THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

COPPER BINDING PROTEINS IN BREAST CANCER: CELLULAR AND MOLECULAR MECHANISMS

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Cover:
[The potential pathways of Cu-binding proteins Atox1 and Memo1 involved in breast cancer cells. **I**: Memo1 binds Cu(I) and protects against Cu-induced ROS generation; **II**: Memo1 might interact with Atox1 via Cu(I) in breast cancer cells; **III**: Atox1 regulates breast cancer cell migration via the Atox1-ATP7A-LOX axis. Structures of Atox1 (PDB: 1TL4), Memo1 (PDB: 3BD0), CTR1 (PDB: 6M97), LOX (LOXL2: PDB: 5ZE3), and ATP7A (ATP7A model [1])]

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ABSTRACT

Cancer is one of the leading causes of human death. However, the mechanisms governing cancer are still not fully understood. Elevated levels of copper (Cu) have been observed in both cancerous tissues and the serum of cancer patients. Cu ions play a crucial role in supporting cancer by activating Cu-dependent enzymes, which promote cancer cell proliferation, angiogenesis, and metastasis. Cu ions can undergo redox reactions, switching between the +1 and +2 oxidation states. Free Cu ions can therefore catalyze the production of reactive oxygen species (ROS), which may damage biomolecules. To avoid such reactions, Cu ions are controlled and transported by dedicated Cu transport proteins in cells.

This thesis focuses on two Cu-binding proteins, antioxidant 1 Cu chaperone (Atox1) and mediator of cell motility 1 (Memo1). Previous studies have shown that Atox1 is upregulated in breast cancer cells and patients. The results of Paper I indicate that Atox1 knockdown significantly reduces the velocity and directionality of breast cancer cell migration. Data in Paper I also show close proximity between Atox1 and the Cu-dependent enzyme lysyl oxidase (LOX) in cells. These results imply that Cu transport in the Atox1-ATP7A-LOX axis is crucial for cancer cell migration. I also investigated the role of an Atox1 homolog, CUC-1, in *Caenorhabditis elegans (C. elegans)* cell migration during development (Paper II). CUC-1 knock-out worms have more developmental defects, implying that Atox1-like proteins contribute to cell migration during development.

Memo1, connected to many oncogenic signaling pathways, was recently suggested to bind oxidized Cu ions and promote ROS generation. In contrast, my work shows that Memo1 binds the reduced form of Cu and protects against Cu-induced ROS generation *in vitro* and in breast cancer cells (Paper III and IV). Memo1 knockdown and external Cu addition significantly decrease cell viability and increase ROS levels in breast cancer cells. This Memo1 functionality may serve as a protective mechanism, allowing cancer cells to handle the increased demand of Cu ions in cancer-related processes.

In conclusion, my research provides important new discoveries for the Cu-binding proteins Atox1 and Memo1 that may be relevant for future cancer treatments.

Keywords: breast cancer, cell migration, Atox1, Memo1, ROS, copper

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Single-cell tracking demonstrates copper chaperone Atox1 to be required for breast cancer cell migration**
Blockhuys Stéphanie, Xiaolu Zhang, and Pernilla Wittung-Stafshede,
Proc.Natl.Acad.Sci.U.S.A. 117, no. 4 (2020): 2014-2019. Doi:10.1073/pnas.1910722117

II. **The *Caenorhabditis elegans* homolog of human copper chaperone Atox1, CUC-1, aids in distal tip cell migration**
Xiaolu Zhang, Stéphanie Blockhuys, Ranjan Devkota, Marc Pilon, and Pernilla Wittung-Stafshede. *Biometals* 33, no. 2 (2020): 147-157. Doi: 10.1007/s10534-020-00239-z

III. **Memo1 binds reduced copper ions, interacts with copper chaperone Atox1, and protects against copper-mediated redox activity *in vitro***
Xiaolu Zhang, Gulshan R. Walke, Istvan Horvath, Ranjeet Kumar, Stéphanie Blockhuys, Stellan Holgersson, Paul H. Walton, and Pernilla Wittung-Stafshede.
*Proc.Natl.Acad.Sci.U.S.A.*119, no. 37 (2022): e2206905119. Doi:10.1073/pnas.220690511

IV. **Memo1 reduces copper-mediated reactive oxygen species in breast cancer cells.**
Xiaolu Zhang, Pernilla Wittung-Stafshede. *Submitted 2023*.

ADDITIONAL PUBLICATION (NOT INCLUDED IN THE THESIS)

I. **Correlation between cellular uptake and cytotoxicity of fragmented α-synuclein amyloid fibrils suggests intracellular basis for toxicity**
Xiaolu Zhang, Emelie Wesén, Ranjeet Kumar, David Bernson, Audrey Gallud, Alexandra Paul, Pernilla Wittung-Stafshede, and Elin K. Esbjörner. ACS Chemical Neuroscience 11, no. 3 (2020): 233-241. Doi: 10.1021/acschemneuro.9b00562

CONTRIBUTION REPORT

I. I performed experiments (PLA and western blot) and contributed to the data analysis.

II. I conceived the idea, planned and performed the experiments, and contributed to the data analysis and paper writing.

III. My co-authors and I conceived the idea, planned and performed the experiments (except surface plasmon resonance experiments), and I contributed to the data analysis and paper writing.

IV. I conceived the idea, planned and performed experiments, contributed to the data analysis, and wrote the initial draft.

PREFACE

This thesis was submitted for partial fulfillment of the degree of Doctor of Philosophy. The original work presented in this dissertation was carried out between February 2019 and May 2023 at the Department of Life Sciences (named Department of Biology and Biological Engineering until January 2023) at Chalmers University of Technology, under the supervision of Professor Pernilla Wittung-Stafshede. The research was funded by the Knut and Alice Wallenberg Foundation, the Swedish Cancer Society (Cancerfonden), and the Swedish Research Council.

Xiaolu Zhang

April 2023

ABBREVIATIONS

Atox1	antioxidant 1 copper chaperone
ATP7A	copper-transporting P-type ATPase A
ATP7B	copper-transporting P-type ATPase B
Cu	copper
CP	ceruloplasmin
CCO	cytochrome c oxidase
CCS	superoxide dismutase
CD	Circular dichroism
CTR1	Cu transporter protein
C. elegans	*Caenorhabditis elegans*
ERBB2	receptor tyrosine-protein kinase erbB-2
ER	estrogen-receptor
E. coli	*Escherichia coli*
FGFR	fibroblast growth factor receptor
GSH	glutathione
HRG	histidine-rich glycoprotein
ICAP	inductively coupled argon plasma
IGF	insulin-like growth factor
IPTG	isopropylthio-β-galactosidase
LOX	lysyl oxidase
LOXL2	lysyl oxidase-like-2
LOXPP	LOX propeptide
Memo1	mediator of cell motility 1
NOX1	NADPH oxidase 1
NTA	nitrilo acetic acid
MT	metallothioneins
PLCγ	phosphoinositide phospholipase C γ
PLA	proximity ligation assay
ROS	reactive oxygen species
RhoA	proteins ras homolog family member A
SEC	size exclusion chromatography
SOD	superoxide dismutase

SHC	Src homology/ collagen
SPR	surface plasmon resonance
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
STEAP	six-transmembrane epithelial antigen of prostate
TGN	trans-Golgi network
UV/Vis	ultraviolet and visible
VEGF	vascular endothelial growth factor

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1. Introduction

Cancer is a complex and multifaceted disease that affects millions of people worldwide. In 2020, it was responsible for around 10 million deaths globally, making it a primary cause of mortality worldwide [2]. The importance of cancer research cannot be overstated. It is key to developing effective prevention, diagnostic, and treatment strategies. Scientific investigations in the filed have also led to the development of various therapies which have significantly improved patient outcomes. However, more work is needed to explore cancer mechanisms.

One promising area of cancer research is the role of the Cu ion, an essential nutrient that cancer cells need in excess compared to normal cells. Several recent studies have suggested that Cu-binding proteins may play a role in cancer development and progression. By understanding how Cu-binding proteins contribute to cancer progression, researchers may identify new ways to target these proteins specifically and counteract cancer disease.

In addition to the potential therapeutic applications, studying Cu-binding proteins in cell biology may also improve our understanding of the underlying mechanisms of disease. For example, cancer is a heterogeneous disease arising from genetic and environmental factors, making it challenging to develop effective therapies for all patients. Identifying specific pathways and mechanisms involved in Cu homeostasis in cancer cells would shed light on how these cells function differently from healthy ones. Cu transport pathways in human cells are tightly regulated to maintain Cu homeostasis in the body. My research focuses on two Cu binding proteins: (1) Antioxidant 1 Cu chaperone (Atox1) and (2) Mediator of cell motility 1 protein (Memo1). Research in this area may eventually lead to new therapeutic targets for cancer treatment and improve our understanding of the underlying mechanisms of the disease. Therefore, continued research on Cu-binding proteins in cancer is important.

2. Metals in Biology

Living organisms require around 25 elements to survive; among those elements, at least 10 are metals [3]. Additionally, the overall content of metal in organisms is relatively low. Humans require ten essential metal ions for normal body function, including sodium, potassium, calcium, magnesium, manganese, iron, cobalt, Cu, zinc, and molybdenum [4]. Sodium, potassium, calcium, and magnesium are "bulk elements" that comprise most body metal ions. The remaining six metal ions are known as "trace elements." "Non-metal constant elements" are carbon, hydrogen, oxygen, and nitrogen. Non-metal elements are used as building blocks for macromolecules of life due to their stable covalent bonds and diverse binding modes, and they are a major component of living organisms. In contrast, metal elements exist in complex with organic molecules in living organisms. Like non-metal elements, metal elements also play an irreplaceable role in the efficient structural and functional integrity of biomolecules.

Metals ions are classified into two broad categories based on their roles in cells. On one hand, calcium, potassium, sodium, and zinc are redox-inactive metals [4]. On the other hand, Cu and iron are redox-active transition metals mainly characterized as static cofactors essential for catalysis. Moreover, research on labile Cu pools indicates that Cu can modify enzyme activity and protein function by reversible binding and reacting with small molecules [5]. Metals are crucial for normal growth and development. Imbalances in metal homeostasis have been related to diseased conditions.

Cu is a crucial transition metal ion (with about 80mg in a 80kg person), playing vital roles in several biological processes such as respiration, angiogenesis, and neuromodulation [3]. However, despite its importance, Cu-binding proteins constitute about 1% of the total proteome in eukaryotes (up to ~30,000) and prokaryotes (up to ~10,000) [6]. Cu(II) sites in proteins can be sorted into three types based on their functions: type I Cu or blue Cu proteins, which play a role in single electron transfer, such as amicyanin. For example, the protein plastocyanin has a typical type 1 Cu center, one Cu(II) ion coordinated with two histidines, one cysteine, and one methionine ligands [7]. Cu-dependent enzymes usually have type II Cu centers serving catalytic sites. Such as Cu/Zn SOD from yeast and four histidine imidazole nitrogen atoms coordinated with Cu(II) ion [8]. Type III Cu sites are dinuclear and participate in oxygen activation and transport [9]. Ceruloplasmin (CP) has all three types of Cu centers. The type III Cu center of CP is one Cu(II) ion coordinated with three histidine residues [10]. The Cu chaperones (with Cu(I) binding sites) belong to a distinct protein category that guarantees

secure and accurate transportation of possibly harmful Cu(I) ions to various indispensable Cu proteins [11]. For example, Atox1 binds with Cu(I) by Cu(I) ion coordinated with two cysteines of the CXXC motif [12].

3. Cu in Health and Diseases

3.1 Cu in human health

Cu is found in various foods, including organ meats, seafood, nuts, seeds, whole grains, and chocolate [13]. In addition, liver and oysters are rich sources of Cu [13]. On the other hand, vegetables and fruits are generally poor sources of Cu, although some, such as potatoes and avocados, contain small amounts of the metals.

Cu is also needed for the activity of many enzymes, such as cytochrome c oxidase (CCO) and lysyl oxidase (LOX) [14]. One of the most important functions of Cu-binding proteins is their role in antioxidant defense. Cu is a component of the enzyme superoxide dismutase (SOD), which helps to neutralize free radicals that can damage cells and tissues [14]. Cu is also incorporated in CP, a protein that plays a role in iron metabolism and antioxidant defense [15]. Cu is also essential for neurological functions, whereby Cu regulates synthesizing neurotransmitters, including norepinephrine, epinephrine, and dopamine via dopamine-β-hydroxylase (DBH); each subunit of DBH binds two Cu ions, and the protein is tetrameric [16].

3.2 Cu proteins and Cu transport pathways

We already know that Cu is an essential trace metal required for proper function of various human enzymes and proteins. However, free Cu ions are toxic to cells since their redox activity could increase ROS generation [17]. Therefore, a network of transporters and chaperones tightly regulates Cu transport and distribution within the cells. Our group previously identified 54 Cu-binding proteins in humans by analyzing the Uniprot.org database, reviewing relevant literature and manual curation, including 12 Cu transport proteins (most involved in Cu transport pathways), 26 Cu-dependent enzymes, and 15 Cu-binding proteins with unknown functions [13].

LOX and LOX-like enzymes belong to the amine oxidase family. LOX is critical in forming crosslinks between elastin and collagen within the extracellular matrix by oxidatively deaminating lysine residues [18]. Initially produced as a proenzyme, LOX must undergo cleavage of the amino-terminal region after exiting the endoplasmic reticulum to become an active enzyme [19]. Given its limited solubility, performing *in vitro* LOX purification and structure analysis is challenging [20]. However, the structure of human lysyl oxidase-like-2 (LOXL2) has recently been determined using X-ray crystallography [21].

CP is a Cu-binding protein synthesized in the liver and secreted into the bloodstream. CP is a Cu transporter in blood. In addition, albumin and transcuprein are also Cu transporters found in the blood plasma, binding Cu directly and with high affinity [22].

Cu is obtained from dietary sources and absorbed by the intestine through Menkes Cu transporting ATPase (ATP7A) [23]. Once in circulation, Cu attaches to the exchangeable pool, which is bound to albumins and amino acids like L-histidine and L-glutamine [23]. As shown in Figure 1, for cellular uptake of Cu(II) to Cu(I), first, Cu(II) has to be reduced to Cu(I) by potential cupric six-transmembrane epithelial antigen of prostate (STEAP) reductases family [24]. Then Cu(I) binds to the Cu transporter protein (CTR1), and CTR1 translocates Cu(I) across the membrane, playing a crucial role in high-affinity Cu uptake [25]. CTR1 is located in two cellular areas: at the plasma membrane and in intracellular vesicles [26]. In specific organs such as the liver, kidney, placenta, and mammary gland, CTR1 is mainly located at the basolateral plasma membrane [27-30]. It retrieves Cu from specific carriers and transports it from blood circulation [31]. In addition to facilitating dietary Cu uptake in the intestine, CTR1 also plays a crucial role in releasing Cu from intracellular vesicles for further utilization [6].

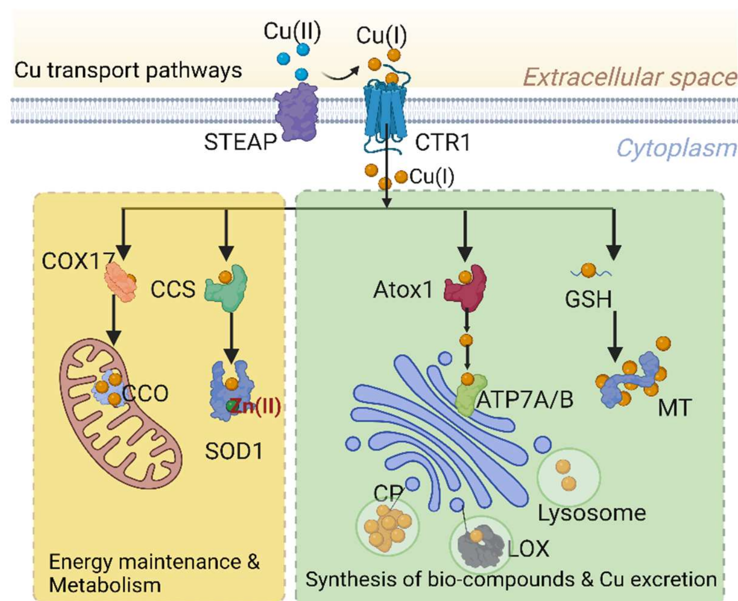

Figure 1. Overview of systemic and cellular Cu homeostasis. Once Cu(II) is reduced to Cu(II) by proteins from the STEAP family, Cu is transported into intracellular through the CTR1. From there, Cu is transferred to chaperones CCS, COX17, and ATOX1, distributing it to various destinations, including cytosolic SOD1 and CCO in the mitochondria, ATP7A/B at TGN. Cu binds to MT and GSH, which act as cellular antioxidants and prevent ROS formation. Within the TGN, Cu is incorporated into Cu-dependent enzymes, such as lysyl oxidase (LOX), which are transported through the secretory pathway. When excess Cu enters cells, ATP7A can also secrete Cu into the extracellular space. *Created with BioRender.com.*

In the first Cu transport pathway, Cu is transported into cells and can be utilized by CCO in the mitochondria to facilitate cellular respiration (Figure 1) [32, 33]. When cytochrome C oxidase Cu Chaperone (COX17) obtains Cu, it delivers Cu to CCO. CCO is the final enzyme in the electron transport chain and functions by coupling electron transfer with cytochrome c oxidation and reducing oxygen, producing water [34]. The Cu_A center on the surface of CCO accepts electrons from cytochrome C and then transfers them to the Cu_B center bound to heme through a series of reactions resulting in its reduction [35]. These redox reactions are pivotal for proton pumping via the mitochondrial membrane and contributing to ATP synthesis [35].

The second pathway for transporting Cu involves the use of a specific chaperone, known as Cu chaperone for superoxide dismutase (CCS), which inserts the Cu directly into SOD1 (Figure 1) [36]. The CCS protein interacts with SOD1 and supports the integration of Cu through the cysteine (CXXC) motif [37]. In addition, CCS also aids in forming the necessary intramolecular disulfide bond that stabilizes SOD1 [37]. SOD1 operates within the cytoplasm and relies on a cupric ion to catalyze superoxide disproportionation [38]. Mammalian SOD1 consists of a homodimer, and each monomer has a metal-binding site at its core for binding Cu and Zn, which are essential for its function [39].

Another Cu-dependent factor, Glutathione (GSH) (Figure 1), an abundant intracellular tri-peptide, has been proposed as an initial Cu acceptor upon Cu entry into cells [5]. Studies have indicated that GSH is a potential physiological carrier of Cu(I) [5]. Initially, it was discovered that Cu absorption kinetics involved GSH binding with Cu before binding with metallothioneins (MT), as revealed by early investigations [40, 41]. The role of GSH in the antioxidant buffering mechanism is pivotal, as it binds with intracellular Cu and is vital to the cellular defense mechanism against oxidative stress [42]. Additionally, GSH balance is associated with the cellular redox status and modulates the Atox1 activity [42].

The regulation of cellular Cu transport heavily relies on ATP7A and ATP7B. These proteins possess comparable structures and roles, sharing approximately 60% similarity in their amino acid sequences [43, 44]. ATP7A/B has a hydrophilic section extending into the cytosol, housing the Actuator (A-domain) and ATP-binding domains [45]. The ATP-binding domain has two smaller domains: the phosphorylation domain (P-domain) and the nucleotide-binding domain (N-domain) [45]. In addition, ATP7A/B has another section of eight transmembrane helices that help shape an intramembranous channel [43, 44]. Finally, the long N-terminal tail has six metal binding domains, allowing them to bind and transport Cu [46].

ATP7A and ATP7B use ATP-dependent phosphorylation and dephosphorylation cycles to move Cu across cellular membranes, which is necessary for many essential Cu-dependent enzymes and for preventing free Cu ions induced toxicity by removing excess Cu (Figure 1) [47].

Figure 1 demonstrates how Atox1 is specifically responsible for delivering Cu to the secretory pathway. After CTR1 transfers Cu to Atox1 [25], Atox1 achieves this objective by interacting with the ATP7A/B metal binding domains via heterocomplex in which Cu is shared between the proteins at the trans-Golgi network (TGN) [48]. ATP7A/B transfers Cu to Cu-dependent enzymes like LOX that travel via the secretory pathway [42]. If the intracellular Cu level gets too high, CTR1 (the high-affinity Cu transporter) is internalized and degrades. On the other hand, ATP7A and ATP7B move from TGN to the plasma membrane and aid in the excretion of Cu from the cell [43, 49].

Our knowledge suggests that Atox1 is a Cu chaperone facilitating Cu transfer into the secretory pathway. This is critical for activating Cu-dependent enzymes involved in various functions, such as neurotransmitter biosynthesis, wound healing, neovascularization, iron efflux, and blood pressure regulation [50, 51]. This thesis presents evidence of Atox1's involvement in breast cancer cell migration. However, before delving into this, the forthcoming section will discuss Atox1's structure and homologs.

3.3 The structure of Atox1 and its homologs

Human Atox1 is a small soluble protein (molecular weight 7.4 kDa) and adopts a ferredoxin-like fold (Figure 2A) [52]. It possesses a single Cu-binding site comprising two cysteine thiols within the CXXC motif. Atox1 consists of a backbone of anti-parallel β-strands (β1-4) that form the core of the protein, with two α-helices (α1 and α2) situated on one side of the sheet (Figure 2A). This site binds one Cu(I) ion with a dissociation constant (Kd) of $2 \times 10^{-17.4}$ M [12]. Sulfur atoms from Cys12 and Cys15 facilitate the Cu coordination, whereas the Met residue remains unchanged and does not serve as Cu-binding ligands [PDB 1FEE] [53]. When Cu binds to Atox1, a subtle change in conformation occurs, resulting in increased rigidity of the β1-α1 coil [54]. The presence of two α-helices on Atox1's surface, which have a positive charge, is essential in Cu chaperone function and interaction with ATP7A and ATP7B's metal

binding domains [55]. Both apo and holo-Atox1 solution structures reveal that Cys15 is wrapped within the Cu-bound α2-helix [54].

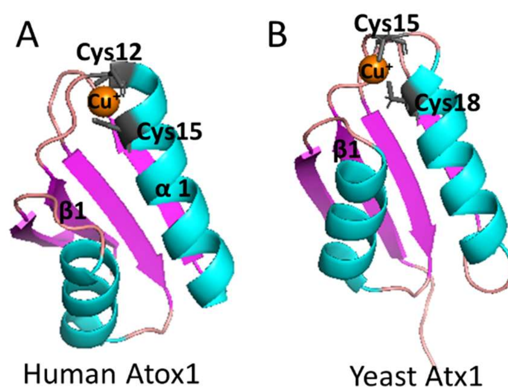

Figure 2. Crystal structures of Atox1 homolog. (A) Human CuAtox1 (1FEE); (B) Yeast CuAtx1 (1FD8). α helices in blue, β strands in magenta, loop regions in pink. Atox1 CXXC motif are displayed as grey stick. Cu ions (orange spheres).

Atox1 homologs are found in most organisms, including yeast and bacteria [56]. The homolog in yeast, called Atx1, was initially discovered in *Saccharomyces cerevisiae*, and they are known to bind to Cu (Figure 2B) and transfer it to the secretory pathway [57]. To investigate the function of Atox1 with Cu in mammals, researchers identified the mouse homolog of Atox1, mAtox1 [56]. The conserved residues and N-terminal metal-binding motif in mAtox1 suggest that it may perform equivalent functions in Cu binding and trafficking to the secretory pathway[56]. Furthermore, the Atox1 homolog, CUC-1, in *C. elegans*, has a significant sequence resemblance to yeast Atx1 [58] and human Atox1 [59], and it also functions similarly to Atx1, indicating that higher eukaryotes possess a Cu trafficking pathway [60].

3.4 Cu in diseases

Cu homeostasis needs to be regulated through Cu transporter proteins. However, Cu deficiency and excess can both lead to various diseases, including Cu deficiency disorders, Wilson Disease, Menkes disease, and cancer.

An inherited disorder affecting Cu metabolism known as **Wilson Disease** leads to the accumulation of Cu in different organs such as the liver, brain, and eyes [61]. The mutation of the ATP7B gene is the main cause of Wilson Disease [61]. ATP7B mutations [62, 63] impede Cu loading of CP, leading to an accumulation of Cu in the cytoplasm [64].

Menkes disease, an uncommon Cu metabolism disorder, is characterized by Cu deficiency in the brain, liver, and kidneys due to X-linked recessive inheritance [65]. ATP7A gene mutations cause Menkes disease, which leads to inadequate supply of Cu to specific enzymes in various organs, resulting in Cu deficiency [65, 66].

Cu-induced oxidative stress reactive oxygen species (ROS), including hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) free radicals and hydrogen peroxide (H_2O_2), are synthesized through the aerobic metabolism of eukaryotic cells and play a crucial role in regulating significant signaling pathways [67].

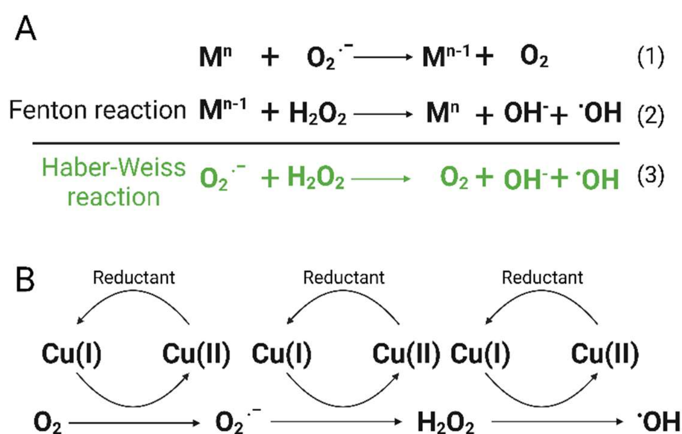

Figure 3. (A) Haber-Weiss Fenton and reaction.(M denotes redox-active metals like iron and Cu, n is the oxidative state) (B) Cu redox cycle to produce ROS. *Created with BioRender.com*

The primary location for ROS production is within the mitochondria through the electron transport chain, which reduces a small portion of O_2 to generate $O_2^{\bullet-}$[67]. Haber and Weiss first discovered that the $^{\bullet}OH$ could be created through the interaction between $O_2^{\bullet-}$ and H_2O_2. This reaction, named the Haber-Weiss reaction (as seen in Figure 3A), requires a metal ion (such as iron or Cu) to act as a catalyst. The net reaction (seen in Figure 3A as reaction (3)), which generates $^{\bullet}OH$, can be separated into two reactions (seen in Figure 3A as reactions (1) and (2)). One of these reactions is the Fenton reaction (reaction (2)). The Haber-Weiss reaction produces the highly reactive hydroxyl radical in various biological systems [68]. Figure 3B shows that the Cu redox cycle initiates with the reduction of Cu(II) to Cu(I) via a reductant such as ascorbic acid, which generates $O_2^{\bullet-}$ that subsequently re-oxidizes to Cu(II). In the presence of H_2O_2, acting as an oxidizing agent, Cu(I) oxidizes to Cu(II), initiating the Fenton-like reactions and generating the $^{\bullet}OH$ radical. ROS damage proteins, DNA, and membranes [69]. The activity of free radicals of ROS in cells can be problematic since they tend to take electrons from other molecules to become stable. For example, exposure of DNA to ROS can cause the oxidation of guanine, resulting in a mutation that can cause double-stranded breaks in nuclear and

mitochondrial DNA, leading to genomic instability [70]. Proteins can also undergo damage when thiol-containing cysteine and methionine residues are oxidized by ROS, leading to structural changes that can lead to loss of function [71]. Furthermore, the exposure of lipids to ROS leads to lipid peroxidation, which can cause cell membrane damage and generate reactive by-products that further damage the cell. Therefore, cells have dedicated Cu transport systems that facilitate the timely and safe delivery of Cu to Cu-dependent enzymes [72-74].

4. Cu in Cancer

Cancer is primarily marked by the rapid expansion of abnormal cells that surpass their established boundaries, capable of infiltrating neighboring organs and tissues, ultimately causing metastasis [75]. The spread of cancer to other parts of the body is a significant cause of death associated with the disease [75]. In 2020, cancer caused almost 10 million deaths globally, making it a top cause of mortality due to a lack of new targets and therapeutics [76]. The most prevalent type of cancer in 2020 was breast cancer [76].

The role of Cu in cancer has been known for many years. Various studies have shown that high Cu levels exist in tumor-bearing mice and individuals with cancer [77, 78]. Elevated levels of Cu in the blood have also been associated with various cancers, including lymphoma, breast, stomach, bronchogenic, and lung cancers, etc. [79]. In addition, increased serum Cu levels were associated with the stage of the cancers [79, 80]. Serum Cu levels may be a biomarker for cancer recurrence and treatment efficacy. However, the mechanism behind increased serum Cu levels in cancer patients is not yet understood. In contrast to increased Cu levels, cancer patients often exhibit lower levels of zinc, iron, and selenium in their serum than healthy individuals [79, 81].

4.1 Cu proteins in cancer

Cu is a crucial element for the proper functioning of various enzymes [82] and has a pivotal role in regulating key processes in cancer progression, including angiogenesis, proliferative immortality, and metastasis [49, 83].

Cu contributes to cancer metastasis through its involvement in a multi-step process that relies heavily on cancer cell migration. First, cancer cells must traverse the tumor stroma and blood vessels to head to metastatic sites [83]. This process necessitates the cancer cells to adjust to, engage with, and modify the extracellular matrix [83]. The cancer cells develop actin-rich plasma membrane protrusions, such as lamellipodia, filopodia, and invadopodia, at the leading edge to facilitate local invasion and migration [83]. Several Cu-dependent proteins, including LOX, Atox1, and Memo1, have been linked to cancer development.

4.1.1 LOX

ATP7A plays a role in cancer metastasis by loading Cu into LOX proenzyme within the secretory pathway before it is released and cleaved into LOX propeptide (LOXPP) and mature LOX [84]. The secretion of LOX by cancer cells modifies the extracellular matrix, forming a

pre-metastatic niche. This niche attracts bone marrow-derived cells and facilitates the spread of cancer to other parts of the body [85]. High levels of LOXL2 in invasive cancers have been linked to metastasis and unfavorable outcomes for those with estrogen receptor-negative breast cancer [86]. Inhibiting LOX and LOXL activity could be a promising therapeutic approach to prevent cancer metastases.

4.1.2 Ceruloplasmin

CP is overexpressed in various breast, ovarian, and prostate cancers [87-89]. High expression levels of CP have been linked to a negative prognosis in patients with breast cancer [87]. This suggests that CP may play a role in cancer progression. CP may contribute to cancer is its ability to modulate the levels of Cu ions in the bloodstream. Studies have demonstrated that CP enhances angiogenesis by triggering the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [90]. Another way in which CP may contribute to cancer is through its role in regulating oxidative stress. CP is an antioxidant that binds and neutralizes free radicals and other ROS [91]. However, studies have also shown that CP can stimulate ROS production in cancer cells, increasing DNA damage and cellular stress [91].

4.1.3 Atox1

Cu-dependent enzymes and Cu-binding proteins may have implications in cancer [92, 93]. Specifically, Atox1, a Cu chaperone, has been implicated in breast cancer cell migration [51], and its expression level is upregulated in various cancer types [94]. Atox1 was observed to accumulate at the border of migrating breast cancer cells [51], and higher expression levels were correlated with shorter survival times in the early stages of breast cancer [95]. Atox1 has also been identified as a potential prognostic biomarker for early-stage and estrogen-receptor (ER)-positive breast cancer [95]. Another study found that Atox1 promoted inflammatory neovascularization [96]. Overexpression of Atox1 in melanoma patients was linked to poor prognosis while knocking it down resulted in decreased cell growth [97] in human melanoma cell lines. Additionally, a tissue microarray analysis of clinical trial data indicated that heightened nuclear translocation of Atox1 was linked to metastatic colorectal cancer and associated with the disease's severity [98].

4.1.4 Memo1 in breast cancer

Memo1 was discovered to interact with pY1227 on the C-terminus of ERBB2 in T47D breast carcinoma cell extracts through unbiased high-pressure liquid chromatography-tandem mass spectrometry (LC-MS) screening [99]. This interaction occurred after histidine-rich glycoprotein (HRG) stimulation and may have been facilitated by the Src homology and collagen 1 (SHC) adaptor protein (Figure 4). In addition, Memo1 also forms a complex with Phosphoinositide phospholipase C γ (PLCγ) in HRG-treated breast cancer cells (Figure 4). Memo1 and PLCγ are required for HRG-induced tumor cell migration, and loss of Memo1 or PLCγ affects cell directionality [100].

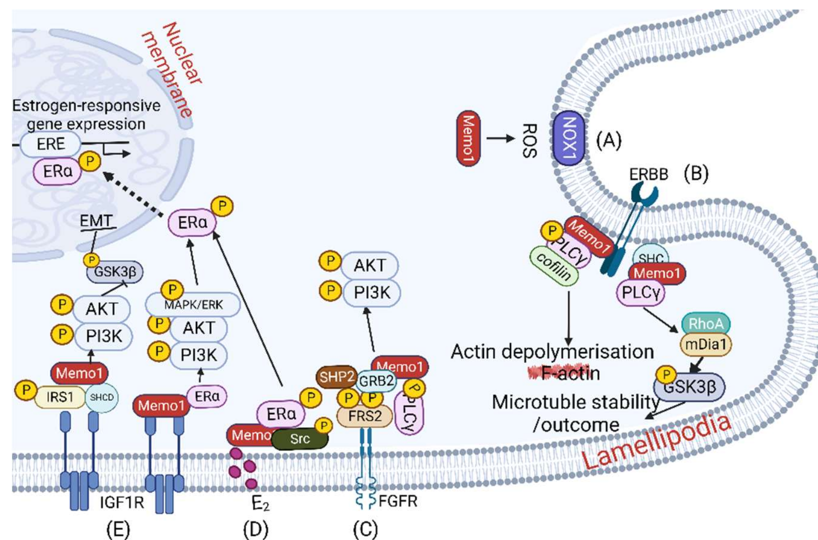

Figure 4. An illustration of Memo1 involved signalling pathways, including the: (A) ROS generation; (B) ERBBfamily (breast cancer cell migration); (C) FGFR-family(cell differentiation,proliferation); (D) IGF1R-family and (E) estrogen receptor (The development and progression of breast cancer). *Created with BioRender.com*

In addition to interacting with the fibroblast growth factor receptor (FGFR) family, Memo1 plays a key role in optimal FGFR signaling, as demonstrated by partial inhibition of FGFR signaling following siRNA-mediated knockdown in cultured cells (Figure 4) [100]. Furthermore, Memo1 has also been found to interact with the insulin-like growth factor (IGF/IGFR) signaling pathway (Figure 4), which regulates cell differentiation, proliferation, and survival [101]. Cells in which Memo1 was knocked down demonstrated decreased migratory capacity in response to IGF-1 stimulation, highlighting Memo1's involvement in the IGF/IGFR signaling pathway [102].

In the search for drug targets for hormone-driven breast cancers, the interaction between growth factors and steroid hormones is essential [103]. The development and progression of breast cancer rely heavily on estrogen and estrogen receptors (ER), with Memo1 playing a vital role in regulating ERα's extranuclear functions through the interaction with IGF1R and ERBB2 (Figure 4) [104]. As a promising link between growth factors and steroid hormone-mediated signaling pathways in breast cancer, Memo1 could be a potential therapeutic target.

Mice with lower Memo1 levels had smaller lung tumor lesions, indicating that reduced Memo1 levels can minimize tumor cell proliferative ability in the lung environment [105]. Furthermore, tumor cells with Memo1 knockdown exhibited more protrusions but fewer motile cells [105]. In both cancerous and non-cancerous breast cell lines, loss of Memo1 significantly impaired cellular migration in wound closure and invasion assays [105]. These findings identify that Memo1 is a crucial controller of cell migration in breast cancer.

Studies of Memo1's function in the actin network identified Memo1 as a binding partner of cofilin-1 – an actin-binding protein (Figure 4) [100]. Furthermore, *in vitro* studies showed that Memo1 stimulates cofilin-induced F-actin depolymerization, and that simultaneous knockdown of Memo1 and PLCγ almost completely blocked migration, indicating that Memo1 and PLCγ converge on cofilin in an ERBB2-initiated migratory pathway [100].

Low Memo1 level induced a more reduced cellular environment [105]. It was further discovered that NADPH oxidase 1 (NOX1) activation through protein kinase C depends on Memo1, which is also necessary for the continued production of O^{2-} following NOX activation[105]. However, contrasting the findings in this breast cancer study [105], *in vivo* ROS-mechanism studies in worms associated Memo1 loss with a more oxidized cellular state [106]. So, it is important to determine how ROS-related functions of Memo1 might integrate with the other identified roles of Memo1 and how Memo1's function influences the redox environment involved in diseases. This thesis aims to solve these questions or at least provide clues about Memo1's role in ROS-related processes.

4.2 The structure of Memo1 and its homologs

Memo1, as determined by Qiu (2008), possesses a single-domain structure that consists of a mixed seven-strand β-sheet surrounded by nine α-helices (Figure 5) [107]. It has been discovered through this study that Memo1 is homologous to LigB, a catalytic subunit that belongs to the catechol dioxygenase LigAB from *Sphingomonas paucimobilis* [108]. LigAB mediates catalytic activity through an iron ion coordinated by specific histidines and a water

molecule [109]. Similarly Memo1 also contains these same conserved histidines (His-49, His-81, and His-192, respectively) but with Glu-242 replaced by Cys-244 [107].

Memo1 crystals were grown under pH 5.5 conditions where the histidine residues were likely to be protonated, making them less capable of coordinating a metal ion [107]. To investigate whether Memo1 bind to metal ions, experiments were performed at neutral pH using X-ray crystallography, UV absorption, and fluorescence [107]. Unfortunately, the tests did not provide evidence to support the idea that Memo1 could bind to metal ions, such as Cu, iron, cobalt, or zinc. However, in experiments where zinc-Memo1 samples were prepared for inductively coupled argon plasma (ICAP) spectrometry, it was found that high zinc concentration could cause Memo1 protein precipitation [107].

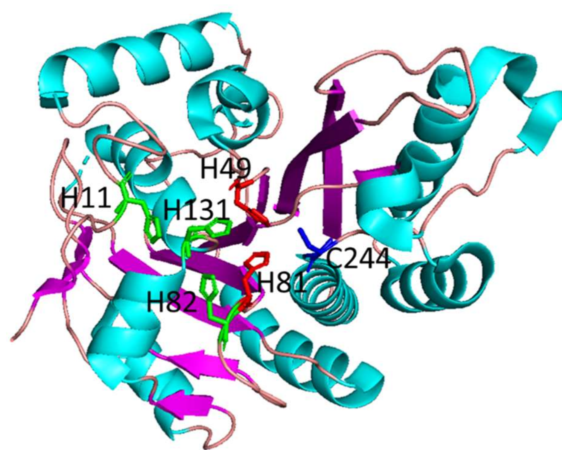

Figure 5. The structure of Memo1 ribbon diagram(PDB: 3BD0)). Putative Cu binding sites: (1) His-49(red), His-81(red), and Cys-244 (blue),(2) His-11(green), His-82(green) and His-131(green)

The metal binding capabilities of Memo1 were long speculated but were not demonstrated until Memo1 was shown to bind Cu(II) and its ability to generate O^{2-} [105]. However, most of the research on the interaction between Memo1 and Cu has been conducted using cell cultures with excessive Cu(II), leaving several unanswered molecular questions regarding this relationship and its link to ROS. The question of how a metal ion might bind with Memo1 was a key motivation in my research question, which aimed to understand how Cu binds to the Memo1 active site. My investigation also led to an explanation of how Memo1 may obtain Cu *in vivo* and how the redox nature of the enzyme could affect cancer cells.

5. Methodology

This section gives a brief description of the techniques which have been used in this thesis. You can find all methods in detail in respective published papers.

5.1 Protein expression and purification

Protein purification is crucial for analyzing key proteins and protein complexes and identifying their interactions. Various protein purification strategies and expression hosts such as *Escherichia coli (E.coli)*, yeast, and mammalian cells can be used to get the purified protein. In work described in the thesis, we have used *E.coli* as the expression host to express our human proteins of interest, Atox1and Memo1

Protein expression

E.coli is a widely used organism for heterologous expression and purification of protein [110]. With numerous molecular tools and protocols available, including an extensive selection of expression plasmids, engineered strains, and cultivation strategies, it is one of the most preferred choices for heterologous protein production.

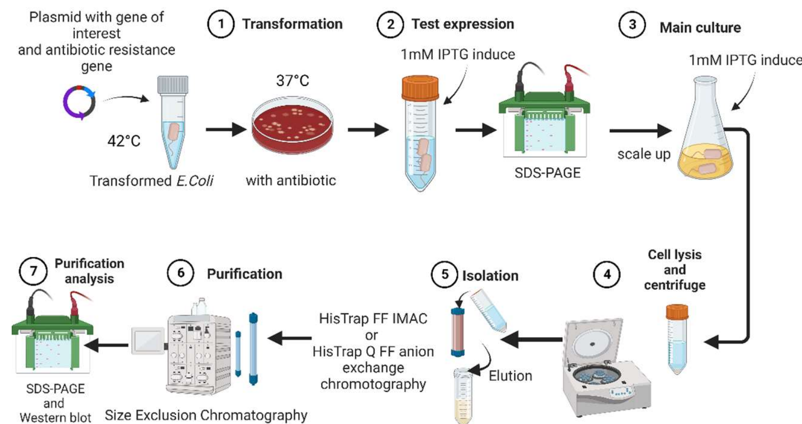

Figure 6. Memo1 and Atox1 recombinant proteins are produced using an *E. coli* as expression host [111]. After transformation, colonies are screened for expression analysis. A single colony is picked and grown with their respective antibiotics. Small test expressions are carried out to optimize and determine the solubility and yield of the protein of interest. The process is then scaled up where 1/100 of an overnight grown culture dilution cells are grown at 37^0 C, induced at O.D of 0.6-0.8, and grown at 20^0 C overnight post-induction. The cells are pelleted by centrifugation, lysed, and the protein's supernatant is then subjected to different chromatography techniques to isolate and purify the protein of interest. Finally, the purified protein is checked for purity and homogeneity using size exclusion, SDS-PAGE and western blot, respectively.

E. coli can be easily transformed with the gene of interest, has fast-growth kinetics, and can achieve high cell densities within a short time frame. Also, the growth media can be made from inexpensive and readily available components making it a suitable expression host.

In the study reported in this thesis, Memo1 and Atox1 were expressed and purified using *E. coli* as host. Plasmid pET-28a and pET-3a harboring the genes for Memo1 and Atox1 were transformed into BL21 (DE3) cells (Novagen) with the appropriate antibiotics Kanamycin and Ampicillin, respectively. A single colony was picked and grown overnight to prepare the primary starter culture, and this was then used for inoculating the secondary culture, 1/100th dilution. The cells were then grown in large flasks at 37^0 C and shaken at 220 rpm until the O.D. reached 0.6-0.8. The culture was then cooled to 20^0 C, induced with IPTG (Isopropylthio-β-galactosidase), and grown overnight at 20^0 C. The cells were harvested by centrifugation and then purified using chromatographic steps(Figure 6).

Protein purification

Purification of protein involves strategies that are determined by the biochemical nature based on size, charge, shape, or affinity [112]. **Ion-exchange chromatography** utilizes the electrostatic interactions between charged protein groups and solid support matrix. The matrix has an ionic group opposite to that of the protein to be separated. This allows the protein binding to the column matrix, which can then be eluted by altering the buffer solution's pH, salt concentration, or ionic strength [113]. **Affinity chromatography** is used to purify various biomolecules, including enzymes, hormones, antibodies, nucleic acids, and specific proteins [114, 115]. The technique involves using a ligand that can form a complex with the target protein, such as aNi-NTA (Nitrilo acetic acid) uses the affinity of Ni^{2+} ion to histidine tag in the protein which can then be eluted using gradient of imidazole eventually releasing the prortein from the column. [115]. This concept is where specific protein that binds firmly to the ligand is retained in the column, while those that can not bind and are feebly associated are washed out in the process. The column bound protein is then released from the column through alterations in pH or the addition of a chelator molecule which removes Ni, thus the binding capacity of the column [115]. **Size exclusion chromatography (SEC)** is based on the hydrodynamic radii of the biomolecule and the separation of these molecules are based on their respective size and dimensions. Larger molecules that can not enter the pores of the matrix elute first. The column cannot resolve these and comes at the exclusion limit called the void volume [116]. The gel comprises spherical beads, each with pores of distinct size distribution.

When subjected to molecules of varying sizes, the smaller ones diffuse into these pores, delaying their flow through the column. Conversely, larger molecules cannot penetrate the pores and are thus eluted out first. Hence, the separation of molecules is contingent on their size, and they are eluted in order of decreasing molecular weight (MW) [116]. SEC is commonly used to purify proteins, polysaccharides, and nucleic acids [116].

SDS-PAGE

SDS-PAGE is a commonly utilized procedure to separate mixtures of proteins in denatured states using an anionic detergent. First, the process denatures the constituent proteins by imparting a negative charge proportional to their molecular weight. Following this, electrophoresis occurs through a porous acrylamide gel matrix, which sorts proteins based solely on their molecular weight [117]. This thesis used it to check recombinant protein purity and the separation of the target proteins from cell lysis after RNA silencing.

5.2 UV-Vis absorption spectroscopy

Ultraviolet and visible (UV/Vis) spectroscopy measures a sample's absorption of ultraviolet and visible light [118]. This technique is widely used to identify a molecule's functional groups or determine a substance's concentration in a solution [118].

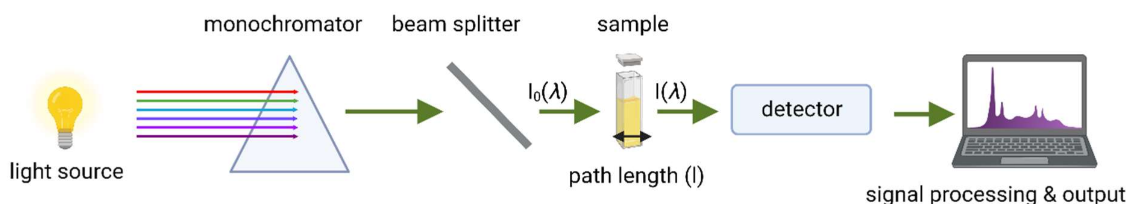

Figure 7. A schematic of the main components in a UV-Vis spectrophotometer. Light passes though a monochromator allowing the specific wavelength light go through (I_0), then splited to go through the sample (I), and collect by a detector. The signal are processing and shown by computer. *Created with* BioRender.com

The sample is exposed to light of different wavelengths (200-800 nm), and the amount of light absorbed is measured and plotted as an absorption spectrum [118]. This spectrum can identify specific functional groups or measure a substance's concentration in a solution. As UV-Visible light is absorbed by molecules, an electronic transition occurs where the energy levels become quantized. This means that the amount of energy required for excitation is fixed, and only electromagnetic radiation with a specific frequency can induce this transition. The absorption of radiation is unique to each substance, allowing for its identification. By absorbing UV light, chemical compounds generate a spectrum that aids in identifying the constituent parts. One can

measure the absorption (A) by ascertaining the intensity of light prior (I_0), and after (I), it passes through the sample (Equation 1) [118].

$$A_{(\lambda)} = log\frac{I_{0(\lambda)}}{I_{(\lambda)}} \tag{1}$$

Suppose a chemical sample has a known molar extinction coefficient (ε), which indicates its ability to absorb light at a specific wavelength. In that case, it can be used to measure the concentration of the sample. The concentration (c) can be calculated by applying the Lambert-Beer law (equation (2)) and knowing the molar extinction coefficient and the optical path length (l) [118]. The chromophore typically used to measure protein concentration is amino acid residues, tryptophan which can be detected at 280nm. The extinction coefficient of these residues can be employed in equation (2) to calculate the protein concentration [118].

$$A_{(\lambda)} = \varepsilon_{(\lambda)}cl \tag{2}$$

As shown in Figure 7, the UV-Vis absorption spectrophotometer has a light source, a monochromator (produces a single spectral line from a broadband light source), a beam splitter, a detector, and a computer for signal processing and output.

5.3 Fluorescence

Fluorescence is a phenomenon in which a molecule absorbs the energy of a photon from a laser or lamp at a specific wavelength, and the electrons of the fluorescent molecule transition from the ground state (S_0) to the electronic singlet states (S_1,S_2) or triplet state (T1) (Figure 8) [119]. Fluorescence microscopy and the assays in this thesis using fluorescence are related to the singlet states.

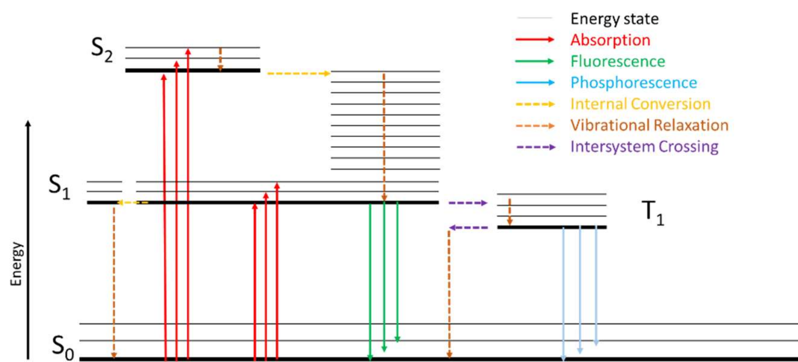

Figure 8. A simplified Jablonski diagram of energy levels and the electronic transition. The horizontal thick lines indicate electronic states, thin lines are vibrational states. Vertical arrows shows radiative process, such as absorption (red), fluorescence (green) and phosphorescence (blue). Dashed line arrows indicates internal conversion (yellow), vibrational relaxation (brown) and intersystem crossing (purple).

The excited state usually lasts very short, ranging from picoseconds to nanoseconds. Then through vibrational relaxation and internal conversion, the molecule relaxes to S_1. When the molecule is at S_1, it will return to the ground state (S_0) by releasing non-radiative (vibrational and collision) and radiative (fluorescence) relaxation. By fluorescence, the molecule returns to the ground state (S_0) by emitting a photon with a longer wavelength than the absorbed photon due to energy loss within the molecule. The molecule structure, the surrounding environment, and temperature determine the molecule returns by which pathway.

There are two types of fluorescence:

1. Intrinsic fluorescence [120]: intrinsic fluorophores such as aromatic amino acid residues within the protein, including tryptophan, tyrosine, and phenylalanine. These amino acid residues can emit fluorescence when excited by ultraviolet (UV) light. This fluorescence is unique to each protein, and the intensity of the fluorescence depends on the protein's conformational state and local environment.
2. Extrinsic fluorescence [121]: fluorophores are external agents, such as fluorescent dyes, modified biochemicals, and antibodies with fluorescent labels, which are utilized to produce fluorescence signals with predetermined spectral properties upon introduction into a sample.

Fluorescence techniques are widely used in biological and medical research for imaging, sensing, and monitoring biological targets.

5.4 Confocal fluorescence microscopy

Confocal fluorescence microscopy is a technique used to produce high-resolution, three-dimensional images of fluorescently labeled specimens [122]. It uses a laser beam to excite fluorophores in a specific plane. The emitted fluorescence is then collected and focused through a pinhole aperture (which eliminates out-of-focus light) onto a detector, creating a sharp image of the specimen. The pinhole aperture in front of the detector is located at the focal plane of the objective lens. It is sized to eliminate any fluorescence outside the focal plane. This removes the contribution of out-of-focus light to the final image, producing a sharper, more detailed image of the specimen. Confocal microscopy can be used to study various biological specimens, including cells, tissues, and whole organisms. It is particularly useful for studying structures that are difficult to visualize with conventional microscopies, such as thick specimens or structures located deep within tissues. In this thesis confocal microscopy is used for 2D single-

cell tracking and cell ROS production studies. Image J software is used to analyze the images data.

5.5 Circular dichroism spectroscopy

Circular dichroism (CD) is a spectroscopic technique used to study the differences in absorption levels of chiral molecules when exposed to left and right circularly polarized light. This phenomenon occurs because of the interaction between the molecules' optical activity and the circularly polarized light, which results in the rotation of the polarization plane of linearly polarized light. CD spectroscopy is highly beneficial when examining the structures and conformations of biological macromolecules, including proteins and nucleic acids, and synthetic chiral compounds [123].

The basic equations for interpreting CD spectra depend on the assumption that the molecules under investigation are chiral and that their electronic transitions are largely dipole allowed. The CD signal can be expressed in terms of the molar ellipticity ([θ]), [θ] is often described in deg cm^2 $dmol^{-1,}$ which is a measure of the degree of asymmetry or chirality in the molecule [123]:

$$[\theta] = 100 \times \theta/(l \times c) \quad (3)$$

where θ is the observed ellipticity in degrees, c is the concentration of the absorbing samples in mol/L, and l is the path length in cm. The molar ellipticity is related to the absorbance difference between left and right circularly polarized light, Δε:

$$[\theta] = \Delta\varepsilon \times 3298.2 \quad (4)$$

where Δε is the extinction coefficient difference between left and right circularly polarized light. The CD spectra of chiral molecules typically consist of several bands corresponding to electronic transitions of specific types of chromophores, such as peptide bonds, aromatic rings, or nucleotide bases. These bands can be assigned to specific structural elements or conformational states of the molecule based on the known CD spectra of similar compounds and theoretical calculations. A chiral amide bond connects each amino acid in a protein. Thus, absorption of these structures are the main contributor to the far UV (180–250 nm) CD spectra of proteins. CD spectra analysis can reveal insights into protein structures and identify proteins' secondary structures. As chromophores align in arrays, their transitions can cause exciton interactions, shifting or splitting the transitions. This phenomenon produces unique CD spectra for each structural element of protein (Figure 9) [123]. For example, proteins with an α-helix

structure Conversely, proteins with a clearly defined anti-parallel β-pleated sheet structure (β-helices) display negative peaks at 218 nm and positive peaks at 195 nm. Random coil proteins, or disordered proteins, exhibit negative peaks around 195 nm and have minimal ellipticity above 210 nm.

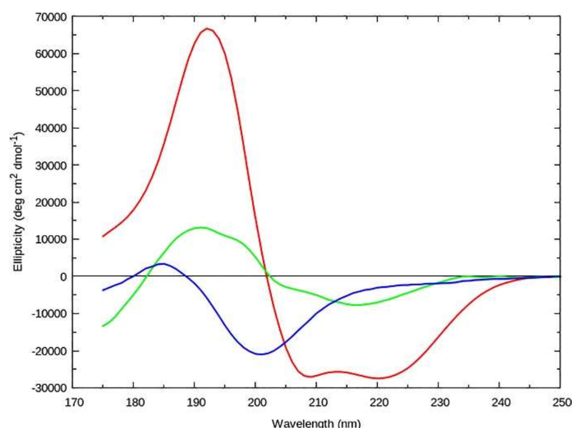

Figure 9. A schematic of different protein secondary structures by CD. α-helix (red), β-sheet (green), and random coil (blue) [124].

This thesis used CD to detect the structure of recombinant proteins and monitor conformational changes caused by mutations and heat. [123].

5.6 Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical technique that measures the binding interactions between biomolecules, such as proteins, peptides, and nucleic acids [125]. As shown in Figure 10, SPR is based on the interaction of light with the collective oscillation of electrons, called surface plasmons, at the interface between a thin layer of metal, usually gold or silver, and a solution containing the analyte of interest [125].

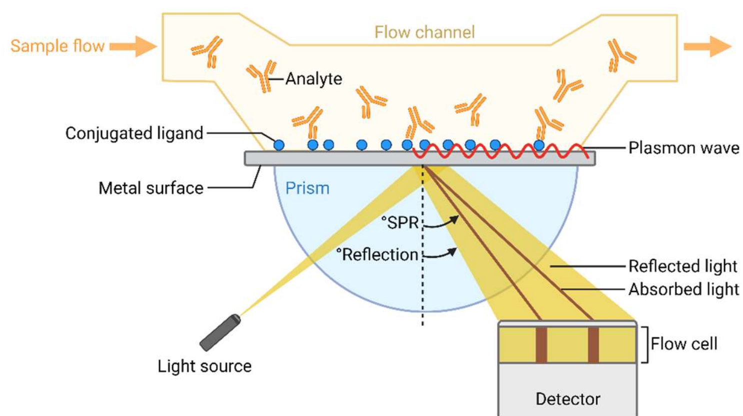

Figure 10. A schematic of SPR [126].

When the light at a specific angle of incidence hits the metal surface, it excites the surface plasmons. As a result, it decreases the reflected light's intensity at a specific angle called the SPR angle. As the analyte binds to the surface-immobilized ligand, it changes the local refractive index and the thickness of the metal layer at the interface, which causes a shift in the SPR angle [125].

By monitoring the change in the SPR angle over time, it is possible to obtain information on the amount of surface-bound molecules. Thus it provides real-time information about the kinetics of the binding interaction, such as the association and dissociation rate constants, equilibrium binding constant, and binding stoichiometry [125]. Additionally, SPR can provide information about the specificity and selectivity of the binding interaction [125].

SPR is a label-free, real-time, and highly sensitive technique that has become a valuable tool in various fields, including drug discovery, protein-protein interactions, and biomolecular interactions. This thesis uses SPR to detect Memo1-Atox1 interaction via Cu.

5.7 Breast cancer cell line

MDA-MB-231 is a human breast cancer cell line that was established in 1978 from a pleural effusion of a 51-year-old woman with metastatic mammary adenocarcinoma [127]. These cells are highly invasive and aggressive and are frequently utilized as a model for investigating the mechanisms underlying breast cancer metastasis [128]. These cells are characterized by their ability to grow well *in vitro*, which makes them a valuable tool for studying tumor biology in the lab [128]. More, MDA-MB-231 cells also express high levels of matrix metalloproteinases and other enzymes. These matrix metalloproteinases and other enzymes degrade the extracellular matrix, facilitate their ability to invade tissues, and metastasize to other body parts [128]. In addition, they resist certain types of chemotherapy and radiation therapy, making them difficult to treat with conventional cancer therapies [128].

5.8 *C. elegans*

C. elegans, as a soil nematode, is commonly used as a model organism in biological research [129]. Worms are usually grown on agar plates using *E.coli* OP 50 as a food source in the lab. It is essential to properly handle and maintain the worms to ensure their viability and usefulness in research. Proper storage conditions and regular monitoring of their growth and health are crucial to obtaining accurate and meaningful results from investigations.

C. elegans are small, and the maximum length of worms is around 1 mm. Growing worms only need a small space with lots of worm populations. Worms can be cryopreserved at - 80°C for years and thawed when needed. We can control the worm development rate by varying the incubation temperature from 12°C to 25°C [130]. *C. elegans* can be synchronized by the bleaching of gravid (carrying eggs) adults. Bleaching gravid adults will kill worms except for embryos so that worms can be synchronized at the same development stage [131].

Comparison of the human and *C. elegans* genomes shows that most human disease genes and pathways are also present in *C. elegans* [132]. Therefore, *C. elegans* is a powerful tool for the pharmaceutical industry and human disease research. Worms are usually evaluated using microscopy [132].

5.9 siRNA silencing and western blot

siRNA silencing uses small interfering RNAs (siRNAs) to specifically target and degrade cellular mRNA molecules, resulting in a reduction or loss of expression of the corresponding protein [134]. The siRNA is designed to complement the target mRNA sequence, and once introduced into the cell, it binds to the mRNA and recruits enzymes that cleave it into small fragments [134]. Cellular machinery degrades these fragments, preventing mRNA translation into protein [134]. The efficiency of siRNA silencing can be assessed using techniques such as western blot.

Western blot is a laboratory technique to detect specific proteins in each sample of biological material such as cells or tissue [135]. The proteins are first separated based on size through a polyacrylamide gel. The gel is then transferred to a membrane, which is blocked with a solution to prevent the nonspecific binding of the primary antibody. The primary antibody is then added to the membrane, binding to the protein of interest. A secondary antibody conjugated to an enzyme or fluorescent molecule for detection is then added, which recognizes and binds to the primary antibody. The enzyme catalyzes a reaction that produces a visible signal, which imaging techniques can detect and quantify [135].

By combining siRNA silencing and western blot, we can specifically silence and sequentially measure the expression level of our proteins of interest in cell cultures.

5.10 Flow cytometry

Flow cytometry is a technique used to analyze individual cells' physical and chemical properties in a heterogeneous mixture [136]. As shown in Figure 11, it passes a suspension of single cells, which presents fluorophores on the surface or inside the cells, through a laser beam, causing light emission at specific wavelengths [136]. A series of photomultiplier tubes then detect this emitted light, each measuring the intensity of the emitted light at a particular wavelength [136].

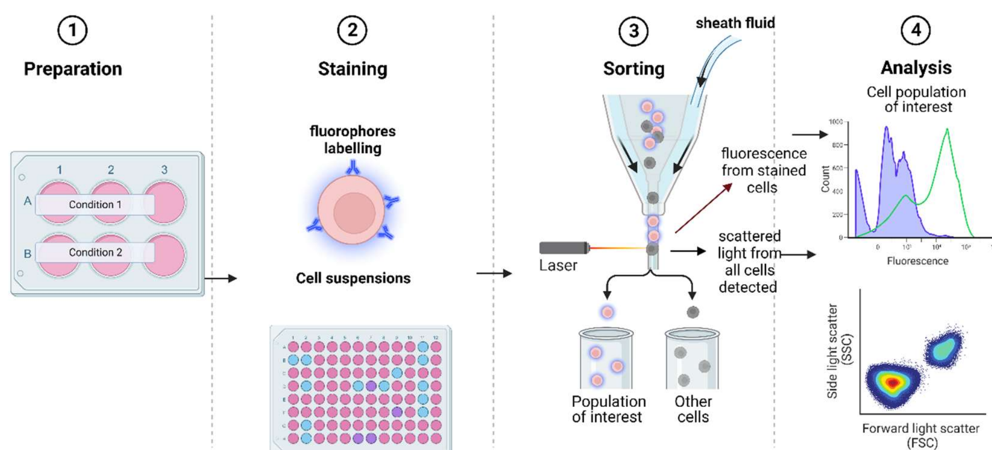

Figure 11 Schematic illustration of the flow cytometry experiment procedure [137].

As each cell passes through the laser beam, its fluorescence and other characteristics, such as size and shape based on the side and forward light scatter, are recorded [136]. These measurements are used to create a scatter plot, where each dot represents a single cell, and the dot's position reflects the intensity of fluorescence at a specific wavelength or the size and shape of the cell [136]. Flow cytometry can analyze various cellular parameters, including cell surface markers, DNA content, cell viability, and intracellular signaling [136].

5.11 Proximity ligation assay

Using the proximity ligation assay (PLA), we can detect and visualize the proximity (<40nm) of two proteins in cells or tissue samples [138]. Figure 12 illustrates the PLA principle. First, after the cells' fixation onto the plate's surface, the primary antibodies of interested proteins, such as Memo1 and Atox1 (Figure 12A), were added to bind to the proteins with high specificity and affinity. Next, as shown in Figure 12B, two secondary antibodies are added to the plate, with one antibody conjugated to a DNA probe and the other to an oligonucleotide.

These secondary antibodies bind to the primary antibodies, creating a sandwich complex where two antibodies surround the proteins.

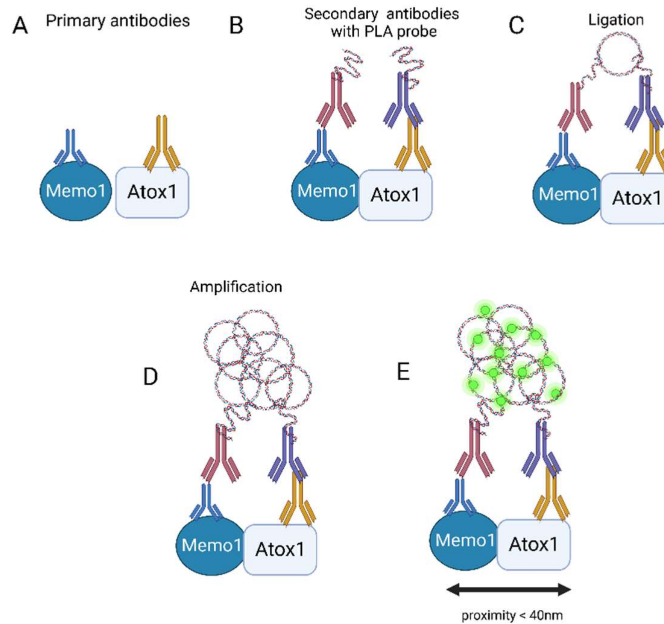

Figure 12 Illustration of PLA principle. (**A**) Additon of primary antibodies ecognize specific proteins (Atox1 and Memo1) in cells. (**B**) Addition of secondary antibodies coupled with PLA probes bind to the primary antibodies. (**C**) Ligation of oligonucleotides, when the distance of PLA probes are shorter than 40 nm. (**D**) Amplification of circular DNA template becomes by DNA polymerase. (**E**) The repeating sequences present in the amplicons are hybridized with fluorochrome-coupled complementary detection oligos. . *Created with BioRender.com*

After the secondary antibodies have been added, a ligase enzyme is added to the plate that catalyzes the formation of a covalent bond between the two oligonucleotides (Figure 12C). This step effectively links the two DNA probes that were introduced by the secondary antibodies. The hybridized DNA strands are then ligated, resulting in a closed circular DNA molecule. Then, DNA polymerase amplifies the circular DNA molecule by utilizing primers that attach to the DNA oligonucleotide sequences (as shown in Figure 12D). Finally, complementary oligos with fluorophores are introduced that bind and generate a fluorescence spot containing numerous fluorophores (as depicted in Figure 12E). The outcome is a fluorescent signal that can be detected through fluorescence microscopy or flow cytometry.

PLA can detect endogenous protein proximity in their natural environment without protein purification or disruption of cellular structures. Therefore, PLA is widely used in research fields such as cancer biology, neurobiology, and immunology to study protein-protein in close proximity. This thesis uses PLA to detect the proximity of Cu-binding proteins.

6. Original work

In this thesis section, the research papers will be summarized and discussed. The first paper (Paper I) explores the roles of Atox1 in breast cancer cell migration and the potential mechanisms involved. This will be followed by a discussion of the investigation into the Atox1 homolog CUC-1 in *C. elegans* cell migration during development (Paper II). Since Memo1 is also reported to be a Cu-binding protein and promotes ROS generation in the breast cancer cell, a study was performed to explore Memo1's Cu binding capacity and the possibility of interaction with Atox *in vitro* (Paper III). Finally, the fourth paper (Paper IV) reports the effect of Cu-Memo1 on ROS generation in breast cancer cells.

6.1 Atox1-mediated cell migration in breast cancer cells and *C. elegans*

The function of Cu transport proteins and Cu chaperones in cancer may extend beyond simply supplying Cu to enzymes [92, 93].

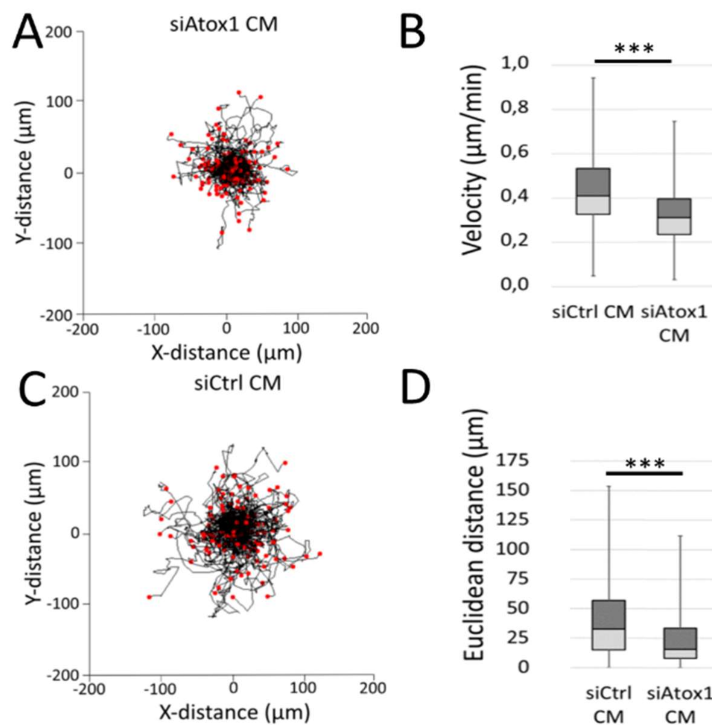

Figure 13. Atox1 siRNA knockdown in MDA-MB 231 breast cancer cells results in reduced cell migration velocity and euclidean distance. (A) & (C)Wind-Rose plots show the tracks of Atox1 silenced (siAtox1) and control (siCtrl) cells, respectively. (B) & (D) Box-whisker plots present migration velocity and euclidean distance of the tracked cells. Error bars indicate the minimum and maximum values for the data sets (the entire range of data is shown in each case). *P*-values for student´s t-test. ***P < 0.001.

For example, Atox1, as demonstrated in section 4.13, localizes to the edges of lamellipodia in breast cancer cells and increases cancer cell migration through an unknown mechanism. In order to further explore this mechanism between Atox1 and migration on a single-cell level, 2D video microscopy was utilized (Paper I). Ten hours of MDA-MB 231 cells single-cell tracking revealed that cells with lower levels of Atox1 exhibited decreased velocity and euclidean distance, as well as less distributed tracks (Figure 13).

In the same study, the effect of ATP7A on cancer cell migration was also examined using ATP7A silencing and video microscopy of 2D single cell migration. While the results showed a decreasing trend in the euclidean distance and velocity upon ATP7A silencing, the differences were not as large and significant as those observed with Atox1 silencing (Paper I). But the similarity in the effects of Atox1 and ATP7A silencing on breast cancer cell migration indicated that these two proteins may function within the same pathway. In addition, Atox1 typically supplies ATP7A with Cu for loading Cu-dependent enzymes, suggesting that this activity may also be involved in cell migration.

According to other scientific reports, the ATP7A-LOX pathway is crucial for breast cancer metastasis [84]. To assess this pathway's activity in our 2D cell migration system, with Atox1 upstream, we used PLA and examined the individual proximity of Atox1 and ATP7A with the LOX proenzyme in MDA-MB 231 cells. As ATP7A brings Cu to the LOX proenzyme, ATP7A-LOXPP interactions were detected (Paper I). Figure 14 reveals an unexpected observation of Atox1-LOXPP in close proximity, suggesting that the transfer of Cu from Atox1 to ATP7A and then to the LOX proenzyme (LOXPP) might be a coordinated sequence of events or that Atox1 might directly interact with LOXPP (Paper I)

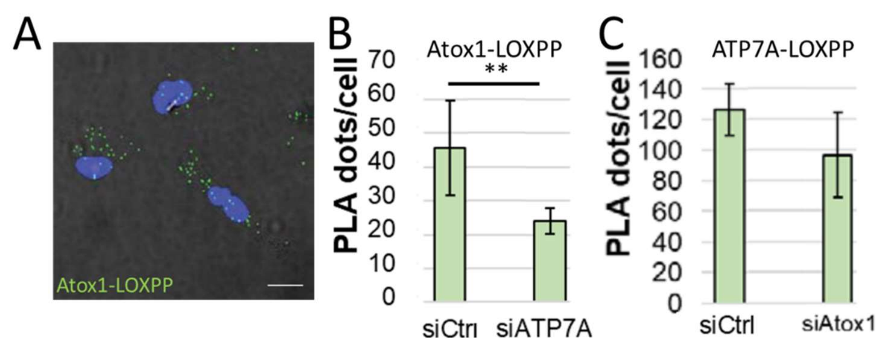

Figure 14. PLA reveals proximity between Atox1, ATP7A and LOXPP. (A) Atox1-LOXPP PLA confocal fluorescence microscopic images. Maximum projection confocal micrograph (blue=DAPI, indicating the nucleus; green=PLA dots) merged with the bright field image. Scale bars indicates 10 µm. (B) Quantitative results (Atox1-LOXPP: n_{Exp}=3 and ATP7A-LOXPP: $n_{FOV/Exp}$=5). Error bars indicate the standard deviations of the mean. *P*-values for student´s t-test. **P < 0.01

Further investigations are required to confirm the specific involvement of LOX with Atox1 and ATP7A in MDA-MB 231 cell migration, despite its known contribution to cancer cell migration and metastasis. Overall, the results of Paper I highlight the crucial role played by Atox1 in driving aggressive breast cancer cell migration. The results suggest that Atox1 mediates such cells' migration by being close to ATP7A and the LOX proenzyme. The purpose of this potential interaction chain is likely to facilitate Cu transport. Cu delivery to the LOX proenzyme is essential for LOX function in extracellular matrix remodeling.

Understanding the mechanisms and pathways of cell migration is crucial. To investigate the Atox1 homolog's role in developmental cell migration processes, the model organism *C. elegans* was used (Paper II). *C. elegans* is a good model organism for studying cell migration *in vivo* due to its translucent body and limited morphology [139]. CUC-1 and CUA-1 are worm homologs to Atox1 and ATP7A/B, respectively, demonstrating Cu transport functions. Furthermore, CUA-1 behaves similarly to ATP7A/B in humans, exhibiting Cu-dependent cell redistribution [60].

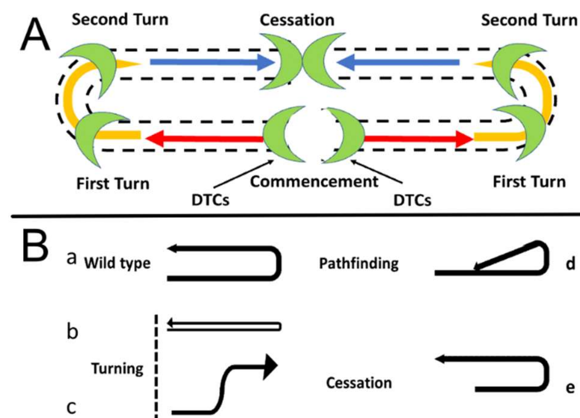

Figure 15 (A) Scheme of the U-shaped migratory path of DTCs cells (green).Phase 1: larval stage 2 (red), phase 2: larval stage 3 (yellow) and phase 3: larval stage 4 (blue). (B) DTCs migration defects patterns;migration direction shown as arrow.

Distal tip cells (DTCs) movement during larval development is responsible for post-embryonic gonad morphogenesis [140]. The two DTCs travel in opposite directions along the ventral basement membrane and turn towards the dorsal side with proper cues. They then turn twice and back towards the midline, forming a U-shaped gonad (Figure 15A). Many genes are involved in the DTC's migration, including the integrin cell adhesion receptor, the netrin guidance system, and matrix metalloproteinases [141, 142]. Malfunctioning DTCs migration can lead to various gonad development defects (Figure 14B), like turning, pathfinding, and cessation issues detectable through microscopy.

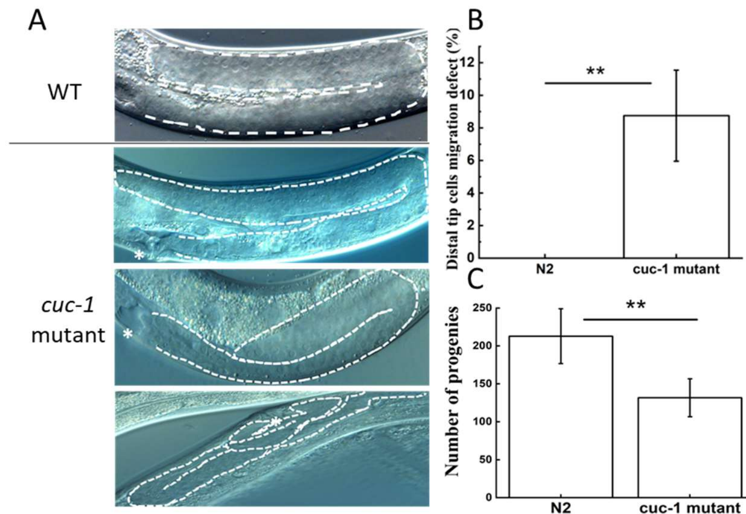

Figure 16. Camparison of *cuc-1* mutant versus wild-type (N_2). (A) Images of DTCs migration defects found in *cuc-1* mutant. Asterisks indicate vulva. White dashed lines indicate gonad shapes. (B) Quantification of DTC migration defects in *cuc-1* mutant (n_{group}=40, three groups in total). (C) Brood size assay (n_{group}=10). *P*-values for student´s t-test. $^{**}P < 0.01$

Paper II utilized the DTCs migration in situ model to study CUC-1, where *C. elegans* with and without the CUC-1 were compared. The *cuc-1* gene deletion resulted in defective DTCs migration patterns and decreased brood size (Figure 16). These findings indicate that CUC-1 (Atox1 homolog) may be involved in developmental cell migration processes besides its traditional function in cytoplasmic Cu distribution.

Paper I and II collectively demonstrate the involvement of Atox1 and its homologs, acting as a Cu chaperone, in cancer cell lines and cell migration in model organism *C. elegans* cell migration. This is potentially facilitated through the Cu transport pathway. However, since Atox1 is involved in cancer cell migration, it is crucial to identify other potential Cu proteins that may interact with it. For example, Memo1, a Cu-binding protein, also affects breast cancer cell migration. Therefore, the next section of this thesis follows a summary of our results for Memo1.

6.2 Memo1 Cu binding and potential interaction with Atox1 *in vitro*

In 2008, a putative metal-binding pocket was identified in Memo1's high-resolution structure, but no metal ion binding was observed [107]. MacDonald's study suggested that Memo1 may be a Cu(II) binding protein that generates ROS and promotes oxidization, but this was only observed in cell cultures by adding excess Cu(II) [105]. Both Qiu's [107] and our study found that excess metal ions (Cu and zinc) can induce Memo1 protein precipitation (Paper III). Hence,

the protein's ability to bind Cu(II) is still unclear. Several unanswered questions remain regarding the molecular interaction between Memo1 and Cu and its connection to ROS.

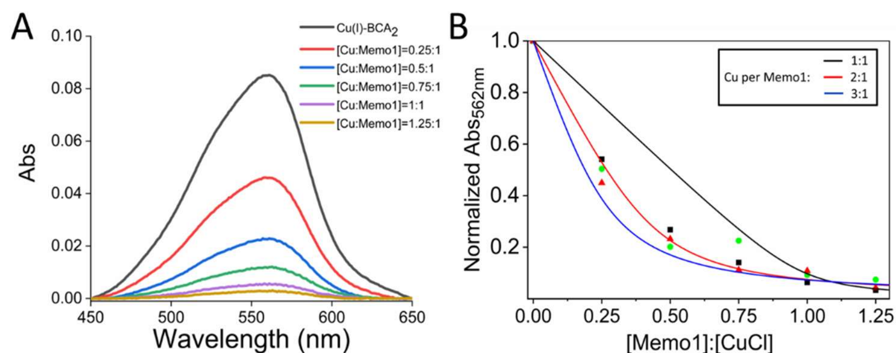

Figure 17. (A) Cu(I)-BCA_2 competition assay with Memo1 at strict anaerobic conditions (B) Normalized absorbance at 562 nm plotted as a function of added Memo1 results for the best fits assuming one (black), two (red) or three (blue) independent Cu(I) site per Memo1 protein.

In Paper III, I purified, folded, monomeric Memo1 protein at pH 7.4, and used various methods, including spectroscopy and titration calorimetry, to confirm Memo1's Cu(II) binding. However, there was no spectroscopic or energetic evidence of this interaction. Subsequently, in order to explore the possibility of Cu(I) binding to Memo1, the Bicinchoninic acid (BCA) ligand was used. BCA is a Cu(I) chelator for competitive Cu(I) binding. The Cu(I)-BCA_2 complex produces an absorption peak at 562 nm. Titrating Memo1 to pre-formed Cu(I)-BCA_2 under anaerobic conditions caused absorption at 562 nm decrease, indicating that Memo1 competes with BCA for Cu(I) (Figure 17A). I fitted the titration data to a simple model (described in Paper III, SI), which suggests that two independent Cu(I) sites might exist (Figure 5) per Memo1 with the same affinity of 4×10^{-15} M (Figure 17B). However, when excess BCA was added to a mixture of Memo1 and Cu(II), it did not form a visible absorption from the Cu(I)-BCA_2 complex. Memo1 can not reduce Cu(II) to Cu(I) (Paper III). This result is in contrast to the findings of MacDonald's Paper [105].

Atox1, which transfers Cu(I) to proteins in the secretory pathway, has been identified as a potential candidate for loading Cu onto Memo1. To investigate the interaction between Memo1 and Atox1, SPR and PLA were used (Figure 18). The SPR results show that Memo1 interacts with Cu(I) loaded Atox1 but not with apo-Atox1, indicating the interaction depends on Cu(I). Furthermore, the affinity between Memo1 and Cu(I) loaded Atox1 was found to be low-micromolar (2.1×10^{-7} M) according to SPR analysis (Figure 18A). Additionally, PLA assay

results (Figure 18B) demonstrate the close proximity of Memo1 and Atox1 in MDA-MB 231 cells.

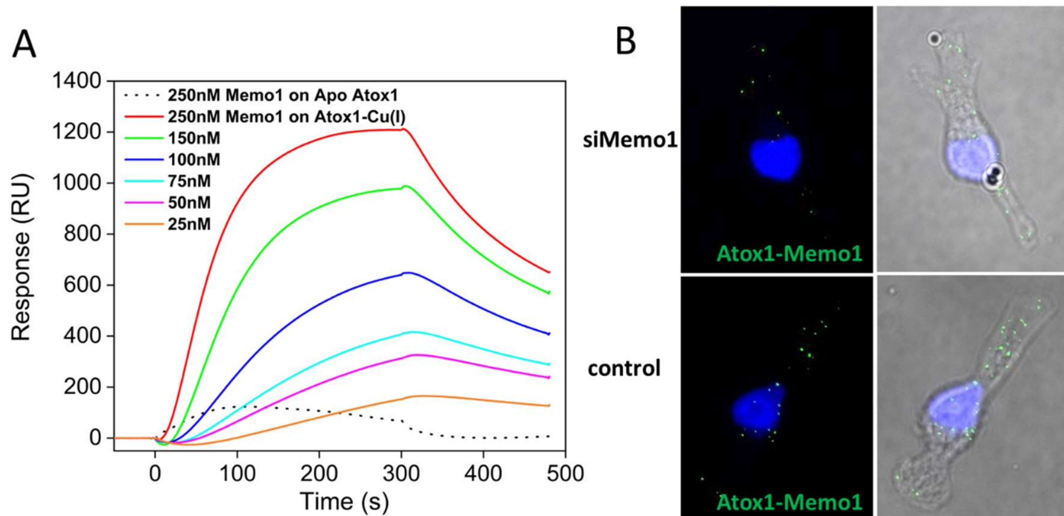

Figure 18. Investigation of Memo1-Atox1 potential interaction (A) Memo1 titration to Cu(I)-loaded Atox1 by SPR (solid curves). Memo1 injected onto to apo-Atox1 surface (dashed curve).(B) Confocal microscopy images of Memo1-Atox1 proximity in Memo1 knockdown (upper panel) and control (lower panel) MDA-MB 231 cells using PLA. Left panel: confocal fluorescence images; Right panel: overlay of the confocal fluorescence images with the bright field images.(blue=DAPI, indicating nucleus; green=PLA dots)

By utilizing ascorbic acid as a reducing agent, absorption changes at 265 nm can be used to monitor the Cu redox cycling process (as illustrated in Section 3.3 Figure 3), which reflects the oxidation of ascorbic acid [143].

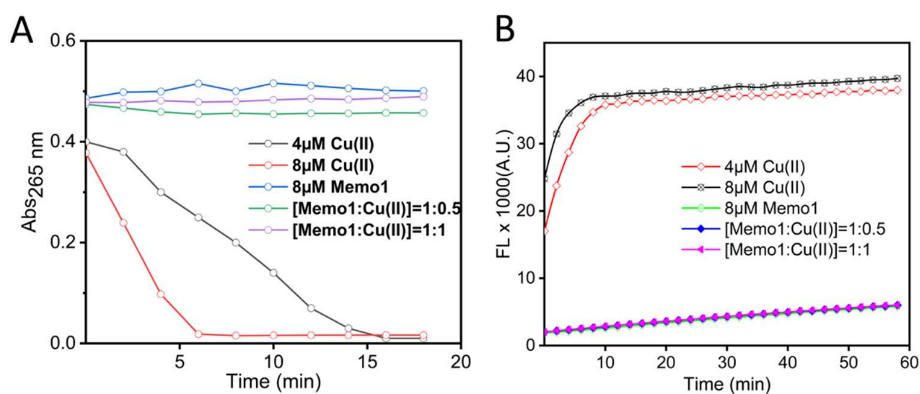

Figure 19 (A) Kinetics of ascorbate acid oxidation catalyzed by Cu in absence and presence of Memo1. (B) Kinetics of hydrogen peroxide production in absence and presence of Memo1 using Amplex Red.

In addition, to track the generation of H_2O_2, Amplex Red can be employed, as it is converted from a non-fluorescent to a red fluorescent product, resorufin, with the presence of H_2O_2 [144].

When Cu(II) is present without Memo1, ascorbate is quickly consumed. This caused a rapid decrease in 265 nm absorption over time and a significant increase in Amplex Red assay fluorescence (Figure 19). However, adding Memo1 to Cu(II) inhibits Cu redox cycling, and H_2O_2 or ascorbate oxidation is not detected (Figure 19). These results support the conclusion that Memo1 binds Cu(I) to prevent the metal ion redox reactions and suppress ROS production (Paper III).

6.3 The effect of Memo1 expression level on breast cancer ROS production

Cancer cells exhibit higher levels of ROS due to their increased metabolic activity compared to normal cells [145]. However, excessive Cu and ROS can kill cancer cells [146, 147]. Therefore, a balanced ROS level is crucial for maintaining cellular homeostasis, which involves ROS production and detoxification [145]. Cu proteins, such as SOD1, are involved in the detoxification of ROS by catalyzing the conversion of $O^{2.-}$ to H_2O_2. The latter is then further detoxified by catalase or glutathione peroxidase [148]. Memo1 has been shown to bind with Cu(I) and prevent Cu redox cycling *in vitro*, thereby decreasing ROS production [Paper III]. A subsequent investigation on how Memo1 influences intracellular ROS was carried out in Paper IV.

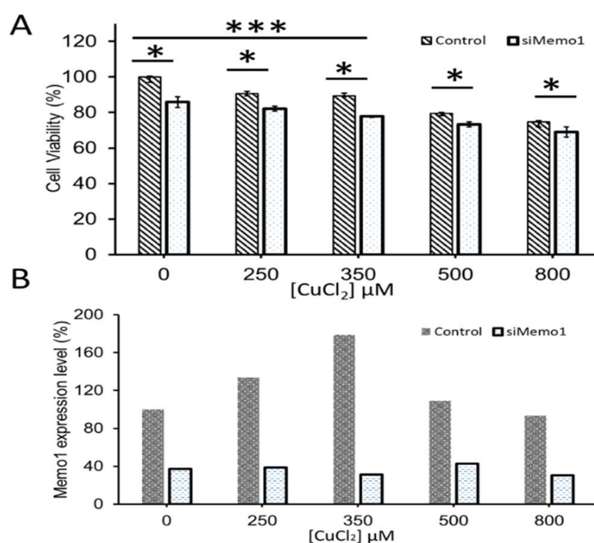

Figure 20. Cancer cell viability and Memo1 expression as a function of Cu. MDA-MB-231 cells were incubated with 0, 250, 350, 500 and 800 μM $CuCl_2$ for 18 hours, followed by MTT assay for (A) cell viability analysis and (B)Western blotting for quantification of Memo1 expression level. Error bar; standard error of the mean (n=4 per condition) *P*-values for student´s t-test. *P<0.05,***P < 0.001

The effect of external excess Cu on cell viability was explored using the MDA-MB-231 breast cancer cell line and Cu levels ranging from 250 to 800 µM. To determine cell viability, we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay that probes active metabolism [149]. The results indicate that incubation of cells with up to 350 µM Cu for 18 hours did not significantly affect cell viability. However, higher Cu levels resulted in a more significant decrease in cell viability (Figure 20A). Furthermore, Memo1 expression levels were examined. While Cu treatment with concentrations up to 350 µM, resulted in an increased Memo1 expression increased, higher Cu levels resulted in decreased Memo1 expression levels, probably related to increased cell death (Figure 20B).

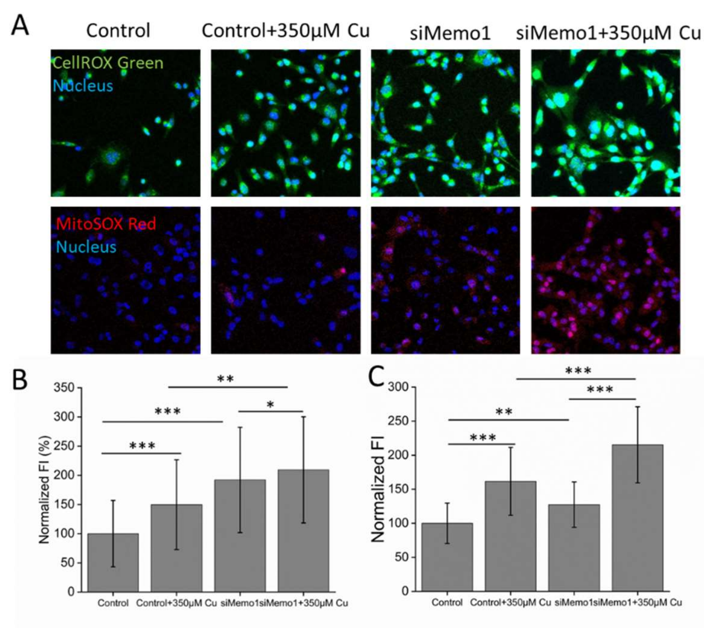

Figure 21. ROS levels as a function of Cu and Memo1. (A) ROS production in control and Memo1 knockdown with and without 18 h $CuCl_2$ incubation. CellROX Green (green) and MitoSOX Red (red) confocal fluorescence images display the production of ROS. Hoechest 33342 stained nuclei (blue). (B,C) Flow cytometry analysis of ROS production in cells (detected as dye fluorescence) with and without Memo1 silencing incubated with and without 350 µM Cu for 18 h. Normalized fluorescent intensities of (B) MitoSOX Red and (C) CellROX Green. *P*-values for student´s t-test. *P<0.05, **P<0.01***P < 0.001

Additionally, ROS levels were measured in cells via three probes under four conditions – normal and knockdown Memo1 expression and Cu levels of 0 or 350 µM. When H_2O_2 levels were measured using the ROS-Glo^{TM} assay [150], upon Cu addition, H_2O_2 levels increased by approximately 50% in Memo1 knockdown cells (Paper IV). To further analyze oxidative state and mitochondrial superoxide levels, the CellROX^{TM} Green and MitoSOX^{TM} Red probes were utilized, respectively. Confocal fluorescence microscopy images and flow cytometry

experiments demonstrated that Cu addition increased ROS levels, further exacerbated by Memo1 knockdown for both ROS probes (Figure 21).

Our study demonstrates that the presence of Memo1 in breast cancer cells is crucial because it limits ROS levels and promotes cancer cell survival (Paper IV). Notably, the reduction of Memo1 expression amplifies these effects significantly (Figure 21). These in-cell outcomes are consistent with the previous *in vitro* studies of purified Memo1 protein (Paper III). Additionally, the loss of Memo1 in *C. elegans* has been linked to increased ROS levels in the organism [106], which supports our presented results. Memo1 may act as a Cu chelator to protect cells against uncontrolled ROS formation, as reported in Paper III and IV.

7. Concluding remarks

Cancer remains a major public health concern and is a major cause of death worldwide, causing immense suffering for patients and their loved ones. Unfortunately, while current cancer therapies have improved significantly in the last couple of decades, they still have limitations that adversely affect patients' quality of life. Furthermore, the available treatments are still not sufficient for all types of cancers. As a result, for some cancer types, the survival percentage of cancer patients is still very low. Therefore, it is crucial to continue researching better cancer diagnosis and therapy to minimize these limitations, improve patient treatment outcomes, understand the mechanism behind cancer triggers and progression and find a cure.

Cu is an essential trace element for normal physiological functions and plays a significant role in cancer. Cu acts as a cofactor for several enzymes involved in cellular proliferation [50] and metastasis, involving migration and invasion of cancer cells [151]. This thesis is a compilation of my efforts to understand the role of Cu-binding proteins in cancer and their potential as a target for the cancer-related process.

The investigation of the role of Atox1 in breast cancer cell migration presented in Paper I reveals that the Cu chaperone activity of Atox1 is involved in the process. Furthermore, the work of Paper I connects the role of Atox1 in breast cancer cell migration to Atox1-ATP7A-LOX Cu transport pathway (Paper I). Paper II of *C. elegans* CUC-1 (Atox1 homolog) studies indicate that Atox1 homologs might also be involved in developmental cell migration processes. Identification of Memo1 Cu binding and the potential interaction with Atox1 *in vitro* (Paper III) proves that Memo1 can bind Cu(I) and that Memo1-Atox1 interaction is one possible route of Cu transfer. Paper III and Paper IV indicate that Memo1 functions as a Cu chelator leading to down-regulation of ROS generation at cellular and molecular levels.

Cu chelation therapy in cancer treatment shows potential. This therapy involves using agents that bind to Cu ions and removes them from the body [49]. Tetrathiomolybdate (TM), initially designed for Wilson's disease [152], is a Cu chelator that depletes systemic Cu levels and has been proven to be effective in inhibiting tumor growth and angiogenesis [153]. However, TM treatment for cancer patients is still in clinical trials. The phase II clinical trial results led to favorable overall survival rates in women with high-risk breast cancer but didn't have similar effects on patients with other cancer types [49, 154]. More studies must be done before Cu chelation can be used as a cancer therapy. Furthermore, this thesis on Cu-binding proteins

Atox1 and Memo1 further supports the potential of Cu-binding proteins as new targets for cancer therapy.

However, further investigation is necessary to understand the molecular mechanisms that underlie the functions of Memo1 and Atox1 in cancer cells. Memo1 has been shown to interact with intracellular LOX [155]. Since Memo1, LOX, and Atox1 bind Cu(I), there may be a relationship between LOX, Atox1, and Memo1 via Cu binding, which might function in breast cancer metastases. Therefore, studying the interaction between Memo1, LOX, and Atox1 may be a promising direction for future research.

In summary, the findings presented in this thesis highlight the importance of Cu and Cu-binding proteins in cancer and suggest that a possible therapeutic intervention can come from targeting these proteins for cancer treatment.

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