Structure-function studies of copper flux across cellular membranes

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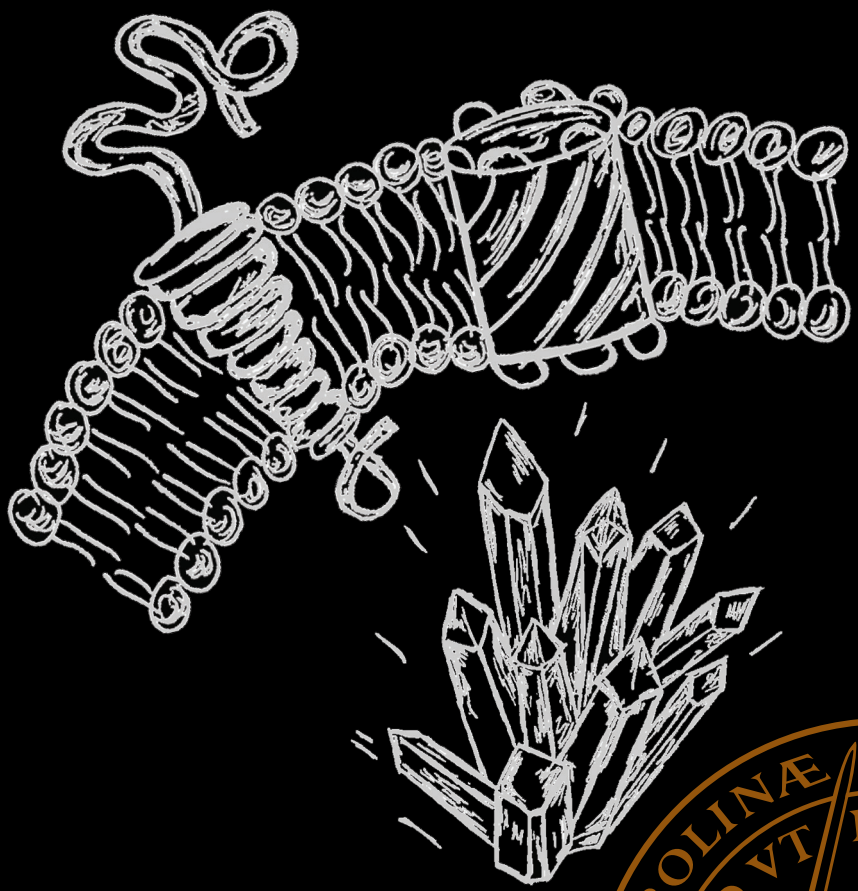

درخت تو گر بار دانش بگیرد

به زیر آوری چرخ نیلوفری را

Structure-function studies of copper flux across cellular membranes

Niloofar Nayeri

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 23rd of March at 13:15 in Segerfalksalen Hall, A building, BMC, Lund

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Durham University

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Abstract: Copper (Cu) is a transition metal that is essential for most organisms. It is for example a co-factor in a number of key enzymes involved in redox reactions, such as respiration, oxidative phosphorylation and nitrification. Yet, due to its redox properties, free Cu can generate toxic reactive oxygen and nitrogen species, cause mismetallation of proteins and destruction of Fe-S clusters. Therefore, organisms have evolved mechanisms for tight regulation of the body and cellular Cu levels. In this process, Cu transporters and channel proteins are critical, providing passage of the metal across cellular membranes. These include but are not limited to the CTR and PcoB/CopB families covered in this work.

Considering the fact that copper homeostasis is so fundamental in physiology, the first part of this work centered on the biochemical isolation and purification of the eukaryotic copper transporter protein family (CTRs). CTRs are the only known Cu importers in eukaryotes and responsible for the initial uptake of the metal and yet remain poorly understood. In this study three challenging CTR targets, human CTR1, human CTR2 and *Candida albicans* CTR were selected, overproduced, membrane solubilized and purified. Acceptable quality of purification and yields were obtained for all targets that open up for downstream structural and functional studies.

The second intention with this work was to shed further light on the structure and function of bacterial outer membrane proteins. In *Escherichia coli* the cytoplasm is kept free of Cu ions primarily by CopA, a P1B-type ATPase that pumps Cu(I) ions to the periplasm, where Cu(I) is then oxidized to less toxic Cu(II) by CueO, a multicopper oxidase, both regulated by CueR, a transcription factor. Many *E. coli* strains living in Cu-rich environments possess an additional Cu resistance system, the pco (plasmid-borne copper resistance) cluster. Indeed, cells expressing the pco genes have been shown to survive in higher Cu concentrations compared to strains lacking these. The Pco determinant consists of seven proteins, PcoABCDRSE, and in this work we successfully structurally determined at 2 Å resolution one of the least studied proteins of this pco system, the outer membrane protein PcoB. We revealed a pathway across the PcoB structure that is compatible with Cu flux, as also supported by complementary functional analysis, by studying a range of structurally guided PcoB mutants. We also employed PcoB as a model protein to develop a giant vesicle-based method with the purpose to investigate ion passage properties of proteins related to Cu homeostasis.

Considering the importance of Cu and the role of the metal as an antibacterial agent, CopB from *Acinetobacter baumannii* was selected for similar efforts as conducted for PcoB. This is relevant as *A. baumannii* frequently infects hospital patients, and its antimicrobial resistance is increasing. Yet it is not clear if CopB operates using similar molecular principles as PcoB. To this end, we managed to crystallize CopB, yielding crystals that diffracted to 6.5 Å.

Collectively, this work sheds further light on copper homeostasis pathways in different organisms, and paves the way for more in depth studies to provide insights valuable for basic and applied sciences.

Key words: Copper homeostasis, Copper transporter family (CTR), PcoB, CopB

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Everything in the universe is within you. Ask all from yourself.

Rumi

Dedicated to my grandparents

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List of publications

Paper I

Nayeri, N., Li, P., Górecki, K., Lindkvist-Petersson, K., & Gourdon, P. (2022). **Principles to recover copper-conducting CTR proteins for the purpose of structural and functional studies.** Protein Expression and Purification, 106213.

Paper II

Li, P.*, Nayeri, N.*, Górecki, K., Becares, E. R., Wang, K., Mahato, D. R., Andersson, M., Abeyrathna, S. S., Lindkvist-Petersoon, K., Meloni, G., Winkel, M. J., & Gourdon, P. (2022). **PcoB is a defense outer membrane protein that facilitates cellular uptake of copper.** Protein Science, 31(7), e4364.

Paper III

Górecki, K., Hansen, J. S., Li, P., Nayeri, N., Lindkvist-Petersson, K., & Gourdon, P. (2022). **Microfluidic-Derived Detection of Protein-Facilitated Copper Flux Across Lipid Membranes.** Analytical Chemistry, 94(34), 11831-11837.

Paper IV

Nayeri, N., Górecki, K., Lindkvist-Petersson, K., Gourdon, P, & Li, P.. **Isolation and crystallization of copper resistance protein B (CopB) from *Acinetobacter baumannii*.** Submitted.

* Authors contributed equally

Author's contribution to the papers

Paper I

I wrote the initial draft of the manuscript and generated all figures, including the data shown in the figures. I expressed and purified all the studied constructs and performed all assays in the paper. I contributed to the methodology, validation and formal analysis.

Paper II

I wrote the first draft of the manuscript. I expressed and purified the mutants. I performed the liposome assays. I participated in discussions and in evaluation of the data. I contributed to the validation, formal analysis, investigation and visualization.

Paper III

I expressed and purified the wild-type and mutant proteins. I participated in data evaluation, discussion and prepared purification figures.

Paper IV

I wrote the initial draft of the manuscript and made all the figures, and generated the data shown in the figures. I expressed, purified and crystallized the protein. I contributed to the methodology, validation, formal analysis, investigation and visualization.

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02/08/2023
Lund – Sweden

Popular science summary

Biological systems have evolved to have a tight control between the internal concentrations of vital elements as compared to the external environment as achieved by a permeable membrane. These elements are crucial to many substantial biological processes. Copper is such element evolved to be involved in various fundamental processes such as energy production, metabolism, and immune response within organelles and cells as the smallest biological compartments. Such a spectrum of function in important biological process makes this element interesting for wide range of applications ranging from for example therapeutic interventions to antibiotics in the farm industry.

Concentration of copper in cells is regulated by some proteins in the membranes of the cells which act like gates and let the copper in or out depending on the need within the cells. For example, in shortage of copper within cells, these copper gates let the copper in the cells to increase the internal concentration to meet the amount of copper required for the cells. On the other hand, high concentrations of copper within cells can be a significant survival threat, and hence these gates let the copper out to reduce excessive amounts within the cells. Interestingly, this significant role in regulation of copper concentration in cells has been exploited for various clinical and industrial interventions. For instance, some anti-cancer agents can be transported through these gates to the cells. In addition, these copper gates can be used as an antimicrobial agent in some bacteria which are harmful to healthcare and farms, and thus eliminate them through exposure to high concentration of copper. Such interventions are obviously the fruit of our understanding from different physical and biochemical properties of these gates resulted by decades of comprehensive research, providing the foundation for targeted manipulation depending on our needs. However, there are various challenges with research on these gate proteins, ranging from technical difficulties to understanding physical, and biochemical properties.

In regards to these challenges, we managed to generate and purify an important type of gate protein available in humans and fungi providing the basic grounds for future structural and functional studies with higher resolution and details. Additionally, we managed to provide a first-ever structural image of one of these protein gates in bacteria with high resolution using top-notch technology which gave us the opportunity to study the mechanisms of function of this particular protein with a neat method resembling its natural environment. Lastly, we managed to overproduce one of these protein gates from a human bacterial pathogen in an experimental model and purify it in the lab for the first time, paving the way for future structural and function analysis of it in more detail. In conclusion, the scientific outcome of the work described in this thesis provides a foundation for various types of research which can ultimately lead to clinical and industrial interventions depending on the need.

Abbreviations

Cu	Copper
CTR	Copper importer protein
Pco	Plasmid-borne copper regulation system
GUV	Giant unilamellar vesicles
CopB	Copper transporter B
3D	Three dimensional
Fe	Iron
Zn	Zinc
ROS	Reactive oxygen species
ATP	Adenosine triphosphate
Cue	Copper efflux
CaCTR	*Candida albicans* copper importer protein
hCTR	Human copper importer protein
SsCTR	Salmo salar copper importer protein
TEV	Tobacco Etch Virus
GFP	Green Fluorescent Protein
His	Histidine
CHS	Cholesteryl Hemisuccinate
RT	Room Temperature
DDM	n-dodecyl-β-D-maltoside
LDAO	Lauryldimethylamine-N-oxide
OG	n-Octyl-β-d-Glucopyranoside
SMA	Styrene maleic acid
SEC	Size exclusion chromatography
F-SEC	Fluorescent size exclusion chromatography
PCR	Polymerase chain reaction
IMAC	Immobilized metal affinity chromatography
LCP	Lipidic cubic phase
3D	hree dimensional
Cryo-EM	Cryo Electron Microscopy
Amp	Ampicillin
IPTG	Isopropyl β- d-1-thiogalactopyranoside
OPOE	octyl polyoxyethylene

Organisms

E. coli	Escherichia coli
S. cerevisiae	Saccharomyces cerevisiae
A. baumannii	Acinetobacter baumannii
C. albicans	Candida albicans

Units

Å	Angstrom
℃	Degrees Celsius
kDa	kilodalton
mM	millimolar
µM	micromolar

Introduction

Cell membrane and membrane proteins

Every living cell and various organelles are shielded by a biological membrane, which shapes them and isolates their inside from extracellular surroundings. Biological membranes consist of a lipid bilayer, typically formed by a double layer of phospholipids. Phospholipids are amphiphilic molecules establishing nearly impermeable barriers to ions and most polar molecules. In addition, membranes prevent exchange of essential nutrients and most solutes. These biological lipid membranes are also encompassing many proteins, classified into two large groups. The first group is known as peripheral membrane proteins, non-covalently bound and dissociable from the surface of the membrane. The second group is tightly integrated into the membrane, known as integral membrane proteins [1], Figure 1. These integral membrane proteins perform selective transmembrane passage of polar molecules and ions to maintain life and cellular homeostasis. This group of proteins is further subdivided into proteins that span the membranes as α-helices or as β-barrels. The former are found mostly in inner membranes, whereas, β-barrels predominantly are localized to the outer membranes of gram-negative bacteria and mitochondria [2].

Previous studies have indicated that in most organisms, 20-30 % of the genome are predicted to encode integral membrane proteins emphasizing their vital role in cell function [3], and yet only 1572 unique molecular structures are published and available [4] which in contrast to their soluble counterparts is highly limited, roughly corresponding to 3.5 % of all the structures deposited in Protein Data Bank [5]. The reason for the limited structural information is, essentially associated with low production levels, protein instability due to its hydrophobicity, and as a result in terms of structural biology, difficulties in generating diffraction-quality crystals and samples suitable for other complementary high-resolution methods.

Due to their accessibility to the cellular out- and insides, membrane proteins are involved in a wide variety of biological processes and function such as molecular/drug transporters signal transduction, energy transduction. Many known human diseases such as obesity, Alzheimer's disease, cancer, and many others are associated with membrane proteins misfolding or malfunctioning [6], and currently, membrane

proteins are the targets of more than 60 % of drugs in clinical use [7-10]. Knowledge of the 3D structure of membrane proteins, including details of how ligands and cofactors are bound, are essential for further studies and future drug discoveries. Despite their fascinating roles in cell function and their importance in therapeutic developments, relatively little is known regarding the structure-function of membrane proteins. Hence, structure-function studies on membrane proteins are crucial for providing valuable knowledge regarding biological processes, as well as for drug design. Structural biology will pave the way towards the understanding of how exactly solutes are transferred, their coordination, and involved residues in the transport, which can then lead to drug development. The main challenge in biophysical and biochemical studies is to maintain the membrane protein stability and function in a lipid-like environment, mimicking their physiological lipid environment.

Membrane proteins are also key players for cellular maintenance of heavy metal levels, the latter required for the function of more than one-third of all proteins. Heavy metal ions such as copper (Cu), zinc (Zn), and iron (Fe) are poorly soluble and highly reactive micronutrients. These trace elements are essential for the function of many enzymes and proteins and are involved in cell growth and proliferation. Nevertheless, an excessive amount of these metal ions could result in metal toxicity, leading to protein degradation, cell malfunctioning, or even cell death [11]. Therefore, all organisms developed mechanisms responsible for keeping the ions concentration at a certain level [12].

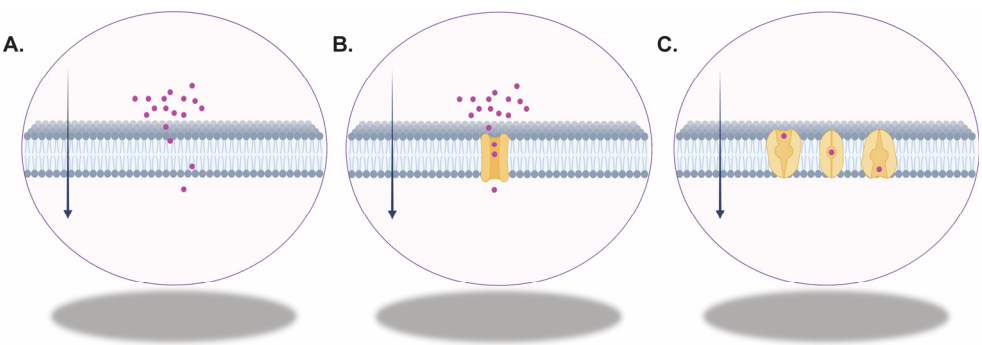

Figure 1. Molecular translocation over the cellular membrane bilayer. A. Passage of molecules over the membrane by passive diffusion. **B.** Flux of the molecules by channels. **C.** Transport of the molecules via transporters. The figure was generated using BioRender.

Copper ion and copper flux

Various types of metals play critical roles in essential regulatory processes in cellular systems. For instance, sodium (Na), calcium (Ca), and potassium (K) which are classified as oxidation/reduction (redox) inactive metals may trigger many signaling processes through fluctuations in their ion pools [13]. Conversely, other metals such as Cu and Fe, when necessary, are protected within active sites of proteins given the risk of reactive oxygen species (ROS) production in catalytic activities they are involved [13]. Cu, in turn, has become one of the most interesting metals for many studies concerning human health given the range of involvement in critical cellular processes.

One of the major processes Cu is involved in is energy production in mitochondria. There, Cu serves as a cofactor in different proteins associated with electron transfer chain. For instance, cytochrome c oxidase, as the final member of respiratory electron transfer chain, takes advantage of Cu to ultimately form a proton gradient across the membrane used as the source of adenosine triphosphate (ATP) production. Interestingly, some of the proteins that are involved in scavenging the produced ROS such as dismutases utilize Cu as a cofactor to minimize the ROS stress within the cells [14].

More recent findings on Cu during recent years has broaden our understanding of its biological role beyond metabolism. For example, direct interaction of Cu with kinases in regulation of signaling pathways has been reported in relation to cancer [15]. Additionally, an inhibitory role of Cu has also been shown in other biological processes such as lipolysis, a cellular process of degrading fat, where signaling activities are interrupted in presence of Cu [16]. Similarly, negative regulation of potassium channels in some immune cells mediated by Cu has been shown to have a suppressor effect on activation on such immune cells [17].

The wide range of Cu involvement in cellular process highlights this metal as a ground for many therapeutic interventions. Interestingly, higher Cu levels in inflamed and malignant tissues mediated by inflammatory cytokines such as interleukin 17 (IL-17) have been noted [13]. Such observations have paved the way for application of therapeutic agents such as Cu-chelators, as such agents have been shown to have antitumor effects in murine models [15].

Copper has a redox potential that can be useful for biochemical reactions [18]; this redox property assist copper to serve as a cofactor in many biological processes such as respiration, iron metabolism, the formation of connective tissue, free radical destruction, however, they can generate damaging free radicals [19, 20]. Therefore, understanding how copper homeostasis and regulation is handled in the cell is critical for both basic and translational sciences, and hence more insight into the molecular

level details is required. In the end, although the majority of studies in this context are focused on identification of biological roles of Cu, the question addressing the regulation of Cu in cells has been challenged less so far and this is the area this thesis is trying to contribute to.

Copper homeostasis

The double-edged nature of copper has resulted in development and evolution of regulatory mechanisms which provide a tight control on its concentration in prokaryotes and eukaryotes [21]. In fact, such mechanisms and pathways ensure contribution of copper to homeostasis and keep the organism from its toxicity which may be generated by uncontrolled accumulation of copper in cells. These regulatory systems are somewhat different among less advanced forms of life such as prokaryotes as compared to more advanced such as eukaryotes. However, in general, the optimal concentration of copper across all organisms is achieved through various regulatory mechanisms including extra/intracellular sequestration, enzymatic detoxification, and metal removal [21, 22].

Copper homeostasis in bacteria

Although some recent works on model bacterial strains have suggested copper importers in such organisms, uptake of copper is known for most of the bacteria to occur through passive diffusion across the cell membrane along its gradient [23]. In this context, electrostatic sequestration of extracellular copper mediated by polysaccharides appears to be one of the mechanisms hampering copper diffusion into the cells [24].

On the other hand, other aspects of Cu homeostasis is orchestrated by several sets of proteins. Among the multiple systems for copper handling in *Escherichia coli (E. coli)* two regulatory pathways are present, the Cus system and Cue system for Cu-resistance and Cu-efflux [25]. The Cue operon encodes two Cu-resistance proteins, CopA, a P1B-type ATPase pumping Cu (I) ions into the periplasm from the cytoplasm [26], and CueO, a periplasmic multicopper oxidase which oxidizes Cu (I) to less toxic Cu (II) [27]. Together, these two proteins act as the first response against Cu stress, keeping the cytoplasm free of unbound Cu ions [25, 26, 28-30].

Copper and antibiotics have been used as growth promoters in pig diets for at least 45 years [31]. Some *E. coli* strains possess additional plasmid-encoded genes. These genes were first isolated from a plasmid, pRJ1004, from the gut flora of pigs fed a high Cu diet [32]. The *pco* gene cluster (plasmid-borne copper resistance) has been

demonstrated to enable bacteria to survive in higher Cu concentrations, compared to the wild-type strain without the Pco system, which accumulates less Cu internally and exhibits higher Cu efflux [32-34]. Homologs of the Pco proteins are often present on chromosomes of other bacteria and were also shown to increase Cu tolerance. However, there is growing evidence for the Pco proteins to be also involved in Cu uptake [33, 35], which hint at a possible contradictory role.

The Pco system in *E. coli* consists of seven genes, pcoABCDRSE [36]. The PcoRS is a two-component regulatory system, analogous to CusRS, sensing the periplasmic Cu concentrations [36]. PcoE is a periplasmic protein able to bind multiple Cu ions, acting as a 'molecular sponge' and thus decreasing the free Cu concentration in the periplasm upon Cu shock [37]. PcoCD was suggested to act together and be responsible for Cu uptake since the expression of only those two proteins leads to Cu hypersensitivity [38]. Indeed, PcoCD is often present in genomes as one fusion protein: a Bacillus subtilis protein YcnJ is highly homologous to PcoCD, and deletion of this protein leads to impaired growth in media with limited Cu concentrations [33].

Similar to PcoCD often acting as one unit, PcoAB was suggested to work together and be the primary actors in pco-dependent Cu resistance [39]. PcoA is a multicopper oxidase, distantly related to CueO, which can also be functionally replaced [40]. PcoB is an outer membrane protein (OMP), known to serve as a Cu-specific porin in bacteria [36, 41, 42]. It was suggested to prevent Cu uptake in the outer membrane [43], however, since the inner membrane Cu-ATPase CopA is necessary for Pco-dependent Cu resistance, PcoB is generally believed to be a Cu-specific porin seizing Cu(II) from the extracellular side [43]. Homologs of PcoAB are often encoded together in genomes of other Gram-negative bacteria, and whereas PcoA is sometimes found alone, PcoB is always accompanied by PcoA, suggesting the interaction between the two and that PcoB requires PcoA for Cu-transport function [44]. For instance, expression of PcoB alone in the absence of PcoA in *Caulobacter crescentus* ΔpcoAB strain did not rescue the Cu-sensitive phenotype [45]. However, PcoC was also shown to be needed for full resistance, and to interact with PcoA, possibly serving as a periplasmic Cu-chaperone [40], Figure 3 A.

As the main mechanism of cytoplasmic detoxification of copper in many bacterial strains, ATPases, which export copper from cytoplasm to periplasm, appear to be another strategy in regulation of copper resistance. This strategy is likewise considered to be conserved among majority of Gram-negative bacteria as their genomes contain at least one Cu (I) -ATPase [21]. This notion is further supported by extra copies of such genes in genome of opportunistic and pathogenic bacteria dealing with copper-driven burdens [46].

Even though it remains unclear how the Pco proteins regulate Cu homeostasis in Gram negative bacteria, this renders them fascinating targets to study their structure

and their importance as antimicrobial targets. Remarkably, recent studies in *E. coli* demonstrated that when bacteria are exposed to copper surfaces it causes DNA degradation by inhibition of respiration, increasing the interest in use of copper as an antimicrobial in healthcare and agriculture [47-50]. Metals as antimicrobials are vital in healthcare and agriculture [49, 50], and copper containing surfaces in hospitals showed an efficient decrease in environmental contaminations [51, 52].

Regarding Gram negative bacteria and their copper regulation as an antimicrobial target, *Acinetobacter baumannii* (*A. baumannii*) is an opportunistic human pathogen [53], predominantly found in hospitals [49, 50]. Their antimicrobial resistance is increasing significantly, leading to restriction in treatments [54-56], and increasing the interest in new therapeutic drugs to treat these multidrug-resistant strains through their outer membrane copper conducting proteins.

In addition to the above mentioned systems for copper ion handling in bacteria, CopB was also introduced as an outer membrane protein dealing with periplasmic copper resistance [41]. Despite the fact that little is known on how exactly CopB protein in *A. baumannii* is mediated, homologous proteins have been genetically identified [47, 57].

Copper homeostasis in eukaryotes

Existence of cellular compartment in eukaryotes which require copper for their biological processes posed an additional evolutionary burden to such cells. Additionally, emergence of multicellular organisms which mandated allocation of copper to specific tissues heightened the evolutionary burdens, necessitating more strictly regulated mechanisms both at the intra and the intercellular level [58].

A considerable fraction of our today-knowledge on homeostasis of copper in eukaryotes comes from decades of study on eukaryotic model organisms such *Saccharomyces cerevisiae* (*S. cerevisiae*), where a group of channels and transporters are found to have vital contribution to copper homeostasis. In this context, copper-specific ATPases play a major role in copper homeostasis as in bacteria. However, unlike prokaryotes in which copper-ATPases are harbored at the plasma membrane, these proteins are often located to intracellular compartments either transporting cytosolic copper to the lumen of organelles in eukaryotic cells or transferring it copper-binding proteins for functional maturation [59, 60]. For instance, they have been reported to be present in Golgi network delivering copper to some copper-dependent oxidases in *S. cerevisiae* [61]. It has also been reported and established that copper-ATPases may relocate from Golgi apparatus to plasma membrane through plasma membrane-fusing vesicles and help as exporting pumps under high intracellular copper stress in mammalian cells [62-64].

Copper transporters (CTRs) which have at least six members among eukaryotes are family of proteins with major contribution to copper homeostasis [65]. These proteins which are not present in prokaryotes has provided adaptive features for eukaryotes. Current models describe that one of the members of the family, namely CTR1, imports Cu (I) ions into the cells [66]. Cu (I) ions are then captured by copper-binding chaperones and distributed among different organelles or various biological pathways where they are required [67]. For instance, Atox1 has been identified to transfer Cu (I) ions to copper-specific ATPases in Golgi apparatus as those mentioned above for downstream functional maturation processes [66].

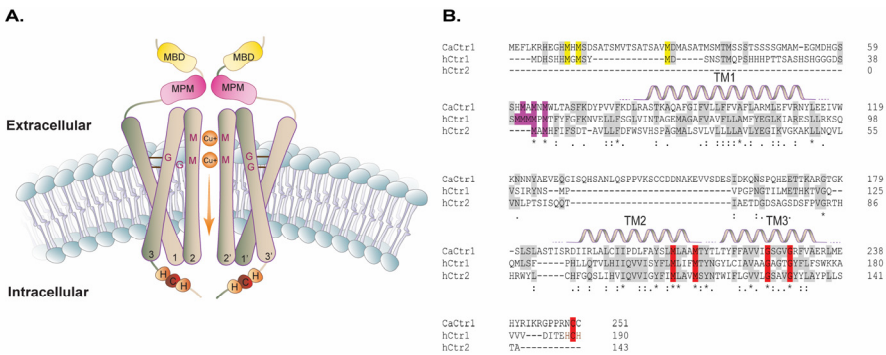

Figure 2. A. Schematic topology of human CTR1 (hCTR1) Paper I, showing only two of the monomers for clarity. The extracellular N-terminus contains several methionine-rich motifs colored in yellow and pink, presumably responsible for Cu (I) binding and uptake. The Cu (I) selectivity filter establishing methionines reside on TM2 adjacent to the extracellular membrane interface. Two Cu (I) ions, interacting with the selectivity filter, are shown as blue spheres. The structurally important GXXXG motif of TM3 is pinpointed. The C-terminus is located on the intracellular side of the cell. It contains a conserved HCH motif, another Cu (I) binding site, perhaps forming an intracellular gate and/or being responsible for Cu (I) passage to intracellular chaperons. The schematic topology of hCTR1 was generated using Adobe Illustrator. **B. Sequence alignment of the three targets.** Metal-binding residues at the N-terminus are highlighted in yellow and purple. Selectivity filter methionines of TM2 and the GXXXG motifs of TM3 are shown in dark red. The conserved HCH motif of the C-termini is pinpointed in red. Secondary structure elements are shown above the alignment, based on the determined structure of AsCTR1 [68]. Alignments were carried out using the multiple sequence alignment tool 'Clustal Omega' with default settings.

Ag (I) shares common characteristics to Cu (I) and showed inhibition of CTR1. However, divalent metal ions were ineffective to provide inhibition in CTR1, therefore, transport of reduced form of copper was suggested to be mediated by CTR1 [69]. Moreover, CTRs are ATP-independent for copper transport and are concentration driven, lacking ATP utilization domain; it was early demonstrated that CTR-mediated Cu flux is not eliminated by ATP synthesis inhibition [70], and the published structure of salmon CTR1 showed no ATP-binding features rather agreeing with features of channels [68].

Despite structural similarities, different CTR members may play a different role such as stability or affinity for copper [65]. For instance, *S. cerevisiae* has been reported to have two high-affinity CTRs and one low-affinity CTR which may be regulated differently according to survival challenges [71-73]. In this context, presence of two high-affinity CTRs in *S. cerevisiae* has been hypothesized as an evolutionary measure considering that more stable CTR3 appears to be a backup for fast-recycling CTR1, ensuring continuous uptake of copper in low-copper environments [65].

In spite of low sequence homology in the CTR family and variable length, they likely share a global overall architecture with common structural features, Figure 2 B. The monomer consists of three transmembrane helices (TM), and multiple metal-binding residues [74-76], Figure 2 A. A low-resolution Cryo-Electron Microscopy (Cryo-EM) structure of human CTR1 (hCTR1) and 3 Å X-ray structure of atlantic salmon (*Salmo salar*) CTR1 (SsCTR1) confirmed that it forms a homo-trimer in the membrane [68, 77]. These structures together delivered details of the architecture and function. There is a highly conserved selectivity filter on the extracellular side of TM2, formed by a MxxxM motif [68, 77], which is crucial for copper coordination [78]. There is a GxxxG (GG4) motif that resides on TMH3 responsible for TMs packing and oligomerization [75, 79]. Interestingly, there is another conserved motif (HCH) at the C-terminus [80]. It is suggested to form an intracellular gate the channel responsible for copper regulation and Cu (I) transition to cytosolic chaperones [68, 77, 81]. This motif is not found in all the CTR family members, implying diversity in function in CTRs, Figure 2 A. Regardless of the available structural information, due to the diversity of the CTR family in regulation and function, still, how exactly copper regulation and transport is achieved among CTR members remains to be elucidated [65]. hCTR1 is a high-affinity copper importer from which copper enters the cells and is localized in the plasma membrane [82, 83]. Human CTR2 (hCTR2) is a homolog of hCTR1 having a shorter N-terminal and lacking CHC motif at its C-terminal, low-affinity copper transporter, localized to endosomal membranes [84-86]. The exact role of hCTR2 is elusive, however, recent studies suggested that it could function in hCTR1 regulation in presence of excess copper, and releasing copper from intracellular copper stores [84, 87]. Another target of the study was Candida albicans CTR1 (CaCTR1), a homolog to hCTR1, with longer N-terminus. *C. albicans* is an opportunistic human pathogen, and CaCTR1 has been selected to be studied due to its possible importance as an antifungal drug target. Therefore, three members of the CTR family have been selected in this study, hCTR1, hCTR2, and CaCTR, to be produced and purified for further structural-functional studies, Figure 2 B.

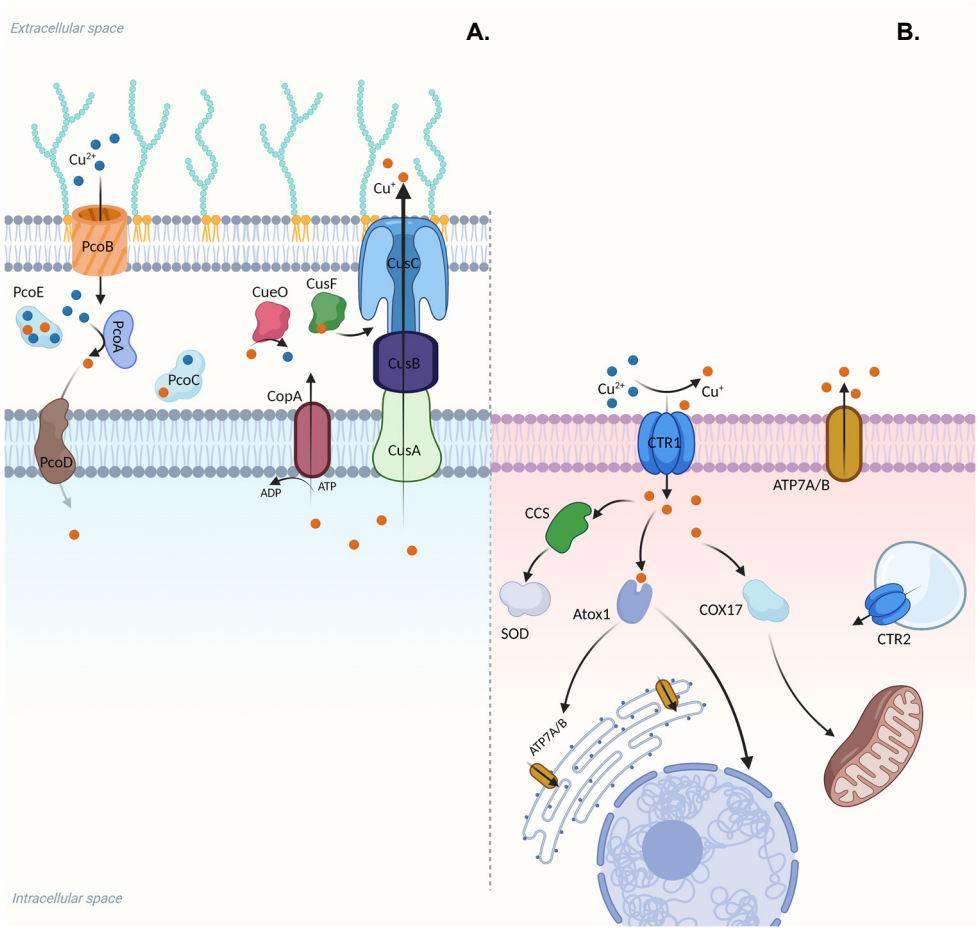

Figure 3. Copper homeostasis in gram negative bacteria and eukaryotes. A. ***E. coli*** **is equipped with multiple systems for copper homeostasis.** CopA is the chromosomally expressed central component for removal of excess Cu (I) from cytoplasm and CueO component is responsible for detoxification in periplasm. At high copper concentration CusABCF system appears to export copper directly from cytoplasm via CusABC and from periplasm through CusF. Another system identified in some bacteria is plasmid-borne copper resistance (pco) system ensuring survival in environments with elevated levels of copper. PcoB is responsible for removal of Cu (II) from extracellular. PcoA is the counterpart of multi-copper oxidase CueO. PcoC binds both Cu (I) and Cu (II) and functions with inner membrane protein PcoD responsible for import of Cu (I) to the cytoplasm. PcoE is located in periplasm and functions as molecular sponge to sequester excess copper in the periplasm. **B. Eukaryotic cell copper homeostasis.** Cu (II) is reduced to Cu (I) in extracellular and transferred to cytoplasm via high affinity copper transporter CTR1. Once in it is delivered to copper requiring proteins via chaperons. ATP 7A/B are P-type copper transporters transporting copper to the lumen of Golgi via receiving it from Atox1. When intracellular copper level is high, P-type ATPases export copper to the extracellular. Low affinity CTR2 is located at the intracellular and may function to release copper from lysosome. The figure was generated using BioRender.

PcoB & CopB - aim of the study

The Pco proteins are important for bacterial copper tolerance and hence highly attractive targets for therapeutic antimicrobial studies. Pco homologues are present in many bacteria, and are also often encoded chromosomally, suggesting a more widespread role in Cu homeostasis. Whereas the functions of the most Pco components have been established, PcoB remains the least studied of the Pco proteins. Therefore, this work, set out to elucidate the function of the outer membrane protein PcoB.

In 2017 World Health Organization (WHO) provided a list of antibiotic-resistant priority pathogens, in which Gram-negative bacteria represent some of the most troublesome organisms to human health, due to their therapeutic challenges and as an multi-drug resistance [88, 89]. Considering the importance of copper and its role as an antibacterial agent and that copper sulfate showed the best bactericidal effect, the interest in new therapeutic drugs makes the PcoB and CopB protein from *E. coli* and *A. baumannii* attractive targets for basic and applied studies. The aim of these studies was structural determination and structural-functional analysis required for poorly biochemically characterized PcoB and CopB to enhance our knowledge of these outer membrane proteins and how they operate in the cells. This represents a key aim for this thesis, and such work may eventually permit efforts to combat drug-resistance among Gram-negative bacteria.

CTRs - aim of the study

Since structural information is limited to a single structure of one specific CTR member and due to the diversity of the CTR family in regulation and function, it remains unresolved how copper regulation and transport to, across the membrane, and from CTRs is achieved among different members of the family [65]. The aim of this study was to isolate CTR targets for downstream structural and functional characterization efforts. This has historically represented a key obstacle in the field, by the lack of structures of the human and many other relevant CTR members, and by the fact that copper facilitated flux has never been demonstrated *in vitro* (using purified protein) for a single CTR member!

A comprehensive structural and functional understanding of CTR members together with processes regulating them under different copper stimuli may also open for therapeutic interventions given their significant role in copper homeostasis. This highlights the need for further in-depth studies of different CTR members. Such structure-function efforts all rely on availability to purified protein, which was the second key purpose of this thesis.

Methodology

Cloning and protein overproduction

In order to understand and study the nature and function of proteins for many aspects of human life, including pharmacology, food and biotechnology industry, tools such as recombinant DNA technologies and protein overproduction emerged [90, 91]. One major bottleneck in protein chemistry is obtaining the desired protein in terms of both quality and quantity for the downstream structural-functional studies. However, recombinant DNA technology provides a platform to overcome these problems.

In this thesis, genes encoding hCTR1, hCTR2 and CaCTR, as well as bacterial outer membrane PcoB and CopB were cloned and expressed. The gene of interests were codon optimized for their respective host cells and purchased. The genes were amplified by PCR and cloned into appropriate vectors for expression in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *E. coli*.

For expression of the CTR targets homologous recombination in *S. cerevisiae* was exploited. DNA for the gene of interest, a TEV-GFP-His_8 PCR fragment and the expression vector pEMBLyes4, double digested with *BamHI* and *HindIII*, were transformed to PAP1500 strain [92]. Transformant cells were grown on synthetic minimal media plate (SD) containing lysine, leucine and confirmed by sequencing.

The expression vector for the bacterial outer membrane targets (PcoB and CopB) was generated through cloning for a bacterial expression platform. Amplified genes were double digested with restriction sites and ligated into pET22b vector. After transformation of the vector harboring the gene of interest into the expression host C43 (DE3), cells were grown on Luria Broth (LB) media plate containing Ampicillin and confirmed by sequencing.

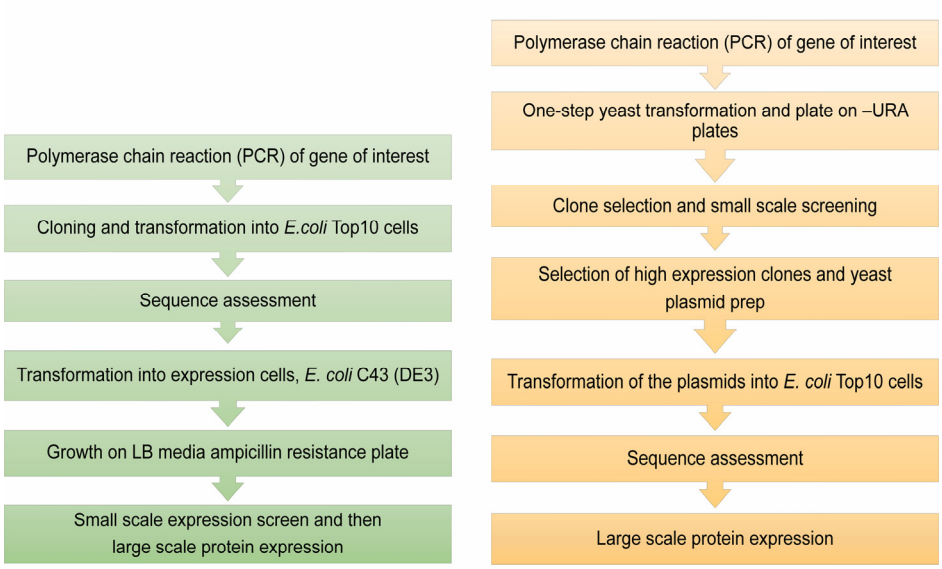

Figure 4. Cloning flow chart for *E. coli* (showing in green) and *S. cerevisiae* (yellow).

Recombinant protein expression in *Saccharomyces cerevisiae*

There are various expression systems for overproduction of membrane proteins including, mammalian cells, insect cells, cell-free expression system, *E. coli* and yeast [93-98]. Among all these platforms, bacterial and yeast systems are the favorite choice for many protein chemists as they provide high protein yields in a cost-effective manner [99]. However, eukaryotic soluble proteins often can be expressed in bacterial platforms. Lack of post-translational modification machinery, crucial for folding and function of eukaryotic membrane proteins, renders yeast-based platforms preferred for eukaryotic proteins [99, 100]. Although, there are several advanced host sources, such as insect and mammalian cell lines, plants, and different yeast organisms. Among these systems, yeast is considered a relatively simple and low-cost system. Moreover, between two commonly used yeast species, *Pichia pastoris* and *S. cerevisiae*, *S. cerevisiae* is well studied genetically and more strains are available to test. Hence, *S. cerevisiae* was selected as a production host for the CTRs, aiming at high protein quality and quantity for downstream applications.

Yeast expression platforms are single cell microorganisms that can be simple to handle in terms of genetic manipulation to perform homologues recombination, with low nutrition requirement and rapid growth. Yeast platforms are also known to perform many post-translational modifications [101, 102]. A novel yeast expression platform of

S. cerevisiae was developed in 2013 [97] and proved delivering high yield of many membrane proteins, including aquaporins, channelrhodopsin-2 (ChR2), human TRP channels and ion channels [97, 103-107]. The yeast strain, PAP1500, together with the expression plasmid pEMBLyex4, possesses Galactose 4 (Gal4) yeast transcription activator, known to be limiting for expression by galactose regulated promotors respectively [108]. Therefore, to overcome this a hybrid galactose inducible gene of GAL10-GAL4 was fused to the yeast strain, increasing the expression level of GAL4 protein used for induction of the CYC-GAL promotor (CG-P), by binding to the region UAS_{gal} , Figure 5 A [109, 110].

In addition to the above mentioned plasmid features, it stimulates cells to obtain an ultra-high plasmid copy number due to the presence of a poorly expressed *leu2-d* gene in combination with absence of leucine in the media [111]; prevention of plasmid-loss as a result of presence of *URA3* and *leu2-d* selection markers [97]. Moreover, green fluorescence protein (GFP) is fused C-terminally to the target gene for assessment of localization, folding marker, solubilization screening, quality and quantity of the target protein. Additionally, gene was tagged with eight histidines in order to facilitate the protein purification, Figure 5 B.

Full length transformed CTR targets were selected on SD media containing lysine and leucine. Single colony of the transformed cell was grown in 5 mL SD media supplemented with lysine and leucine at 30 °C for 18 hours. To ensure the high copy plasmid number over leucine starvation, cells were grown in 100 mL SD media supplemented with Lys at 30 °C for 30 hours. Next, cultivation was continued in 800 mL SD media supplemented with 0.5 % glucose, 3 % glycerol, 1x Lund dropout (-Iso/-Ura) at 30 °C for 18 H. The protein expression was induced later by lowering the temperature to 25 °C and adding 200 mL induction media (1 x YNB, 3 % glycerol, 1 x dropout, 2 % galactose). Protein production and localization was monitored by fluorescence microscopy and cells were typically harvested after 72 hours.

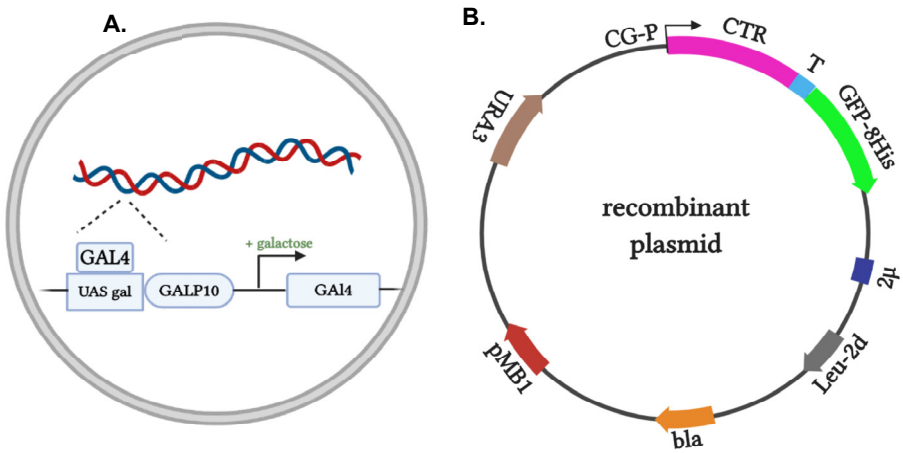

Figure 5. Overproduction platform for CTR targets in S. cerevisiae. A. The production strain PAP1500 overexpress the Gal4 transcriptional activator in the presence of galactose. Expression of CTRs is controlled by the same system and initiation of expression of Gal4 is resulting in expression of CTRs. B. Schematic view of pEMBLyex4 vector. The vector consists of a galactose inducible promotor (CGP), poorly expressed leu-2d gene and URA3 selection marker for the prevention of the plasmid loss, high plasmid copy number in media lacking leucine and GFP fusion for assessment of localization and solubilization. The figure was generated using BioRender and Adobe Illustrator.

Recombinant protein expression in *Escherichia coli* (*E. coli*)

Protein expression in bacteria is straightforward and hence *E. coli* is one the most frequently used and well-characterized expression platforms for production of recombinant proteins. *E. coli* expression is swift, cost-effective and robust and there are established protocols available for production of diverse range of protein targets and for labelling for structural studies [112, 113]. In this work one of the commonly used *E. coli* expression platforms was used for the overproduction of bacterial outer membranes. However, as each target is different, it requires optimization and different strategies to acquire successful large scale protein production.

The pET22b vector having strong bacteriophage T7 promoter, and *E. coli* C43 (DE3) strain, broadly used for membrane protein expression [114], were used for overproduction. Full length truncated and mutants of PcoB and CopB were selected on Luria Broth (LB) media containing Ampicillin (Amp) as a selection marker. A single colony of the transformants was grown in 5 mL LB media supplemented with Amp at 37 °C for 18 h. Next, 1 L LB media containing Amp was inoculated for 18 h and incubated first at 37 °C for around 3 - 4 h (until the optical density at absorbance 600 nm reached 0.5), subsequently the temperature was reduced to 25 °C and induction

was applied by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Finally, cells were harvested by centrifugation.

Membrane protein solubilization & Detergent screen

Cell disruption is an important step for downstream processes to release cellular material such as DNA, RNA and proteins by breaking the cell membrane. The method for cell lysis is influenced by the cell type and the target molecules of study. To break resilient plasma membrane and cell wall of bacteria typically mechanical methods like sonication (high-power ultrasound at low frequencies) or presses (high-pressure) with high-pressure are employed. For yeast cells with more robust cell wall, mechanical methods such as high-pressure homogenizers and glass beads have been shown efficient, particularly after multiple applications.

Whilst the biological membrane is ruptured, to study the desired individual membrane protein it needs to be isolated in their native and stable form from the membrane. In order to study structural and functional characterization of integral membrane proteins, the step is traditionally achieved by detergents to maintain the proteins in a water-soluble state. Detergents are amphipathic molecules consisting of a polar hydrophilic head and a non-polar hydrophobic tail [115]. Due to the nature of these amphiphilic molecules by increasing the detergent concentration, hydrophobic tails disrupt the lipidic membrane and extract integral membrane proteins, forming water-soluble detergent-lipid-protein complexes that can be used downstream structural studies. However, the approach of using detergents is challenging; it is a time consuming and requires a costly screening approach to identify the right detergent type, maintaining the protein structure and function. It can disrupt interactions within protein itself resulting in aggregation [116]. Additionally, importance of lipid has been reported in the function/crystallization of some membrane proteins, e.g., transporters and GPCRs [117-120]. Furthermore, lipid removal by detergents may cause the loss of lateral membrane pressure having an impact on protein function [121].

To combat above mentioned issues regarding the application of detergents, new approaches capable of membrane protein stabilization and extraction were developed; e.g., styrene maleic acid (SMA) polymer [122], amphipols [123], nanodiscs [124] and others. However, while these new methods are attractive complements to detergents for extraction of membrane protein extraction and purification and enhancing the protein stability, detergent still represent the first choice for many structure-function studies of membrane proteins.

As variety of commercially different detergents are available. Therefore, screening is required for making the right choice for solubilization and purification of the desired membrane protein. For membrane proteins N-dodecyl-β-D-maltoside (DDM) is the most used detergent for purification and crystallization [125, 126]. Another used detergents is lauryldimethylamine-N-oxide (LDAO) and its application has grown as the first membrane protein structure was solubilized and solved in this detergent [127]. Octyl-β-D-glucoside (OG) is another choice considering the fact that it forms small protein-detergent complex suitable for crystallization [126]. A review on top ten detergents used for membrane protein purification and crystallization has been reported previously and detergents used in this study were selected based on those studies [128].

Importantly, the selected detergent efficacy in solubilization is assessed by extraction efficiency on the target protein and not necessarily stabilizes the protein, therefore further screening and optimization is required. Moreover, as observed for many other membrane proteins, addition of cholesteryl hemisuccinate (CHS) throughout the solubilization and purification can considerably increase the solubilization efficiency [129]. Consequently, CHS has been included for screening and solubilization procedure in this work.

Fluorescence detection size exclusion chromatography

In order to assess the solubilization screening, the gene of interest can be fused to GFP and initial detergent screening solubilization efficiency can be verified through measurement of the total GFP signal to the remaining GFP signal after removal of insolubilized material. Upon successful solubilization and identification of suitable detergents, it is necessary to examine the monodispersity in the selected detergent and condition of the protein of interest, indicative of the stability for structural characterization of the target [130]. Size exclusion chromatography (SEC) is one of the most well-founded methods to screen for this parameter. Typically, well-behaved, stable and monodisperse protein sample yields a single symmetrical peak after SEC, whereas unstable and polydisperse sample elutes as multiple, asymmetric or aggregated peaks.

Additionally, the GFP-tag allows for quantification of the target membrane protein and localization by live cell imaging, and it functions as a folding reporter [131]. Combination of SEC and such a fluorescent tag permits fluorescence-detection size-exclusion chromatography (F-SEC), which allows for small scale SEC analysis using minute amounts of sample [132].

Protein purification

Following expression and solubilization, other proteins important for cellular function are also expressed and solubilized. Therefore, it is crucial to isolate the protein of interest from impurities and the most common approach is protein purification through chromatography. Proteins are having different characteristic features and can be separated from each other based on their size and shape, solubility, charge and binding affinity [133]. The most commonly purification techniques are column chromatography such as, affinity chromatography and SEC.

Affinity chromatography is the most effective and efficient technique among all and immobilized metal affinity chromatography (IMAC), is an one of the most employed affinity chromatography method. In this method proteins are typically tagged with to 6-8 histidine residues (His-tag) with highly specific interaction mechanism to divalent metal ions, such as cobalt and nickel in the resin. This method is frequently performed as an initial step of the protein purification. SEC is a fundamental tool in protein biochemistry and separates molecules based on their size. Additionally it can be used for buffer exchange, on column refolding and detection of changes in protein structure molecules including non-homogeneity and aggregation [134]. SEC often serves as a polishing step and to indicate if the quality of the protein sample suitable for further experiments and downstream structural-functional studies.

In this thesis proteins were initially purified using pre-packed nickel columns. Afterward, GFP-His-tag fusion were removed via TEV protease treatment. Non-pure samples and un-cleaved proteins were further purified by a powerful purification strategy, reverse Ni-affinity purification. Subsequently, to acquire a homogenous protein sample and to remove aggregations and contaminations, samples were subjected to SEC purification and analysis with SDS-PAGE and Coomassie staining for further structural-functional investigations.

Crystallization

Unravelling the three-dimensional (3D) structure of membrane proteins is a key factor for further structural-functional studies and for downstream drug discoveries. Currently, X-ray crystallography, nuclear magnetic resonance (NMR) and cryo-electron microscopy (cryo-EM), are three main methods being used for 3D structural determination. NMR is commonly used to study targets with molecular weight below 50 kDa and it requires labelling of the protein [135]. On the other hand, cryo-EM is another powerful tool to determine the 3D structure of proteins in their near native

condition which has become more widely applicable and increased the pace of membrane protein structural determination [136]. Cryo-EM is capable of resolving structures of large protein complexes that were difficult to crystallize and it came with the detection threshold of 100 kDa and in the last few years its size limit was enhanced to around 50 kDa [137-139]. Despite of all the advantageous and disadvantages of NMR and cryo-EM techniques, X-ray crystallography remains applicable and powerful to study a broad range of 3D structure of the proteins specially membrane proteins.

X-ray crystallography remains one of leading technique for structural determination and to generate atomic models of proteins [140]. The process of crystallization for obtainment of well-diffracting crystals to yield high resolution structures is highly variable and time consuming, and this notion is further underscored for membrane proteins. The major bottlenecks for membrane protein crystallography are associated with; a) poor overexpression levels making it difficult in generating milligrams of pure protein and with high monodispersity, b) extraction and solubilization difficulty, c) instability and loss of function, and d) generation of well-diffracting crystals [141, 142].

The intension is formation of 3D membrane protein crystals and two basic types of membrane protein crystals can be formed, type I and type II [141]. In crystal packing type I, mainly produced during crystallization *in meso* (lipidic cubic phase (LCP)), ordered 2D crystals are stacked where lipid-protein and protein-protein interactions are assembled. On the other hand, crystal packing type II is formed from detergent solubilized samples (*in surfo*) using vapor diffusion or batch. These crystals are produced by polar protein-protein interactions which protrude from the detergent micelle [1, 141, 143].

Once membrane proteins are purified and solubilized in detergents, crystals are obtained with standard crystallization techniques as their soluble counterparts and vapor diffusion by sitting/hanging drop is most frequently applied [1, 144]. Vapor diffusion method is based on evaporation of the water or any volatile component. The protein solution is mixed with low concentration of precipitant and placed above the solution with higher concentration of precipitant and crystal formation is achieved as water diffuses from the droplet, increasing the concentration of the protein yielding in protein crystals [145], Figure 6 A. Well-ordered crystals formed by vapor diffusion are typically type II crystals and very fragile due to the presence of detergent micelles and their large solvent content [145].

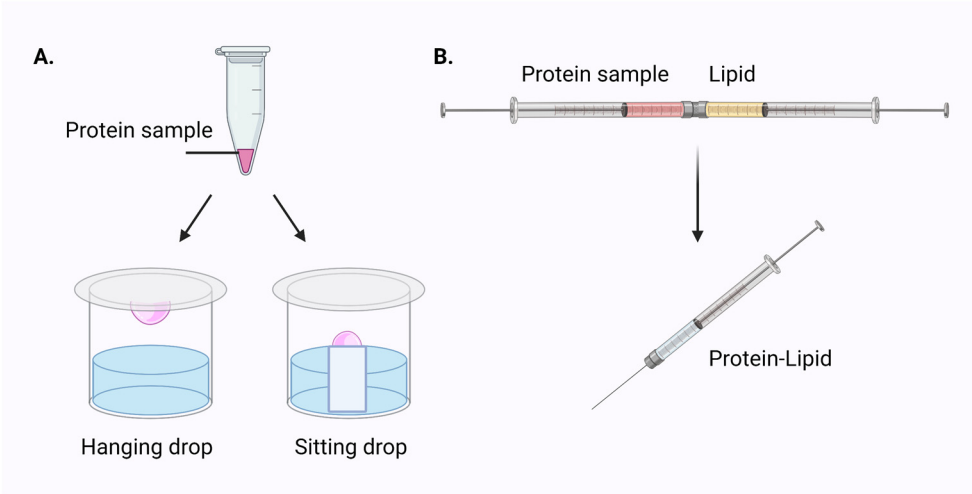

Figure 6. Schematic representation of vapor diffusion crystallization and LCP crystallization. A. Detergent solubilized protein sample is mixed with crystallization buffer and crystallization drops are set either as hanging drop or sitting drop. **B.** Detergent solubilized protein sample is mixed with lipid using two Hamilton syringes and a coupler and afterward protein-lipid mixture drop is set overlayed with buffer on a glass sandwich LCP plates. The figure was generated using BioRender.

To overcome the obstacles associated with detergent solubilized membrane proteins crystallization another method providing more native-like membrane environment was specially developed and introduced in 1996, LCP or *in meso* crystallization [146] and many GPCR structures have been solved using this technique [147]. In this method detergent solubilized membrane protein is mixed at a certain lipid-protein ratio and temperature to form regular bi-continuous bilayers and as the protein concentrates crystal growth happens [1, 145], Figure 6 B. Crystals formed in LCP are type I 3D crystals, small but relatively well-diffracting [148].

Unfortunately, there is not a universal platform for crystallizing all membrane proteins, and each membrane protein has unique characteristics and a wide screening must be performed for each target.

In this study, targets were subjected to both *in surfo* and *in meso* crystallization. N-terminally truncated PcoB crystals were obtained in 0.4 % C_8E_4 detergent by hanging drop vapor diffusion, which diffracted to 2 Å. In addition, full length detergent purified CopB target was subjected to LCP and *in situ* hanging drop vapor diffusion in deterrent detergents. Samples solubilized in 0.5 % OPOE detergent resulted in small crystals by hanging drop vapor diffusion, which diffracted to 3.5 Å.

Functional characterization

Beside structural determination of membrane proteins, study at the molecular level and how exactly these macromolecules are contributing to cellular homeostasis is both of great interest and hampered due to the complexity of the biological membrane. One of the most effective way of studying membrane proteins' function is to keep them in a membrane-like environment to retain their structure and function. Thus, focusing on membrane proteins' reconstitution into artificial lipid environment serves as an essential tool to analyze the function, and structure-based models of mechanisms.

A variety of membrane mimics have been developed and incorporation of detergent purified membrane proteins into lipid vesicles has allowed for detailed functional studies [149]. The most studied membrane model systems are Small spherical liposomes (0.1 – 0.2 μm) and giant unilamellar vesicles (GUV) (10 – 100 μm) that have been widely used recently to study the function of integral membrane proteins [150, 151]. Liposomes are multilayer lipid bilayers and relatively small, whereas GUVs are observed using optical microscopy and their size correlates to that of biological cells (1 – 100 μm) [152].

In this work, PcoB was evaluated functionally in liposomes and GUVs. Traditionally, Cu (II) flux assays are measured using fluorophores such as Fluozin-1 or -3, which are being activated by addition of Zn (II) and quenched by addition of Cu (II). Consequently, detergent purified PcoB forms were reconstituted in vesicles with trapped fluorophore dye. Subsequently, the flux of copper across the liposomes was assessed through addition of buffer containing Cu^{2+} that quenched the dye inside the liposomes, Figure 7.

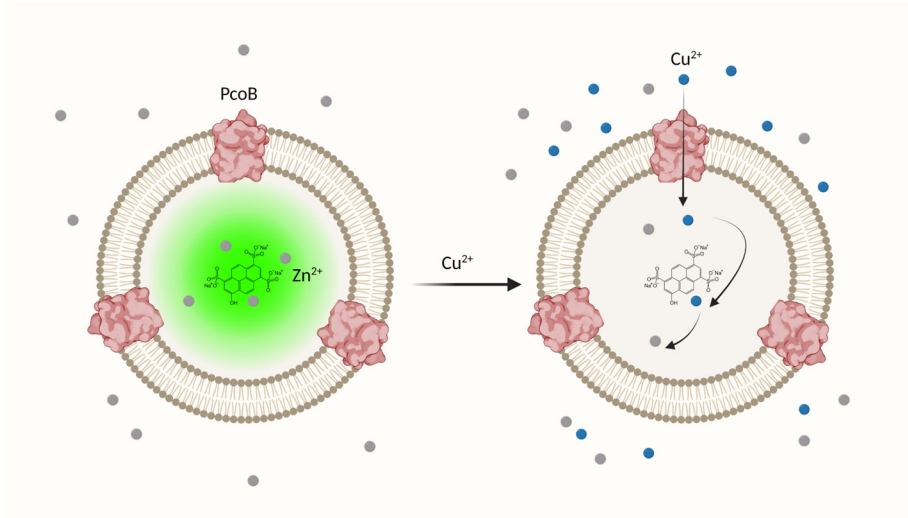

Figure 7. Schematic view of PcoB reconstitution into liposomes. Liposomes are formed with fluorophore dye trapped inside. PcoB is reconstituted into liposomes and through addition of Cu^{2+} containing buffer, quenching of the dye is measured as a result of Cu^{2+} import via the PcoB channel. The figure was generated using BioRender.

Results and discussion

Paper I

The first part of the program cantered on the overproduction of the eukaryotic CTR family. The objective was successfully established using a *S. cerevisiae* platform with yield, homogeneity, and purity appropriate for downstream structural efforts.

CTRs were first identified and characterized in a study on iron deficiency of *S. cerevisiae* [153]. CTR1 is present in all eukaryotic organisms [82]. CTR1 is known as the only high-affinity copper uptake in eukaryotes, localized to the plasma membrane [154]. CTRs are not directly linked to human diseases, nevertheless, embryonic lethality of CTR1 knockout in mice indicated that CTR1 is an important factor for human health [155-157]. Moreover, CTR1 is also an important factor for the uptake of Cisplatin or cis-diamminedichloridoplatinum (II) (cis-Pt(NH3)2Cl2), a platinum-based anticancer drug, across the cellular membrane [158-160].

In this study, a low-cost, and efficient *S. cerevisiae* overproduction platform was employed for CTR targets. The approach of the study was production, localization test, solubilization screen, and protein purification of challenging CTR targets. Sequences were codon-optimized for *S. cerevisiae* and constructed in high copy number plasmid fused to TEV protease, four glycine, and one serine (G4S) linker, GFP-8His tag, to facilitate localization, quality control, and purification of CTR channels. Imaging of live yeast cells expressing CTR targets fused to GFP fluorescence visualized the protein expression and accumulation in the plasma membrane and intracellular membranes. To identify proper detergents and conditions to extract the proteins from crude membrane, a detergent screen was performed utilizing six different detergents with and without CHS, 4°C, and room temperature (RT) for solubilization. The results presented in Figure 8 A, indicate effective solubilization in n-dodecyl-β-D-maltoside (DDM) – 4 °C, lauryldimethylamine Oxide (LDAO)-RT, DDM: CHS-RT, for hCTR1, hCTR2, and CaCTR respectively. Next, the monodispersity of the solubilized samples was examined using F-SEC as previously described [161], Figure 8 B.

Even though, with the plenty of data in the F-SEC analysis the intention with the F-SEC analysis was not to in detail assess all conditions, but rather find indications of conditions that may be suitable for the large-scale efforts and accordingly, the above mentioned conditioned were selected for each target.

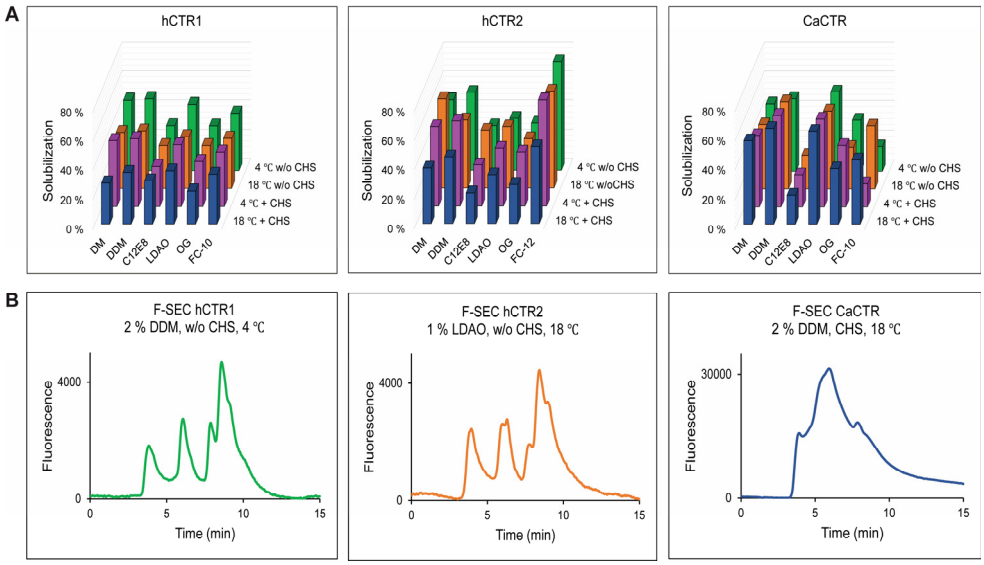

Figure 8. Detergent screen of hCTR1, hCTR2, and caCTR. A. The membrane of the cells was solubilized for 2 hours with six different detergents at 4 degrees and RT and estimated by GFP fluorescence. **B.** FSEC analysis of the solubilized samples. Effective conditions among all for each target, are indicated.

Subsequently, samples were subjected to affinity-based protein purification. The G4S-GFP-8His tag was cleaved off followed by reverse affinity chromatography to remove the protease and the tag. Then, samples were evaluated using SEC, and the quality of the samples was analyzed by SDS-PAGE, Figure 9.

The multimeric state of membrane proteins can be difficult to elucidate, and may even vary in a reversible manner along the purification procedure. In general, the SEC profiles revealed peaks with shoulders, perhaps representing the oligomerization state of the proteins, congruent with the trimers and tail-to-tail dimer-of-trimers previously detected for CTRs [162, 163]. CTRs are known to display monomers, dimers, trimers and even dimer of trimers [77, 164]. In order to separate trimers from dimer of trimers 1 mM DTT was supplemented to the purification buffer and in Figure 9 panel B, I and II represent dimer of trimers and trimers, respectively. This is not the case in Figure 8 were only solubilization of the targets were assessed. Finally, the investigated samples are also rather different in respect to sample purity. Based on these earlier studies on a CTR target later successfully employed for structural studies, we expect the main fractions indicated with dotted lines in Figure 9, to represent trimers.

Lastly, conventional yield and purity for downstream structural-functional analysis were obtained. The yields are compatible with *in vitro* liposome studies, however assessment of Cu (I) flux or transport is notoriously difficult and challenging. Conversely, the quality of the samples is suitable for downstream cryo-EM studies.

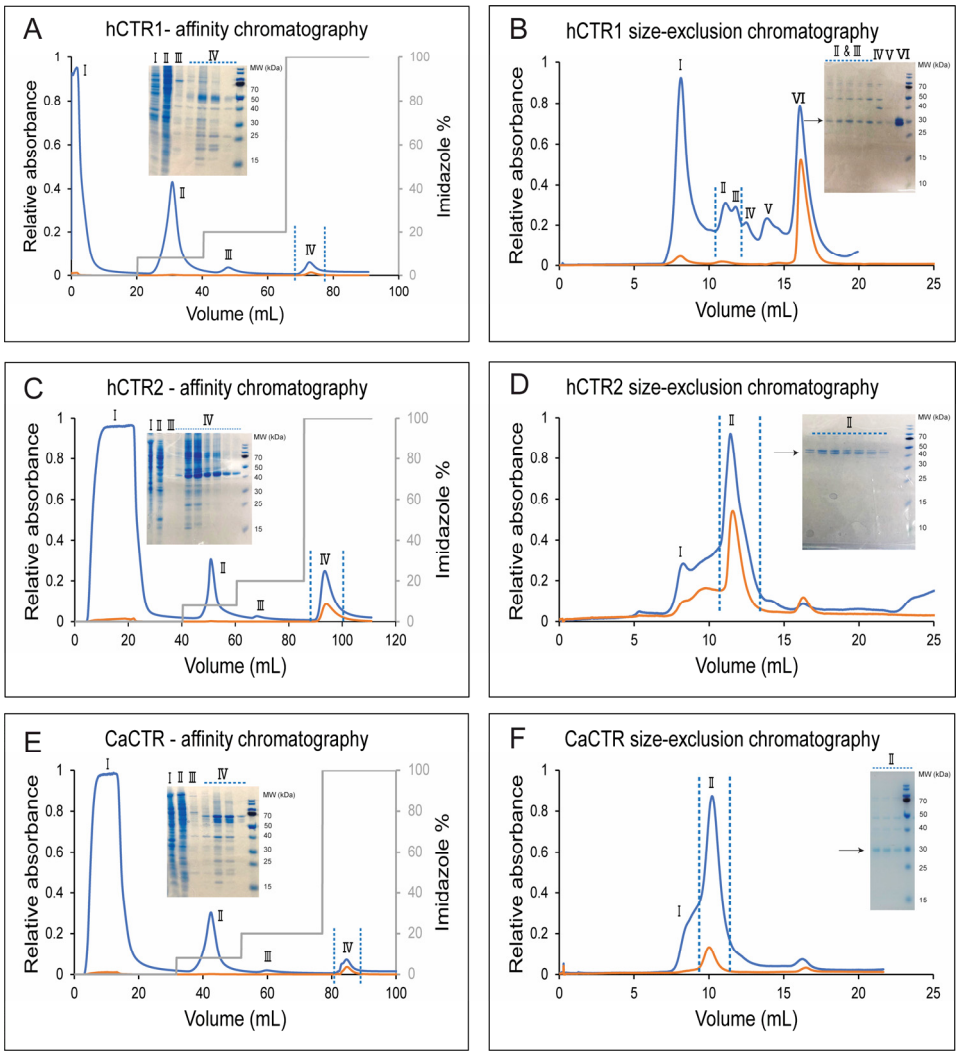

Figure 9. Purification of the studied CTR proteins. Blue and orange profiles indicate the relative absorbance at 280 nm and 500 nm (the latter is a signal for GFP). **A, C, and E.** Affinity protein purification, performed using immobilized metal ion affinity chromatography (IMAC). IMAC profiles for the eluted proteins and corresponding percentage of imidazole concentrations used for elution (in gray). The peaks are labeled on the corresponding chromatograms and SD-PAGEs with Roman numbers. The desired protein is shown with dotted blue lines on the chromatogram and SDS-PAGEs. **B, D and F.** Size-exclusion chromatography (SEC) profiles of the three targets in their selected solubilization condition and detergents. Coomassie-stained SDS-PAGE of the indicated monomeric peaks (shown using dotted lines in the chromatograms and SDS-PAGEs) of the SEC purification indicating efficient yield and quality of the samples. Different oligomerization states are shown with Roman numbers on the chromatograms and their corresponding SDS-PAGEs.

Papers II & III

The second intention of the PhD project was the structural determination of PcoB, with complementary functional analysis. The main focus here was to shed light on the overall architecture and to demonstrate how the ion flux is established and pinpointing structurally and functionally significant residues in the pore. The PcoB gene was synthesised and codon optimized. The N-terminal His tag followed by a TEV protease cleavage site were introduced into the PcoB gene after signal peptide to facilitate the protein purification. The protein was overproduced and extracted from *E. coli* outer membrane with a two step extraction. Afterward, however, the full-length purified PcoB yielded poor diffraction crystals and therefore the N-termini truncation PcoB $_{\Delta 27\text{-}81}$ was cloned and purification resulted in reasonable yields and homogeneity after SEC purification for outer membrane protein expression.

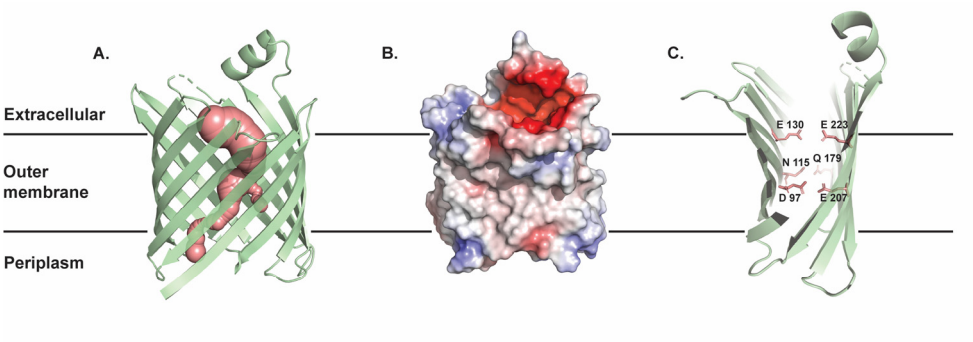

Figure 10. Structure of PcoB. A. Structure of PcoB showing a β-barrel architecture and a pore through the channel (salmon). **B.** Electrostatic surface in accordance with extracellular copper uptake (negative surface on the outside). **C.** Side view of the structure and proposed Cu selectivity filter (and gate) shown in stick representation. The figures were generated using PyMol and Adobe Illustrator.

Three-dimensional crystals of purified PcoB protein were produced in different detergents but the crystals diffracted to limited resolution. After wide-ranging screening and optimization, structure of PcoB $_{\Delta 27\text{-}81}$ protein in C8E4 detergent in Cu (II)-free state at 2 Å resolution was determined. Initial attempts to solve the PcoB structure by molecular replacement using different β-barrel structures failed. In order to solve the phase problem, mutations were generated for selenomethionine (SeMet) labelling protein expression. The SeMet-based single wavelength anomalous diffraction data set was collected SeMet-based single-wavelength (0.9798 Å) anomalous diffraction (SAD) experiment was performed to solve the phase problem and the structure was achieved using X-ray crystallography.

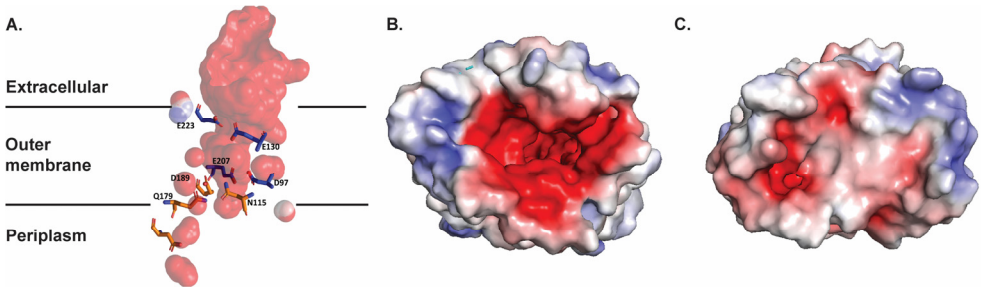

Figure 11. The surface charge distribution of the protein. The charge distribution in the surface map is displayed in red and blue. Negatively and positively charged electrostatic surfaces are shown in red and blue respectively. **A.** Highly negatively charged pore (in red) and structurally important residues along the pore. **B.** Extracellular side of the channel and **C.** Periplasmic side of the channel. Figures are generated using PyMol and Adobe Illustrator.

We revealed PcoB is an outer membrane porin consisting of a 12-stranded antiparallel β-barrel, having N- and C-termini located in the periplasm, Figure 10 A. Strikingly, the pore is highly electronegative, suggesting that electrostatics might play a key role in the Cu passage, Figure 10 B & 11 B & C. The structure revealed that two highly conserved pairs of negatively charged residues, E130-E223 and D97-E207, are forming possible gates of the protein in the pore Figure 10 C & 11 A.

To investigate the functionality of PcoB we were additionally able to demonstrate Cu flux across the membrane through reconstitution of PcoB into Giant unilamellar vesicles (GUVs) and liposomes. This approach facilitates Cu homeostasis investigations and overcomes the established difficulties with assessing flux of Cu across biological membranes. Evaluation of Cu-flux is a difficult matter, due to rapid delivery of heavy-metals, bursting proteoliposomes, and toxic metal effects. Nevertheless, we developed two separate protein copper flux assays, one using protein reconstituted in GUVs, Figure 12 C & D, and one with protein in liposomes, Figure 11 A & B. The experiment was designed in a way that, a fluorescence membrane-impermeable dye was trapped in the reconstituted lipids. Hence, it indicated that PcoB facilitates Cu (II) flux and in order to identify functionally and/or structurally important residues along the pore, open and close mutations were subjected to a proteoliposome assay. A wide range of screening on structurally important residues was applied and several point mutations were grown and purified. After extensive screening from growth to purification and as well as successful reconstitution, D97-E207 was mutated to K97-E207 to form a salt bridge and hinder the Cu flux as a close mutation. Additionally, a double mutation of E187A-Q179A was applied at the periplasmic side of the protein, which was illustrated to be open *in silico*. The data demonstrated that a double mutated protein at the gate

region showed an entirely open channel, whereas a single mutation showed an entirely closed channel, not allowing the passage of the ions, Figure 12 A & B.

Concurrently, we were able to reconstitute the protein in GUVs, based on the prior knowledge of other membrane protein reconstitution in artificial vesicles. We managed to obtain high quality vesicles in 5-20 µm size. Incorporation of the protein into the vesicles was assessed by labeled PcoB to Pacific Blue NHS ester and showed effective incorporation. The principle here was akin that of the liposome assay that, a fluorescence membrane-impermeable dye was trapped in the GUVs and Cu (II) flux was measured by the quenching of the dye-Zn complex over addition of Cu containing buffer whereases in control samples, GUVs with no protein, a slight decrease of the FL was observed. We employed the E252A mutant as non-conducting control for GUVs measurements, Figure 12 C & D.

Nevertheless, as the conclusion in regards of the details in flux and Cu delivery in GUVs was not satisfactory, microfluidic delivery of Cu was established. This method is characteristically used in microbiological assays, however we managed to apply the technique for Cu flux measurement of an ion channel protein. The method requires low volume of the sample less than 50 µl and enables the reliable measurement of different Cu concentrations. It is beneficial for Cu flux studies as the Cu containing buffer is pumped slowly via a syringe, thereby reducing Cu precipitation issues and uneven Cu concentration, and providing smooth Cu delivery resulting in accuracy of the measurement. Taken together, our results propose a neat and feasible method for study membrane proteins link to Cu (II) handling.

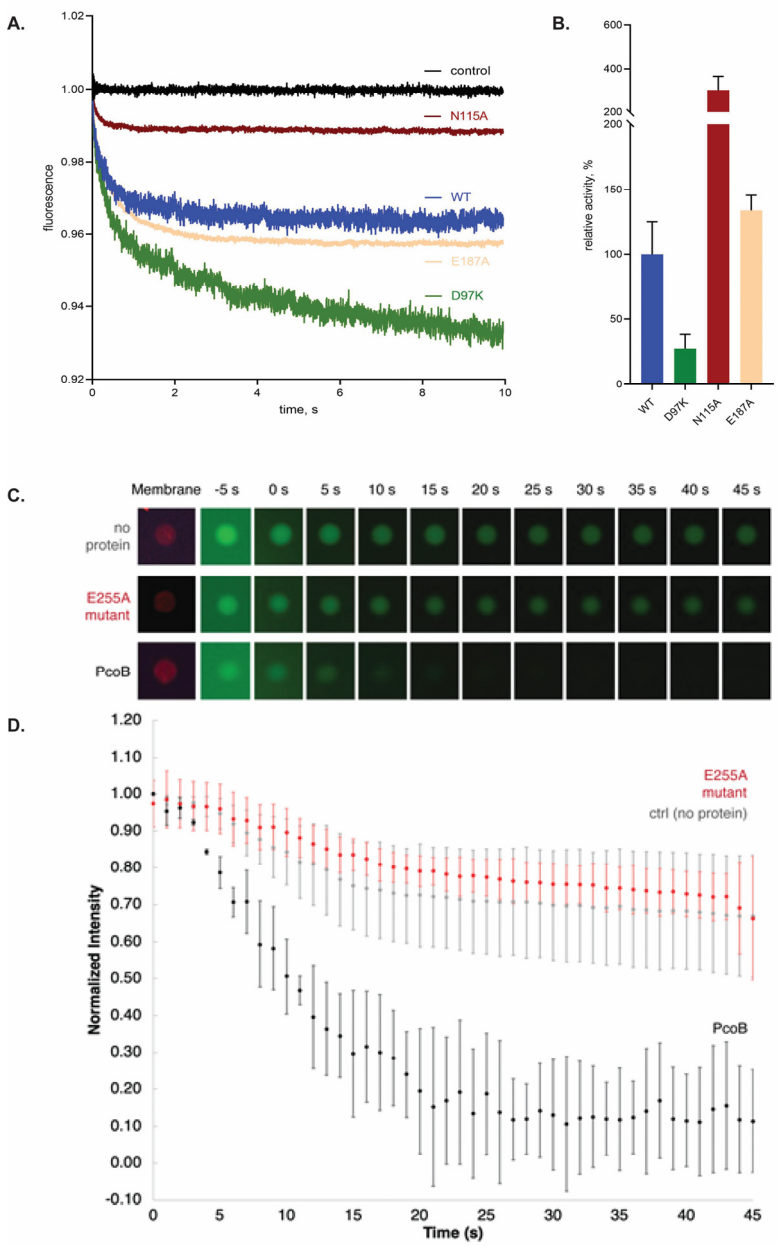

Figure 12. Copper flux assays using proteoliposomes and GUVs. A. Proteoliposomes containing PcoB (WT), open and close mutants. Reconstituted protein facilitates passive Cu flux and exhibited Cu-channel function. **B.** Bar diagrams of the relative activity of the investigated PcoB mutants with wild type PcoB-containing. **C.** GUVs showing quench of the fluorescence dye due to flux of copper ions into the GUVs. **D.** PcoB-containing GUVs (black circles), PcoB mutant E255A containing GUVs (red circles), and control vesicles (gray) are shown.

Later, a growth study of the cells harboring wild-type PcoB in media containing high Cu concentration showed sensitivity towards Cu ions in comparison to its control (vector without PcoB). Subsequently, to indicate the defense mechanism of the desired protein, the entire Pco system conferring all Pco genes, was studied *in vivo*. This indicated a change in the morphology of the cells, suggesting the sequestration of excess copper in the periplasmic part of the cells, Figure 13.

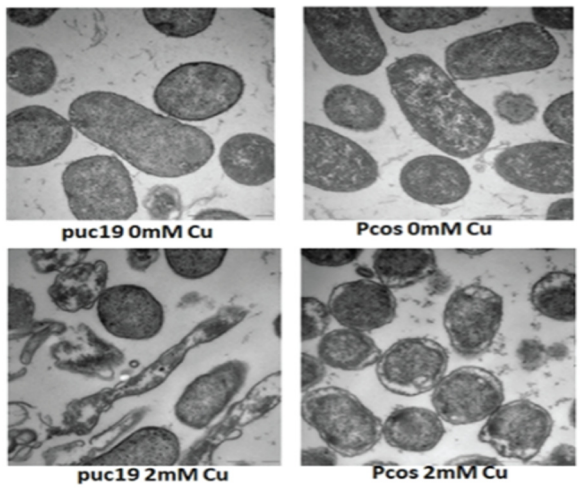

Figure 13. Electron microscopy of the cells harboring the entire Pco system at high copper concentration. Showing copper sequestration in the periplasmic region of the Pco genes expressed cells.

All in all, our data indicated that PcoB is an outer membrane protein facilitating Cu (II) flux and involved in bacterial Cu tolerance. The Pco system was suggested to be a defence mechanism and PcoB was suggested to be an efflux membrane channel. However, considering the fact that the structure was solved in its closed state and molecular electrostatics surfaces showed that the channel is narrowed at the periplasmic side, and less negatively charged, suggesting PcoB can attract and import copper ions using electrostatic forces. Conversely, there is a notion that Pco system works as periplasmic Cu defense mechanism [26] and our results were in line with that notion in a sense that cells harboring *pco* operon showed somehow copper tolerance, Figure 13.

Paper IV

Moreover, the third objective was related to a protein with homology (72 % similarity) to PcoB, the outer membrane protein CopB from *A. baumannii*, which has been suggested to serve as a copper pore [165]. This is relevant considering the importance of copper and its possible role as an antibacterial target. There is no structural and functional information available on CopB, therefore, the focus was on production and crystallization to eventually characterize the structure and function.

The gene was ordered and cloned into a pET22-b plasmid using the *NdeI* and *XhoI* restriction sites and with the PcoB signal peptide followed by 6x Histidine and a TEV cleavage site at its N-termini. The plasmid was transformed into the *E. coli* C43 (DE3) expression strain using the heat shock transformation method. Following cultivation, cells were harvested, disrupted, and the protein was extracted from the crude membrane and solubilized in 2 % Elugent. Next, affinity-based purification in 0.04 % LDAO, followed by TEV cleavage, and reverse affinity chromatography was applied. Subsequently, to increase the likelihood of generating crystals, detergent exchange and screening were applied to the sample. The protein displayed satisfactory monodispersity in five different detergents on small scale (0.1 mg protein), Figure 14.

The well behaved and monodisperse samples were obtained in OPOE, NG, C8E4, Cymal-6 and LDAO, whereas the other detergents appeared to have higher amount of aggregation. Consequently, OPOE, C8E4, Cymal-6 and LDAO were reproduced in large-scale (1L) purifications protein.

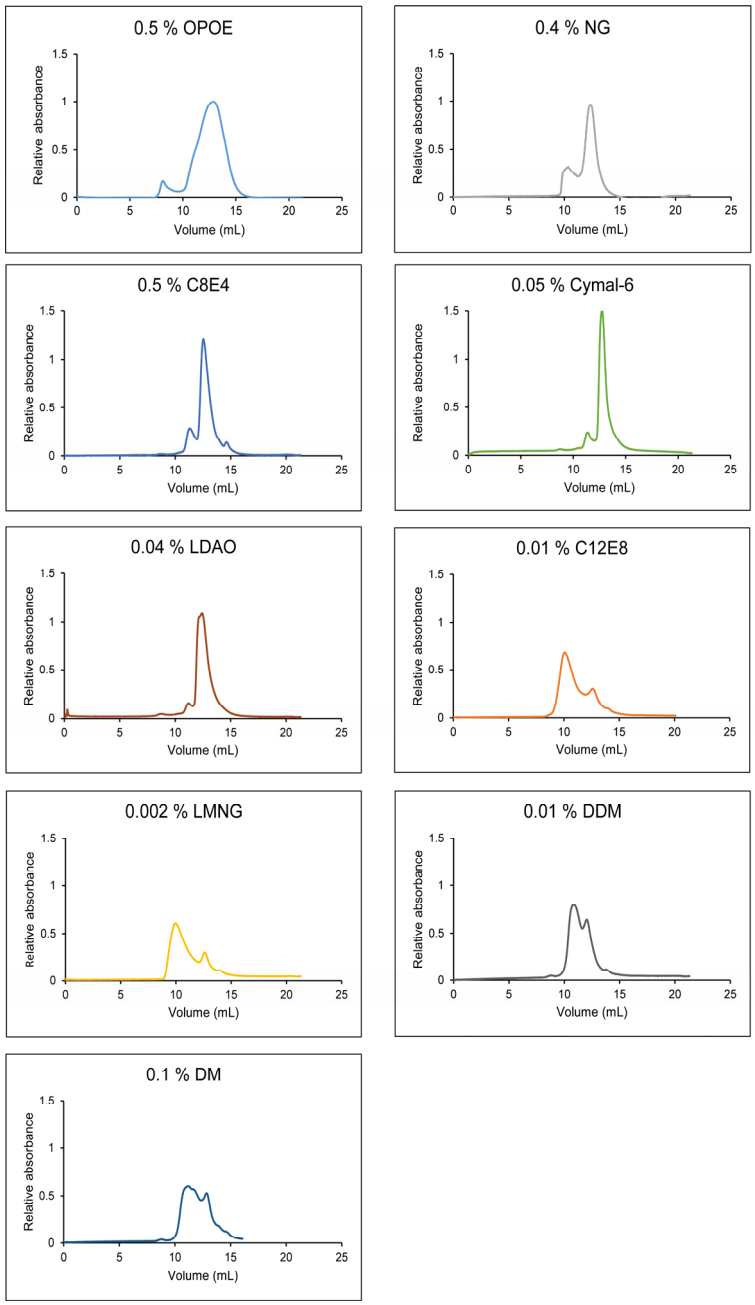

Figure 14. Size-exclusion detergent screen chromatograms of CopB in nine detergents. Each graph shows a detergent and its solubilization condition. All graphs are set to the same scale. The Y-axis shows the relative absorbance 280 and the X-axes the volume (mL).

Well behaved protein samples in OPOE, Cymal-6, LDAO and C8E4 were subjected to crystallization using different commercially available membrane protein crystallization kits, using vapor diffusion and LCP methods. Particularly, OPOE, LDAO and C8E4 are commonly used for outer membrane protein crystallization [166]. After an extensive amount of crystallization in different detergents and conditions, initial crystals were obtained in 0.5% OPOE detergent, 0.35 M NaCl, 0.1 M Tricine pH=8.5, 28% w/v PEG1000, which diffracted to 6.5 Å, Figure 15 B & C. Further optimization of the conditions was performed using additive screens. Well-formed crystals, still small, were obtained in the same condition containing 0.1 M manganese (II) chloride tetrahydrate.

The purified samples were of high purity congruent with downstream functional studies, such as the liposome assay developed for PcoB. This will be critical considering that the Cu flux of CopB protein is poorly characterized. Moreover, further optimization of crystals will be required for structural determination.

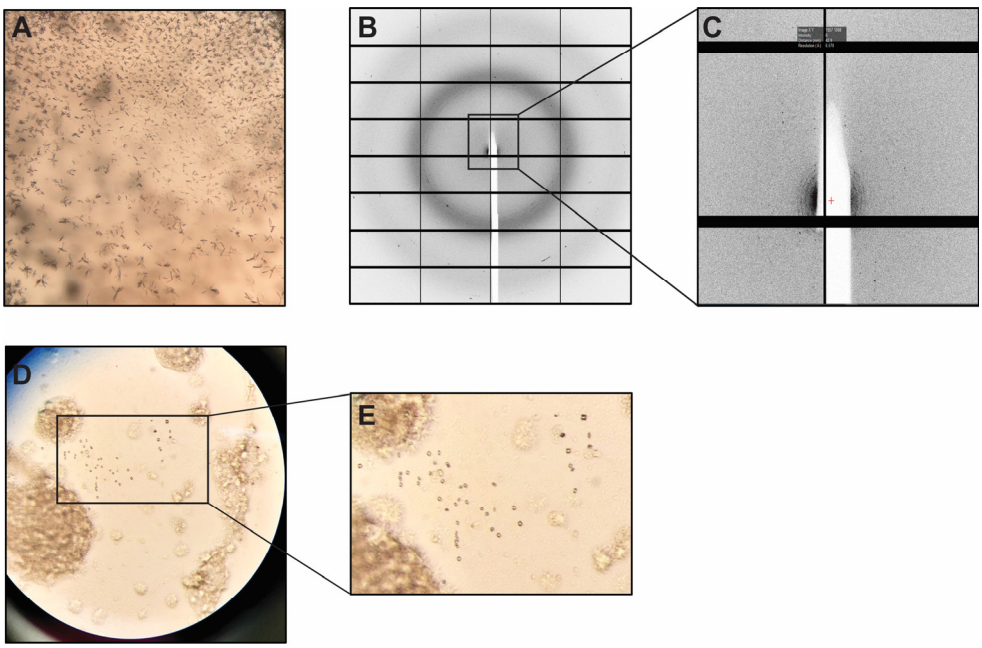

Figure 15. A. Initial crystals of CopB in 0.05% OPOE, obtained in hanging drop, MemPlus screen. **B and C.** Diffraction pattern of initial crystals which was diffracted to 6.5 Å. **D and E.** Optimized crystals in additives.

Concluding remarks and outlook

The work in this thesis provides a valuable foundation for downstream efforts on copper conducting proteins in the membranes of bacteria, yeast and human, and supports the use of structural and functional characterization methods to study copper transport mechanisms.

We revealed principles to recover and purify eukaryotic Cu importers (CTRs), opening up for downstream structural determination efforts on human and pathogenic targets. The core issue relates to the molecular mechanism underlying the copper flux and how the conductance is regulated which is largely unknown today.

It has been suggested that the C-termini of CTR1 blocks the copper import in response to excess copper through conformational changes. Moreover, the extracellular histidine- and methionine-rich N-termini is essential for function and form metal-binding domains, which also have been proposed to control the function through gating [167]. However, the structural basis for these mechanisms, if at all present, remain to be elucidated. Additionally, another unsettled topic to be studied is how copper donating and accepting proteins associate with CTRs. It has been suggested that at the C-termini there is a conserved motif, HCH, serve as an intracellular gate and involved in copper passage [68, 167]. This motif is missing in hCTR2, nonetheless, this is one of many aspects of the basic function of CTRs that remain poorly understood. A detailed understanding of the copper delivery to and from CTRs necessitates high-resolution structures of full-length versions of these proteins with and without interacting copper donors/acceptors.

The latter point requires functional assessment of the purified targets, however, it is notoriously difficult to assess Cu flux or transport across cellular membranes *in vitro*, and even more for the reduced form, Cu (I) [168]. In fact, still no functional assessments of CTR mediated Cu (I) conductance has been demonstrated *in vitro*, despite a low-resolution structure of hCTR1 and a high-resolution structure of SsCTR.

Highlighting the significance of our findings, the behavior of our CTRs is the same or even better than the few CTR targets that have been structurally characterized [68, 77]. Interestingly, a single high-resolution structure is available from *Salmo salar*, but no high-resolution structure is present for the human members. Therefore, X-ray crystallography or cryo-EM high-resolution data of the studied CTRs in this thesis in

the absence or presence of copper and in complex with interacting proteins will further increase the understanding of their physiological function. The recent finding that CTRs are linked to human diseases further highlights the importance of this particular protein family [169] and successful overproduction and purification of CTRs presented in this this work makes a contribution to this end.

Additionally, in this work we successfully crystallized the Cu-specific outer membrane porin PcoB, and determined its structure to 2 Å resolution. Moreover, despite the established complications in the assessment of Cu flux across biological membranes, we managed to detect and quantify PcoB-facilitated Cu passage using GUVs and liposomes. However, while our findings illustrated structurally important residues responsible for shuttling Cu through the protein, further studies on the directionality of the copper flux of PcoB are required. It is also poorly understood why full resistance and Cu-transport of the Pco system may depend on the presence of two other components PcoC and PcoA along with PcoB [40, 44]. Possible methods to study these protein-protein interactions are for example pull-down assays, MST or Far Western Blot. Collectively, high-resolution structures of full length PcoB with interacting proteins, open structure of PcoB, and open/close mutants will shed further light on function and regulation of the Pco system. These studies could potentially be done with cryo-EM or X-ray crystallography.

Similar to other β-barrel channels the function and mechanism of the PcoB is still under debate and it remains its physiological and basic function remain debated. Is PcoB an antiporter? What is the directionality of the PcoB function? More importantly, how a Cu channel, historically known to be involved in defence mechanism through exporting activity, is hypothesized by our data to have importing activity remains as an interesting question for future structural and functional studies.

Moreover, in this study another outer membrane protein CopB from *A. baumannii*, a homolog to PcoB, was successfully produced, and resulted in initial crystals that diffracted to 6.5 Å resolution. Sequence alignment of CopB to PcoB shows that important residues differ structurally and comparison of PcoB to CopB Alpha fold suggests differences in the architecture within the pore. Unveiling the first high-resolution structure of CopB, is thus a key to the fundamental research questions related to how copper homeostasis in *A. baumannii* is conducted through the protein; how Cu regulation is achieved and in what way copper is accepted and donated to the two sides of the outer membrane. The strategy to address these questions will center on X-ray crystallography and biochemical characterization of this protein. In this regard, optimization of the CopB crystals (presented in this work) by changing protein concentration, seeding or temperature of the set ups have the potential to contribute to this shortage. To shed further light on the functional characteristic of this target, liposome studies and investigation of Cu flux could be of interest, however only low

CMC detergents are compatible with liposome assay, and hence not all the studied detergents in this work are suitable for such applications.

In conclusion, our findings on the studied proteins in this work significantly increase our understanding of the structural and functional basics of Cu homeostasis proteins and provides a foundation for further structure-function studies. The obtained knowledge may thus open new avenues for more in-depth studies of the investigated proteins as a contribution to the ultimate drug discovery goal.

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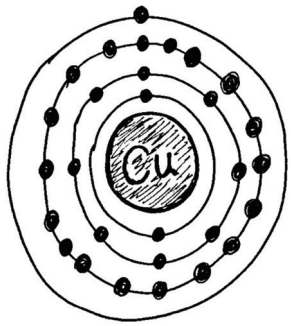

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