived from Y4, H13, H20 and N21 residues in the peptide. This spectral range is focused for the following Cu-luminal loop peptide interaction studies. The acquisition of a two-dimensional 1H-13C HSQC spectrum increased the spectral resolution and provided significant support to the assignment of proton resonance signals. When compared to L-WT, the L-H2A and L-M3A peptides had minor changes in the chemical shift pattern whereas L-M3H2A peptide (Figure 28) had slightly more changes than the L-H2A or L-M3A peptide, but overall the mutated peptides retain the structural characteristics of L-WT.

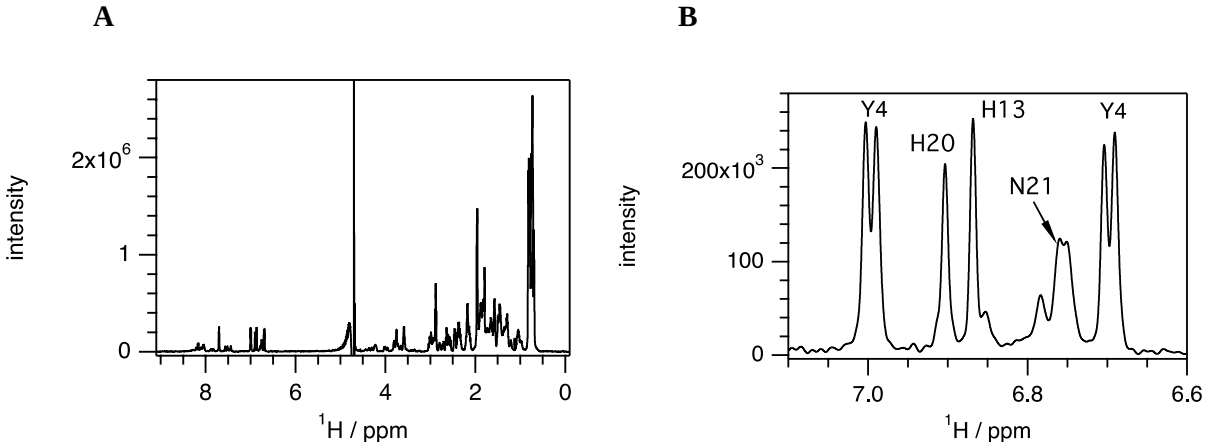


Figure 26: One-dimensional proton NMR spectra for L-WT peptide. (**A**) Chemical shifts for all protons of the wild type peptide. (**B**) Chemical shifts of the selected aromatic and side chain resonances only. Associated assignment is shown by using the one letter code for amino acids following position in primary sequence.

Interaction of luminal loop peptide with Cu

Cu interaction with L-WT peptide (1:1 Cu:peptide) showed profound changes in the chemical shift pattern noticed by decrease in H13 and loss of H20 signals (Figure 27). Further, addition of Cu (2:1 Cu: peptide) amplified the changes in the chemical shift observed during 1:1 Cu-peptide, and in addition had a slight change in Y4 signal (next to putative Cu ligand M5). This suggests that there is more than one Cu-binding site in L-WT peptide.

As a next step, the diffusion coefficient is determined from NMR diffusion spectra. Both apo-L-WT and Cu bound peptide had a similar diffusion coefficient that corresponds to the hydrodynamic radius of an unstructured monomeric peptide. This indicates that the unstructured L-WT peptide is monomeric in solution both in the presence and absence of Cu. Thus, L-WT peptide had at least two Cu-binding site while remaining monomeric state in a solution.

Addition of Cu to L-H2A or L-M3A peptide induced site-specific changes in the chemical shift pattern and increasing the Cu amounts (2:1) showed no significant change in the chemical shift pattern which implies both peptides have only one Cu-binding site. The diffusion coefficient determined for L-H2A and L-M3A peptide provides evidence that the peptides remain in the same monomeric state when it is bound to Cu.

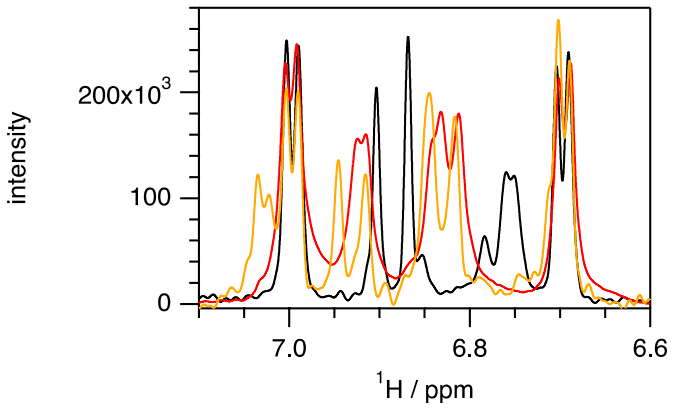


Figure 27: Interaction of Cu with luminal loop peptide. One-dimensional proton NMR spectra for L-WT (refer to figure 26B) in absence (black) and in presence of one Cu ion (red) or two Cu ions per peptide (orange).

The chemical shift pattern of L-M3H2A peptide did not change upon addition of Cu which suggest that L-M3H2A peptide does not bind Cu (Figure 28). Further, it implies that some of the histidine and methionine residues in the L-WT peptide are needed for Cu binding. Moreover, the results obtained from the *in vitro* NMR spectroscopy match with the *in vivo* yeast results which showcase that a combination of histidine and methionine residues is essential for Cu binding in the segment of the luminal loop.

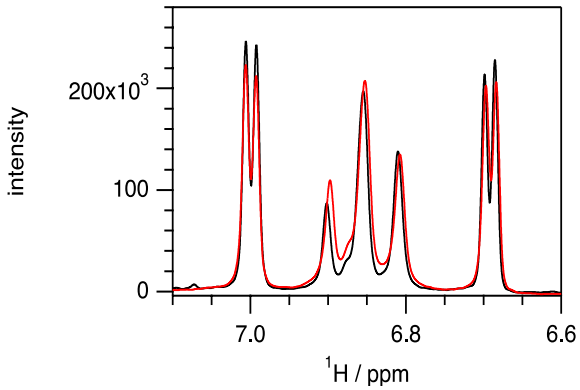


Figure 28: Interaction of Cu with L-M3H2A peptide. One-dimensional proton NMR spectra of L-WT peptide and the L-M3H2A variant. Replacing of all histidine and methionine residues with alanine residues induces changes in the chemical shift pattern L-M3H2A (black). Note that chemical shift values and signal width are almost as seen for the aromatic protons of Y4 (Figure 26B) are fully retained. The addition of one Cu to L-M3H2A (red) did not change the chemical shift pattern compared to the absence of Cu (black).

Though there may be differences between the *invitro* studies using only the luminal loop segment as a peptide, and the *in vivo* expression of the full length ATP7B protein and its variants, several implications can be made from the obtained results when combined together. Notably, replacing all histidine and methionine residues in the luminal loop peptide abolishes Cu binding which is in accordance with the results obtained from the *in vivo* studies in yeast. Inability of M3H2A (all five mutations) variant of ATP7B to transport Cu in yeast is due to the abolished Cu binding in the luminal loop peptide. The Cu transport efficiency of H2A and M3A variants in yeast is less than in the wildtype ATP7B but the NMR spectroscopic data show that the L-H2A and L-M3A peptide binds one Cu ions which implies that specific Cu-binding sites or the number of Cu ions that binds simultaneously is vital for the ATP7B function. Taken all together, it appears that the Cu-binding sites require a combination of histidine and methionine residues and more than one Cu-binding sites are present in the luminal loop.

Another study showed the presence of two distinct Cu-binding sites in ATP7A which comprise that combination of histidine and methionine residues, in accordance with our findings for ATP7B. Mutation on the Cu-binding residues in ATP7A resulted in the inhibition of dephosphorylation and Cu-release functions (Barry et al. 2011). Cu-binding sites in transmembrane were suggested to include three sequential Cu-binding sites noticeably, a Cu entry site that involves charged residues, central Cu-binding site involving conserved CPC motif (ion transduction) and Cu release site which involves D765 residue and plus four nearby methionine residues (Schushan et al. 2012). Our work adds a final Cu exit site in the luminal loop segment of ATP7B involving combination of histidine and methionine residues. This Cu exit site adds to the previous three Cu-sites in transmembrane.

3.4 Mutation causing Dysfunctional ATP7B

OBJECTIVES

This study aimed to assess the effect of WD mutation in MBDs and to probe mutation-induced structural dynamic effects at the atomic level.

MOTIVATION

From our previous studies on MBDs, the MBDs (MBD 5 & MBD 6) close to the membrane are found to be essential for Cu delivery to the transmembrane Cu-site and have parallel routes for Cu delivery. Blocking Cu-sites in MBD56, impaired the Cu transport activity. The MBD56 are known to be the hotspot for WD mutations in MBDs. Moreover, the MBD56 are more conserved in all eukaryotic Cu-ATPase. It is important to understand how the WD mutation in the MBD56 affect the protein function. Moreover, it is necessary to have knowledge on the effect of the WD mutation in the MBDs, since it is one of the key domain in ATP7B (involved in regulatory interactions, interaction with Atox1, Cu delivery to the transmembrane site). So far, the Δ*ccc2*Δ*atx1* yeast system has shown great potential to unravel the roles of MBDs and luminal loop. Using the same system, we investigate the effect of common WD mutation in MBD56 in Cu transport activity. In addition, we employed *in silico* MD to probe the deviation in the structural dynamics that cause protein dysfunction (Paper IV (Shanmugavel et al. 2019)).

METHODS

WD causing ATP7B Mutants: The disease-causing ATP7B mutants namely L492S, A604P, R616W, and G626A (Figure 29) were constructed. The mutations were introduced into the full-length ATP7B by quickchange site-directed mutagenesis kit using the previously constructed p426GPD-ATP7B plasmid as a template. The yeast complementation assay and growth analysis were carried out as mentioned in methods part in Chapter 3.1. Immunofluorescence method was used to visualize the localization of ATP7B in yeast cells (refer Paper II & IV) (Shanmugavel et al. 2019). The location of the WD mutations in MBD56 are shown in Figure 29. The list of WD causing ATP7B mutant are shown in Table 6.

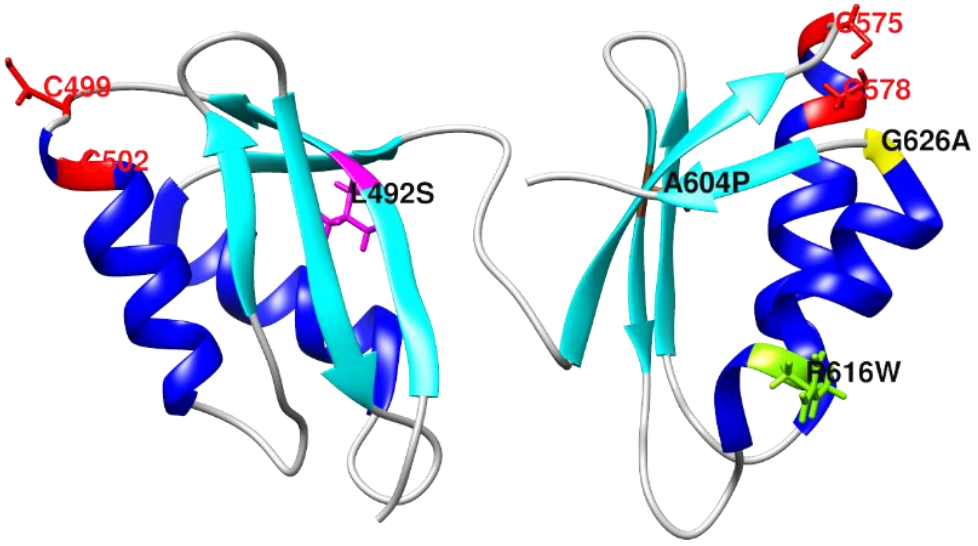


Figure 29: Secondary Structure of MBD 56 of ATP7B. High-resolution NMR structures of MBD56 domains (PDB ID: 2EW9). The Cu-binding cysteines are indicated in red. The WD mutants: L492S (indicated in pink); A604P (in brown); R616W (in green); and G626A (in yellow).

Molecular Dynamics

Molecular dynamics (MD) is a computational simulation technique that uses Newton's equations of motion to study the physical movement of atom or molecules. The biological activity is the result of time dependent interactions between molecules and these interactions occur at the interfaces such as protein-protein, protein-ligand. Time dependent and time independent microscopic behavior of a molecule can be calculated by molecular dynamics simulations. This method is one of the principal tool for modelling proteins, nucleic acids and their complexes and extensively used to investigate the structure, dynamics and thermodynamics of biological molecules. MD simulations have provided detailed information on the protein stability, folding, conformational changes, enzyme reactions, ion transport in biological systems, molecular recognition and drug design. They are also used in determination of structures from NMR and X-ray crystallography. MD simulations approximate the interactions in the system using simplified models. The method allows the prediction of the static and dynamic properties of molecules directly from the underling interactions between the molecules (Karplus and McCammon 2002). A molecular dynamic simulation requires description of the function (also known as potential function) by which the particles in the simulation will interact. In biology, the potential function is referred as molecular mechanics force field. The force field refers to the functional form and parameters sets used to determine the potential energy of a system. MD simulation permit us to gain insight into situations that are impossible to monitor experimentally. The MD simulation is evolved such that the present simulation time are very close to biologically relevant ones (Hospital et al. 2015).

In the current study, we took advantage of MD simulations to examine the WD mutation-induced structural dynamics effects at the atomic level. The *in silico* systems were prepared based on the solution structure 2EW9 (a two-domain structure of the human ATP7B MBD56). The WD mutants: L492S, A604P,

G626A and R616W were *in silico* mutated. Single-domain systems were constructed by removing the linker between MBD5 and MBD 6. The MD simulations were performed on the created single-domain systems for 1.5μS in NPT conditions (i.e. constant pressure and temperature).

RESULTS AND DISCUSSION

Role of disease-causing mutations in ATP7B mediated Cu-transport

The growth rate of the yeast strain expressing L492S mutant is an exact match with the yeast strain lacking ATP7B and Atox1 proteins (Figure 30) which signifies L492S mutant have absence of Cu transport activity. The R616W yeast had marginally higher growth rate as compared to the other variants but significantly decreased growth than the wildtype-ATP7B. This explains that Cu transport in R616W strain is impeded and not completely inhibited. Notably, the A604P yeast strain had better growth than L492S and R616W yeast but substantially reduced growth rate than the wildtype-ATP7B. The Cu transport competence was not significantly reduced in G626A yeast in comparison to other disease-causing mutants (Figure 30). Based on the Cu transport, the results indicate that L492S, and R616W are more severe mutations than A604P, and the G626A is a mild mutation.

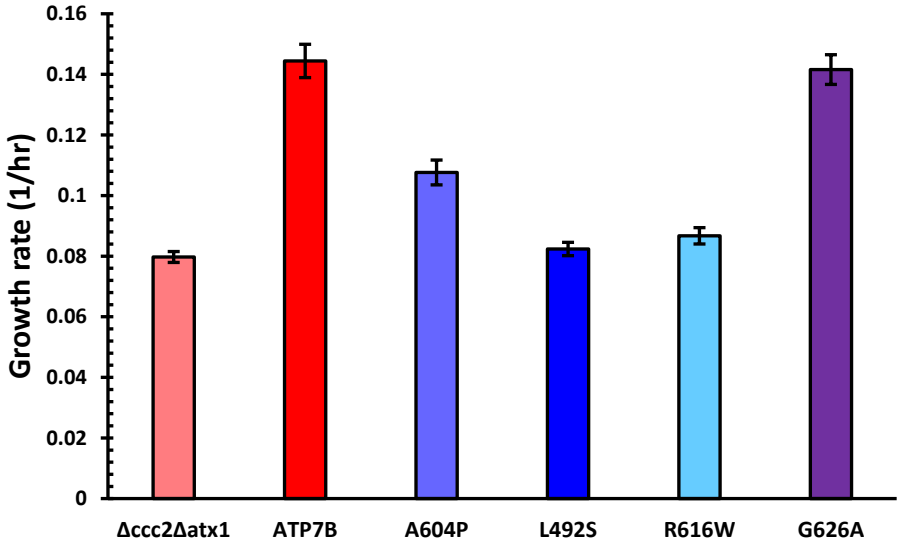


Figure 30: Wilson disease mutants in Δ*ccc2*Δ*atx1* yeast. Growth rates of the Δ*ccc2*Δ*atx1* yeast strain complemented with wildtype Atox1 and WD-causing ATP7B mutants in Fe-limited conditions. Error bars are based on the calculation of weighted errors from six biological replicates.

Cellular localization of WD-causing ATP7B mutants

The expressed protein is evenly distributed throughout the yeast cell in a dispersed pattern in wildtype-ATP7B yeast. All ATP7B mutants except L492S had localization similar to wildtype-ATP7B (Figure 31). The localization of L492S protein predominantly appears to be concentrated in the ER, surrounding the nucleus. Western blot analysis confirmed that the expression levels of all ATP7B variants

in yeast were similar to that of wildtype-ATP7B. In L492S mutant, the lack of Cu transport appears due to improper biosynthesis, thereby trapping in the ER. The decrease in Cu transport in R616W and A604P mutant is a result of mutation-induced protein dysfunction.

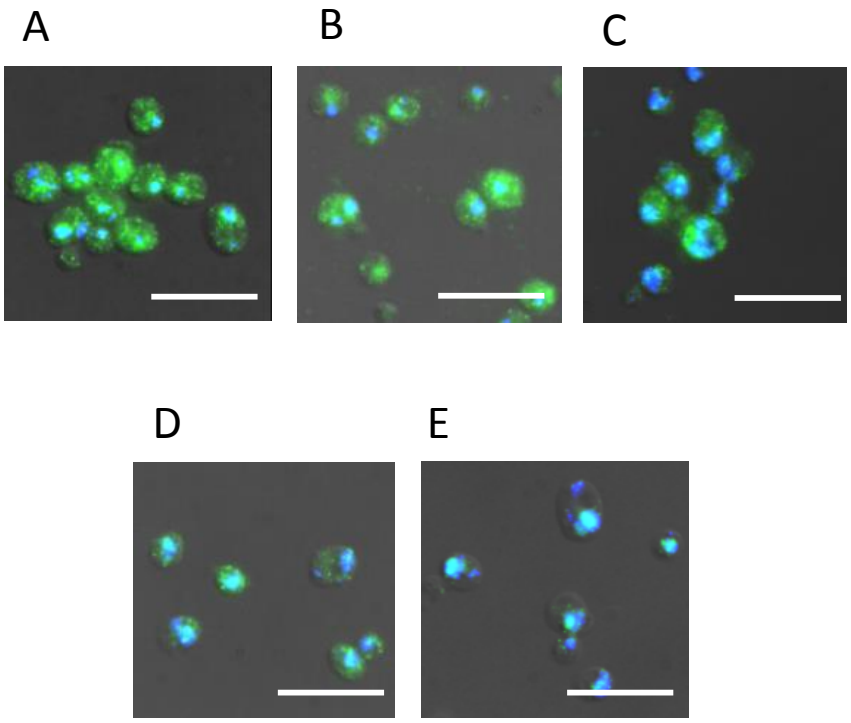


Figure 31: Cellular localization of WD ATP7B mutants. The immunostaining of ATP7B mutants in yeast cells using ATP7B-specific antibodies. Nuclei were visualized by DAPI staining. Scale bars 2.5 μm. (*A*) *Δccc2Δatx1* yeast with wildtype ATP7B as control; (*B*) the G626A mutant; (*C*) the A604P mutant; (*D*) the R616W mutant; and (*E*) the L492S mutant.

Molecular dynamics simulations of WD-causing mutant MBDs

MD simulations were performed on six single-domain systems; namely wildtype-MBD5 ($MBD5_{WT}$), L492S-MBD5 ($MBD5_{L492S}$), wildtype-MBD6 ($MBD6_{WT}$), A604P-MBD6 ($MBD6_{A604P}$), R616W-MBD6 ($MBD6_{R616W}$), and G626A-MBD6 ($MBD6_{G626A}$). The simulations were carried out for three replicas of 500 ns per each simulation time (total 1.5 μs). The root mean square fluctuations (RMSF) values are mapped onto the corresponding average structures from the MD simulations to observe the deviation in the structural dynamics due to mutation.

For all WD mutations, the observed dynamics changes were structurally distant from the mutation site, and no major changes in the local structure were observed near the mutation site All four WD mutants presented altered fluctuations in the Cu-binding loop and loop between β2 and β3 that faces the Cu-binding loop. Increased structural dynamics in the Cu-binding loop was observed for $MBD5_{L492S}$ (Figure 32). The $MBD5_{L492S}$ had altered dynamics throughout the domain and less secondary structure, compared to $MBD5_{WT}$

The increased alterations and reduced secondary structure appear to be the cause of improper processing of full-length ATP7B in L492S yeast.

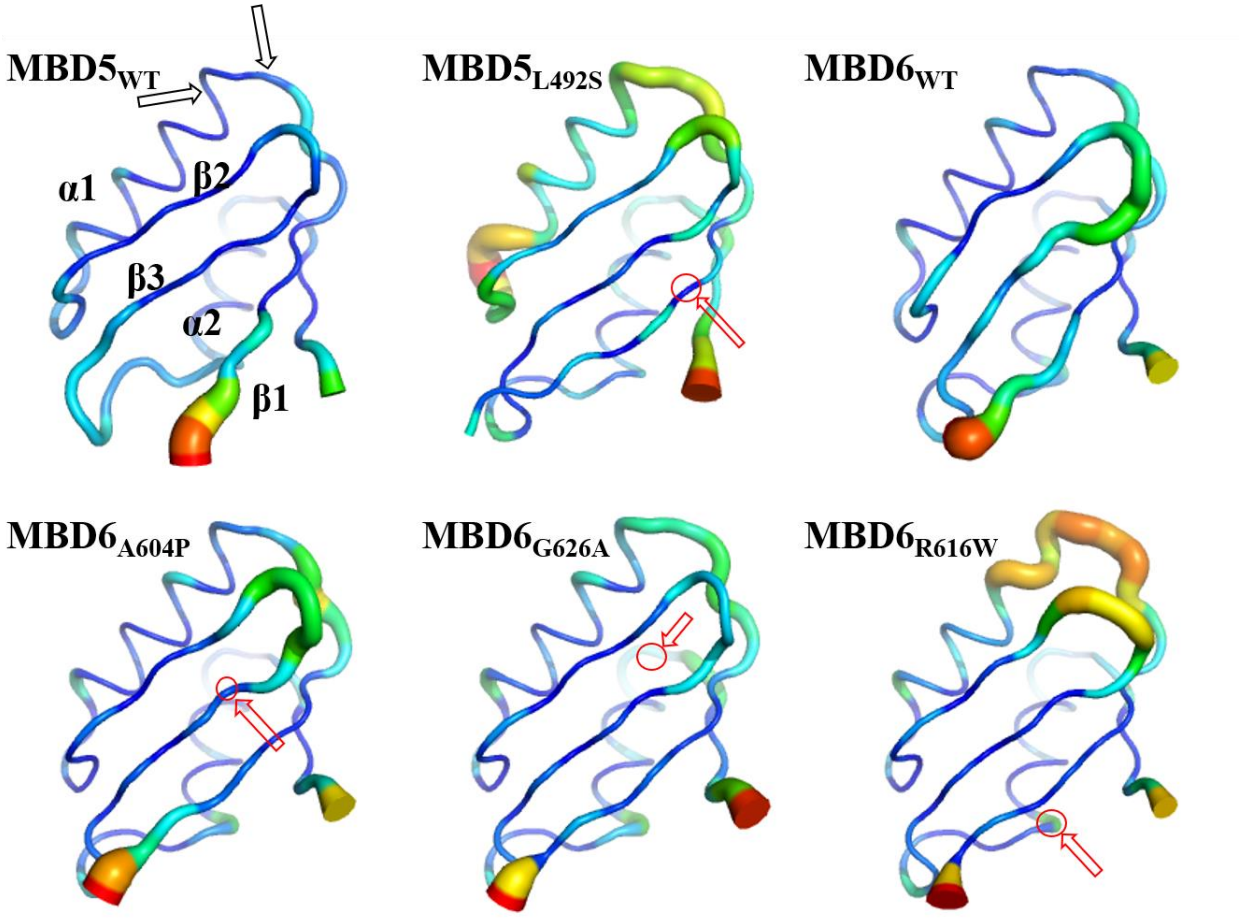


Figure 32: Structural mapping of RMSF values for single-domain systems. The RMSF values (average from three replicas) are mapped on the protein backbones. The thin and blue ribbon gradually changes to thick and red ribbon with the increasing RMSF values. All six constructs are placed in a similar orientation. The two Cu-binding cysteines are indicated by black arrows on the wild-type WD5 structure. The mutated residues on mutant systems are pointed out by the combination of red circles and arrows.

$MBD6_{A604P}$, $MBD6_{R616W}$, and $MBD6_{G626A}$ had increased RMSF fluctuations in the Cu-binding loop and lower RMSF fluctuations in selected parts of the domain compared to $MBD6_{WT}$. $MBD6_{R616W}$ had substantially increased RMSF fluctuations in Cu-binding loop and decreased secondary structure compared to other MBD6 system. In contrast to $MBD6_{R616W}$, the $MBD6_{A604P}$ and $MBD6_{G626A}$ had increased secondary structure than $MBD6_{WT}$. Although the $MBD6_{WT}$ showed decreased secondary structure, the Cu-binding loop was relatively stable based on the RMSF data. Certain dynamic fluctuations in the MBD seem to be essential for ATP7B functional interactions with other proteins.

Decreased Cu transport competence in A604P and R616W yeast appeared to be the result of increased dynamics in the Cu-binding loops. The increased Cu-binding loop dynamics may affect the efficiency of Cu delivery to MBD6 from other MBDs/Atox1 and also affect the Cu delivery from the MBD6

to the transmembrane site. The efficient Cu transport in G626A yeast seems to have no major effect on the structural dynamics. However, the slight changes in the secondary structure and RMSF of $MBD6_{G626A}$ domain may influence other aspects of ATP7B function.

There are limited data on the properties of WD mutations in ATP7B. There are very few studies reporting on the biophysical-molecular studies of WD mutations in MBDs (Kumar et al. 2017). Cu transport is the most common and direct way to study WD mutations. Out of four WD mutations, three variants showed reduced Cu transport activity in yeast. The MD simulations indicated clearly the effects of WD mutation in the MBD, in regard to dynamics in the Cu-binding loop and overall fluctuations in the MBD. The A604P, R616W, and G626A missense mutations were also observed in Menke's disease protein ATP7A and described as disease-causing mutation. The I492T in ATP7A protein (L492S in ATP7B) is known to be a disease-causing mutation (Tumer 2013).

The reason that the G626A mutation is linked to WD in patients cannot be established from our work but may be due to modulation of other aspects of ATP7B function. ATP7B interactions with the COMMD1 was found decreased for G626A ATP7B variant (de Bie et al. 2007). Similar to G626A in ATP7B, a similar mutation in ATP7A has been reported to have trafficking problems in high Cu levels but has normal transport competence (Kim, Smith, and Petris 2003). However, the trafficking ability of G626A ATP7B has been reported to be similar to wildtype-ATP7B (Braiterman et al. 2014). The protein dysfunction in G626A appears to involve changes in interactions with regulatory proteins, such as COMMD1 (de Bie et al. 2007).

4 SUMMARY

Cu transport proteins are required to maintain Cu homeostasis and to supply Cu to enzymes in cells. Dysfunction of Cu transport proteins may lead to anomaly in Cu homeostasis that may result in Cu-related disorders. WD protein, ATP7B, is responsible for the loading of Cu to Cu-dependent enzymes in the secretory pathway. Several questions around ATP7B function are not answered to date. With a focus to learn more about ATP7B Cu transport, we asked basic questions around ATP7B mechanisms.

In this work, we built upon previous knowledge and established an advanced yeast model system that aid in unravelling the mechanism of Cu transport in ATP7B. In **Chapter 3.1**, we present the development of a yeast model system which allows us to probe the Cu flow through human Atox1 and ATP7B. In **Chapter 3.2**, we demonstrate that our new yeast model system is a valuable tool to study the roles of the MBDs in ATP7B for Cu transport. We found that the presence of at least one MBD is required in ATP7B for Cu transport and (in the absence of Atox1) individual MBD can act as a chaperone. Furthermore, the data support that MBD1-3 forms a regulatory unit and the Cu-site in one of MBD5 or MBD6 is essential for Cu transport by ATP7B.

We then shifted our focus to a luminal loop segment of ATP7B, which has not received much attention but speculations suggest a pivotal role in the function of ATP7B. Employing the yeast system together with *in vitro* biophysical tools, mainly NMR, we analyzed residues in the luminal loop segment of ATP7B (**Chapter 3.3**). The results from this study show that the luminal segment of ATP7B can bind Cu via histidine and methionine residues and therefore suggested that ATP7B has a Cu-exit site in the luminal loop segment.

Another interesting question which intrigued me during the course of my research was how mutations in ATP7B causes protein dysfunction. Extending the application of the yeast system in combination with *in silico* MD simulations, we shed light on how WD mutations may cause protein dysfunction. In **Chapter 3.4**, we describe the effect of WD mutations in some MBDs of ATP7B. The reduction in Cu transport ability was linked to changes in structural dynamics in the mutated domains.

Though we have investigated important aspects of Cu transport process in ATP7B, many research questions are still unexplored. Until high-resolution structural information of full-length ATP7B is available, the yeast based assay adds value to our understanding of mechanistic aspects. Looking forward, our in house yeast model system is a useful platform to explore Cu transport further and associated phenotypic effects of WD mutations.

5 CONCLUDING REMARKS AND OUTLOOK

Cu metabolism dysfunction is implicated in an increasing number of diseases such as WD, aceruloplasminemia, cancer, and neurodegeneration (De Freitas et al. 2003). WD is a genetic disorder that affects Cu-transport due to mutations in ATP7B, a protein responsible for regulation of Cu levels in liver cells. To explore underlying processes leading to Cu transport dysfunction, yeast is useful model organism since Cu transport proteins and pathways are highly conserved from yeast to humans (De Freitas et al. 2003).

Among all types of yeast, the baker's (budding) yeast (*S. cerevisiae*) is widely used as a model organism to establish mechanisms related to human diseases (De Freitas et al. 2003). This is due to its well characterized Cu transport pathway and that many biochemical pathways are conserved among eukaryotes. The application of a yeast complementation assay for the study of ATP7B provides a tool to assess putative Cu transport dysfunction of various mutants. This assay has previously been used to study ATP7A, ATP7B, and also used as a diagnostic tool for WD (Papur, Terzioglu, and Koc 2015; Cater et al. 2004). Other Cu-transport assays typically requires the usage of radioactive Cu (^{64}Cu) isotope which has a short half-life (12.7 hours) or purified proteins. Yeast complementation assays are widely adapted to understand the function of proteins and have been applied for studying the mammalian transport protein Nramp2 (Natural resistance associated macrophage protein) involved in iron deficiency in human (Pinner et al. 1997) and CFTR (cystic fibrosis transmembrane conductance regulator) that relates to cystic fibrosis (Teem et al. 1993).

Earlier studies using yeast assays have provided significant information about disease-causing mutations in ATP7B, the importance of MBDs, phosphorylation, ATP binding site, and Cu-binding regions of ATP7B (Forbes, Hsi, and Cox 1999). These studies were carried out in Δ*ccc2* yeast strain thus relying on yeast Atx1. In order to include Atox1-ATP7B interactions, we performed experiments using Δ*ccc2*Δ*atx1* yeast strain complemented with both ATP7B and Atox1. Incorporation of wildtype ATP7B and Atox1 in this strain enables transport of Cu in iron-limited medium and can be used to probe functionality of variants. This system may be a valuable tool to explore other Cu transport proteins and Cu-dependent processes. Genotype-phenotype correlations in WD have not been established, and due to this, treatment of WD patients is often delayed. The development of yeast phenotypic parameters for various ATP7B mutants could help to establish genotype-phenotype correlations in WD.

In our work, we used the yeast system to probe the Cu relay path through MBDs in ATP7B using a range of truncated and mutated variants (Paper I and III). There are numerous studies on the cytosolic and

transmembrane domains in ATP7B (Nilsson et al. 2013; Mondol, Aden, and Wittung-Stafshede 2016) but less attention is given towards the luminal section of ATP7B (Kohn et al. 2018). In this thesis, apart from MBDs we also focused on the luminal section of ATP7B that links TMD1 and TMD2 and has putative Cu-binding residues. In our yeast system, we investigated the putative role of luminal loop Cu-binding residues in ATP7B for Cu transport (Paper II), together with *in vitro* NMR spectroscopic experiments. The combined results from these studies imply that ATP7B has a Cu-exit site in the lumen segment, similar to recently found in ATP7A (Barry et al. 2011). We also assessed if selected disease-causing mutations affected Cu transport by ATP7B. A significant fraction of WD mutations is located in MBD56 and we focused on some of these. We delineated effects of disease-causing mutations in MBD5 and MBD6 using our yeast system together with *in silico* MD simulations of individual domains. Alterations in structural dynamics of mutated domains appeared to influence ATP7B Cu transport (Paper IV).

In the future, the methods established in this work could be used to probe Cu transport by other WD-causing ATP7B variants, the role of Atox1 mutants and to investigate the Cu transport of different Cu-ATPases and chaperones. The yeast system may also be used to test drugs for WD (Materia et al. 2012; Chandhok et al. 2014; Aggarwal and Bhatt 2018; Schilsky 2005) and MNK (Kreuder et al. 1993), and effects of helper proteins such as heat shock proteins that may rescue ATP7B from misfolding due to mutations (Materia et al. 2012). It may also be possible to exploit the yeast model system to characterize more in depth the physiology of mutant variants involving mitochondrial damage, oxidative damage, and lipid metabolism, all of which have been observed in WD patients (Lichtmannegger et al. 2016; Liu et al. 2014; Stattermayer et al. 2015; Wright et al. 2009).

Cu dyshomeostasis in PD patients may be linked to genetic mutations (*e.g.* A1135Q) of ATP7B (Desai and Kaler 2008; Waggoner, Bartnikas, and Gitlin 1999). Cu transfer by A1135Q-ATP7B can be studied using our yeast system. PD patients have decreased expression of Ctr1, but the reason behind IT is not known (Castillo-Gonzalez et al. 2017). Interestingly, a recent study reported the interaction of Atox1 with α-syn *in vitro* (Horvath, Werner, and Wittung-Stafshede 2018). Constitutive expression of α-syn in our yeast system may allow us to probe the pathway that causes downregulation of Ctr1 and examine the role of Atox1 in α-syn amyloid formation. Atox1 dysfunction and overexpression of Ctr1 have been reported to enhance Aβ phenotype in *Drosophila* (Sanokawa-Akakura et al. 2010). By exploiting this yeast system, the effect of Cu transport proteins in regards to Aβ aggregation can be investigated. Prion proteins are known to cause oxidative damage in cells (Giampietro et al. 2018). The oxidative activity of prion proteins in the presence of Cu transport proteins can be determined by utilizing this system together with mitochondrial activity assays. Moreover, the yeast system can be used to study consequences on Cu transport upon interactions with the anti-tumor agent cisplatin (known to bind ATP7B (Leonhardt et al. 2009) and Atox1 (Palm et al. 2011)).

To conclude, my experiments with the developed yeast model system have provided information about the roles of MBDs, the role of luminal loop, and the effects of WD-causing mutations of ATP7B, and shows great potential for investigating human Cu transport in greater detail. In the future, this yeast model system may be used for studying other disorders that relates to imbalances in Cu homeostasis, such as cancer and neurodegeneration.

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