COPPER-INDUCED ACTIVATION OF TRANSCRIPTION FACTOR EB AND ITS DUAL EFFECT ON LYSOSOMAL EXOCYTOSIS

by

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Transition metals toxicity is an important factor in pathogenesis of numerous human disorders, including neurodegenerative diseases. Lysosomes have emerged as important factors for transition metal toxicity because they can handle transition metals via endocytosis, autophagy, absorption from the cytoplasm and exocytosis. Transcription factor EB (TFEB) regulates lysosomal biogenesis and the expression of lysosomal proteins in response to lysosomal and/or metabolic stresses. Since transition metals cause lysosomal dysfunction, I proposed that TFEB may be activated in order to drive gene expression in response to transition metal exposure and may influence transition metal toxicity. I found that transition metals copper (Cu) and iron (Fe) activate overexpressed TFEB and stimulate the expression of TFEB-dependent genes in TFEB-overexpressing cells. Specifically, in cells that show robust lysosomal exocytosis, TFEB was cytoprotective at moderate levels of Cu exposure, decreasing oxidative stress as reported by the expression of *HMOX1* gene. However, at high levels of Cu exposure, particularly in cells with low levels of lysosomal exocytosis, activation of overexpressed TFEB was toxic, increasing oxidative stress and mitochondrial damage. In addition, I found that Cu has a dual effect on lysosomal exocytosis: when cells were exposed to Cu for a prolonged period of time, lysosomal exocytosis was reduced, whereas brief exposure to Cu activated lysosomal exocytosis. Cu-induced lysosomal exocytosis depends on calcium (Ca) and the lysosomal SNARE VAMP7. Furthermore, depletion of ATP7B, a Cu pump recently shown to be involved

in lysosomal exocytosis, suppressed the basal lysosomal exocytosis, but did not affect the ability of Cu to activate lysosomal exocytosis. ATP7B knockdown was associated with sustained oxidative stress. The removal of Ca from the extracellular medium, but not the extracellular addition of Ca channel blocker lanthanum (La) suppressed the Cu-dependent component of the lysosomal exocytosis. Based on these data, I conclude that the TFEB-driven gene network participates in the cellular response to transition metals and that exposure to Cu promotes lysosomal exocytosis in order to avoid the toxic effects associated with prolonged exposure to Cu.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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1.0 INTRODUCTION

1.1 OVERVIEW

Trace levels of transition metals are essential for life and they participate in many cellular functions. However, high levels of transition metals are toxic and may cause oxidative stress and eventually lead to cell death. Therefore, cells have evolved mechanisms to tightly regulate transition metals levels. Among these mechanisms, the lysosome plays a central role by storing and excreting transition metals. Additionally, in recent years, the gene network involved in lysosomal biogenesis and function, which is controlled by transcription factor EB (TFEB), has been established. TFEB is activated in response to stress and its discovery has positioned the lysosome as a signaling organelle able to respond to different stress conditions. However, to date, it is not known whether TFEB is activated in response to stress induced by transition metals. Furthermore, it is not clear how metals affect lysosomal function and regulation. Over the course of my doctoral studies, I have been interested in understanding how lysosomes respond to stress induced by transition metals, particularly copper. Specifically, I have investigated the role of TFEB in copper regulation. In addition, my research has focused on investigating how copper regulates lysosomal exocytosis, a process required for elimination of copper from the cell. The data obtained from my research has greatly contributed to the understanding of how lysosomes regulate transition metal homeostasis.

In the following chapters I will discuss the current literature and the results obtained during my doctoral research. This dissertation is divided as follows: Chapter 1 includes detailed background information regarding my research; Chapter 2 describes the experimental approaches and materials used to carry out my research; Chapters 3 and 4 include the results obtained during my doctoral research, the former is currently *in press* in the *Biochemical Journal*, while the last was published in *Cell Calcium* [1]. Finally, Chapter 5 contains the conclusions of the work presented here and different approaches to address the questions that arose from my research.

1.2 TRANSITION METALS IN BIOLOGY

Transition metals are essential to carry out different cellular functions. They include copper (Cu), iron (Fe), zinc (Zn), cobalt (Co), and nickel (Ni), among others. Under physiological conditions, they can exist in different valence states, this property gives them the versatility to participate in numerous reactions as enzymatic cofactors and play critical roles in cellular respiration [2]. Several defects have been associated with unbalanced levels of transition metals [2]. Thus the existence of regulatory mechanisms is crucial to maintain cellular transition metals homeostasis. Transition metal deficiency often results in loss of enzymatic activity, while overdose causes oxidative stress by generating reactive oxygen species (ROS) that lead to cell death [2, 3]. Furthermore, accumulation of transition metals has been associated with numerous neurodegenerative disorders and lysosomal storage disorders [2, 4].

In this section I will explain the importance of transition metals in cellular function as well as their regulation and diseases in which their deregulation is involved. Since the focus of

my dissertation is the role of lysosome in Cu regulation, the following introductory section is centered on the role of Cu in cellular functions and diseases.

1.2.1 Role of transition metals

Transition metals are required at very low levels for many cellular functions (Figure 1). In humans, Cu is found at concentrations ranging from 1.4 to 2.1 mg/kg [2]. Normal Cu levels in serum have been reported to range between 18.1 and 31.5 μM [5, 6]; however, under pathological conditions serum Cu concentrations have been found at 205 μM [7]. Although Cu toxicity is rare, there are many genetic conditions and diseases that can result in elevated concentrations of Cu [2, 8]; some of these diseases will be discussed in section 1.2.3. In addition, environmental exposure to Cu can result in high levels of Cu and toxicity. Cu levels in drinking water have been reported to be as high as 160 μM and in river waters Cu concentrations can reach 90 mM [9]. At the cellular level, the impact of Cu exposure may depend on different factors, such as cell type, concentration and length of exposure to Cu. In an effort to understand how cells respond to different levels of Cu, Song *et al* carried out a transcriptome study in hepatic HepG2 cells exposed to different concentrations of Cu [9]. They found that Cu concentrations of 100 and 200 μM activated the expression of genes involved in the normal physiological response to transition metals, such as metallothionein isoforms. On the other hand, exposure to higher levels of Cu of 400 and 600 μM increased the expression of genes associated with toxicological responses, indicating that these concentrations induce Cu toxicity [9]. In addition, they found that longer exposure (24 hours) increased Cu toxicity [9].

Fe is more abundant than Cu in humans and body concentrations of Fe are around 50 mg/kg. Typically, most of it is found in hemoglobin in blood cells and in myoglobin in muscle [10]. In addition, Zn is the most abundant trace metal in the cell, with cytoplasmic concentrations ranging between 100 and 500 μM [11].

In the cell, transition metals are mostly found bound to proteins. Studies have shown that one in four proteins contain a metal ion and most of these coordinating metals are transition metals [12]. There are about 30 Cu-containing proteins; most of them are extracellular proteins with the exception of a few cytoplasmic proteins that are involved in protection and Cu detoxification [2]. Moreover, due to the ability of Cu to cycle between oxidized and reduced states, a large number of enzymes utilize Cu to carry out electron transfer reactions [2]. Most of these Cu-utilizing enzymes are involved in redox reactions and energy production in the mitochondria, including cytochrome c oxidase, also known as complex IV of the mitochondrial electron transport chain [2, 13]. In Cu-containing proteins, Cu can be found in three types of Cu centers. In type 1 Cu centers (or blue Cu centers), Cu is coordinated between two nitrogen atoms and sulfur atoms; type 1 Cu centers are found in proteins involved in electron transfer reactions, such as cupredoxins [2]. Type 2 Cu centers have Cu bound by two or three nitrogen or oxygen atoms; these Cu centers are often found in proteins involved in the catalysis of reactive oxygen species (ROS) [2]. Finally, Cu can be found in type 3 Cu centers, where Cu binds to oxygen or hydroxyl groups; they are found in multicopper oxidases [2, 13]. Although Cu is not an abundant metal, it plays an essential role in redox reactions involved in energy production and detoxification of metals.

Fe-binding proteins are also essential to carry out several reactions in the cell. Fe can be inserted into protoporphyrin IX, forming the heme prosthetic group found in proteins such as hemoglobin, myoglobin, and cytochromes, or it can be assembled into Fe-S (iron-sulfur) clusters which can be found in several proteins, including ferredoxins, nitrogenases, and hydrogenases [2, 14, 15]. In addition, Fe can bind proteins directly to the polypeptide chain, these Fe-binding proteins are known as non-heme Fe proteins [2]. Due to the variety of Fe binding proteins, Fe is essential for many cellular functions, such as, oxygen transport, cellular respiration, and DNA synthesis [16]. Figure 1 summarizes the cellular functions in which transition metals are involved.

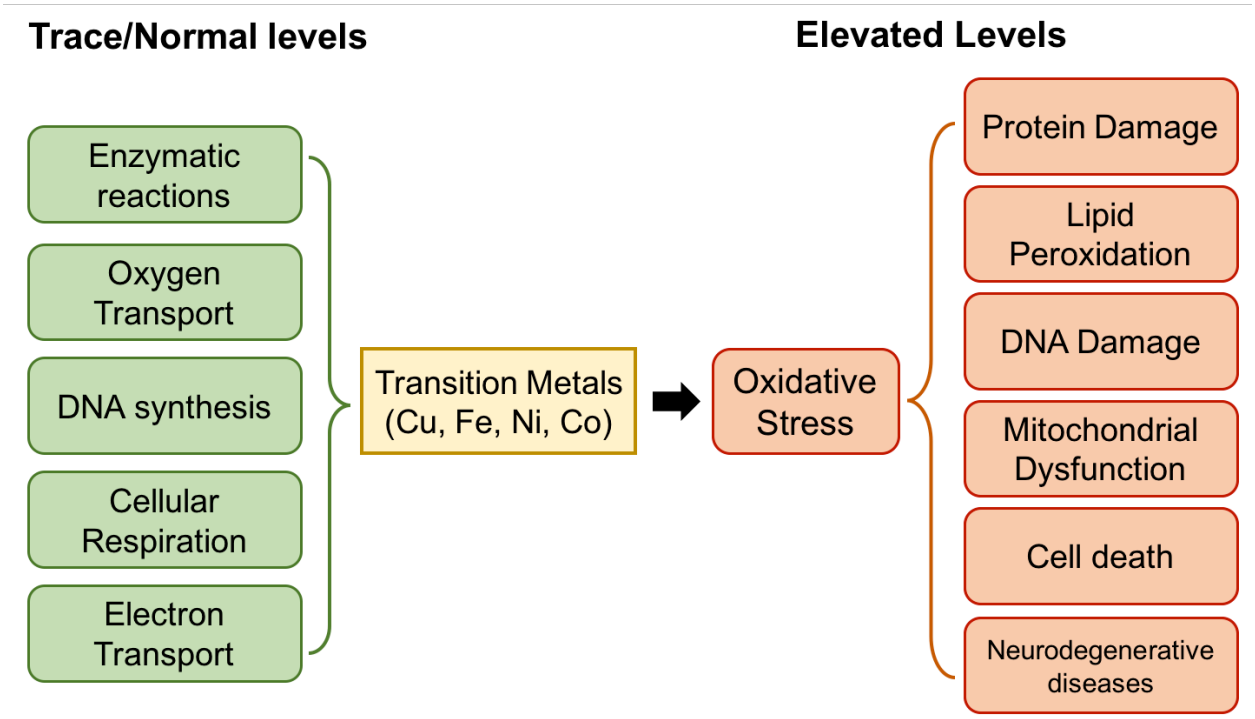

Figure 1. Role of transition metals in biology

Diagram summarizing the role of transition metals in human biology. Trace levels of transition metals are essential to carry out many cellular functions (green boxes). Elevated levels of transition metals cause oxidative stress, resulting in cellular damage and cell death (red boxes). Importantly, transition metals toxicity has been associated with numerous neurodegenerative diseases.

1.2.2 Regulation of cellular transition metals

As depicted in Figure 1, high levels of transition metals can cause oxidative stress. The same redox versatility that makes transition metals essential for many cellular reactions also makes them excellent catalysts for the production of ROS. In particular, both Cu and Fe can participate in Fenton reactions, in which hydrogen peroxide breakdown produces the extremely reactive hydroxyl radical [17]. In order to avoid the toxic effects of free transition metals, cells have evolved mechanism to regulate their levels. One of these mechanisms is the transcriptional regulation of metallothioneins (MTs), cysteine-rich metal binding proteins that chelate free cytoplasmic transition metals in order to maintain their homeostasis [18, 19]. MTs expression is regulated by metal-responsive transcription factor-1 (MTF-1) [20]. MTF-1 is activated in response to several stress conditions, principally transition metals, but also hypoxia and oxidative stress [20]. Once MTF-1 is activated, it binds to the metal responsive element (MRE) in the promoter region of response genes inducing their transcription; among these genes are MTs [20]. MTs expression has been suggested to be the first cellular response to transition metals, including Cu [9, 20]. Another mechanism the cells employ in order to regulate cytoplasmic levels of transition metals is the transport of metals into membranous organelles, such as the Golgi apparatus and the lysosome, through specific metal transporters [10, 17, 21, 22]. The role of the lysosome in regulation of transition metals will be discussed in section 1.3.3.

1.2.3 Transition metals and disease

As previously mentioned, the cellular levels of transition metals are tightly regulated in order to avoid toxicity. Thus, it is not surprising that defects in regulation mechanisms would give rise to

diseases. Many of the diseases associated with transition metals are caused by mutations in the genes that regulate the levels of metals [2, 8]. Table 1 shows a list of diseases caused by mutations in genes involved in Cu homeostasis. Interestingly, mutations in two P-types ATPases cause diseases with opposite phenotypes: ATP7A mutations cause Menkes disease, which is characterized by Cu deficiency [2, 8, 21, 23]; on the other hand, ATP7B mutations cause Wilson's disease, whose main characteristic is the accumulation of Cu in the liver [2, 8, 21]. This difference may be related to the cellular localization of these two Cu transporters. ATP7A localizes to the plasma membrane transporting Cu inside the cell [24], whereas ATP7B is found in intracellular organelles, such as the lysosome, where its main function is to remove cytoplasmic Cu [22].

Cu is also involved in diseases where metal regulation pathways have not been directly affected by mutations. One example is the participation of Cu in Alzheimer's disease. Cu has been found in plaques and neurofibrillary tangles, both characteristic of Alzheimer's disease, interacting with amyloid precursor protein and β-amyloid protein [25]. Cu-induced oxidative stress has been proposed as a factor in the pathology of Alzheimer's disease [8, 21]. Cu accumulation has also been observed in brains of patients with Huntington disease [26]. Huntington disease is caused by mutations in the gene encoding huntingtin (htt) protein, resulting in the expansion of glutamine repeats causing the aggregation of the mutant htt protein. It has been proposed that increased Cu bound to htt leads to oxidative stress and neurodegeneration, both hallmarks of Huntington disease [8, 26]. Oxidative stress is also an important component of Parkinson's disease [8]. Mutations in the gene coding for α-synuclein are associated with the aggregation of α-synuclein into Lewy bodies in the brain. Such protein aggregation causes oxidative stress and could lead to cell death. It has been shown that Cu accelerates the

aggregation of α-synuclein, suggesting a possible link between Parkinson's disease and Cu imbalance [8, 27]. Another group of neurodegenerative diseases associated with Cu is prion diseases [8]. The cause of prion diseases is abnormal folding of modified prion proteins (PrPs). PrPs have sites of high affinity for Cu and *in vitro* studies have shown that Cu enhances stabilization and infectivity of PrPs. For these reasons, it has been hypothesized that Cu plays a role in the pathogenesis of prion diseases [28, 29]. Finally, Cu has also been shown to be elevated in models of Niemann-Pick C disease, a lysosomal storage disorder characterized by accumulation of cholesterol in the lysosome and increased oxidative stress [30].

Table 1. Diseases caused by mutations in copper-related proteins

Disease	Gene mutated	Protein function	Characteristics	References
Wilson's	*ATP7B*	Cu transporter	Accumulation of Cu in liver and brain. High levels of free circulating Cu. Hepatic cirrhosis and basal ganglia degeneration	[2, 8, 21]
Menkes	*ATP7A*	Cu transporter	X-linked recessive disorder. Cu deffiency and low levels of circulating ceruloplasmin	[2, 8, 21, 23]
Aceruloplasminemia	Ceruloplasmin	Multi-Cu oxidase. Transports Cu in plasma	Accumulation of iron. Degeneration of retina and basal ganglia. Diabetes mellitus	[2, 8, 31, 32]
Amyotrophic lateral sclerosis (ALS)	*SOD1*	Cu/Zn superoxide dismutase	Muscle atrophy, respiratory problems	[2, 8, 33]

1.3 THE MANY FUNCTIONS OF THE LYSOSOME

Lysosomes were first discovered by Christian de Duve in 1955 while studying insulin metabolism in the liver using differential subcellular fractionation [34, 35]. This fortuitous discovery awarded him the Nobel Prize and opened new ways of studying and understanding cellular organelles. Lysosomes were first identified as membranous organelles rich in hydrolytic enzymes, capable of degrading cellular material [35]. These organelles have an acidic lumen containing over 60 hydrolases that become active at low pH; they also have about 50 membrane proteins involved in the transport of substances to the inside of the lysosome and from the lysosome to the cytoplasm, acidification of the lysosomal lumen, and fusion of the lysosome with other membrane compartments [36, 37]. An interesting aspect of the lysosome morphology is the presence of a polysaccharide layer on the inner side of the lysosomal membrane, known as glycocalyx [36]. The main function of glycocalyx is to protect the lysosomal membrane from the action of the hydrolases present in the lumen. By this way, the acidic and lytic environment of the lysosome is efficiently isolated from the rest of the cell [36]. The acidic nature of the lysosomal lumen (pH 5) is maintained by the action of the vacuolar proton (H^+) pump ATPase (v-ATPase), a protein complex that pumps H^+ into the lysosome using the energy of ATP hydrolysis [38, 39].

Since its discovery, and due to the lytic nature of the luminal hydrolases, the lysosome has been considered an organelle mainly involved in the degradation and recycling of cellular material. However, research has shown that the lysosome is also involved in several cellular processes, such as exocytosis, membrane repair, and signaling [35, 36]. Thus, it is not surprising that defects in lysosomal proteins can lead to diseases. Lysosomal storage disorders (LSDs) are a

group of rare inherited diseases with an overall incidence of 1 in 5000 births [36]. About 60 LSDs have been identified; they are caused by mutations in genes coding for proteins involved in lysosomal function and are characterized by the accumulation of undegraded material in the lysosome, which often leads to neurodegeneration [36]. Thus, understanding the many functions of the lysosome may help in developing treatments for these diseases. Throughout this section I will discuss lysosomal functions focusing on autophagy and degradation, lysosomal exocytosis, and regulation of transition metals by the lysosome. The role of the lysosome as a signaling organelle will be discussed in section 1.4 as part of the transcriptional regulation of lysosomal function.

1.3.1 Autophagy and degradation

As mentioned above, the lysosome contains hydrolytic enzymes involved in the degradation of cellular material, including proteins, carbohydrates, lipids, and nucleic acids, as well as organelles and extracellular material. Extracellular material, including soluble proteins and plasma membrane receptors, arrives at the lysosome mainly via endocytosis. Endocytosed material is passed from early endosomes to late endosomes, which eventually fuse with the lysosome [36, 40]. The degradation process takes place in the lysosomal lumen through the action of hydrolases. The acidic pH of the lysosome is not only required for the action of the digestive enzymes, but it is also needed for the membrane fusion events required to deliver cargo to the lysosome [36]. This process of endocytosis followed by lysosomal degradation is very important to terminate the signaling of some plasma membrane receptors [36].

Delivery of material to the lysosome also takes places through a process known as autophagy, a catabolic pathway employed by cells to capture and degrade cytoplasmic material. So far, three types of autophagy have been described: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy [36]. Microautophagy refers to the process by which cytoplasmic material is directly engulfed by the lysosome, which uses protrusions or invagination of its membrane to capture the material to be degraded [36, 41]. During CMA, proteins are unfolded by the action of molecular chaperones and translocated to the lysosomal lumen through the lysosome-associated membrane protein 2A (LAMP2A) [36, 42]. Also, CMA is responsible for the degradation of 30% of cytosolic proteins when cells are deprived from nutrients for a prolonged period of time [42]. Finally, macroautophagy, from here referred to as autophagy, requires the formation of an autophagosome, a double-membrane organelle that fuses with the lysosome to initiate the degradation process [36, 43].

Autophagosome biogenesis starts with the formation of a small sac known as a phagophore or isolation membrane, which engulfs a portion of the cytoplasm. Once the autophagosome is formed, its outer membrane fuses with the lysosome originating a different compartment called the autolysosome, where the degradation process takes place [43]. The molecules generated from this degradation process, such as amino acids, are transported back to the cytoplasm where they can be recycled to generate new molecules or used for energy production [36, 43]. Thus, autophagy plays an important role maintaining cellular homeostasis, especially under nutrient starvation to maintain amino acids levels [43]. In addition, autophagy can be induced in response to other cellular stressors, such as hypoxia, energy depletion, and high temperatures [43].

1.3.2 Lysosomal exocytosis

Lysosomal exocytosis is the process by which the lysosome releases its content by fusing with the plasma membrane. It was originally described as a means of repairing plasma membrane injuries by recruiting lysosomal membrane to seal damaged plasma membrane [44]. Lysosomal exocytosis has also been implicated as a defense mechanism against bacterial infections [45]. For a long time it was thought that lysosomal exocytosis was a process only occurring in specialized secretory cell types, however, it is now known that lysosomal exocytosis can occur in almost any cell type [36, 46]. Importantly, lysosomal exocytosis plays a role in cellular clearance by eliminating undigested intralysosomal material [47].

The fusion of lysosomes with the plasma membrane is regulated by calcium (Ca) and depends on a specific set of SNARE components [46, 48, 49]. This fusion machinery involves the vesicular SNARE (v-SNARE) VAMP7 that is found on the lysosomal membrane along with the Ca sensor synaptotagmin VII. On the plasma membrane, SNAP23 and syntaxin 4 (types of t-SNAREs) are responsible for the fusion with lysosomes [48]. In addition, several Rab proteins localized to the lysosomal membrane have been shown to be important for the process of lysosomal exocytosis [36, 48, 50]. Furthermore, it has been shown that lysosomal Ca release through the lysosomal channel MCOLN1 is required for lysosomal exocytosis [47]; however, my recent published data show that lysosomal exocytosis also requires the entry of extracellular Ca [1]. Finally, lysosomal exocytosis has been recently shown to be important in regulating Cu homeostasis in the cell [1, 22]. The function of lysosomal exocytosis in transition metal regulation will be discussed in the following section.

1.3.3 Lysosomal regulation of transition metals

Lysosomes play an important role in regulating the levels of transition metals in the cell. As shown in Figure 2, transition metals enter the cell through specific plasma membrane transporters [51, 52] or through endocytosis of metal-binding proteins [53]. In addition, some transition metals can enter the cell bound to specific receptors. This process has been previously studied for Fe, which enters the cells bound to transferrin through endocytosis of the transferrin receptor [51, 52]. Once inside the cell, endocytosed metal-binding proteins follow the endocytic pathway, are degraded in the lysosome, and the protein-bound metal is released to the lysosomal lumen [17, 54, 55]. Another way of transition metals delivery to the lysosome is through specific metal transporters on the lysosomal membrane (Figure 2). The action of such transporters is important to remove free transition metals from the cytoplasm in order to avoid their toxic effects. An example of this is the regulation of cytoplasmic Zn levels. It has been shown that Zn transporters ZnT2 and ZnT4 are present in the lysosomal membrane [56, 57], and their suppression or overexpression causes deregulation of Zn homeostasis [56, 58, 59]. The regulation of Cu levels also involves a lysosomal Cu transporter. It has recently been shown that ATP7B localizes to the lysosomal membrane and is required to transport Cu from the cytoplasm into the lysosomes [22, 60, 61]. Finally, transition metals are also delivered to the lysosome through autophagy of metal-rich organelles, such as the mitochondria [10].

Due to the acidic and chemically active environment of the lysosomal lumen, accumulation of transition metals promotes Fenton-like reactions, generating ROS. As a consequence of ROS generation, the lysosomal membrane is disrupted and lysosomal enzymes are released to the cytoplasm, leading to cell death [17, 55]. In addition, transition metals induce

the buildup of lipofuscin, a product of peroxidation of material under degradation, which can be detrimental to the cell as it inhibits lysosomal function and autophagy [17, 54, 55]. Therefore, transition metals need to be taken out of the lysosome in order to avoid their toxicity inside the lysosome. One manner by which this is achieved is the action of transition metals transporters on the lysosomal surface that transport excess metals from the lysosomal lumen back to the cytoplasm. Two of these transporters are DMT1, which transports Fe and other metals [62, 63], and MCOLN1, whose loss appears to affect the distribution of Zn and Fe between the lysosomes and the cytoplasm [56, 64-66]. In addition, the human copper transporter 2 (hCTR2) has been shown to localize to the lysosomal membrane and be responsible for the transport of Cu from the lysosome to the cytoplasm [67]. Most importantly, lysosomal exocytosis has been shown to be an important mechanism for Zn and Cu removal [1, 22, 68]. In the case of Cu, lysosomal exocytosis is activated in order to excrete Cu from the cell. It has been shown that this process requires the action of ATP7B, which is translocated to the lysosomal membrane in order to transport Cu inside the lysosome [22]. Moreover, ATP7B is required for the transport of lysosomes towards the plasma membrane, as it has been shown to interact with the p62 subunit of the motor protein dynactin, facilitating lysosomal transport towards the apical pole and subsequently release of Cu, as seen in hepatic cells [22].

In summary, the lysosome plays a central role in the regulation of transition metals by storing and eliminating metals through lysosomal exocytosis. Deregulation of this process can result in increased ROS production and oxidative stress, followed by lysosomal membrane permeabilization and cell death. Therefore, understanding how the lysosome regulates transition metals is fundamental for finding treatments options for diseases associated with transition metal accumulation.

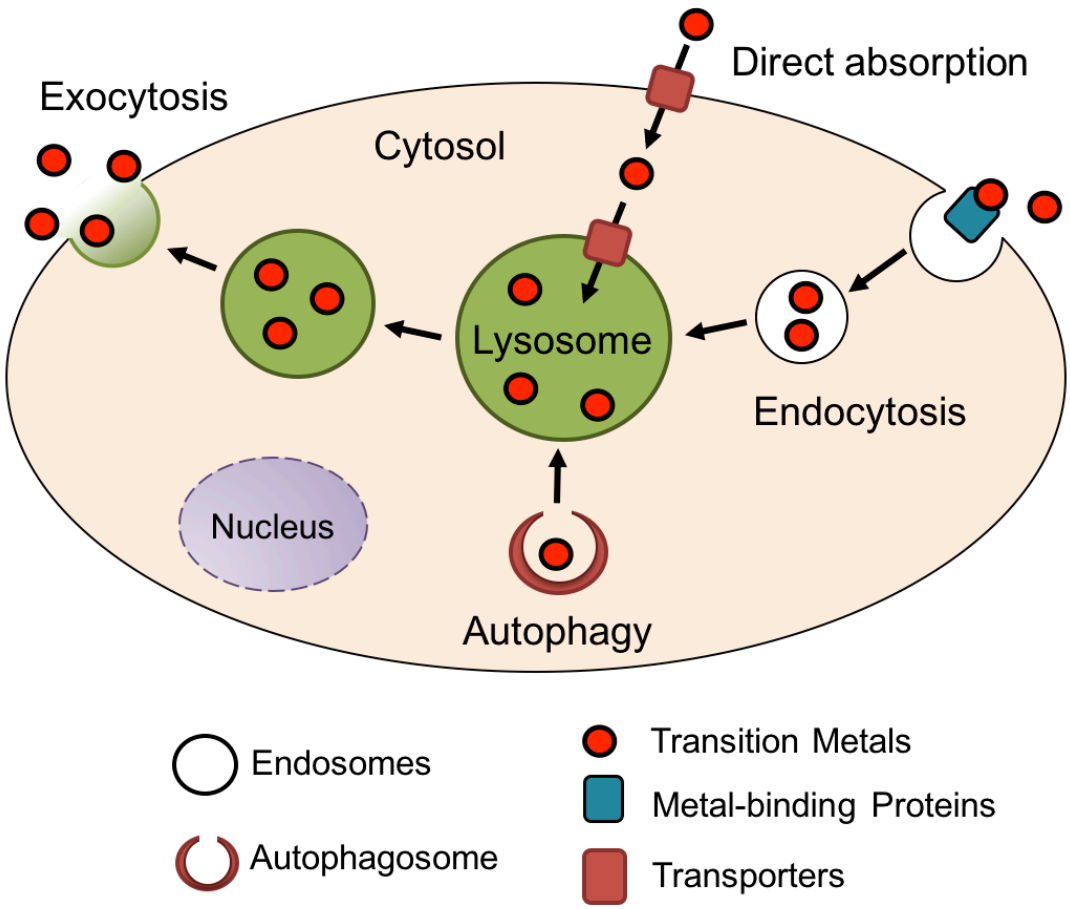

Figure 2. Lysosomal regulation of transition metals

Model of the regulation of transition metals by the lysosome. Transition metals enter the cell via endocytosis of metal-binding proteins or through plasma membrane transporters. Endocytosed proteins follow the endocytic pathway and are degraded by the lysosome. Autophagy of organelles also delivers transition metals to the lysosome. In order to remove transition metals from the cytoplasm, transporters on the lysosomal membrane transport metals into the lysosomal lumen. Lysosomal exocytosis is required to excrete transition metals that have been stored in the lysosome.

1.4 TRANSCRIPTIONAL REGULATION OF LYSOSOMAL FUNCTION

In recent years, a gene regulatory network has been proposed to control the function and biogenesis of lysosomes [69-71]. Transcription factor EB (TFEB) was initially described as the main transcription factor involved in this network [71]. In addition, recent studies have also implicated transcription factor E3 (TFE3) as responsible for regulating the expression of autophagy-related genes [72]. Both TFEB and TFE3 are transcriptional activators of genes involved in lysosomal function and biogenesis. Their overexpression and/or activation are sufficient to increase the expression of such genes and stimulate lysosomal biogenesis, autophagy, and lysosomal exocytosis [69-71]. In contrast, the transcriptional repressor ZKSCAN3 has been proposed to decrease the expression of lysosomal genes and suppress autophagy, working in coordination with TFEB [73]. These findings have opened a broad spectrum of possibilities to study the lysosome as a function of cellular status and have given new perspectives to treatment of lysosomal diseases.

Throughout this section, I will discuss and summarize the current knowledge about transcriptional regulation of lysosomal function and its therapeutic impact.

1.4.1 The MiT/TFE subfamily of transcription factors

The microphtalmia-transcription factor E (MiT/TFE) subfamily belongs to the family of basic helix-loop-helix (bHLH) leucine zipper transcription factors. In this subfamily, TFEB and TFE3 are included as well as the closely related proteins MiTF and TFEC. These four transcription

factors bind DNA both as homo- and heterodimers and all possible heterodimer combinations have been observed *in vitro*, but they are not necessarily *in vivo* [74]. Expression analysis in different human tissues has shown that these transcription factors are differentially expressed and the expression of each of them is regulated in a tissue-specific manner [74]. This aspect is very important to consider, especially when working with cultured cells. As it will be discussed in Chapter 3, low activity of both TFEB and TFE3 is detected in several cell lines, making the study of endogenous TFEB and TFE3 difficult. For this reason, and as discussed throughout this section, most of the studies regarding TFEB function, including my own research, have been done using recombinant TFEB [47, 70, 71, 75].

It has been shown that alternative splicing regulates the expression of these transcription factors [74]. In the case of human TFEB, seven alternative 5' exons have been found, originating seven transcripts (*TFEB-A* to *TFEB-G*) that encode the same protein [74]. All these TFEB transcripts are expressed in different tissues, but the level of expression of each transcript varies depending on the tissue. *TEFB-D* is the only transcript whose expression could not be detected by RT-PCR, probably due to very low levels of expression [74]. *TFEB-A* is mostly expressed in the placenta, which is in agreement with other studies suggesting a crucial role of TFEB in placental development in mice [76]. Expression of *TFEB-B* and *TFEB-C* can be detected in most tissues at similar levels, however *TFEB-C* is not expressed in the liver [74]. *TFEB-E* and *TFEB-G* are almost exclusively expressed in the brain, while *TFEB-F* is mostly expressed in the spleen. By contrast, only one transcript for the *TFE3* gene has been identified, showing similar levels of expression in all tested tissues [74].

Three splice variants for human TFEC have been identified (*TFEC-A* to *TFEC-C*); all of them originated from three alternatives 5' exons, with *TFEC-C* encoding a shorter protein, which is thought to act as a negative regulator of other MiT/TFE members [74]. TFEC transcripts have a limited pattern of expression: *TFEC-A* is mostly expressed in testis, thymus, trachea, colon, and prostate; *TFEC-B* can be detected in several tissues, except in heart and liver, while *TFEC-C* is only expressed in kidney and small intestine [74].

MiTF is one of the most well studied transcription factor of this subfamily, in terms of expression pattern and function. There at least eight isoforms of MiTF originated from several alternative 5' exons. These isoforms are differentially regulated in tissues and can be found in melanocytes, heart, and mast cells [77-80]. In addition, MiTF plays an important role in melanocyte development and regulation of pigmentation [80]. Mutations in the human gene encoding MiTF give rise to Waardenburg syndrome, which is characterized by hypopigmentation and deafness [80].

bHLH leucine zipper transcription factors, including all members of the MiT/TFE subfamily, bind the consensus DNA element E-box [74, 81]. The E-box consensus sequence is CANNTG, where N represents any nucleotide. The basic region of bHLH leucine zipper proteins recognizes and binds to the E-box. There are many transcription factors that recognize and bind to the E-box and regulate the expression of genes involved in developmental processes, such as cellular differentiation and lineage commitment [81]. In addition, E-box-binding transcription factors play important roles regulating development of different organs and tissues, including muscle, heart, pancreas, and neuronal cells [81]. Together, this evidence makes the E-box an important transcriptional regulator element.

1.4.2 TFEB and the CLEAR network

In an effort to identify a transcriptional regulator able to coordinate cellular status with lyosomal activity, Sardiello *et al* carried out a promoter analysis of 96 human genes encoding lysosomal proteins (from now referred to as lysosomal genes), identifying a 10 base pair (bp) palindromic motif, GTCACGTGAC [70]. This consensus sequence was also found in promoter regions of genes involved in lysosomal biogenesis and function and named Coordinated Lysosomal Expression and Regulation (CLEAR) element [70]. Interesting, the E-box sequence, CANNTG, is contained within the CLEAR element, which suggested that members of the MiT/TFE subfamily might regulate the expression of lysosomal genes. Next, Sardiello *et al* tested whether overexpression of TFEB, TFE3, TFEC, and MiTF affected the expression of lysosomal genes. Surprisingly, overexpression of TFEB, but not other MiT/TFE members, resulted in increased mRNA levels of lysosomal genes in both HeLa and human embryonic kidney 293 (HEK-293) cells; enhanced activities of the lysosomal enzymes Cathepsin D, β-glucosidase, and β-glucoronidase were also observed after TFEB overexpression [70]. In addition, overexpression of TFEB resulted in increased number of lysosomes [70] and autophagosomes [71], indicating that TFEB regulates both lysosomal biogenesis and autophagy. Increased expression of genes involved in autophagy has also been shown to be a consequence of TFEB overexpression [71].

Additional analysis of CLEAR-containing genes, using chromatin immunoprecipation (ChIP) assays, confirmed that TFEB binds to the CLEAR element in the promoter region of lysosomal genes and other genes involved in lysosomal function and biogenesis [69, 70]. Genome-wide studies in TFEB-overexpressing HeLa cells showed that TFEB directly regulates the expression of 471 genes, most of them being lysosomal genes or genes involved in lysosomal

function [69, 70]. Table 2 summarizes TFEB target genes with known participation in several lysosomal functions, including genes coding for hydrolases, lysosomal membrane proteins, proteins involved in lysosomal acidification, proteins involved in autophagy, and proteins involved in lysosomal biogenesis [69]. Interestingly, further analysis revealed that TFEB target genes are involved in a variety of cellular functions, including several biochemical and signaling pathways, DNA metabolism, and cell metabolism [69]. It is important to note that some of the genes regulated directly by TFEB, as shown by chromatin immunoprecipitation assays (ChIP), are involved in the oxidative stress response and redox metabolism, such as *HMOX1* (heme oxygenase 1), *GSTO1* (glutathione S-transferase omega 1), and *GSR* (glutathione reductase) [69, 70], which makes TFEB a good candidate to regulate metal-induced oxidative stress. These findings not only suggest a role of TFEB as a master regulator of lysosomal function and biogenesis, but also indicate that TFEB plays an important role regulating several cellular functions. Consequently, the lysosome may play a central role regulating such cellular functions.

Table 2. Genes involved in lysosomal function that are direct targets of TFEB

Category	Gene name
Lysosomal hydrolases and accessory proteins	*ASH1, CTSA, CTSD, CTSF, GAA, GALNS, GBA, GLA, GLB1, GNS, GUSB, HEXA, HEXB, IFI30, NAGLU, NEU1, PLBD2, PPT1, PSAP, SCPEP1, SGSH, TPP1*
Lysosomal membrane	*Clorf85, CD63, CLCN7, CLN3, CTNS, MCOLN1, SLC364A1, LAMP1, TMEM55B*
Lysosomal acidification	*ATP64P1, ATPV0A1, ATP6V0B, ATP6V0C, ATP6V0D1, ATP6V0D2, ATP6V0E1, ATP6V1A, ATP6V1B2, ATP6V1C1, ATP6V1D, ATP6V1E1, ATP6V1G1, ATP6V1H*
Non-lysosomal proteins involved in lysosomal biogenesis	*NAGPA, GNPTG, IGF2R, M6PR, BLOC1S1, BLOC1S3, HPS1, HPS3, HPS5, SUMF1*
Autophagy	*BECN1, GABARAP, HIF1A, NRBF2, PRKAG2, RAB7A, RRAGC, SQSTM1, STK4, UVRAG, VPS8, VPS11, VPS18, VPS26A, VPS33A, VPS35, WDR45*

Table modified from Palmieri *et al*., 2011 [69]

1.4.3 TFEB activation and lysosomal stress

TFEB overexpression seems to be sufficient to increase the expression of lysosomal genes, but there are other stimuli that activate TFEB function, therefore inducing the expression of lysosomal genes (Figure 2). Most of these stimuli cause lysosomal stress and affect lysosomal function. One important stressor is the accumulation of undegraded material in the lysosome. It has been previously shown that the expression of lysosomal genes, and other genes involved in cholesterol metabolism and intracellular trafficking, is increased in response to sucrose [82, 83]. Sucrose enters the cell through fluid-phase endocytosis and accumulates in the lysosome where it cannot be degraded, resulting in swollen lysosomes and lysosomal biogenesis [82]. Based on this evidence, Sardiello *et al* tested whether TFEB is activated upon sucrose exposure. Addition of

sucrose to the culture medium of HeLa cells stably expressing TFEB, resulted in an increase in TFEB binding to CLEAR element of lysosomal genes as well as an increase in the expression levels of these genes [70]. In addition, they also showed that sucrose stimulated the gradual translocation of TFEB from the cytoplasm to the nucleus [70]. In untreated cells, TFEB is mostly found in the cytoplasm, whereas after 24 hours of sucrose treatment it is predominantly localized to the nucleus, which correlates with the time point at which the expression of the CLEAR network genes starts increasing in response to sucrose [70]. Thus, nuclear localization of TFEB is required for its transcriptional activation.

Since the sucrose-induced phenotype is similar to the one observed in lysosomal storage disorders (LSDs), in which accumulation of undigested material is a common feature, Sardiello *et al* decided to assay the localization of TFEB in embryonic fibroblast from three different mouse models of LSDs: Mucopolysaccharidoses types II and IIIA (MPSII and MPSIIIA) and Multiple Sulfatase Deficiency (MSD) [70]. These embryonic fibroblasts were transiently transfected with TFEB and in all cases TFEB was found in the nucleus [70]. Therefore, accumulation of undegraded material in the lysosome induces TFEB activation, resulting in increased expression of lysosomal genes.

Another lysosomal stressor that has been shown to cause the activation of TFEB is nutrient starvation [71, 75, 84, 85]. Autophagy is activated during nutrient starvation in order to provide energy to the cell from the degradation of cytoplasmic material [86]. It has also been shown that starvation increases the expression of autophagy genes [87, 88]. Settembre *et al* showed that removal of nutrients from medium of HeLa cells resulted in increased expression of the CLEAR network genes [71]. The authors also showed that nutrient starvation caused nuclear

translocation of recombinant TFEB in stably transfected HeLa cells [71]. In addition to these observations, nutrient starvation induces the dephosphorylation of TFEB, which is highly phosphorylated under normal conditions [71, 75, 84, 85]. TFEB dephosphorylation is another important mechanism of TFEB activation and is required for its nuclear translocation. This was demonstrated by using a TFEB mutant unable to be phosphorylated, which is always localized to the nucleus [84, 85].

Increasing the pH of the lysosome also stimulates the activation of TFEB. Bafilomycin and chloroquine are two compounds that change the lysosomal pH and inhibit lysosomal function, primarily autophagy. Bafilomycin inhibits the vacuolar ATPase (v-ATPase), which is in charge of maintaining the acidic pH of the lysosome, while chloroquine is a weak base that accumulates in the lysosome and raises its pH [84]. Both compounds have been shown to induce nuclear translocation of TFEB and induce its dephosphorylation [75, 84].

In summary, there are three approaches to assay TFEB activation: TFEB dephosphorylation, nuclear translocation of TFEB, and increased expression levels of the CLEAR network genes. Under conditions of nutrient abundance and low lysosomal pH, TFEB is inactive and resides in the cytoplasm, whereas under conditions of nutrient starvation, lysosomal accumulation of undigested material, and high lysosomal pH, TFEB is dephosphorylated and translocated to the nucleus where it activates the expression of the CLEAR network genes. TFEB activation is summarized in Figure 3.

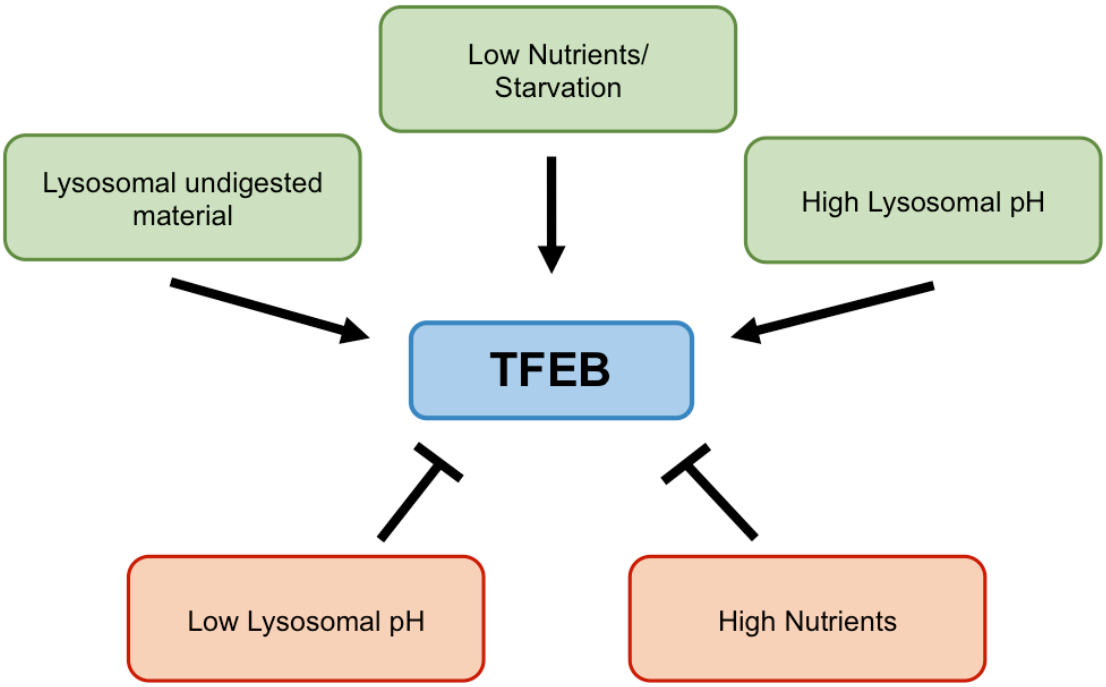

Figure 3. TFEB activity is regulated by environmental cues

Diagram summarizing the different stimuli that regulate TFEB activity. Normal cellular conditions, including abundance of nutrients and low lysosomal pH (red boxes), inhibit TFEB transcriptional activity. On the other hand, nutrient starvation and lysosomal stress, induced by the accumulation of intralysosomal undigested material or high lysosomal pH (green boxes), activate TFEB transcriptional activity.

1.4.4 TFEB activity is regulated by mTORC1

As mentioned, TFEB activation depends on its dephosphorylation. TFEB has been shown to be phosphorylated in two different serine, S142 [71] and S211 [75, 84, 85]; however, only S211 has been shown to be responsible for the modulation of TFEB activation [75, 84, 85]. Several studies have shown that the mammalian target of rapamycin (mTOR) phosphorylates TFEB in S211, causing its retention in the cytoplasm [75, 84, 85] (Figure 3).

mTOR is a serine/threonine kinase involved in the regulation of several cellular processes, including autophagy, cell growth, proliferation, and cell cycle; consequently mTOR is implicated in diseases in which those cellular process are deregulated, such as cancer, metabolic diseases, and aging [89]. mTOR is part of two different complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In both complexes, mTOR is the catalytic subunit and is accompanied by other accessory proteins. As shown in Figure 4, mTORC1 components are the regulatory-associated protein of mTOR (RAPTOR) and a negative regulator known as PRAS40, which is a 40 kDa proline-rich Akt substrate. On the other hand, mTORC2 components include the rapamycin-insensitive companion of mTOR (RICTOR) PROTOR1 and PROTOR2 (protein observed with RICTOR1), and the mammalian stress-activated map kinase-interacting protein 1 (mSIN1). In addition, both complexes share two other proteins, a positive regulator known as mLST8 (mammalian lethal with SEC13 protein 8) and the negative regulator, DEPTOR (DEP domain-containing mTOR-interacting protein) [89]. Although both mTORC1 and mTORC2 have similar components and they could potentially regulate the same cellular process, it has been shown that mTORC1 is regulated by nutrients, growth factors, energy, and cellular stress, while mTORC2 has only been shown to be regulated by growth factors [89].

mTORC1 activity and localization are regulated by amino acids and the Rag proteins, a family of small guanosine triphosphatases (GTPases) [90-92]. In the absence of amino acids, mTORC1 is found in the cytoplasm, but it is translocated to the lysosomal membrane when amino acids are added to the cell medium [91]. Rag proteins are required for the lysosomal localization of mTORC1, but not for its catalytic activation [91]. It has been shown that the protein complex Ragulator interacts with Rag proteins on the lysosomal surface and that they act as a scaffold for mTORC1 [90]. At the lysosomal membrane, mTORC1 interacts with Rheb, a small GTP binding protein, which in turn activates the kinase activity of mTORC1 [91, 93]. It has been proposed that the purpose of the lysosomal localization of mTORC1 in response to amino acids is to facilitate the interaction with its activator Rheb [92]. Thus, at the lysosomal membrane, mTORC1 has the ability to sense nutrient availability and elaborate a signaling response accordingly. In fact, it has been shown that mTORC1 can sense amino acid levels from the lysosomal lumen, as the removal of lysosomal amino acids prevented the recruitment of mTORC1 to the lysosomal membrane [94]. Moreover, the lysosomal pump v-ATPase interacts with Ragulator in an amino acid-dependent manner and it has been shown that v-ATPase activity is required for mTORC1 recruitment to the lysosomal membrane [94].

The fact that mTORC1 regulates autophagy, among other cellular functions, localizes to the lysosome, and senses amino acid levels, makes it a good candidate to regulate TFEB function. In support of this idea, in 2012 several groups identified mTORC1 as a regulator of TFEB function [75, 84, 85] (Figure 5). Under conditions of nutrient abundance, mTORC1 phosphorylates TFEB at S211. Since mTORC1 is localized to the lysosomal membrane, TFEB needs to be recruited to the lysosomal membrane as well. In fact, lysosomal localization of TFEB

has been observed under normal conditions [75, 84, 85, 95] and it has been shown that, similar to mTORC1, TFEB recruitment to lysosomes requires the action of Rag proteins [95]. In addition, phosphorylation of TFEB by mTORC1 induces TFEB interaction with 14-3-3 proteins, retaining phosphorylated TFEB in the cytoplasm [84, 85]. On the other hand, it has been demonstrated that the use of mTORC1 inhibitors, such as rapamycin and Torin-1, cause dephosphorylation of TFEB and its translocation to the nucleus, therefore causing TFEB activation [75, 84, 85]. Similarly, inhibition of mTORC1 via nutrient starvation can also activate TFEB [75, 84, 85]. Interesting, not only is TFEB regulated by mTORC1, but also it has been shown that MITF is recruited to the lysosomal membrane via Rag proteins, where it is phosphorylated by mTORC1 [95]. Accordingly, mTORC1 inhibition induces MITF translocation to the nucleus [95]. Thus, cellular conditions that cause the inactivation of mTORC1 would, in turn, activate TFEB or other members of the MiT/TFE family. This evidence suggests an important role of the lysosome as a signaling organelle able to regulate different cellular functions.

Recent evidence suggests that mTORC1 inhibition may not be the only mechanism to activate TFEB [96]. It has been shown that TFEB is dephosphorylated by the action of calcineurin, a phosphatase that requires Ca for its activity [96]. The authors showed that starvation stimulates the release of Ca from the lysosome through the lysosomal channel MCOLN-1; then, Ca release activates calcineurin and, therefore, TFEB [96]. This mechanism of TFEB activation would also result in increased lysosomal biogenesis and autophagy [96].

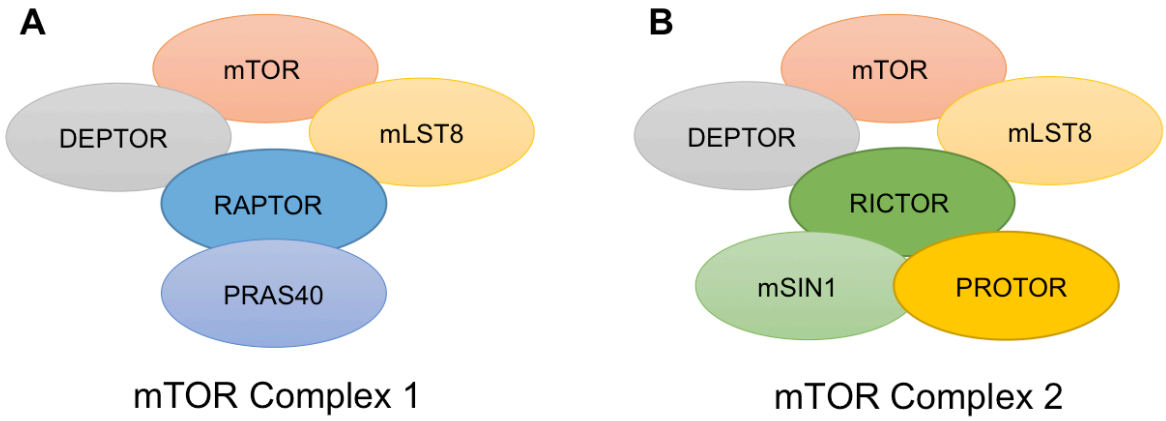

Figure 4. mTOR is the catalytic subunit of two multi protein complexes

A. Diagram representing mTOR Complex 1 (mTORC1). Unique components of mTORC1 include RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa). B. Diagram representing mTOR Complex 2 (mTORC2). mTORC2 has three proteins not found in mTORC1: RICTOR (rapamycin-insensitive companion of mTOR), PROTOR (protein observed with RICTOR), and mSIN1 (mammalian stress-activated map kinase-interacting protein 1). Both mTORC1 and mTORC2 share DEPTOR (DEP domain-containing mTOR-interacting protein) and mLST8 (mammalian lethal with SEC13 protein 8). Figure adapted from Zoncu *et al*, 2011 [89].

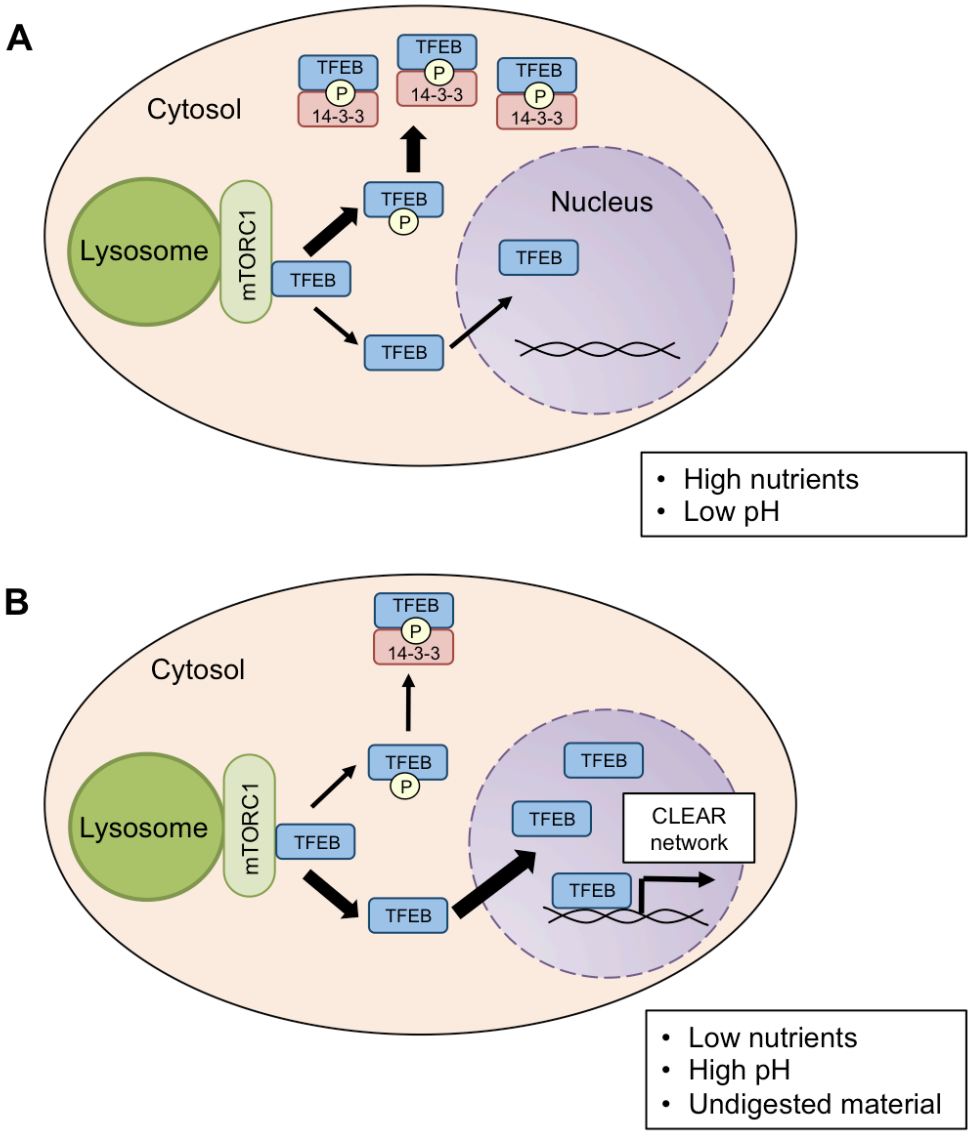

Figure 5. Mechanisms of TFEB activation

A. TFEB is inactivated under optimal lysosomal conditions of low pH and nutrient abundance. mTORC1 phosphorylates TFEB; phosphorylated TFEB binds to 14-3-3 proteins and is retained in the cytoplasm. B. TFEB is activated under lysosomal stress caused by high pH, nutrient starvation, and accumulation of intralysosomal undigested material. mTORC1 inhibition or the phosphatase calcineurin (not shown) decrease the levels of phosphorylated TFEB, allowing TFEB translocation to the nucleus and inducing the expression of CLEAR network genes. Figure adapted from Roczniak-Ferguson *et al*, 2012 [84].

1.4.5 TFEB as a therapeutic target

One of the main physiological consequences of TFEB overexpression and/or activation is cellular clearance [47, 69]. Cellular clearance refers to the removal of accumulated material in the lysosome through lysosomal exocytosis and autophagy. Since storage of undigested material in the lysosome is a common hallmark of LSDs, TFEB has been proposed as a therapeutic target for this type of diseases [47].

TFEB overexpression in mouse embryonic fibroblasts (MEFs) obtained from murine models of the LSDs, MSD and MPSIIIA, resulted in increased lysosomal exocytosis and clearance of glycosaminoglycans (GAGs) [47]. GAGs accumulate in the lysosome of MSD and MPSIIIA neurons, leading to neurodegeneration. Therefore, TFEB overexpression would reduce neurodegeration associated with these diseases [47]. In addition, TFEB-induced lysosomal exocytosis was able to reduce the levels of intralysosomal lipofuscin in a murine model of Batten disease, a type of LSD characterized by the accumulation of lipofuscin in neurons [47]. TFEB overexpression has also been shown to induce cellular clearance in Pompe disease [47, 97]. Pompe disease is a type II glycogen storage disease characterized by the accumulation of glycogen in the lysosome [97]. Overexpression of TFEB in fibroblasts from a Pompe disease patient resulted in the reduction of accumulated glycogen [47]. Additionally, overexpression of TFEB in immortalized myotubes from a mouse model of Pompe disease reduced lysosomal size and glycogen storage due to enhanced lysosomal exocytosis [97]. Finally, TFEB-induced autophagy was also shown to be involved in glycogen clearance in muscle of a mouse Pompe disease model [97].

In addition to LSDs, TFEB overexpression has also been proposed as a therapeutic target for diseases in which aggregation of proteins is an important aspect of their pathogenesis [98-101]. One of those diseases is Parkinson disease, in which the aggregation of α-synuclein is associated with the loss of dopaminergic neurons. The aggregation of α-synuclein has also been associated with accumulation of autophagosomes and reduction of lysosomal markers in dopaminergic neurons, suggesting that impared lysosomal function is affecting the clearance of aggregated α-synuclein [102-104]. For this reason, Decressac *et al* sought to investigate whether TFEB overexpression could alleviate the toxic effects of α-synuclein aggregation [100]. Injection of TFEB-containing adeno-associated virus into substantia nigra of an α-synuclein transgenic mouse, resulted in stimulation of autophagy and reduction of motor impairment in the α-synuclein transgenic mice [100]. In addition, the authors showed that pharmacological activation of TFEB, via rapamycin-induced inactivation of mTORC1, resulted in increase autophagy and reduction of motor impairment in a Parkison disease mouse model [100].

TFEB overexpression has also been shown to alleviate alpha-1-anti-trypsin (ATT) deficiency [101]. Mutant ATT aggregates in the endoplasmic reticulum of hepatocytes, causing liver damage, which is characterized by increased apoptosis and fibrosis [101]. Overexpression of both TFEB and mutant ATT in MEFs resulted in reduced aggregation of mutant ATT due to increase autophagy [101]. In addition, a TFEB-containing vector injected in a mouse model of ATT deficiency, resulted in decreased levels of mutant ATT and reduction of liver damage, suggesting that TFEB gene transfer is an efficient method to alleviate the symptoms associated with ATT deficiency [101]. Furthermore, TFEB has been shown to be a therapeutic target for Huntington's disease [98, 99]. Huntington's disease is a neurodegenerative diseases characterized by aggregation of mutant huntingtin (htt); TFEB overexpression in Neuro2a cells

transfected with mutant htt, resulted in decreased aggregation of htt [98]. Finally, TFEB overexpression has been shown to attenuate hypoxia-induced cardiomyocyte death by increasing lysosome biogenesis [105].

In summary, several studies have shown that stimulating lysosomal functions by overexpressing TFEB corrects defects and decreases toxicity associated with both LSDs and diseases characterized by the protein aggregates. However, these studies have not addressed the possible toxic effects of TFEB overexpression in systems in which increasing lysosomal volume can be more damaging than beneficial. This topic will be further discussed in Chapter 3.

1.4.6 Regulation of lysosomal function by TFE3

Although the initial studies describing the CLEAR network were only able to identify TFEB as transcriptional regulator of lysosomal function and biogenesis [70], new evidence has also implicated TFE3, another member of the MiT/TFE subfamily, as a transcriptional regulator of lysosomal function [72]. Similar to TFEB, nutrient starvation or inhibition of mTORC1 induced nuclear translocation of both endogenous and recombinant TFE3, suggesting that mTORC1 also regulates TFE3 activation [72]. In fact, it was shown that TFE3 interacts with mTOCR1 at the lysosomal surface through binding Rag proteins, resulting in phosphorylation of TFE3 by mTORC1 [72]. Phosphorylated TFE3 is retained in the cytoplasm by interacting with 14-3-3 proteins; however under conditions of lysosomal stress, starvation, or inhibition of mTORC1, TFE3 phosphorylation is reduced and it is translocated to the nucleus [72]. Similar to TFEB, nuclear TFE3 binds to the CLEAR element and activates the expression of lysosomal genes and

genes related to autophagy, resulting in increased lysosomal biogenesis and autophagy [72]. Interestingly, regulation of lysosomal genes by TFE3 is independent of TFEB, as TFE3 overexpression in HeLa cells stably expressing a shRNA against TFEB resulted in increased expression of lysosomal genes [72]. Finally, TFE3 overexpression has been shown to enhance lysosomal exocytosis and cellular clearance in a model of Pompe disease [72].

In addition, it has also been shown that TFE3 regulates the Golgi stress response and it is activated in response to Golgi stress. Similar to TFE3 response to lysosomal stress, under conditions of Golgi stress TFE3 is dephosphorylated and translocated to the nucleus where it activates the expression of Golgi-related genes [106]. Therefore, it is possible that MiT/TFE transcription factors play an important role in different cellular mechanisms of stress response.

1.4.7 ZKSCAN3, a master repressor of lysosomal function

So far, I have discussed how transcription factors positively regulate lysosomal function. However, it has been shown that autophagy is also negatively regulated by ZKSCAN3 [73]. ZKSCAN3 belongs to the family of zinc finger transcription factors containing a Kruppel-associated box (KRAB) and SCAN domains; members of this family are transcriptional repressor that play important roles in many cellular functions, such as apoptosis and cell proliferation [107]. It has been shown that knockdown of ZKSCAN3 results in inhibition of cell growth due to induction of senescence [73, 108].

Since activation of autophagy and mTORC1 inhibition have been shown to mediate the transition from cell proliferation to senescence [109], Chauhan *et al* investigated if ZKSCAN3 repression can induce autophagy, along with senescence [73]. Inhibition of ZKSCAN3 by shRNA in the bladder cancer cell line UC13 resulted in increased number of autophagosomes and autophagy flux, whereas ZKSCAN3 overexpression caused inhibition of autophagy [73]. Additionally, ZKSCAN3 repression increased lysosomal biogenesis, which was diminished when ZKSCAN3 was overexpressed [73]. Therefore, ZKSCAN3 negatively regulates autophagy and lysosomal biogenesis, opposite to what MiT/TFE transcription factors do.

Since ZKSCAN3 belongs to a family of transcriptional repressors [107], the authors sought to investigate whether ZKSCAN3 represses the expression of genes involved in autophagy [73]. Microarray analysis showed that over 60 genes involved in autophagy and lysosomal function were upregulated when ZKSCAN3 was depleted using shRNA [73]. In addition, ChIP assays indicated that ZKSCAN3 binds to the promoter regions of those genes [73]. Interestingly, it was shown that lysosomal stress, such as nutrient starvation and inhibition of mTORC1 by Torin-1, inactivates ZKSCAN3 causing its translocation to the cytoplasm [73]. As shown in Figure 6, under normal conditions ZKSCAN3 is found in the nucleus, repressing the expression of autophagy and lysosomal genes; however, ZKSCAN3 inhibition or depletion results in increased transcription of autophagy and lysosomal genes, as well as in induction of autophagy and lysosomal biogenesis [73]. Furthermore, since TFEB and ZKSCAN3 seem to regulate autophagy in an opposite manner, it has been proposed that these two transcriptional regulators may act together [73].

Transcriptional regulation of lysosomal function is a complex process that is regulated by environmental cues, such as nutrient availability and lysosomal status. When studying the function of MiT/TFE transcription factors, it is important to consider the fact that expression of lysosomal genes is being repressed by ZKSCAN3. There is not enough evidence to assume that all TFEB activators would, in turn, inactivate ZKSCAN3. Thus, TFEB activation may not always result in high levels of lysosomal genes expression if ZKSCAN3 is efficiently repressing the expression of those genes.

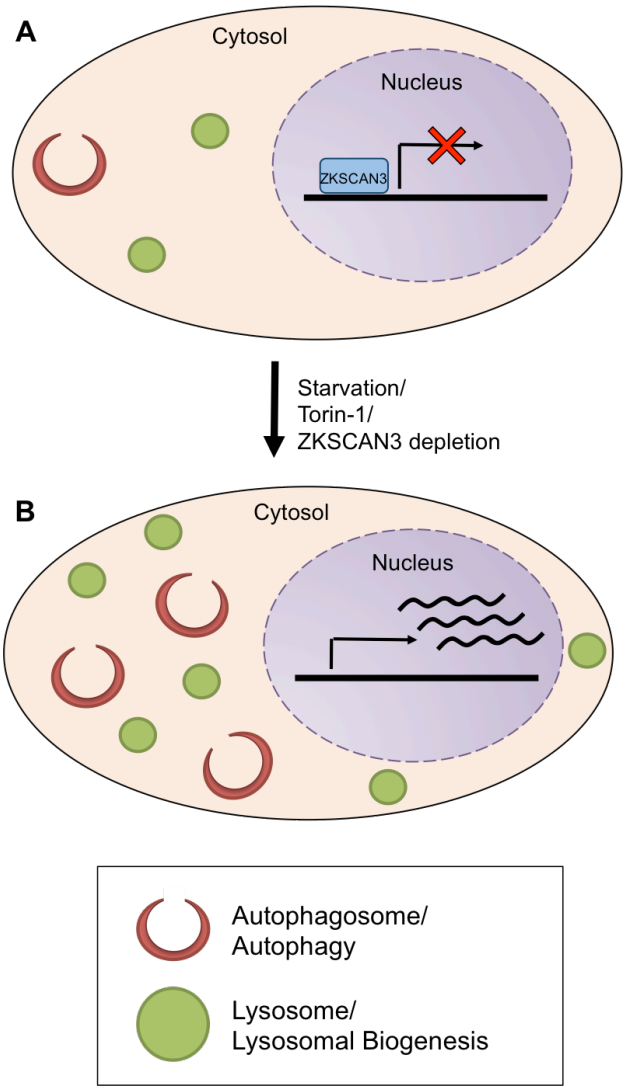

Figure 6. ZKSCAN3 represses autophagy and lysosomal biogenesis

A. Under normal conditions, ZKSCAN3 localizes to the nucleus, repressing the expression of lysosomal and autophagy genes. Autophagy and lysosomal biogenesis are repressed by ZKSCAN3. B. Nutrient starvation and Torin-1 inactivate ZKSCAN3, which is translocated to the cytoplasm. Under these conditions, autophagy and lysosomal biogenesis, as well as expression of genes involved in those processes, are activated. The same effect is observed when cells are depleted from ZKSCAN3 by RNA interference. Figure adapted from Chauhan *et al*, 2013 [73].

1.5 DISSERTATION AIMS

In Chapter 1, I have summarized and discussed the current literature about transition metals homeostasis and lysosomal function and regulation. One important aspect of lysosomal function is that recent evidence has positioned the lysosome as a central factor regulating transition metals toxicity. Lysosomes handle transition metals via endocytosis, autophagy, and absorption from the cytoplasm. In addition, I have discussed the role of TFEB as a master regulator of lysosomal function and biogenesis, which is activated in response to lysosomal and/or metabolic stresses. Transition metals can cause lysosomal dysfunction; therefore they are potential lysosomal stressors and TFEB activators. However, there are no studies addressing the role of TFEB in transition metal regulation. During my doctoral research I have investigated whether transition metals, particularly Cu, activate TFEB and if TFEB activation influences transition metal toxicity.

The importance of lysosomal exocytosis in Cu homeostasis has been recently explored. In fact, studies show that Cu exposure induces the translocation of the Cu pump ATP7B to the lysosome followed by lysosomal exocytosis [22]. However, whether and how Cu regulates the process of lysosomal exocytosis is currently unknown. These questions have also been the focus of my doctoral research.

The specific aims of my dissertation are the following:

Aim 1: Determine whether transition metals activate TFEB. I propose that transition metals, particularly Cu, can induce lysosomal stress; therefore, they can activate TFEB. To measure

TFEB activation I looked at the expression of CLEAR network genes in cells overexpressing TFEB and treated with Cu. In addition, I examined TFEB dephosphorylation and nuclear translocation in response to Cu exposure, as well as lysosomal biogenesis and autophagy as consequences of TFEB activation. Finally, I explored the role of TFEB in metal toxicity, finding that TFEB activation can increase toxicity if cells cannot efficiently excrete Cu.

Aim 2: Determine whether Cu regulates lysosomal exocytosis. Recent evidence suggests that short exposure to Cu (8 hours) stimulates lysosomal exocytosis; however my data indicate that prolonged exposure to Cu (24 hours) inhibits lysosomal exocytosis. Thus, I propose that Cu has a dual role on lysosomal exocytosis depending on the length of the exposure. How does Cu regulate lysosomal exocytosis? To answer this question, I examined whether Cu-stimulated lysosomal exocytosis has the same requirements of basal exocytosis, such as Ca and SNARE function. If Cu-stimulated exocytosis requires Ca and SNAREs, then Cu may be regulating a membrane fusion step.

2.0 EXPERIMENTAL PROCEDURES

2.1 CELL CULTURE

Human embryonic kidney 293 (HEK-293) cells and HeLa cells were maintained in growth medium, containing DMEM (Dulbecco's modified Eagle's medium; Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Retinal pigmented epithelial 1 cells immortalized with hTERT (RPE1) were maintained in DMEM/Ham F12 (1:1 mix; HyClone, Logan, UT) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). All cells were grown at 37ºC in the presence of 5% CO_2.

2.2 TREATMENTS

For transition metal treatments, cells were incubated either with 100 μM $CuCl_2$ or 100 μM $FeCl_2$ in growth medium for the indicated times. For sucrose treatment, cells were incubated in growth medium supplemented with 100 mM sucrose for 24 to 48 hours. Control cells were left untreated. Nutrient starvation was performed by incubating cells in EBSS, supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for the indicated time. Glutathione (GSH) treatments were performed with 2 mM GSH in growth medium for the indicated times. For Torin-1 treatments, cells were incubated for 1 hour with 250 nM Torin-1 in growth medium. For lysosomal

exocytosis assays using Butyl hydroperoxide (TBHP), cells were incubated for 1 hour with 400 μM TBHP (Invitrogen, Carlsbag, CA) in regular buffer. For $LaCl_3$ experiments, cells were pre-treated for 5 min with the indicated concentration of $LaCl_3$ in regular buffer. $LaCl_3$ was removed, added back and kept in the medium for the length of the assay.

2.3 TRANSFECTIONS

2.3.1 cDNA transfections

pCMV-TFEB-3XFLAG and pCMV-3TAG4-TFE3 plasmids were kindly provided by Dr. Rosa Puertollano (NIH). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbag, CA). Transfections were performed as described by manufacturer's protocol using 0.5 μg of cDNA and 1.5 μL of Lipofectamine 2000 per well of a 12-well plate. Briefly, cells were seeded at subconfluency and transfected the next day (at 70% confluency) either with pCMV-TFEB-3XFLAG (TFEB), pCMV-3TAG4-TFE3 (TFE3), or empty pcDNA3 vector (mock). Media was changed 16 to 24 hours later. Under my experimental conditions, transfection efficiency exceeded 80%.

2.3.2 siRNA transfections

VAMP7, ATP7B, and Control (universal negative control #1) siRNAs were acquired from Sigma-Aldrich (St Louis, MO). Cells were transfected using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbag, CA). Transfections were performed following manufacturer's protocol using siRNAs at a final concentration of 125 nM and 3 μL of Lipofectamine RNAiMAX per

well of a 12-well plate. Briefly, cells were seeded at subconfluency and transfected next day (at 70% confluency) with siRNA for either VAMP7 or ATP7B, or control siRNA. Media was changed 16 to 24 hours later. Experiments were carried out 48 hours after transfection. siRNA efficiency was measured by qPCR.

2.4 RNA EXTRACTION AND cDNA SYNTHESIS

HEK-293 or HeLa cells were seeded in 12-well plates, transfected, and treated as indicated. Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbag, CA) according to manufacturer's protocol. For cDNA synthesis, 2 μg of total RNA were incubated with 0.5 μg of oligo$(dT)_{18}$ primer (IDT, Cralville, IA), dNTPs, RNase inhibitor, and MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA) . Negative RT controls were performed by not adding MuLV Reverse Transcription to the reaction mix.

2.5 REAL-TIME QUANTITATIVE PCR

qPCR experiments were carried out using 1:500 dilutions of cDNA, 2X SYBR Green (Fermentas, Glen Burnie, MD), and 4 μM primer mix per 10 μl reaction. Table 3 shows the primer sequences used for gene expression analysis. All primers, with the exception of *CTSB* primers, were obtained from IDT (Cralville, IA). Primers for *CTSB* were obtained from QuantiTect Primer Assay (Qiagen, Venlo, Netherlands). To ensure amplification of cDNA only, all IDT primers were designed to span exons and negative RT reactions were performed as control. The Relative Quantification method on the 7300 Real Time System (Applied

Biosystems, Foster City, CA) was used to perform qPCR. Samples were amplified with the following program: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 sec followed by 60°C for 1 min. Samples were run in triplicates. At least 3 biological replicates were performed per condition. Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as the difference between the target genes and the expression of the endogenous gene, *RPL32*; $\Delta\Delta^{Ct}$ values were calculated relative to untreated controls. Data are presented as fold of change.

Table 3. Primers used for gene expression analysis

Gene Symbol	Species	Forward Sequence	Reverse Sequence
RPL32	Human	5'- CAACATTGGTTATGGAAGCAACA-3'	5'-TGACGTTGTGGACCAGGAACT-3'
CSTD	Human	5'-GCTGATTCAGGGCGAGTACATGAT-3'	5'-TGCGACACCTTGAGCGTGTA-3'
LAMP1	Human	5'-GGACAACACGACGGTGACAAG-3'	5'-GAACTTGCATTCATCCCGAACTGGA-3
HMOX1	Human	5'-GAGACGGCTTCAAGCTGGTGAT-3'	5'-CCGTACCAGAAGGCCAGGTC-3'
SOD1	Human	5'-CAAAGGATGAAGAGAGGCATGT-3'	5'-CTTCAATAGACACATCGGCCA-3'
ATP7B	Human	5'- GTGGGCAATGACACCACTTT-3'	5'- TGGGTGCCTTTGACATCTGA-3'
CTSB	Human	QT00088641*	QT00088641*

* QuantiTect Primer Assay, Qiagen

2.6 NUCLEAR EXTRACTIONS

Nuclear fractions were prepared as previously described [85]. Briefly, cells were grown in 60 mm dishes, transfected, and treated as indicated. Cells were washed two times with 1X cold PBS and transferred to a microcentrifuge tube. Cell suspensions were centrifuged at 300 x g for 5 min at 4ºC. Cell pellets were resuspended in NP-40 Lysis Buffer (10 mM Tris pH 7.9, 140 mM KCl,

5mM $MgCl_2$, 1mM DTT, 0.5% (v/v) NP-40) supplemented with phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF, 100 μM β-glycerophosphate) and protease inhibitors (Protease Inhibitor Cocktail III, Calbiochem, Gibbstown, NJ) and incubated for 15 min on ice. Cytoplasmic fractions were obtained by centrifuging lysed samples at 1,000 x g for 5 min at 4ºC. Nuclear pellets were washed two times with NP-40 Lysis Buffer and resuspended in Nuclear Lysis Buffer (25 mM Tris pH 7.4, 0.5% (v/v) Triton X-100, 0.5% (w/v) SDS) supplemented with phosphatase and protease inhibitors. Nuclear fractions were sonicated 3 times for 10 seconds each. Cytoplasmic and nuclear fractions were incubated for 5 min at 100ºC in 2X Laemmeli sample buffer (BioRad, Hercules, CA). Samples were loaded on a 10% precast TGX polyacrylamide gel (BioRad, Hercules, CA) and run at 250 V for 40 minutes. Proteins were transferred to nitrocellulose membrane (BioRad, Hercules, CA). Nitrocellulose membranes were blocked in 10% milk in TBS-T for 1 hour. All primary antibodies were incubated overnight at 4ºC in 1% milk in TBS-T. To detect TFEB-3xFLAG, mouse anti-FLAG antibody (M5, Sigma-Aldrich, St Louis, MO) was used at 1:2,000 dilutions. For GAPDH, rabbit anti-GAPDH antibody was used at 1:20,000 dilutions. HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Amersham, Piscataway, NJ) were used at 1:20,000 and 1:1,500 dilutions respectively.

2.7 WESTERN BLOT

For LC3 and CCS western blots, cells were grown on 6-well plates, transfected, and treated with the specified compounds. Cell were washed once with cold 1X PBS. Lysis buffer (20 mM Hepes pH 7.4, 75 mM NaCl, 1.5 mM MgCl2, 2 mM EGTA, 2 mM DTT, and 0.5% v/v Triton-X100), supplemented with protease and phosphatase inhibitors, was added to each well and cells were

incubated for 1 hour at 4ºC on a shaker. Cells were scraped, transferred to a tube, and centrifuged at 16,000 x g for 10 min at 4ºC. Supernatant was collected and equal amount of protein per condition were incubated at 100ºC for 5 min in 2X Laemmeli sample buffer (BioRad, Hercules, CA). Samples were loaded on a 12% TGX polyacrylamide gel (BioRad, Hercules, CA), run at 250 V for 40 min, and transferred to PVDF membrane (Millipore, Billerica, MA). Rabbit anti-CCS antibody was a kind gift from Dr. Dennis Thiele (Duke University) and was used at a 1:500 dilution and incubated overnight at 4ºC. For LC3 detection, rabbit anti-LC3 antibody was used diluted 1:1000 and incubated overnight at 4ºC. HRP-conjugated anti-rabbit secondary antibody was incubated for 1 hour at room temperature. Immunodetection was performed with the Luminata Forte HRP substrate (Millipore, Billerica, MA). Band intensities were measured using ImageJ (NIH, Bethesda, MD).

2.8 CONFOCAL MICROSCOPY

All confocal imaging was performed on the stage of Leica TCS SP5 confocal microscope equipped with a 63X oil immersion objective.

2.8.1 Lysotracker Red Staining

HEK-293 cells were seeded on coverslips and loaded with Lysotracker Red (Invitrogen, Carlsbag, CA) for 15 min at 37ºC in regular buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/L glucose). Hoechst dye was added to the cells for 5 min. Cells were washed once and kept in regular buffer before imaging. Images were obtained using a

561 nm laser line for Lysotracker Red and a 405 nm laser line for Hoechst dye. Laser intensity and gain were kept the same for all images.

2.8.2 Lipid Peroxidation

Lipid peroxidation assay was performed as previously described [64]. Briefly, HeLa cells plated on glass coverslips were treated for 3 hours with 100 μM $CuCl_2$ in growth medium and incubated with 5 μM Bodipy 581/591 C11 (Invitrogen, Carlsbag, CA) in regular buffer for 30 min at 37°C. Cells were then washed in regular buffer and imaged. Images were acquired using 488 and 561 nm laser lines. Spectral detection was set at 498-544 nm and 580-620 nm for green and red fluorescence, respectively. Lipid peroxidation is detected as a shift from red to green emission. Images are shown as merge of red and green channels. Red and green fluorescence intensities were measured using ImageJ (NIH, Bethesda, MD) and red to green ratios were calculated.

2.9 LYSOTRACKER QUANTIFICATION

To analyze Lysotracker images, ImageJ software (NIH, Bethesda, MD) was used. To determine the size of lysostracker-positive vesicles (lysosomes), threshold was applied to 8-bit images. Binary images were created and watershed process was used to separate particles at very close proximity. The particle analysis tool was used to quantify the size and number of individual lysosomes. Particles with an area smaller than 1 μm^2 were excluded because they may represent slices of lysosomes instead of whole lysosomes. To quantify aggregation of lysosomes, watershed was not applied to binary images, avoiding the separation of lysosomal complexes.

Particles larger than 7 μm^2 were considered aggregations of three or more lysosomes. Quantification data were plotted and analyzed using GraphPad Prism software (La Jolla, CA).

2.10 TRANSMISSION ELECTRON MICROSCOPY

To analyze monolayers of cells by TEM, HEK-293 cells were plated on 6-well plates, transfected with either pcDNA3 (mock) or TFEB, and treated with 100 μM $CuCl_2$ for 48 hours. Monolayers were then fixed with 2.5% glutaraldehyde in PBS, pH 7.4, for 1 hour at room temperature and washed three times in PBS. Post-fixation was carried out for 1 hour in 1% OsO_4 with 1% potassium ferricyanide, followed by three washes in PBS. Fixed cells were dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 90%, 100%). Then, monolayers were incubated in epon resin three times for 1 hour each. To embed monolayers, epon-filled plastic capsules were inverted over the monolayer and let polymerize overnight at 37ºC, followed by 48 hours at 60ºC. Samples were then trimmed, sectioned, and post-stained. Imaging was performed using JEOL 1011CX Transmission Electron Microscope equipped with high resolution AMT digital camera Images at the Center for Biological Imaging of the University of Pittsburgh (CBI).

2.11 β-HEXOSAMINIDASE ACTIVITY ASSAY

To determine the effect of short exposure to Cu (up to 3 hours) HeLa cells were plated on 12-well plates. The day of the experiment, cells were washed once with regular buffer and incubated with 250 μL of $CuCl_2$ diluted in regular buffer. Buffer was collected at the indicated times. For

prolonged Cu treatments (24 hours), cells were washed with regular buffer and 250 µl of regular buffer was added to each well. Buffer was collected at the indicated times. Collected samples were incubated with 300 µl of 3 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (N9376, Sigma-Aldrich, St Louis, MO) for 30 minutes at 37ºC in 0.1 M citrate buffer (0.1 M sodium citrate, 0.1 M citric acid, pH 4.5). Reactions were stopped by adding 650 µl borate buffer (100 mM boric acid, 75 mM NaCl, 25 mM sodium borate, pH 9.8) and the absorbance was measured in a spectrophotometer at 405 nm. To determine total cellular content of β-hexosaminidase, cells were lysed with 250 µl of 1% Triton X-100 in PBS and after a 10,000 x g spin for 5 minutes at 4ºC, 25 µl of the cell extracts were used for the enzyme activity reaction. Enzyme activity was determined as the amount of 4-nitrophenol produced. Absorbance was calibrated with different amounts of 4-nitrophenol (N7660, Sigma-Aldrich, St Louis, MO) in 0.1 M citrate buffer.

2.12 FLOW CYTOMETRY SURFACE LAMP1 ASSAY

HeLa cells plated on 6-well plates were incubated with 100 µM $CuCl_2$ in regular buffer for 1 hour at 37°C or left untreated (control). Cells were trypsinized and washed in PBS before fixing in 1% paraformaldehyde (PFA) for 30 minutes. To detect cell surface LAMP1, fixed cells were incubated with CD107a LAMP1 antibody APC conjugated (Life Technologies, Carlsbag, CA) in 5% BSA in PBS for 30 minutes. Cells were washed and resuspended in PBS prior to analysis in BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.13 JC-1 ASSAY

In order to assess for mitochondrial health, mitochondrial membrane potential was measured using the potential-sensitive dye JC-1 (Invitrogen, Carlsbag, CA). JC-1 assays were performed as previously described [64]. Briefly, cells plated on a 6-well plate were transfected with either TFEB-3XFLAG or empty vector and treated with $CuCl_2$ for 48 hours at the indicated concentrations. Cells were loaded for 30 minutes at 37ºC with 1:1000 dilution of JC-1 in growth medium. Cells were rinsed twice with growth medium and 0.05% trypsin (Invitrogen, Carlsbag, CA) was added to detach cells. Cells were then pelleted at 300 x g for 5 minutes and were rinsed twice with cold PBS. Cell pellets were resuspended in regular buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/L glucose) and measurements were recorded at 485/530 nm for green and 535/590 nm for red, using a fluorometer. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a positive control.

2.14 STATISTICAL ANALYSIS

Statistical significance was calculated using a two-tailed, unpaired Student's t-test with $p<0.05$ considered statistical significant and it is denoted with an asterisk (*). In some cases, two asterisks (**) are used to denote $p<0.01$. Data are presented as mean ± standard error of the mean (SEM) of three independent experiments (unless otherwise indicated).

3.0 TRANSITION METALS ACTIVATE TFEB IN OVEREXPRESSING CELLS

3.1 INTRODUCTION

Low levels of transition metals, such as Cu and Fe, are essential to carry out several functions in the cell; however, exposure to high levels of these metals are detrimental for cell survival [2]. High dietary uptake or inhalation of transition metals causes conditions such as cirrhosis and dementia [110]. The loss of cellular components involved in the regulation of transition metals homeostasis results in metal overload and is a root cause of several human diseases, such as Wilson's and Menkes diseases [111-113]. Beyond that, transition metals are key factors in the pathogenesis of stroke, Alzheimer's and Parkinson's diseases, among others [2, 114-121]. In fact, accumulation of transition metals is thought to cause neurodegeneration through the production of ROS catalyzed by transition metals [122-124]. Thus, the use of metal and ROS chelation is considered a treatment option for neurodegenerative diseases [117].

Transition metals enter the cell through plasma membrane transporters [51, 52] and by endocytosis of free and protein-bound metals [53], which may follow the endocytic pathway or be mediated by binding to specific receptors, as is the case for the transport of Fe, which binds transferrin to enter the cell [51, 52]. Metals separate from the binding proteins in the lower endocytic pathway due to low pH and proteolytic activity present in these membranous compartments. Autophagy of metal-bound proteins is another means by which transition metals

are delivered into the lysosomes. An alternative pathway of metal entry into the lysosomes is through metal transporters present in membranes of intracellular organelles, including lysosomes. These transporters play an important role evacuating potentially toxic metals from the cytoplasm into intracellular organelles. Indeed, lysosomal uptake and exocytosis of Zn is critical for its detoxification [68]. Confocal immunohistochemistry and subcellular fractionation suggest the presence of the Zn transporters ZnT2 and ZnT4 in the lysosomal membrane [56, 57]. Their suppression or overexpression causes cellular and lysosomal Zn mishandling [56, 58, 59]. Similar evidence exists for Cu as well, since proteomic analysis confirms localization of ATP7B in the lysosomal membranes [60, 61]. Recently published data show that Cu absorption from the cytoplasm into the lysosomes through ATP7B is followed by Cu clearance via lysosomal exocytosis [22]. My recently published data show that brief exposure to Cu activates lysosomal exocytosis to facilitate the expulsion of this metal [1]. Based on this evidence, lysosomes appear to serve as a cellular metal sink that absorbs and detoxifies transition metals.

The removal of transition metals from the lysosomes is a function of the ion transporters. The best characterized is DMT1 (Slc11a2) [62]. Among other metal transporters implicated in this process is TRPML1, whose loss appears to affect the distribution of Zn and Fe between the lysosomes and the cytoplasm [56, 64-66]. Lysosomal exocytosis has emerged as a key mechanism of Zn and Cu removal [1, 22, 68]. If metal delivery to the lysosomes exceeds its clearance, as is likely to happen during high dietary metal uptake, metals build up in the lysosomes. Although the account of metal effect in lysosomes is far from being complete, it is clear that the ensuing lysosomal deficiencies can be explained by direct inhibition of the lysosomal enzymes and transporters by transition metals and by ROS-mediated formation of lipofuscin [17, 54, 55]. The detrimental effect of transition metals on the lysosomes suggests

that, at least under certain conditions, lysosomes themselves are the targets of transition metal toxicity.

The expression of the components that regulate lysosomal function is under the control of TFEB and its relatives such as TFE3 [69-72]. TFEB binds to the promoter regions of genes containing the Coordinated Lysosomal Expression and Regulation (CLEAR) element [47, 69, 70] and has been implicated as a master regulator of lysosomal biogenesis and autophagy. These transcription factors are regulated by mTORC1, which senses lysosomal status and nutrient availability and regulates protein synthesis and autophagy [75, 84, 85]. Under normal conditions (low lysosomal pH and high nutrients), TFEB is inactivated by mTORC1-dependent phosphorylation, leading to binding of the proteins 14-3-3 and retention of TFEB in the cytoplasm [84]. Inhibition of mTORC1 leads to an increase in the dephosphorylated form of TFEB and its translocation to the nucleus. In the nucleus, TFEB promotes the activation of a set of genes driving lysosomal function and autophagy including genes coding for lysosomal hydrolases such as cathepsin B and cathepsin D (*CTSB* and *CTSD*, respectively), structural proteins such as LAMP1 (*LAMP1*) and many others [47, 69, 70]. This feedback loop mediated by TFEB upregulates lysosomal and autophagic activity in response to metabolic cues. The fact that upregulation of the TFEB-driven gene network was demonstrated in lysosomal storage disorders and can be induced by lysosomal inhibitors suggests that deficits of the lysosomal function are reported through this gene network as well.

TFEB overexpression has been recently used to correct cellular and tissue pathologies in a range of diseases including Huntington's and Parkinson's diseases [47, 99-101]. Such effect can be explained by TFEB-induced stimulation of various aspects of the lysosomal function,

including lysosomal exocytosis, that may compensate or correct the abnormalities underlying these conditions. It is unclear whether this principle is universal. Indeed, it can be argued that under certain condition, making more lysosomes may enhance the effects of the toxin. This idea finds support in the recent evidence of increased cancer drug retention in the lysosomes of TFEB-overexpressing cells [125]. In the course of the studies presented in this Chapter, I sought to answer whether TFEB senses the lysosomal deficiencies caused by transition metals, particularly, Cu. I found that transition metal exposure activates overexpressed TFEB, increasing the expression of lysosomal genes. While this effect was cytoprotective at moderate Cu exposure, at high levels of Cu exposure it was associated with increased oxidative stress and mitochondrial damage, which were especially pronounced in cells with observable low levels of lysosomal exocytosis. The central conclusion from of my studies is that cytoprotective function of TFEB requires robust lysosomal exocytosis, in the absence of which TFEB activation may become toxic. Together, these findings identify TFEB as a player in the response to transition metal toxicity and suggest that under some conditions, the effects of TFEB overexpression may enhance toxicity.

3.2 RESULTS

3.2.1 Activation of recombinant TFEB and expression of lysosomal genes in cells treated with copper

The activation of TFEB has been linked to many factors that induce lysosomal stress. Lysosomes play an important role in transition metal homeostasis, and thus I reasoned that lysosomal stress could be induced by metal overload, since lysosomal storage disease phenotype was reported in some models of Cu exposure [126]. Lysosomal stress activates TFEB by suppressing its phosphorylation by mTORC1 [84, 85]. To test TFEB activation, I used mock- and TFEB-3XFLAG-transfected HEK-293 cells; 24- or 48-hour-long exposure to 100 mM sucrose was used as a positive control for TFEB activation [70]. This system provides excellent resolution of the mechanisms of TFEB activation. Figure 7A shows Western blot analysis of cytoplasmic and nuclear fractions of TFEB-3XFLAG transfected HEK-293 cells. GAPDH and LAP2 were used as cytoplasmic and nuclear markers, respectively. Under normal control conditions, TFEB is predominantly phosphorylated and concentrated in the cytoplasmic fraction and the nuclear fraction contained relatively low levels of phosphorylated TFEB (lanes 1 and 2, respectively; TFEB phosphorylation status was confirmed using lambda phosphatase, Figure 7B). Exposure of HEK-293 cells to 100 μM $CuCl_2$ resulted in the appearance of a fast migrating band in the nuclear fraction (Figure 7A, lane 4), indicating that dephosphorylated TFEB has been translocated to the nucleus of Cu-treated cells. This was confirmed, as in cells treated with 100 mM sucrose TFEB is dephosphorylated in both cytoplasmic and nuclear fractions and TFEB is more abundant in the nuclear fraction (Figure 7A, lanes 5 and 6). Together, these data indicate that Cu exposure is associated with activation of recombinant TFEB: it causes TFEB

dephosphorylation and consequent nuclear translocation, which, in turn, results in increased expression levels of lysosomal genes in response to Cu exposure.

TFEB activation should result in an increased expression of the CLEAR network genes. mRNA levels of genes previously assigned to the CLEAR network: *CTSD, LAMP1*, and *CTSB*, were analyzed using qPCR. In accordance with the previously published results where recombinant TFEB was used to show activation [69-71], the endogenous levels of TFEB in HEK-293 cells are insufficient to cause measurable response: when 48-hour-long exposure to 100 mM sucrose was used as a positive control as previously described [70], a decrease in *CTSD, LAMP1* or *CTSB* mRNA was detected. However, when TFEB-3XFLAG was transiently expressed in HEK-293 cells, the expression of *CTSD, LAMP1*, and *CTSB* genes was significantly increased by the sucrose treatment (Figure 7C; 2.17 ± 0.27, 2.24 ± 0.13, 2.07 ± 0.29 fold increase, respectively; mean ± SEM of 3 to 4 independent experiments). In addition, nutrient starvation stimulated the expression of lysosomal genes in a TFEB-dependent manner (Figure 8). Also, TFEB-overexpressing cells showed increased basal expression of these genes. qPCR readouts presented in Figure 7 are normalized to the basal mRNA levels in mock- and TFEB-transfected cells. Cells transfected with an empty vector did not show an increase in mRNA levels of these genes in response to sucrose (Figure 7C), indicating that the response to sucrose is specific to TFEB expression.

Figure 7D shows that exposure of mock-transfected HEK-293 cells to 100 μM $CuCl_2$ for 24 or 48 hours did not increase the expression of these CLEAR network genes; indeed, a decrease in corresponding mRNA was detected. However, TFEB-overexpressing cells showed a significant increase in *LAMP1*, *CTSB*, and *CTSD* mRNA when cells were treated with Cu for 24

hours (1.92 ± 0.33, 1.91± 0.33, 1.45 ± 0.10 fold increase, respectively; 3 to 4 independent experiments) or 48 hours (1.98 ± 0.16, 2.4 ± 0.20, 1.87 ± 0.23 fold increase, respectively; 3 to 4 independent experiments). This Cu concentration is within the range commonly used to study Cu transport and toxicity [1, 22, 127]. Additionally, exposure of TFEB cells to 100 μM $FeCl_2$ also resulted in an increased expression of lysosomal genes (Figure 8B). TFE3, a TFEB relative that has been shown to regulate the expression of lysosomal genes [72, 128], seems to be activated by Cu as well. Figure 8C shows that similar to TFEB, TFE3 overexpression prevented the decrease in expression of *LAMP1*, *CTSB*, and *CTSD* in response to Cu; however, the magnitude of increase was smaller than the one observed in TFEB-transfected cells. Furthermore, I tested whether Cu was able to activate endogenous TFEB in HeLa cells. The mTORC1 inhibitor and TFEB activator Torin-1 was used as a positive control [75, 84, 85, 129]. Figure 9 shows that both Cu and Torin-1 induced a modest increase in the expression of *CTSD* and *LAMP1*, suggesting that the endogenous levels of TFEB in these cells, as well as in HEK-293 cells, are very low. Therefore, the increased expression of lysosomal genes observed in TFEB-overexpressing cells is specific to TFEB expression. In conclusion, these data suggest that a TFEB-dependent (and likely TFE3-dependent) mechanism mediates activation of the CLEAR network genes in response to transition metals.

Next, since TFEB activation is associated with lysosomal biogenesis, I sought to investigate how the exposure to Cu affects the lysosomal compartment in HEK-293 cells transfected with either TFEB or mock control (empty vector).

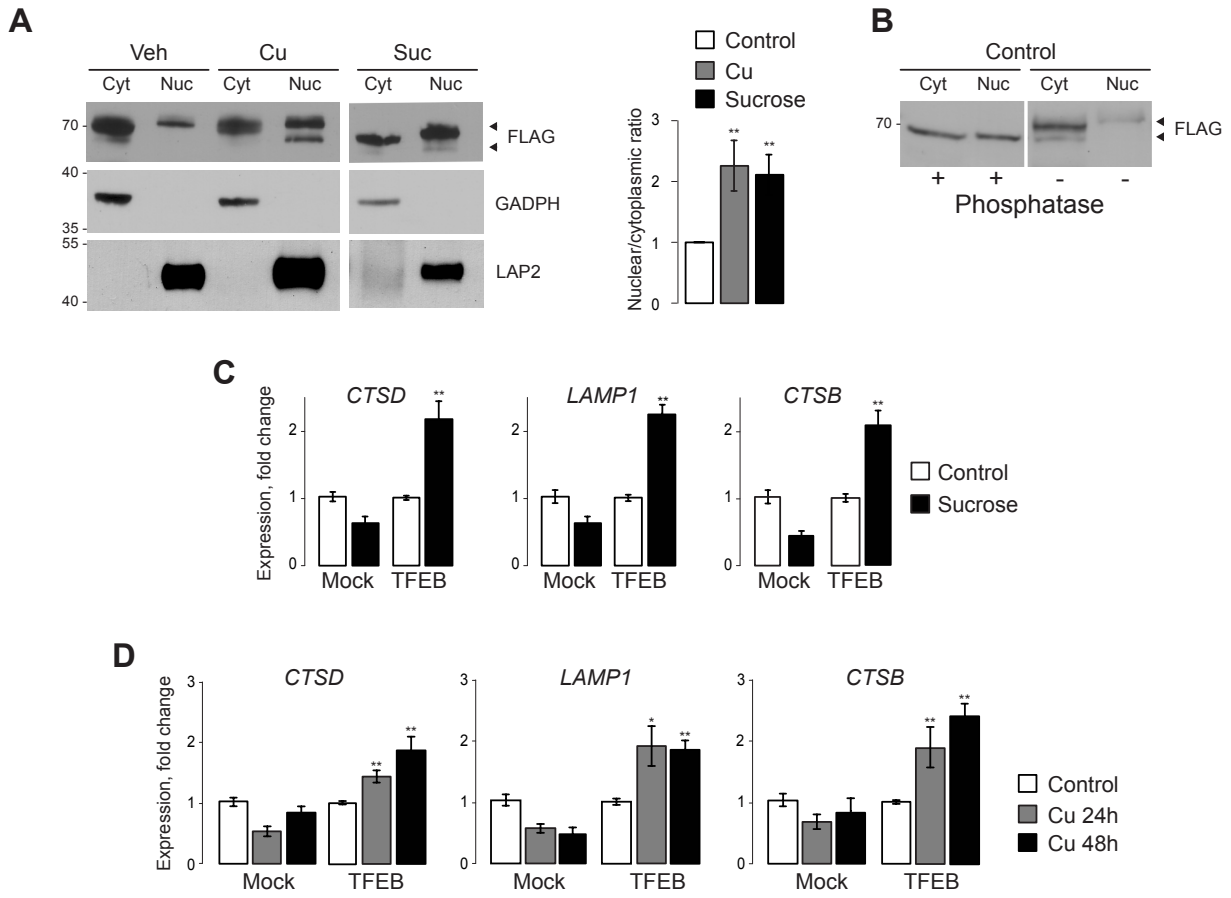

Figure 7. Copper induces the activation of TFEB

A. Western blot analysis of nuclear/cytoplasmic fractionation of TFEB cells treated with either 100 μM $CuCl_2$ or 100 mM sucrose for 48 hours. GAPDH and LAP2 were used as cytoplasmic and nuclear markers, respectively. Image is representative of three independent experiments. The quantification of these experiments is represented in the column graph. B. Western blot analysis showing the effect of lambda phosphatase on TFEB motility, indicative of TFEB dephosphorylation in Cu-treated cells. C and D. qPCR analysis of mock and TFEB cells treated with either 100 mM sucrose for 48 hours (C) or 100 μM $CuCl_2$ for 24 or 48 hours (D). Both sucrose and Cu activate the expression of TFEB-regulated genes *CTSB, CTSD* and *LAMP1*. Values represented as mean ± SEM of three to four independent experiments; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*) and $p<0.01$ (**) considered significant.

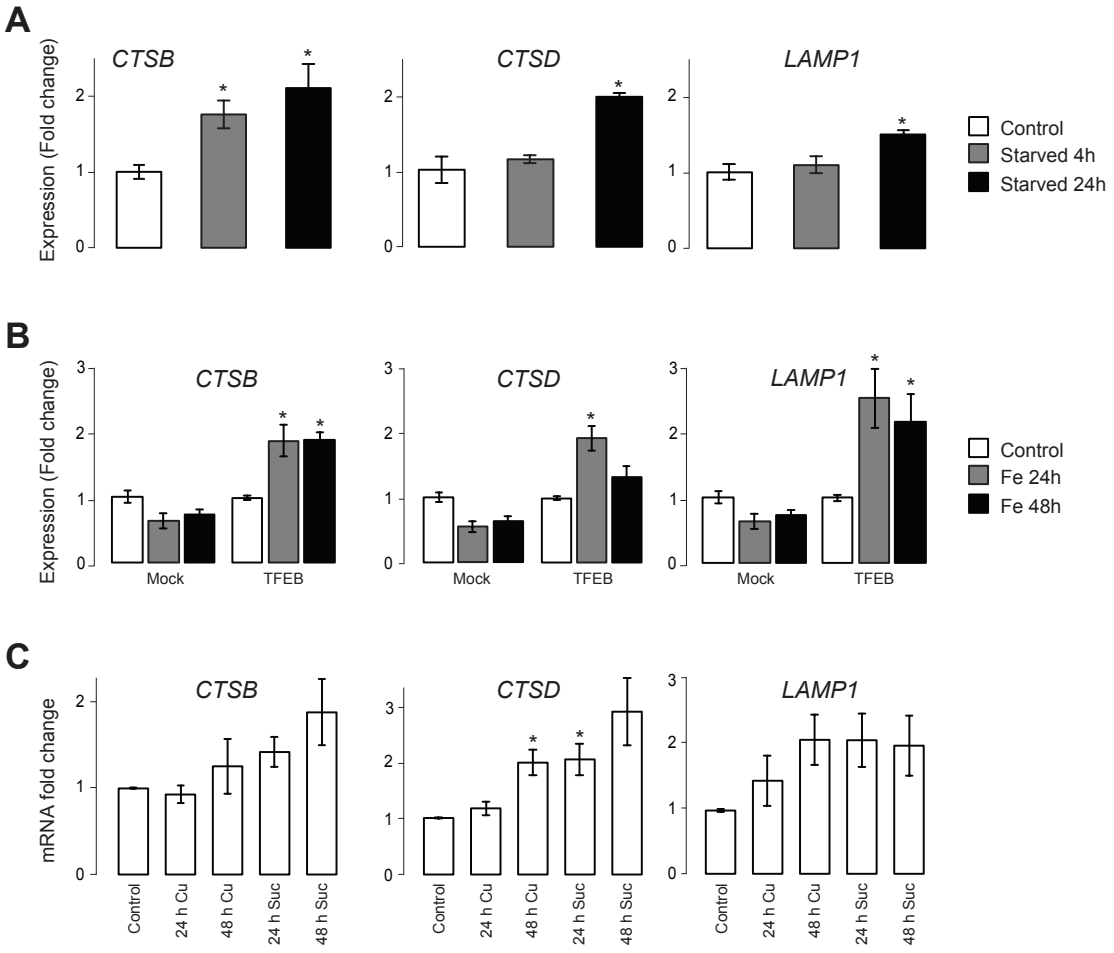

Figure 8. Nutrient starvation and iron induce the expression of lysosomal genes

A. TFEB- and mock-transfected HEK-293 cells were incubated in EBSS buffer for 4 and 24 hours. qPCR analysis shows an increase in the expression of TFEB-regulated genes *CTSD*, *LAMP1*, and *CTSB*. Data were normalized to the values in mock samples. B. TFEB- and mock-transfected HEK-293 cells were treated with 100 μM $FeCl_2$ for 24 or 48 hours. qPCR analysis shows that Fe activates the expression of TFEB-regulated genes *CTSD*, *LAMP1*, and *CTSB*. C. qPCR analysis of TFE3-transfected HEK293 cells treated with 100 μM Cu and 100 mM sucrose (Suc) as a positive control. After normalization to *RPL32*, the data were normalized to the values in mock-transfected cells under the same conditions. Values represented as mean ± SEM of three to four independent experiments.

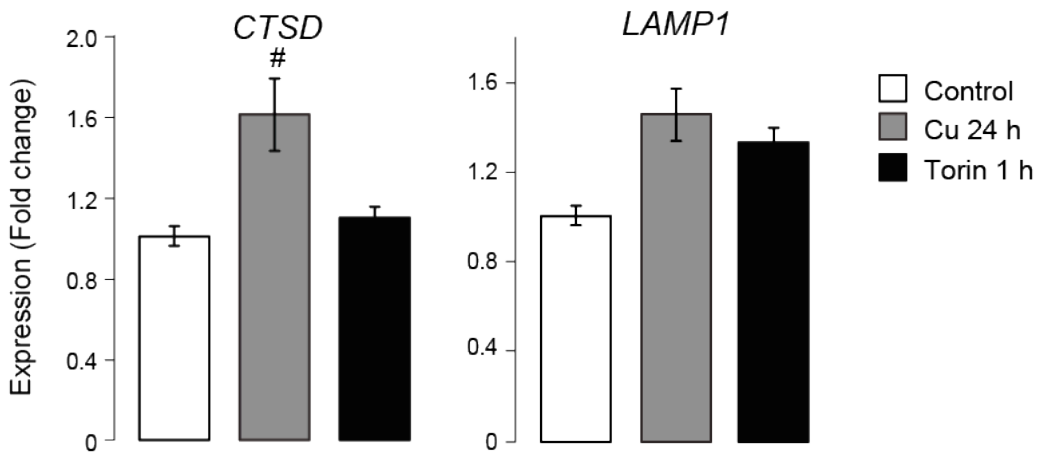

Figure 9. Upregulation of lysosomal gene expression in HeLa cells exposed to copper or Torin-1

qPCR analysis of *CTSD* and *LAMP1* in HeLa cells treated with 100 μM $CuCl_2$ for 48 hours or with 250 nM Torin-1 for 1 hour. Only the experiments (n=3) in which Torin-1 showed an increase in lysosomal gene expression were used to show the effect of Cu. Data were normalized to the corresponding untreated controls, which were taken as 1. Values represented as mean ± SEM. # represents p=0.052.

3.2.2 Copper induces aggregation of lysosomes

In order to investigate the effect of Cu on lysosomes, cells were exposed to 100 μM $CuCl_2$ for 24 hours and acidic organelles, including lysosomes, were stained with Lysotracker Red followed by confocal live cell imaging (Figures 10 and 11). In order to quantify Lysotracker-positive vesicles, I developed a protocol that allows me to distinguish between clustered and individual lysosomes (see Chapter 2 and Figure 10). Image analysis revealed that TFEB-transfected cells have significantly higher number of lysosomes (particles bigger than 1 μm^2) than mock-transfected cells (Figure 11A; 165.3 ± 6.3 vs 122.0 ± 5.6 lysosomes per image, respectively; 2 separate experiments, 3 images each; $p<0.01$), ostensibly due to increased TFEB-driven lysosomal biogenesis. When cells were treated with Cu, the number of lysosomes was not affected in mock-transfected cells, but it was significantly increased in TFEB-transfected cells (Figure 11B; count increased to 353.7 ± 25.21 lysosomes per image; 2 separate experiments, 3 images each; $p<0.01$ when compared to TFEB-transfected, untreated cells). Incubation with 100 mM sucrose, which has been previously described as a lysosomal stressor and TFEB activator [70], was also able to increase the number of lysosomes in both mock and TFEB-transfected cells (Figure 11B). Cu-treated cells showed increased number of lysosomal aggregates (Figure 11C). Interestingly, the number of Cu-induced lysosomal aggregates was increased by TFEB-overexpression (Figure 11C, D; 14 ± 0.58 and 30.67 ± 3.33 aggregates per image in mock- and TFEB-transfected cells treated with Cu, respectively; 2 separate experiments, 3 images each; $p<0.01$). These data indicate that Cu causes the reorganization of lysosomes by inducing their aggregation and this effect is enhanced by the overexpression of TFEB.

In addition to the observed changes on lysosomal number and organization in response to Cu, I observed effects on autophagy. I analyzed the levels of autophagosome-bound LC3-II by Western blot (Figure 12A, B). TFEB-transfected HEK-293 exposed to Cu showed an increased LC3-II/LC3-I ratio, indicating a higher number of autophagosomes in response to Cu. Furthermore, transmission electron microscopy revealed that exposure to 100 μM $CuCl_2$ for 48 hours induced the accumulation of storage bodies, including multivesicular bodies and large autophagic vesicles, in both mock- and TFEB-transfected HEK-293 cells (Figure 13).

In summary, by using three different functional readouts: gene expression, lysosomal buildup and autophagic markers, I was able confirm activation of recombinant TFEB by Cu in this system.

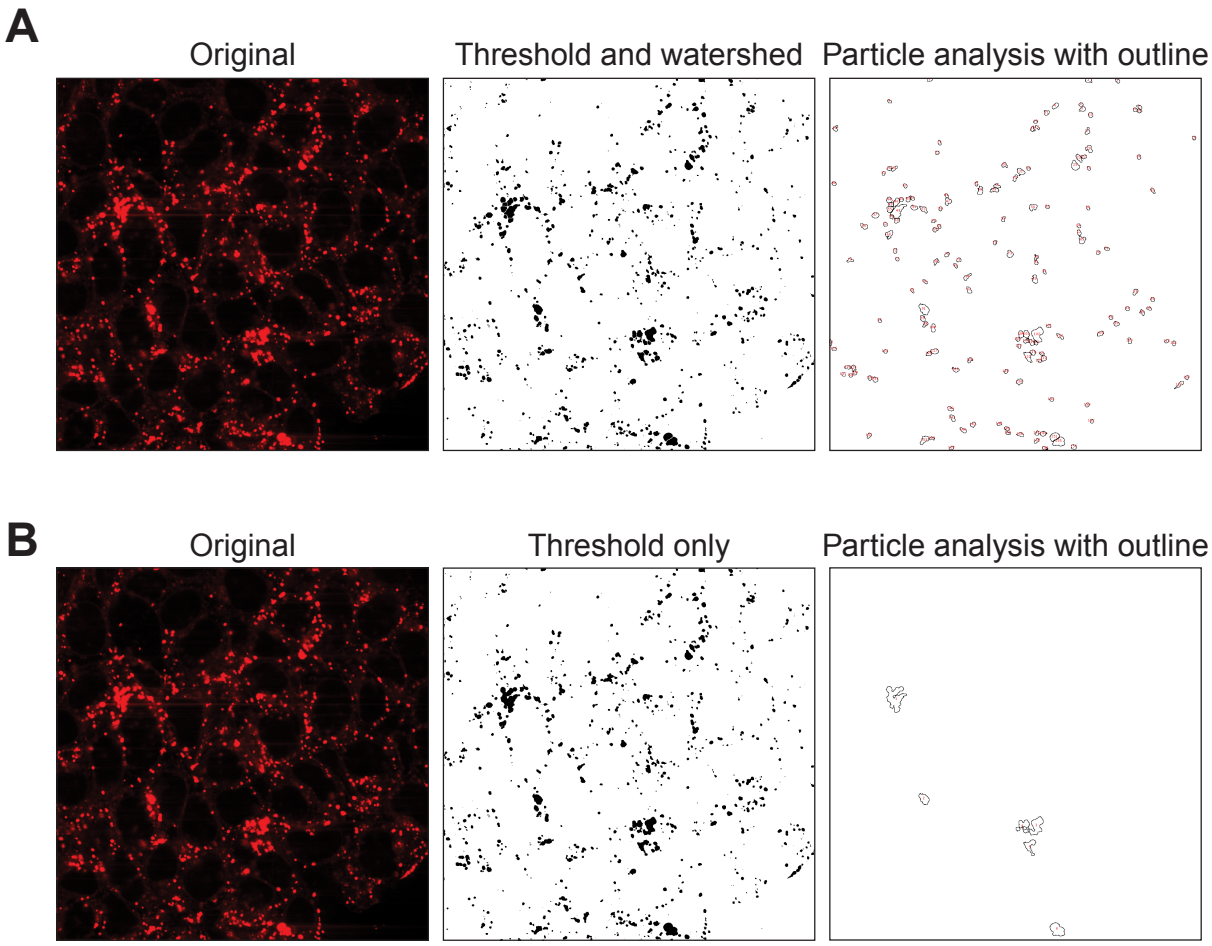

Figure 10. Quantification of Lysotracker-positive vesicles

A. Example of quantification of individual lysosomes using ImageJ. Original confocal image (first panel) was converted to a 8-bit image and threshold was applied. Image was then converted into a binary image and wathershed function was applied to separate clustered particles (middle panel). Lysosomes were quantified using the Analyze Particle function, excluding particles smaller than 1 μm^2, as they probably represent sections of lysosomes instead of whole lysosomes. An image showing the outline of the counted particles was originated (last panel). B. Example of quantification of aggregated lysosomes using ImageJ. Original images were processed as in A, without applying watershed. Lysosomal aggregates were quantified using the Analyze Particle function, excluding particles smaller than 7 μm^2. Particles larger than 7 μm^2 were considered aggregations of lysosomes (last panel).

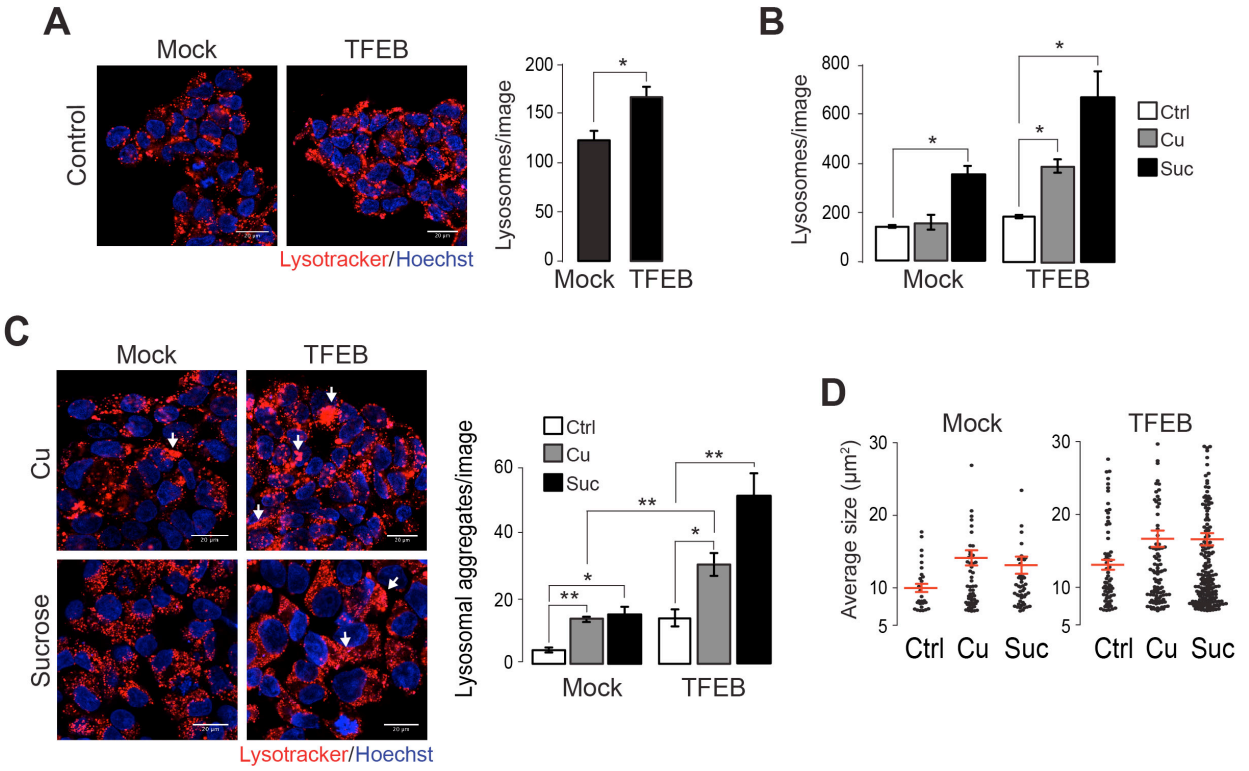

Figure 11. Copper induces aggregation of lysosomes

A. HEK-293 cells were transiently transfected with TFEB-3XFLAG (TFEB) or an empty vector (mock) and lysosomes were stained with Lysostracker (red) and Hoechst dye was used to stain nucleus (blue). Graph represents the number of individual lysosomes per image analyzed as discussed in Chapter 2 and in Figure 10. B. The number of individual lysosomes is increased in TFEB cells treated with 100 μM $CuCl_2$ for 24 hours or with 100 mM sucrose for 24 hours. C. Confocal images of mock and TFEB cells treated with 100 μM $CuCl_2$ or 100 mM sucrose stained with lysostracker (red) and Hoechst dye (blue). Arrows indicate lysosomal aggregation. Graph represents the number of lysosomal aggregations per image. D. Distribution of the size of lysosomal aggregates (in μm^2) of mock and TFEB cells treated with either $CuCl_2$ or sucrose analyzed as in the Chapter 2 and in Figure 10. Red lines represent the mean value ± SEM for each condition. All images were analyzed using ImageJ. Values represented as mean ± SEM of two independent experiments, six images total; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*) and $p<0.01$ (**) considered significant.

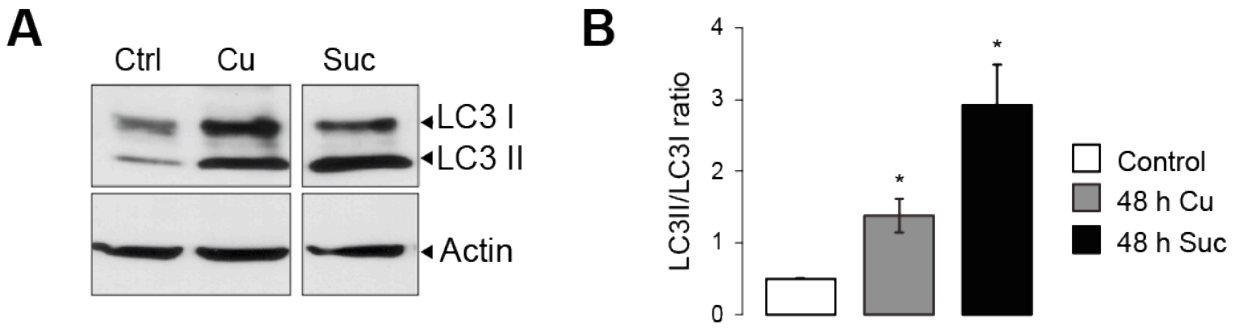

Figure 12. The effect of copper on autophagy

A. Western blot analysis showing the levels of autophagy marker LC3-I (cytosolic) and LC3-II (autophagosome-bound) in mock- and TFEB-transfected cells exposed to 100 μM Cu or 100 mM Sucrose (Suc). Image is representative of three independent experiments. B. Graph representing band intensity quantification of the Western blots.

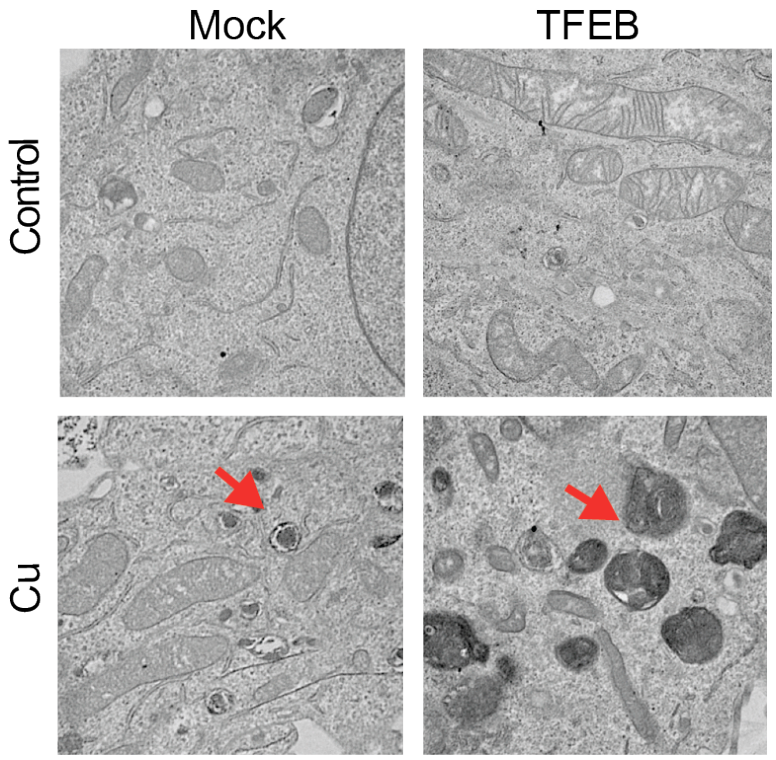

Figure 13. Copper induces the formation of storage bodies

Transmission electron microscopy (TEM) images of mock- and TFEB-transfected HEK-293 cells treated with 100 μM $CuCl_2$ for 48 hours. Red arrows indicate storage bodies. Note that storage bodies are larger in TFEB-transfected cells treated with Cu. Images are representative of two independent experiments, total of 10 to 20 images per condition. Images were taken using a JEOL 1011CX Transmission Electron microscope at the Center for Biological Imaging (CBI) of the University of Pittsburgh.

3.2.3 The impact of TFEB on copper-induced oxidative stress

TFEB overexpression has been proposed to be therapeutic in models of several diseases including Huntington's, Parkinson's and Pompe diseases [47, 99-101], ostensibly due to activation of lysosomal biogenesis and clearance. Transition metals catalyze the formation of ROS through a Fenton's reaction favored by the acidic lysosomal environment [130]. ROS damage lysosomal membranes, likely causing the release of lysosomal digestive enzymes and cell death [131]. High levels of ROS can cause the formation of lipofuscin as a consequence of peroxidation of non-degraded material, and impair autophagy [130]. Since lysosomes handle Cu and other transition metals including Fe and Zn [22, 56, 64, 65, 68, 132-134], I sought to answer whether increased Cu retention in the lysosomes of TFEB-overexpressing cells increases toxicity by increasing ROS production. The toxicity induced by transition metal is a contributing factor to cell death in many pathologic conditions, including neurodegenerative diseases [112, 114, 116, 135-147], the same conditions against which TFEB overexpression was proposed to be protective.

In order to measure ROS production, I analyzed the expression of *HMOX1* gene, which encodes for the enzyme called heme oxygenase-1. *HMOX1* expression is induced by ROS via the NRF2 transcription factor that binds to an antioxidant response element (ARE) in the *HMOX1* promoter. As an additional control, I analyzed the expression of the *SOD1* gene coding for superoxide dismutase, which is activated by metal-regulated transcription factor 1 (MTF-1) [148]. Figure 14A shows the results of qPCR analysis for *HMOX1* in TFEB and mock transfected HEK-293 cells after 24 or 48 hours treatment with 100 μM $CuCl_2$. In both cases, mock-transfected cells showed a significant increase in *HMOX1* expression after Cu exposure for

24 hours (2.44 ± 0.67 fold increase, 3 independent experiments) and 48 hours (2.87 ± 0.47 fold increase; 3 independent experiments). HEK-293 cells expressing TFEB showed even larger *HMOX1* response: I detected 4.28 ± 0.58 fold increase after 24 hours, and 6.99 ± 0.99 fold increase after 48 hours (t test $p<0.01$, n=3, Figure 14A). *SOD1* response showed the same *HMOX1* trend (Figure 14B). Cu did not induce the expression of *SOD1* in mock cells; however the expression of *SOD1* was significantly increased in TFEB-transfected HEK-293 cells treated with Cu (1.51 ± 0.12 fold increase, $p<0.05$). The effect of Cu was concentration-dependent as treating the cells for 48 hours with increasing concentrations of $CuCl_2$ (0, 1, 10, and 100 μM) revealed that *HMOX1* expression gradually increased with Cu (Figure 14C). These data suggest that TFEB-overexpressing cells have an increased response to ROS induced by prolonged exposure to high levels of Cu.

What is the nature of the TFEB-dependent component of the Cu effect on *HMOX1*? In order to answer this question, I treated mock- and TFEB-transfected HEK-293 cells with 100 mM sucrose for 48 hours and measured the expression of *HMOX1*. Figure 14D shows that exposure to sucrose significantly increases *HMOX1* expression in TFEB, but not in mock-transfected cells (5.95 ± 0.65 fold increase; 3 independent experiment; t test $p<0.01$ compared to untreated, or treated mock-transfected cells, in which HMOX1 mRNA showed 1.56 ± 0.30 fold increase; independent experiments; t test $p=0.083$). These data indicate that either *HMOX1* expression is directly controlled by TFEB or that sucrose-induced lysosomal deficits raise ROS. Analysis of *HMOX1* promoter revealed the presence of a consensus CLEAR element near the *HMOX1* promoter (Figure 15). The consensus sequence GTGCACTG is found at position -11 from the transcription initiation site for *HMOX1*. Furthermore *HMOX1* is listed among the genes

directly regulated by TFEB in the earlier studies that identified TFEB as a master regulator of lysosomal function [69, 70].

To gauge the contribution of ROS-dependent and CLEAR-dependent components of *HMOX1* response to Cu, I analyzed this response in the presence of the antioxidant glutathione (GSH) along with 100 μM $CuCl_2$ for 8 hours. The short exposure to Cu was to eliminate the possible compensatory effects of expression of genes whose products play an antioxidant role. Figure 14E shows that co-incubation with GSH almost completely abolished the effect of Cu on *HMOX1* expression, suggesting that after 8 hours of Cu exposure the expression of *HMOX1* is mainly activated by ROS, and that at least at this time point TFEB does not directly activate *HMOX1*. To test whether or not ROS are required for the activation of *HMOX1* expression after long Cu exposure, I pre-treated TFEB transfected cells with 100 μM $CuCl_2$ for 40 hours, followed by co-incubation with GSH and Cu for additional 8 hours. At this time point, GSH was able to significantly reduce the levels of *HMOX1* expression induced by Cu, but it did not completely abolish the effect of Cu as cells treated with both Cu and GSH showed significantly higher levels of *HMOX1* compared to untreated cells (Figure 14E). These data suggest that after long Cu exposure TFEB may regulate *HMOX1* expression in a ROS-independent manner. Since this timeframe is compatible with the time required for the TFEB-dependent activation of CLEAR network genes by Cu, I conclude that within the longer timeframe of exposure, TFEB has a direct effect on the expression of *HMOX1*, a gene whose product is an antioxidant.

ROS are damaging to several key cellular components, including mitochondria. With this in mind, I analyzed the effect of Cu on mitochondrial membrane potential, as readout of overall cellular health. Cells were treated with 1, 10, or 100 μM $CuCl_2$ or left untreated and analyzed in

a fluorometer, using the mitochondrial membrane potential sensitive fluorescent dye JC-1. In the presence of mitochondrial membrane potential, JC-1 accumulates in the mitochondria resulting in a shift of fluorescence emission from green to red. Loss of mitochondrial membrane potential is detected as a decrease in the red to green ratio of JC-1 [149, 150]. Interestingly, basal mitochondrial membrane potential was significantly increased (by 44.00 ± 9.62%, n=4; Figure 16A) in TFEB-transfected cells compared to cells transfected with an empty vector, indicative of healthier mitochondria in TFEB-overexpressing cells under the resting conditions. This hyperpolarization of mitochondria can be attributed to a faster turnover of damaged mitochondria through TFEB-induced autophagy. This is consistent with the role of autophagy and lysosomes in maintaining healthy mitochondria [151, 152]. CCCP, an uncoupler of mitochondrial electron transport chain, was used as a control; it significantly decreased the mitochondrial membrane potential in both mock and TFEB-treated cells (Figure 16A).

Cu caused depolarization of mitochondrial membrane in a dose-dependent manner in both mock and TFEB-transfected cells. Consistent with the higher levels of oxidative stress in TFEB-overexpressing cells exposed to Cu, the hyperpolarization observed in TFEB cells was not sufficient to prevent the loss of membrane potential caused by Cu. In fact, I observed that the decrease in membrane potential was exacerbated to a greater degree in TFEB-overexpressing cells than in cells transfected with an empty vector (Figure 14B). At 1 μM $CuCl_2$, the mitochondrial membrane potential of mock-transfected cells remained unaffected whereas it was decreased by 8.36 ± 1.59% in TFEB cells (n=3, $p<0.01$). Mitochondrial membrane potential of cells exposed to 10 μM $CuCl_2$ was decreased by 9.03 ± 0.83 in mock and 19.02 ± 3.79% (n=3, $p=0.06$) in TFEB cells, respectively. The highest loss of membrane potential was observed when cells where treated with 100 μM of $CuCl_2$ and the drop in membrane potential was significantly

higher in TFEB cells than in mock cells (45.11 ± 5.03% vs 23.48 ± 3.89% decrease in TFEB and mock cells, respectively; $p<0.05$). These data suggested that during prolonged exposure of HEK-293 cells to high levels of Cu TFEB does not protect against Cu toxicity; in fact, TFEB overexpression seems to increase the toxic effects of prolonged Cu exposure in these cells.

The data described show that Cu induces the activation of recombinant TFEB, and such activation has a profound effect on the lysosomal status and on oxidative stress. This is consistent with magnified effect of Cu exposure on oxidative stress in TFEB-overexpressing cells. Similar to the recently published data on increased cancer drug sequestration and retention in the lysosomes of TFEB overexpressing cells [125], I propose that Cu-induced lysosomal stress activates overexpressed TFEB and lysosomal biogenesis, increasing Cu retention in the lysosomes and, therefore, ROS and oxidative stress. This is critically important for the value of TFEB upregulation and stimulation as a therapy against oxidative stress and metal toxicity – factors involved in a range of conditions including stroke and several neurodegenerative diseases [112, 114, 116, 135-147].

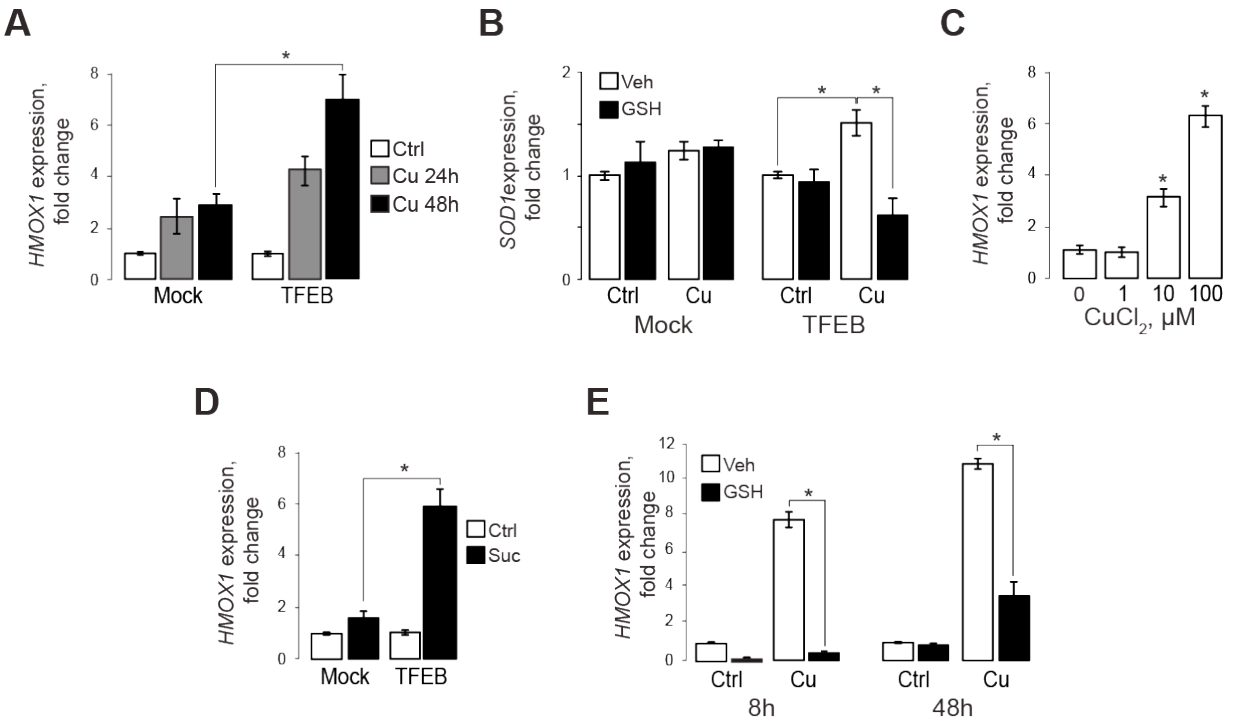

Figure 14. Copper increases the expression of *HMOX1* in HEK-293 cells

A. qPCR analysis of *HMOX1* expression of mock or TFEB HEK-293 cells exposed to 100 μM $CuCl_2$ for 24 or 48 hours. B. Cu induces the expression of *SOD1* mRNA in TFEB cells, but not mock cells, treated with 100 μM $CuCl_2$ for 8 hours in the presence or absence of 2 mM GSH. C. qPCR analysis showing that *HMOX1* expression depends on $CuCl_2$ concentration (0, 1, 10, 100 μM $CuCl_2$) in TFEB-transfected cells. D. Expression of *HMOX1* mRNA is increased by sucrose in TFEB-transfected cells shown by qPCR analysis. Values represented as mean ± SEM of three independent experiments; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ considered significant (*) (A-C). E. *HMOX1* expression in TFEB-transfected cells upon treatment with 100 μM $CuCl_2$ for 8 or 48 hours with or without 2 mM GSH for 8 hours. Values represented as mean ± SEM of two independent experiments. Values represented as mean ± SEM of two independent experiments; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ considered significant (*).

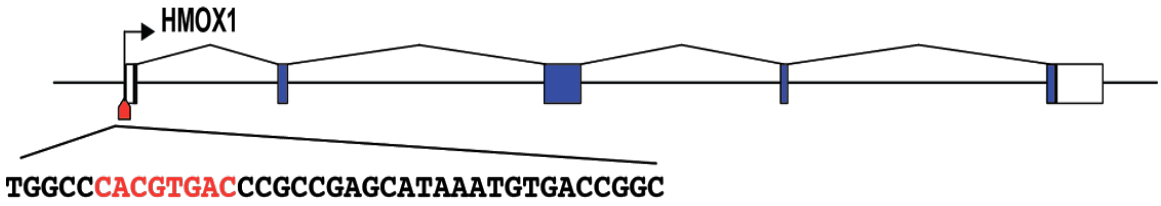

Figure 15. *HMOX1* promoter contains CLEAR sequence

Promoter analysis of *HMOX1* gene. Blue boxes represent exons. Arrow represents the transcriptional start site of *HMOX1*. Consensus CLEAR sequence found in the promoter region of *HMOX1* is shown in red and is found at position -11 from the transcriptional start site. Promoter analysis was carried out using GenePalette software.

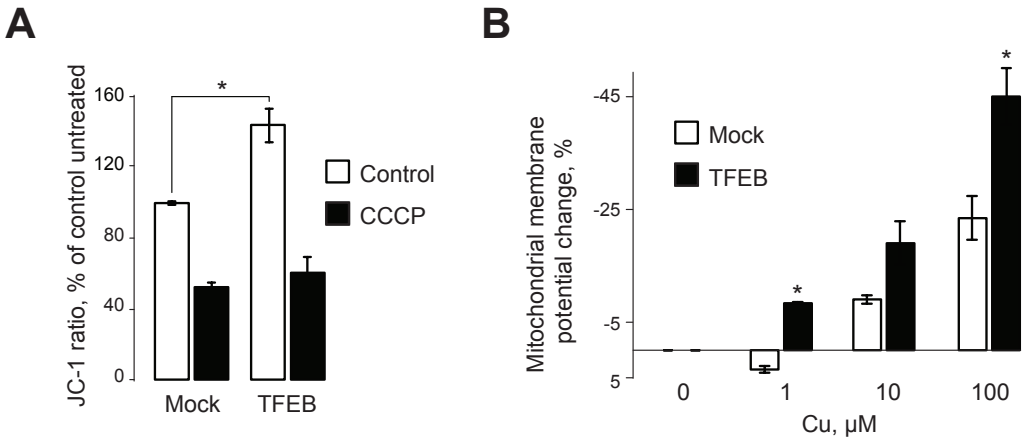

Figure 16. Copper decreases mitochondrial membrane potential

TFEB and mock HEK-293 cells were treated with 0, 1, 10, 100 μM $CuCl_2$ for 48 hours and mitochondrial membrane potential was assessed using JC-1 dye. A. Red/green JC-1 ratio shows that TFEB-tranfected cells have higher mitochondrial membrane potential than mock-transfected cells. B. Reduction of mitochondrial membrane potential is dependent on CuCl2 concentration in both mock and TFEB-transfected cells. Plots represent the difference between JC-1 ratio of no Cu and Cu for mock and TFEB-cells. * represents statistically significant difference (a two-tailed, unpaired t-test with $p<0.05$) relative to the values recorded in similarly treated mock-transfected cells.

3.2.4 TFEB does not affect cytoplasmic levels of copper

Since TFEB overexpression increased toxicity in HEK-293 cells exposed to Cu, as observed in Figure 17, I tested whether the cytoplasmic levels of Cu were altered by TFEB overexpression. For this purpose, I measured the protein levels of the Cu chaperone to superoxide dismutase (CCS), as an indirect method to measure cytoplasmic Cu [153, 154]. CCS delivers Cu to superoxide dismutase and it has been shown that in the presence of high levels of Cu the proteasome-dependent degradation of CCS is induced [155], as Cu is more available to superoxide dismutase. Figure 17 shows that overexpression of TFEB did not affect the reduction of CCS levels after 24 hours exposure to 100 μM $CuCl_2$, as both mock- and TFEB-overexpressing HEK-293 cells presented reduced CCS levels by 60%. In addition, I measured the mRNA levels of *MT2A* in both mock- and TFEB-overexpressing HEK-293 cells (Figure 18). Cells were treated with 100 μM $CuCl_2$ for 24 hours, followed by RNA extraction. In addition, chase experiments were done in which RNA was collected after 24 hours following Cu removal. As expected, the expression of *MT2A*, whose protein product is a metallothionein, increased in response to Cu in both mock- and TFEB-transfected cells. The levels of *MT2A* mRNA were not significantly different between mock- and TFEB-transfected cells. Moreover, removal of Cu from the medium resulted in the decrease of *MT2A* expression in both mock and TFEB-overexpressing cells, again, with no significant difference between them. Together, these data suggest that TFEB overexpression does not cause a major retention of Cu in the cytoplasm. Instead, I propose that the increased ROS response and mitochondrial damage observed in response to TFEB overexpression are a consequence of higher Cu loading into the lysosomes, whose total volume rise as a consequence of TFEB activation.

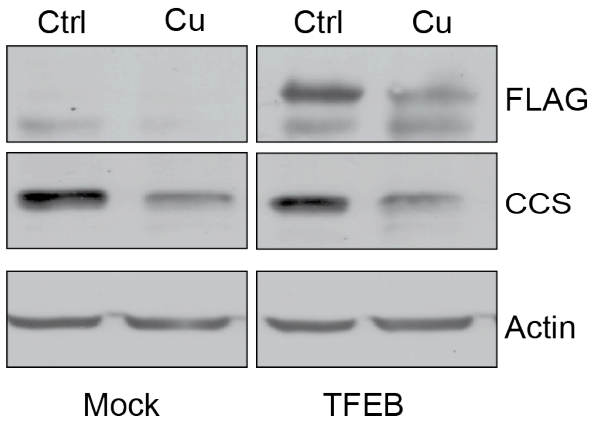

Figure 17. CCS levels are not affected by TFEB upon copper exposure

Western blot analysis of CCS levels in mock- and TFEB-transfected HEK-293 cells after exposure to 100 μM $CuCl_2$ for 24 hours. Actin was used as loading control. Image is representative of 4 independent experiments.

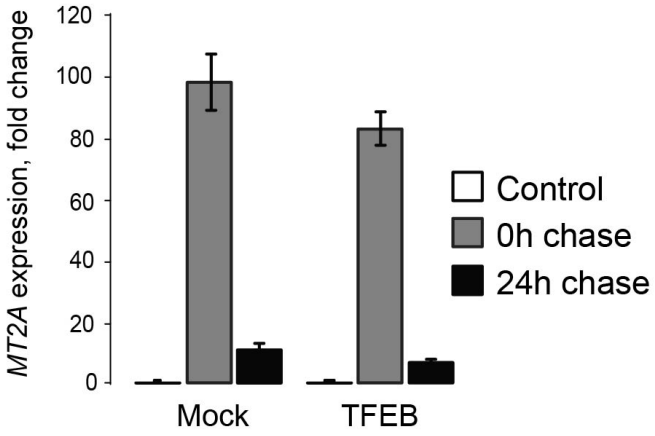

Figure 18. TFEB does not affect *MT2A* expression in response to copper

qPCR analysis of *MT2A* levels in mock- and TFEB-transfected HEK-293 cells after exposure to 100 μM $CuCl_2$ for 24 hours (0h chase). After 24 hours of Cu exposure, Cu was removed from the medium and cells were incubated with fresh medium for another 24 hours (24h chase). The expression of *MT2A* was not significantly different between mock- and TFEB-transfected cells. Values represented as mean ± SEM of three independent experiments.

3.2.5 TFEB, oxidative stress, and lysosomal exocytosis

TFEB was shown to have therapeutic effect for several diseases [47, 99-101], ostensibly by enhancing cellular clearance of toxic compounds. One of the proposed mechanisms of such clearance was enhanced lysosomal exocytosis [97, 156]. The recently published data [22], including our analysis of Zn and Cu handling [1, 68], strongly indicates a key role for lysosomal exocytosis in clearance of transition metals. Why was recombinant TFEB toxic when HEK-293 cells were exposed to Cu? To answer this question, I proposed that lysosomal exocytosis is a rate-limiting step in the TFEB-dependent lysosomal clearance process, and in TFEB-overexpressing HEK-293 cells, increased sequestration and retention of Cu in the lysosomes is not effectively countered by lysosomal exocytosis, leading to enhanced oxidative stress. It is possible that TFEB will have more cytoprotective effects in cells with high rates of lysosomal exocytosis. Not aware of a way to specifically upregulate lysosomal exocytosis by pharmacological or genetic means, I sought to test our model using cells with higher lysosomal exocytosis rates. HEK-293 cells do not appear to have a robust lysosomal exocytosis process as indicated by exocytosis of the lysosomal enzyme ß-hexosaminidase [48] (ß-Hex, Figure 19A). In contrast, HeLa cells showed robust ß-Hex and transition metal exocytosis [1, 68] and these cells were chosen to supplement the data obtained with HEK-293 cells.

Figure 19B shows that when HeLa cells were exposed to 1, 10 or 100 μM of Cu for a period of 1, 3 or 16 hours, mock-transfected cells displayed increased *HMOX1* mRNA levels, indicative of increased oxidative stress. Mock-transfected cells showed measurable increase in *HMOX1* mRNA at 1 μM Cu after 1-hour exposure (1.54±0.09 fold increase, $p<0.05$, $n=3$). Under these conditions, such an increase was not observed in TFEB-transfected HeLa cells. Sixteen-

hour long exposure to 100 μM Cu induced a significant increase in *HMOX1* mRNA level in mock-transfected cells: 10.30 ± 0.94 fold increase ($p<0.05$, $n=3$), while such increase averaged only 5.53 ± 0.47 fold in TFEB-transfected cells. These data suggest that in HeLa cells TFEB has a cytoprotective effect at short exposure and low levels of Cu.

Interestingly, the trend did not persist at longer exposure times as 24-hour long exposure to Cu caused the same change in *HMOX1* expression in mock- as in TFEB-expressing cells (Figure 19C). Lysosomal exocytosis is regulated by ionic events including Ca-dependent SNARE interaction [48], and it is possible that Cu interferes with some aspect of SNARE function. The importance of lysosomal exocytosis for Cu detoxification is illustrated in Figure 19D: in HeLa cells, the response of *HMOX1* to Cu is accentuated by the siRNA-dependent knockdown of the lysosomal-plasma membrane SNARE VAMP7. Figure 19E shows that prolonged exposure to high concentrations of Cu inhibits ß-Hex exocytosis. In accordance with the previous evidence, TFEB overexpression stimulates ß-Hex exocytosis, but it does not eliminate the inhibitory effect of high Cu exposure (Figure 19E). Based on these results, I propose that TFEB increases the lysosomal capacity for exocytosis and, with it, TFEB increases Cu detoxification. However, under chronic exposure to high Cu levels, TFEB effect may be toxic due to an increase in the lysosomal Cu absorption capacity and a decrease in their exocytosis. This is a novel aspect for the Cu toxicity.

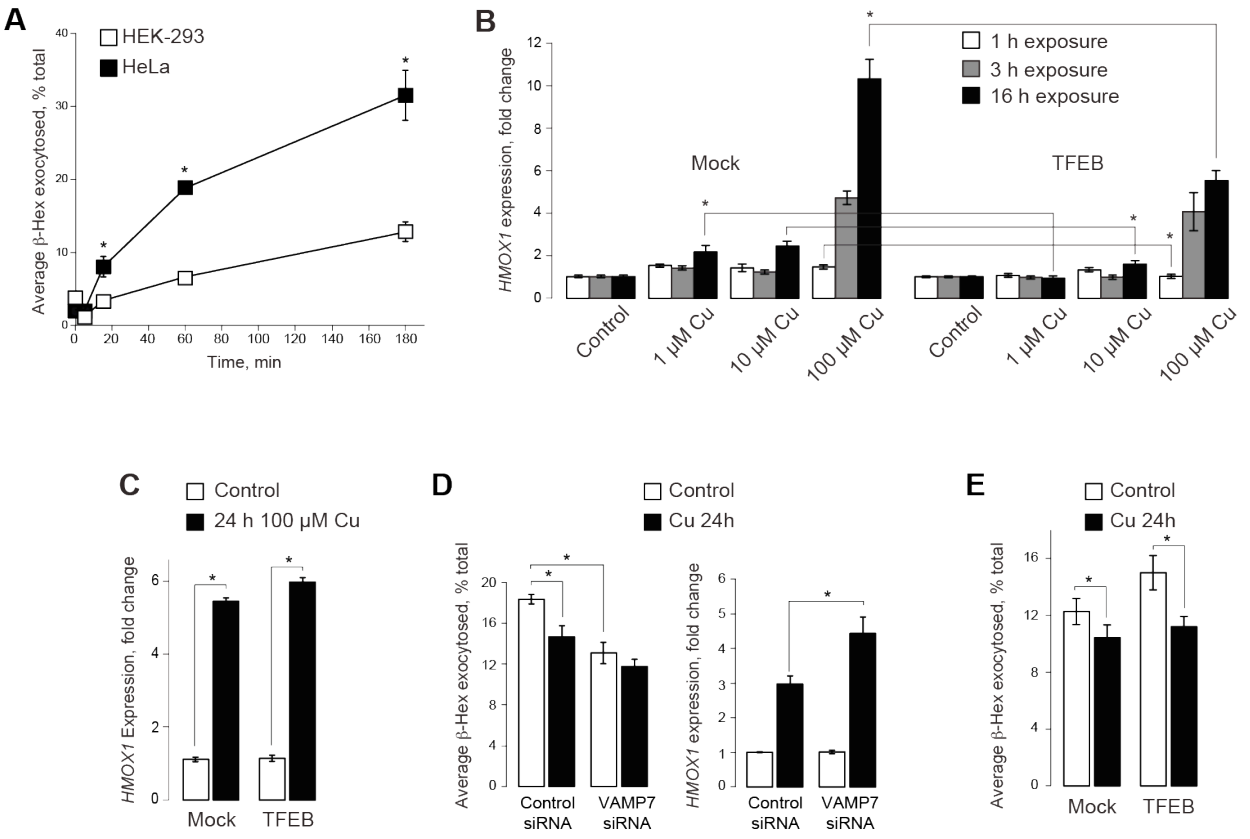

Figure 19. TFEB decreases oxidative stress induced by moderate copper exposure in HeLa cells

A. Time course of ß-Hex exocytosis assay in HEK-293 and HeLa cells ß-Hex levels in the medium were measured at 0, 5, 15, 60, and 180 min. HeLa cells exhibit a higher rate of lysosomal exocytosis than HEK-293 cells. B. qPCR analysis of *HMOX1* expression in mock- or TFEB-transfected HeLa cells exposed to 1, 10 and 100 μM $CuCl_2$ for 1, 3 and 16 hours. Data were normalized to the corresponding untreated controls, which were taken as 1. C. The same experiments performed at 24-hour time point, with 100 μM Cu. D. ß-Hex exocytosis (left panel) and *HMOX1* expression (right panel) in HeLa cells treated with Cu as a function lysosomal exocytosis, which was suppressed using VAMP7 siRNA. ß-Hex levels in the medium were measured after 1 hour of exocytosis. E. Lysosomal exocytosis (ß-Hex exocytosis) in mock- and TFEB-transfected HeLa cells treated with Cu. Values represented as mean ± SEM of three independent experiments; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*) considered significant.

3.3 DISCUSSION

In the course of the studies described in this chapter, I have shown that exposure to transition metals activates recombinant overexpressed TFEB. Cu exposure was associated with TFEB dephosphorylation, resulting in activation of TFEB-dependent transcription. I have also shown that TFEB regulates the expression of *HMOX1*, a gene involved in the response to oxidative stress. Although *HMOX1* has been previously identified as being part of the network of TFEB-regulated genes, this is the first time that *HMOX1* expression and oxidative stress have been directly linked to TFEB activity. Additionally, our data contribute to better understanding of the therapeutic potential of TFEB activation or overexpression by identifying some of the margins for its effects.

I show that recombinant TFEB protects against Cu toxicity at moderate levels of Cu exposure, but it is more toxic at high levels of exposure; in fact, in HEK-293 cells, TFEB overexpression aggravated the Cu-induced loss of membrane potential despite increasing mitochondrial membrane potential under basal conditions (Figure 16). It does not seem likely that such effect includes cytoplasmic Cu, as I was unable to detect measureable differences in cytoplasmic Cu between mock- and TFEB-transfected cells using the protein levels of CCS (Figure 17).

The toxic effect of TFEB overexpression in HEK-293 cells is likely due to their lack of efficient lysosomal exocytosis (Figure 19A). In contrast, cells with robust lysosomal exocytosis, such as HeLa cells, are more likely to manifest cytoprotective function of TFEB. Although transition metals are effectively secreted with lysosomal exocytosis [22, 68], it is possible that

Cu interferes with that exocytosis mechanism in several ways. Others and I have recently shown that brief (1-8 hours) exposure to Cu activates lysosomal exocytosis [1, 22]. However, prolonged exposure to Cu seems to suppress lysosomal exocytosis in HeLa cells as shown in Figure 19E. It is possible that short and long exposures to Cu have different effects on various components of the lysosomal exocytosis machinery.

I have also shown that Cu exposure induces dephosphorylation of exogenous TFEB, and subsequent nuclear translocation, resulting in increased expression of lysosomal genes (Figure 7). It is important to note that phosphorylated TFEB was also found in the nucleus of control and Cu-treated cells, suggesting that the nuclear translocation of TFEB does not necessarily require its dephosphorylation (Figure 7A). The mechanism by which Cu activates TFEB remains to be elucidated. A likely mechanism of TFEB activation by Cu is the lysosomal deficits in Cu-treated cells. mTORC1 activity requires functional v-ATPase [94]. Inhibition of the ATPase by Cu should cause mTORC1 inhibition and TFEB activation. Furthermore, it has been shown that changes in lysosomal positioning can affect mTORC1 activity [157]. In fact, Korolchuk *et al* showed that clustering of lysosomes due to disrupted transport leads to a reduced mTORC1 activity [157]. Since TFEB is activated when mTORC1 activity is inhibited, defects in lysosomal transport could induce the activation of TFEB. Therefore, the aggregation of lysosomes could be a consequence of disrupted lysosomal transport in response to Cu, inducing the inactivation of mTORC1. In this scenario, increased number of lysosomes will result in more lysosomal aggregates, in agreement with the increased number of aggregates observed in TFEB-overexpressing cells treated with Cu (Figure 11C). An effect of Cu (or Cu-induced ROS) on lysosomal traffic may account for the reported effects of Cu on lysosomal exocytosis.

Interestingly, in HEK-293 cells TFEB overexpression caused an increase in mitochondrial membrane potential (Figure 16). It is possible that this observation is due to up regulation of mitophagy of damaged mitochondria as a consequence of TFEB overexpression. Additionally, TFEB overexpression could increase mitochondrial biogenesis, as it has been recently shown [158, 159], resulting in a higher mitochondrial membrane potential due to increased number of mitochondria. Another possibility is that TFEB directly regulates mitochondrial genes associated with membrane potential; although there is no evidence showing direct regulation of such genes by TFEB, genes involved in the TCA cycle and mitochondrial oxidative phosphorylation have been listed as direct targets of TFEB [70]. It is not surprising that TFEB can regulate biogenesis and gene expression of organelles other than the lysosome; in fact, TFE3, a TFEB relative, has been shown to regulate the expression of genes involved in the Golgi apparatus function and stress response [106]. This evidence supports our idea that TFEB can regulate different cellular processes, as discussed below.

In addition to the above observations, my data show that TFEB may be involved in the response to oxidative stress. The earlier studies describing the gene network regulated by TFEB have identified several genes that contain TFEB-binding sequence or CLEAR element and they are involved in different cellular pathways; however, studies have been focused on the genes that regulate lysosomal-associated processes. Among the genes that are not directly related to the lysosome is *HMOX1*. It should be noted that *HMOX1* and other genes involved in oxidative stress response, such as *GPX1* (Glutathione peroxidase1) and *GSTO1* (Glutathione S-transferase omega 1), are found in the original set of genes identified as TFEB-dependent [69, 70]. The fact that *HMOX1* promoter region contains a CLEAR sequence suggests an exciting possibility that beyond its role in lysosomal biogenesis, TFEB may drive a broader stress response mechanism,

involving antioxidant genes. Indeed, I have shown that Cu induces expression of *HMOX1* in a ROS-dependent manner; however, my data also indicate that only a fraction of *HMOX1* expression depends solely on TFEB activation. This is evidenced by the increase on *HMOX1* mRNA levels observed in TFEB overexpressing cells treated with sucrose and by the fact that GSH did not completely abolish the expression of *HMOX1* after long exposure to Cu (Figure 14E). Furthermore, the basal levels of *HMOX1* in TFEB overexpressing cells were 30% higher than in cells transfected with an empty vector (not shown). With this in mind, I cannot rule out the possibility that TFEB controls the function of other transcription factors involved in the response to oxidative, such as MTF-1 and NRF2, the latter known to regulate the expression of *HMOX1* as well. The role of TFEB in the oxidative stress response is an interesting aspect that has not been explored before. Future studies should focus on the role of TFEB in oxidative stress, especially because there is increasing evidence showing the relationship between autophagy and ROS [160, 161].

The expression of lysosomal genes is regulated not only by TFEB. It has recently been shown that TFE3 regulates the expression of genes involved in autophagy and is activated in response to lysosomal stress [72, 84, 128]. TFE3 also binds to promoter regions containing the CLEAR sequence, thus it may control the expression of TFEB-regulated genes as well. As shown in Figure 8C, TFE3 overexpression reversed the drop in gene expression after Cu treatment; however the increase in gene expression in response to Cu was somewhat lower than the one observed in TFEB-transfected cells. At present, I do not know the reason for the lower magnitude of TFE3-dependent response to Cu. Since both TFEB and TFE3 seem to receive input from the same TFE3-dependent signaling pathway, it is likely that some events or processes downstream of TFEB/TFE3 activation are responsible for these differences. Such processes may

include efficacy of transcriptional activation, or some other regulatory mechanisms converging on these transcription factors downstream of their activation. As shown in Figures 7 and 8, in mock-transfected cells, Cu exposure causes a loss of mRNA corresponding to several lysosomal genes. Whether or not oxidative stress is a factor in the differences between TFEB and TFE3 efficacy remains to be elucidated.

A related issue exists with the status of native TFEB/TFE3 responses in our model. TFEB overexpression has been used to study its activation, and the resulting upregulation of the lysosomal gene network in the vast majority of reports utilize cell cultures. It is important to note that it was not only Cu that showed low activation of endogenous TFEB; accordingly, I was unable to consistently detect activation of the lysosomal gene network using native TFEB stimulated by starvation, sucrose or TFEB activator Torin-1 [129] in HEK-293, HeLa, RPE-1 or HUH-7 cells, consistent with low levels of TFEB in these cells, and with previously published results [84, 85, 95, 101, 162]. In experiments in which such activation was detected, I was also able to detect activation of lysosomal gene expression by Cu (Figure 9), however, the small amplitude and poor reproducibility of these responses precluded its further analysis. The reasons for these effects are unclear, but very low levels of native TFEB/TFE3 in these cells have been suggested, and it is in agreement with what has been observed in different human tissues [74]. Their scarcity may become important if oxidative stress does, indeed, affect stability of mRNA corresponding to the lysosomal-associated genes, resulting in the loss of mRNA as shown in Figures 7 and 8. It is possible that cultured cells are not prepared to respond to nutrient starvation or lysosomal stress because they normally grow in optimal conditions of nutrient abundance, thus they do not maintain high basal levels of TFEB. This may make them less likely to respond to other lysosomal stressors, including Cu.

The data described here suggest that Cu and Fe activate overexpressed TFEB and this activation leads to measureable change in lysosomal status and the ability of cells to fight oxidative stress caused by transition metal exposure. I show that the full cytoprotective effect of TFEB manifests only when cells have robust lysosomal exocytosis. Indeed, TFEB overexpression seems to increase transition metal toxicity in cells with low lysosomal exocytosis rates. These data suggest that cytoprotective function of TFEB fully manifests when lysosomal exocytosis is effective, which is not the case for all cell types. It is tempting to suggest that therapies involving TFEB activation or upregulation should also focus on pharmacological stimulation of lysosomal exocytosis.

3.4 ACKNOWLEDGEMENTS

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4.0 BRIEF EXPOSURE TO COPPER ACTIVATES LYSOSOMAL EXOCYTOSIS

4.1 INTRODUCTION

Lysosomal exocytosis was originally described as a means of repairing plasma membrane via recruitment of the lysosomal membrane to a place of membrane damage [44]. Lysosomal fusion with the plasma membrane depends on a specific set of SNARE components [48], suggesting a regulated process. Therefore, the significance of lysosomal exocytosis likely extends beyond pathological conditions of membrane rupture, possibly including response, adaptation, or signaling involvement. The latter idea finds support in the recent series of evidence on the role of lysosomes in transition metal extraction from cells [22, 163].

Transition metals such as Fe, Zn and Cu enter cells via plasma membrane transporters or via endocytosis followed by absorption through lysosomal/endosomal transporters [2, 124, 145]. While all cells require some levels of transition metals, an excessive exposure to transition metals is toxic, necessitating their tight regulation. In the cytoplasm, transition metals are bound to chelating proteins, exported via plasma membrane transporters or absorbed into organelles, which is followed by exocytosis. Among the transporters implicated in transition metal absorption into lysosomes are the Zn transporters ZnT2 and ZnT4 (SLC30A2 and SLC30A4), and a Cu transporter ATP7B. Suppression of these transporters was shown to significantly affect Zn and Cu handling [22, 163].

Lysosomal transition metal importers are regulated in a variety of ways. ATP7B, the Cu transporter whose loss is responsible for Wilson's disease [112], responds to Cu exposure by moving from the trans-Golgi to the lysosomes [22, 60]. Cu absorption by the lysosomes is followed by its extraction from the cells via SNARE-dependent lysosomal exocytosis [22]. Thus, the main mechanism of ATP7B regulation appears to be translocation to the lysosomes or perhaps formation of the new population of ATP7B-bearing lysosomes. In addition, ATP7B interacts with the p62 subunit of dynactin, facilitating lysosomal transport towards the apical pole of hepatic cells where Cu is released [22]. ZnT transporters, especially ZnT2, have been shown to translocate to the lysosomes in response or in parallel to Zn exposure, and structural determinants of such translocation have been proposed [164]. At the same time, the transcription of genes coding for several ZnTs is regulated by the transcription factor MTF-1, which responds to Zn and other transition metals [165]. These data show that lysosomal metal uptake capacity is regulated by the cytoplasmic transition metals. Whether or not Cu regulates the rate of the lysosomal metal extraction has not been consistently explored. This is the main question of this study.

4.2 RESULTS

4.2.1 Activation of lysosomal exocytosis by short exposure to copper

In Chapter 3 I have demonstrated the effects of long-term exposure to Cu on lysosomal function; importantly I have shown that Cu decreases lysosomal exocytosis and induces oxidative stress (Figure 19). Once absorbed by the cell, Cu enters the lysosome through ATP7B and is then excreted via lysosomal exocytosis. Therefore, blocking lysosomal exocytosis may result in Cu accumulation and metal toxicity. Recent published data indicate that short-term exposure to Cu induces the transport of lysosomes to the plasma membrane, where Cu is released [22]. This response to short-term exposure to Cu would prevent its accumulation and toxic effects. However, it is not clear whether Cu regulates the rate of lysosomal metal extraction.

To analyze the dependence of lysosomal exocytosis on Cu, I measured the release of the lysosomal enzyme β-hex from HeLa cells in the presence of physiological levels of free Ca (1 mM), which is required for the exocytosis process. HeLa cells were incubated in fresh buffer and β-hex levels were measured in the extracellular medium, followed by measuring β-hex content in the total cellular lysate. Figure 20A shows that cells gradually released β-hex, and at the 1-hour time point, cells released about 20% of their β-hex content, which is in line with the previously published data [163]. The addition of 100 μM $CuCl_2$ to the extracellular medium significantly increased the β-hex exocytosis rate (Figure 20A). At the 1-hour mark, the amount of β-hex released by Cu-treated cells was 41.9% higher than in control cells ($n=3$, $p<0.05$). The effect was concentration-dependent, as exposure to 1 and 10 μM $CuCl_2$ had no effect on β-hex release (Figure 20D). Flow cytometry analysis revealed that the plasma membrane levels of lysosomal

protein LAMP1 were increased when cells were treated with 100 μM $CuCl_2$ for 1 hour (Figure 20B), which is in agreement with the β-hex data. In addition, lysosomal exocytosis was increased in retinal pigment epithelial cells 1 (RPE1) exposed to 100 μM $CuCl_2$ for 1 hour (Figure 20C, 367.7% increase, n=3, $p<0.05$). Together, these data indicate that Cu stimulate lysosomal exocytosis.

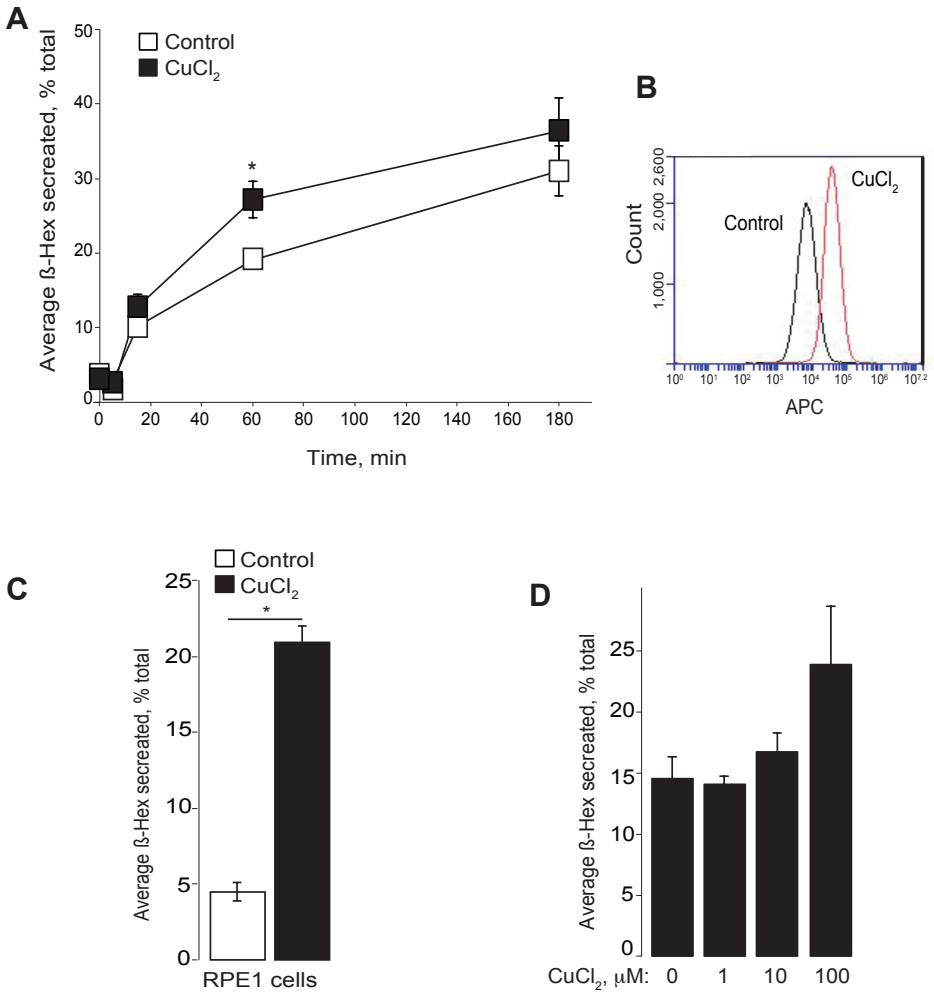

Figure 20. Copper stimulates lysosomal exocytosis in HeLa and RPE1 cells

A. HeLa cells were exposed to 100 μM $CuCl_2$ for 5, 15, 60, and 180 min or left untreated (control). β-hex activity was measured in the extracellular medium at the specified times. β-hex activity in the medium is normalized to the total cellular β-hex activity content. Cu significantly increased lysosomal secretion at 60 min. B. Flow cytometry analysis of LAMP1 at the plasma membrane. HeLa cells were treated with 100 μM $CuCl_2$ for 1 hour, fixed, and incubated with anti-LAMP1 antibody APC-conjugated. Graphs represent APC fluorescence intensity at the plasma membrane, which is increased by Cu. C. Cu stimulates the release of β-hex in RPE1 cells treated for 1 hour with 100 μM $CuCl_2$. D. The effect of Cu on lysosomal exocytosis is dose dependent. HeLa cells were treated with 1, 10, and 100 μM $CuCl_2$ for 1 hour. Values represented as mean ± SEM percent of total β-hex activity of three independent experiments an the statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*).

4.2.2 Copper stimulates VAMP7 and calcium-dependent lysosomal exocytosis

In previously published studies by our laboratory, it has been showed that β-hex release is enhanced by intracellular Ca and suppressed by the removal of SNARE components, which is in agreement with the SNARE/Ca-dependent model of lysosomal exocytosis [163]. Accordingly, my data show that a SNARE-dependent process mediates the stimulatory effect of Cu on lysosomal exocytosis, since 48 hour-long VAMP7 knockdown in HeLa cells reduced the basal and Cu-induced lysosomal exocytosis by 13 and 30%, respectively (Figure 21A, $n=3$, $p<0.05$).

In order to analyze the dependence of lysosomal exocytosis on Ca, intracellular Ca levels of HeLa cells were increased using the Ca ionophore, ionomycin. Additionally, Ca-free buffer was used to reduce extracellular Ca levels of HeLa cells. Figure 21B shows that ionomycin increased both basal and Cu-induced lysosomal exocytosis by 27% and 49% respectively ($n=3$, $p<0.05$). The fact that ionomycin was more effective in stimulating lysosomal exocytosis when Cu was present suggests that Cu facilitates a Ca-dependent step of the lysosomal exocytosis. In addition, incubation of cells with a Ca-free buffer reduced the basal lysosomal exocytosis by 28% ($n=3$, $p<0.05$) and prevented the stimulation of lysosomal exocytosis by Cu (Figure 21C). In order to further explore this outcome, I used a broad plasma membrane Ca channel blocker, Lanthanum (La) [166]. Pre-incubation of cells with 0.1 mM $LaCl_3$ suppressed both basal and Cu-stimulated lysosomal exocytosis; however, $LaCl_3$ effect on the basal exocytosis was higher than on the Cu-stimulated exocytosis (60% vs 36% reduction in control and Cu treated cells, respectively, $n=3$, $p<0.05$, Figure 21D).

High levels (1-10 mM) of La inhibited both basal and Cu-dependent aspects of the lysosomal exocytosis (Figure 21D). Taken together, these data confirm the dependence of lysosomal exocytosis on extracellular Ca. The different effects of Ca-free buffer and La on the ability of Cu to activate lysosomal exocytosis suggest two possibilities regarding the mechanism of its effect on lysosomal exocytosis. First, Cu may activate a plasma membrane channel whose sensitivity to La is low. Although experiments using the Ca dye Fura-2am did not show any measureable Ca influx in response to Cu addition (not shown), it is possible that such influx is very small and localized. Second, Cu may facilitate a Ca-dependent step in lysosomal exocytosis, making it possible to happen under the conditions of suppressed extracellular Ca influx at cytoplasmic Ca levels slightly below normal.

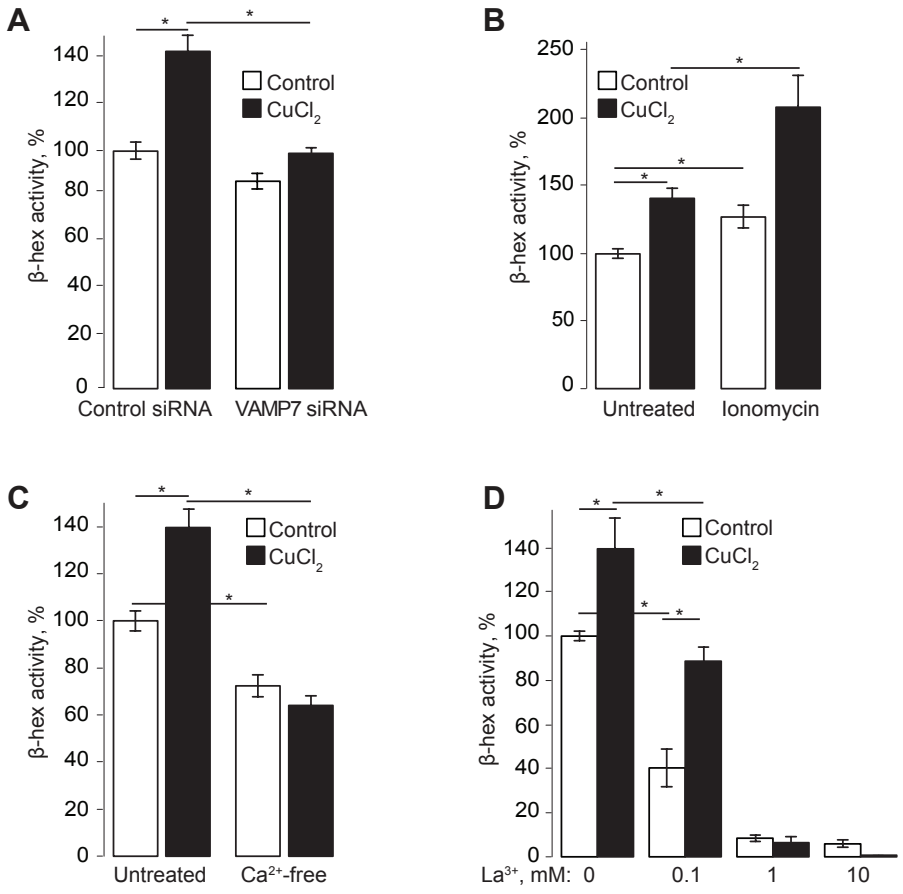

Figure 21. Copper increases VAMP7 and calcium-dependent lysosomal exocytosis

A. HeLa cells were transfected with VAMP7 or control siRNA 48 hours before the experiment was performed. Cells were treated for 1 hour with 100 μM $CuCl_2$ in regular buffer or left untreated. β-hex activity was measured in extracellular medium at 1 hour. VAMP7 siRNA reduces both basal and Cu-induced lysosomal exocytosis. B. Addition of the Ca ionophore, ionomycin, for 15 min increased both basal and Cu-induced lysosomal exocytosis, observed as a decrease in β-hex activity in extracellular buffer at 1hour. C, D. Extracellular Ca is required for lysosomal exocytosis. Incubation of cells in Ca-free buffer reduced β-hex activity in extracellular medium and prevented Cu-induced exocytosis after 1hour (C). Addition of $LaCl_3$ reduced lysosomal exocytosis in a dose-dependent manner. Cells were exposed to 0.1, 1, and 10 mM $LaCl_3$ for 1 hour in the presence or absence of Cu (D). Values represented as mean ± SEM percent of β-hex activity to control cells of three independent experiments and the statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*).

4.2.3 Lysosomal exocytosis and oxidative stress

In order to document the physiological impact of lysosomal Cu uptake and exocytosis I knocked down the lysosomal Cu transporter ATP7B using siRNA (Figure 22A). Cu catalyzes the production of reactive oxygen species [124, 167, 168], which are toxic. Evacuation of Cu and Zn via lysosomal exocytosis was proposed to be a key component of transition metal detoxification [22, 163]. Thus, I reasoned that reducing the evacuation of Cu, by preventing lysosomal Cu uptake, would induce oxidative stress. Figure 22B shows that a 48-hour-long ATP7B knockdown is associated with a measurable increase in the ability of Cu to induce oxidative stress, as indicated by increased heme oxygenase 1 expression (*HMOX1* gene), in response to Cu (23.5 fold increase in control siRNA cells vs 42.6 fold increase in ATP7B siRNA cells; $n=3$, $p<0.05$). *HMOX1* expression is a reliable tool to measure oxidative stress [64, 169] and it is associated with increased lipid peroxidation in cells treated with Cu (Figure 23). Further analysis of ATP7B-knockdown cells suggests that Cu affects lysosomal exocytosis from the cytoplasm. Figure 22C shows that ATP7B knockdown noticeably suppressed basal lysosomal exocytosis, but did not eliminate the stimulatory effect of Cu on lysosomal exocytosis: in ATP7B-knockdown cells, the gain of β-hex release in response to Cu was indistinguishable from that in cells transfected with a control siRNA (26.3% and 34.4% increase, respectively; $n=3$). Therefore, Cu affects lysosomal exocytosis by interfering with membrane fusion from the outside of the lysosome and not by affecting the lysosomal lumen.

The inhibitory effect of ATP7B knockdown on lysosomal exocytosis is likely mediated by the resulting oxidative stress induced by Cu in the cytoplasm, as exposure to oxidative stress induced by tert-Butyl hydroperoxide (TBHP), inhibited β-hex exocytosis as well, indicating that

oxidative stress inhibits lysosomal exocytosis (Figure 22D). Based on these results I propose that Cu stimulates lysosomal exocytosis to accelerate Cu extraction and prevent oxidative damage.

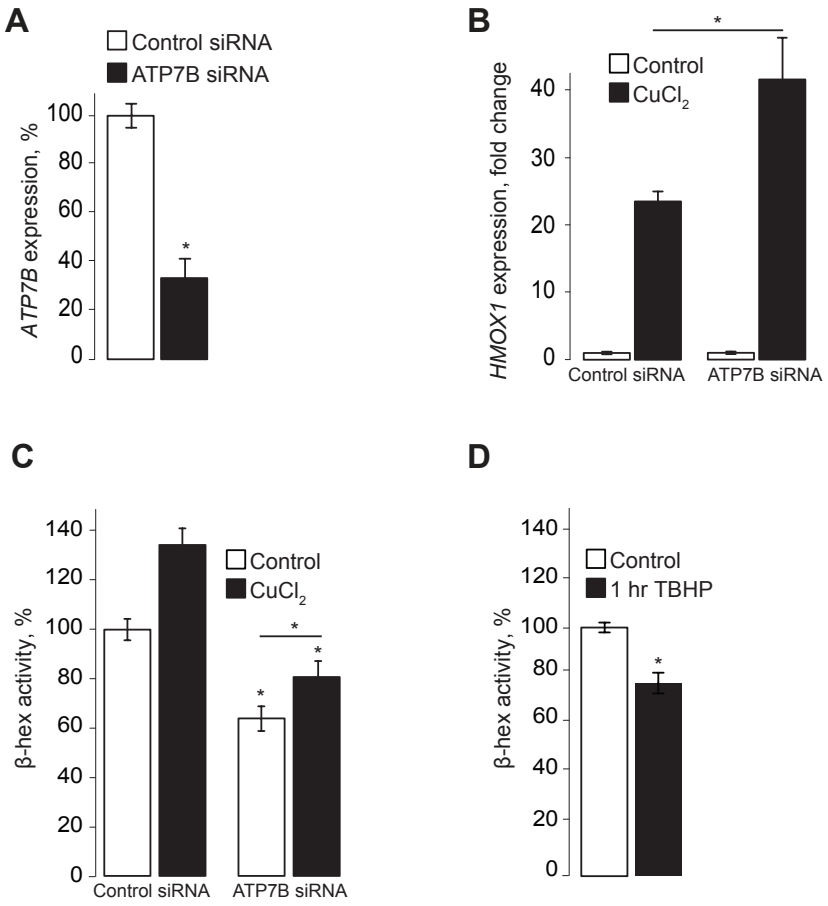

Figure 22. Preventing copper evacuation through lysosomal exocytosis increases oxidative stress in HeLa cells

A, B. qPCR analysis shows that transfection of HeLa cells with ATP7B siRNA for 48 hours significantly reduced *VAMP7* mRNA levels (A) and increased the expression of *HMOX1*, a ROS-responsive gene, in cells treated for 4 hours with 100 μM $CuCl_2$ in growth medium, compared to cells transfected with a control siRNA (B). Values represented as mean ± SEM percent expression of control siRNA (A) or fold change (B). C. Both basal and Cu-induced lysosomal exocytosis was significantly reduced in ATP7B knockdown cells. D. Induction of oxidative stress with TBHP decreased β-hex activity in extracellular medium of HeLa cells, indicating that oxidative stress reduced lysosomal exocytosis. Values represented as mean ± SEM percent of β-hex activity in control cells (untreated); three independent experiments; statistical significance was calculated using a two-tailed, unpaired t-test with $p<0.05$ (*).

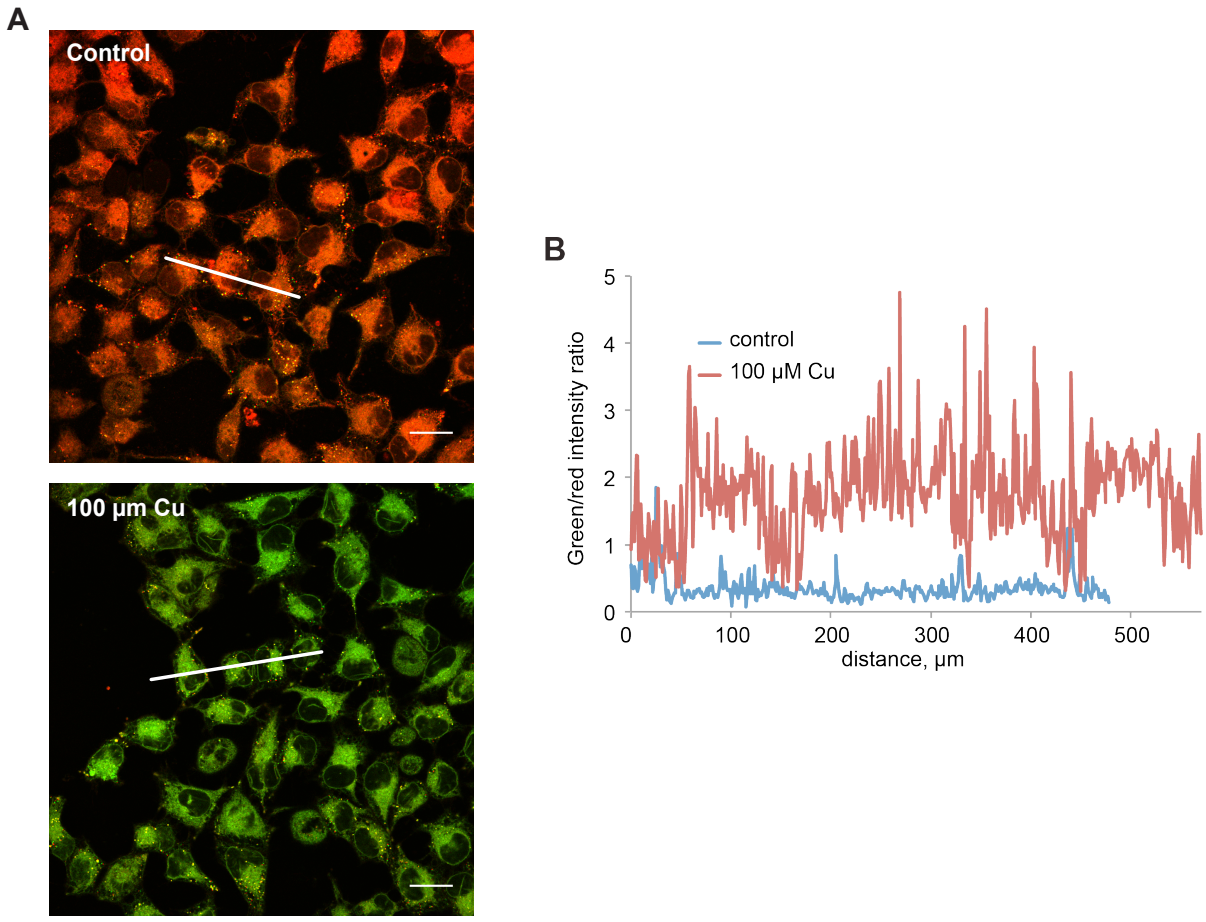

Figure 23. Exposure to copper induces lipid peroxidation in HeLa cells

A. Merge images of live HeLa cells treated for 3 hours with 100 µM $CuCl_2$ and incubated with 5 µM Bodipy 581/591 C1. Lipid peroxidation is detected as a shift from red to green emission. Images are shown as merge of red and green channels. Scale bar represent 24 µm. B. Red and green fluorescence intensities were measured using ImageJ (NIH) along the lines indicated in white in Panel A, and green to red ratios were calculated. Higher green to red ratio is indicative of lipid peroxidation as observed in Cu-treated cells.

4.3 DISCUSION

Lysosomes have emerged as key determinants of transition metal detoxification as lysosomal exocytosis was shown to be indispensable for removal of Cu and Zn from cells. Although the molecular determinants of metal excretion via lysosomal exocytosis have been delineated [22, 163], the functional relations between transition metals and exocytosis are not well understood. The active increase in transcription and translocation of lysosomal metal transporters in response to transition metal exposure suggests a sophisticated relationship between transporters and lysosomal exocytosis.

I showed that exposure to Cu stimulates lysosomal exocytosis. I also showed that Cu-stimulated lysosomal exocytosis requires Ca. While the Ca release through the lysosomal ion channel MCOLN1 was suggested to drive lysosomal exocytosis, it is unlikely to contribute to the Cu-dependent lysosomal exocytosis. First, MCOLN1 does not conduct Cu and does not seem to be activated by Cu [66]. Second, siRNA-driven MCOLN1 knockdown did not affect the Cu-dependent component of the lysosomal exocytosis (not shown). Finally, stimulation of the lysosomal exocytosis by Cu is inhibited by removal of extracellular Ca (Figure 21C) or by addition of extracellular $LaCl_3$ (Figure 21D), suggesting involvement of a plasma membrane Ca channel activated by Cu and poorly sensitive to La. Information on the effect of Cu on plasma membrane channels is limited (see summary in a recent review [166]). TRPA1 and some members of the TRPM family are among candidates for the role of such a channel.

On the other hand, the fact that it was not possible to detect any measurable spike in cytoplasmic Ca in response to the extracellular Cu application suggests a possibility of Cu

inducing a very local Ca influx, or an effect on La-insensitive Ca transporter. Finally, it is possible that Cu sensitizes the machinery responsible for lysosomal fusion with the plasma membrane to Ca. I find that the Cu-dependent component of the lysosomal exocytosis persists in ATP7B-deficient cells (Figure 22B), making it unlikely that Cu exerts its effect via ATP7B-dynactin interaction only. However, the stimulation of lysosomal exocytosis by Cu was absent in VAMP7-depleted cells (Figure 21A), again underscoring the possible role of Cu in Ca-dependent aspects of the lysosomal exocytosis. Whether or not Cu regulates the Ca-dependence of other components of the membrane fusion machinery remains to be answered.

The effect of ATP7B knockdown on lysosomal exocytosis is intriguing. ATP7B not only mediates lysosomal Cu uptake, it also facilitates the transport of lysosomes to the plasma membrane [22]. The reduction of lysosomal exocytosis in control cells transfected with ATP7B siRNA (Figure 22C) can be a consequence of the latter. Furthermore, reducing ATP7B protein levels affects Cu homeostasis in two ways: lysosomal Cu uptake is reduced and evacuation of lysosomal Cu is prevented. Under these circumstances even low levels of Cu present in growth medium can induce oxidative stress and further affect lysosomal exocytosis. Taken together with the increased oxidative stress in ATP7B-knockdown cells (Figure 22B) and with the suppression of the lysosomal exocytosis by oxidative stress, it provides more evidence for a dynamically regulated cytoprotective system driven by lysosomal uptake of transition metal followed by their exocytosis. Beyond their role in cellular digestion, the data presented here redefines lysosomal function as cytoprotective organelles.

4.4 ACKNOWLEDGEMENTS

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5.0 CONCLUSIONS AND FUTURE DIRECTIONS

For a long time, the lysosome was perceived as the digestive system of the cell, mainly because of its ability to degrade macromolecules and organelles through autophagy. However, new evidence had positioned the lysosome as an organelle involved in regulating several cellular processes, including handling of transition metals [36, 71, 75]. The discoveries of the CLEAR network and the transcriptional regulation of lysosomal function by MiT/TFE transcription factors have opened new ways of studying the lysosome and its role in stress response [71, 72, 75, 84, 85, 128]. Throughout my doctoral studies, I have been focused on understanding how transition metals, particularly Cu, affect lysosomal function and how lysosomes respond to the toxic effect of transition metals. Importantly, I have been interested in whether TFEB is activated in response to Cu and how Cu affects lysosomal exocytosis, a process that is required to regulate cellular Cu levels. Two main conclusions can be made from my work: 1) Cu activates transcriptional activity of TFEB and 2) lysosomal exocytosis is differentially regulated by Cu depending on the length of Cu exposure. My findings provide new insights to the current understanding of the role of the lysosome in Cu homeostasis. In addition, my studies on TFEB-overexpressing cells suggest that increasing TFEB activity is not always beneficial to the cell, contrary to what it has been proposed for TFEB as therapeutic agent. A detailed description of my work's conclusions and the questions raised from it are discussed below.

5.1 COPPER ACTIVATES TFEB-GENE NETWORK

As discussed in Chapter 1, TFEB activation can be assessed by three experimental approaches: TFEB dephosphorylation, nuclear translocation of TFEB, and increased expression of CLEAR network genes. In addition, increased lysosomal biogenesis and autophagy can also indicate TFEB activation. The data presented in Chapter 3 shows that Cu induces TFEB activation based on those three experimental approaches. As shown in Figure 7A, 48 hours exposure to 100 μM $CuCl_2$ resulted in both nuclear translocation and dephosphorylation of TFEB in HEK-293 cells transfected with TFEB. Since phosphorylated TFEB is also found in the nuclear fractions (Figure 7A), it is possible that dephosphorylation of TFEB is not the only requirement for its nuclear translocation; instead reduced interaction of TFEB with 14-3-3 proteins may result in less retention of TFEB in the cytoplasm. Thus, further studies should focus on determining how Cu affects the interaction of TFEB with 14-3-3 proteins in the cytoplasm.

When expression of CLEAR network genes was analyzed in TFEB-overexpressing HEK-293 cells, I observed that Cu was able to induce the expression of *CTSD*, *LAMP1*, and *CTSB*, indicating that TFEB is transcriptionally active in response to Cu (Figure 7D). The increase in gene expression in response to Cu was only observed in TFEB-overexpressing cells, but not in mock-transfected cells, indicating that Cu is specifically activating TFEB. These data also suggest that the endogenous levels of TFEB are not enough to induce a transcriptional response to Cu or other TFEB activators, such as sucrose. Figure 7C shows that sucrose induced the expression of CLEAR network genes only when TFEB was overexpressed, because increased gene expression was absent in mock-transfected cells treated with sucrose.

Interestingly, both Cu and sucrose treatment induced a decrease in gene expression in mock-transfected HEK-293 cells. This phenomenon was also observed when mock-transfected cells were starved (Figure 8A), however TFEB overexpression was able to rescue the decreased mRNA levels of *CTSB*, *CTSD* and *LAMP1*. In addition, exposure to 100 μM $FeCl_2$ resulted in increased gene expression CLEAR network genes in TFEB-overexpressing HEK-293 cells, but not in mock-transfected cells. Altogether, these data indicate that overexpressed TFEB is activated by exposure to Cu, resulting in increased gene expression of lysosomal genes. Future studies on this matter should be focused on finding a better cell system in which endogenous TFEB activity can be detected. As discussed in Chapter 1, the expression of TFEB is regulated by alternative splicing in a tissue-dependent manner and its expression levels vary in different tissues [74], thus it is possible that HEK-293 cells do not constitute the best model to study endogenous TFEB. In fact, HEK-293 cells are kidney-derived cells and it has been shown that the expression of most of TFEB variants is low in the kidney [74]. Other cells lines have been used to study endogenous TFEB, such as HeLa cells, however under my experimental conditions, the activation of endogenous TFEB by Torin-1 or Cu was not consistent in HeLa cells. Figure 9 shows an example in which both Torin-1 and Cu were able to induce the expression of lysosomal genes *CTSD* and *LAMP1*.

Although my data show that Cu activates exogenous TFEB, the mechanism of TFEB activation is not clear. Additional studies are required to determine whether Cu directly activates TFEB. One possible scenario is that Cu inhibits mTORC1 activity. In order to address this, mTORC1 kinase activity and localization should be assessed in response to Cu. Since ZKSCAN3 has been shown to negatively regulate the expression of lysosomal genes [73], the activity of ZKSCAN3 should be tested in cells exposed to Cu. If Cu inactivates ZKSCAN3,

inducing the expression of lysosomal genes, then ZKSCAN3 should be translocated from the nucleus to the cytoplasm in response to Cu (See model in Figure 6). In addition, Cu exposure induced lysosomal biogenesis and the aggregation of lysosomes (Figure 11). Lysosomal biogenesis in response to Cu may be a direct consequence of TFEB activation; however, why lysosomes were clustered is not totally clear. One possibility is that Cu affects the transport and movement of lysosomes in the cell. Future studies should focus on exploring the effects of Cu on lysosomal movement. Figure 24 shows a model of TFEB activation in response to Cu and its consequences in lysosomal distribution and biogenesis.

An important conclusion obtained from my work is that TFEB is involved in the response to Cu-induced oxidative stress by directly regulating the expression of the antioxidant gene *HMOX1* (Figures 14 and 15). Future studies should be designed to answer whether TFEB is activated in response to oxidative stress from other sources, not only metals. Finally, my work has shown that TFEB overexpression can enhance Cu-induced toxicity in cells that lack of an efficient lysosomal exocytosis process. As shown in Figure 16B, TFEB overexpression resulted in increased loss of mitochondrial potential in response to Cu in HEK-293. To further understand the effect of Cu on mitochondria depolarization of TFEB-overexpressing cells, future studies should use live cell imaging techniques to quantify the number of depolarized mitochondria in TFEB-overexpressed cells. In addition, the effect of TFE3 overexpression in mitochondrial depolarization should also be addressed.

Since TFEB has been suggested as a potential therapy for several diseases, it is important to consider that TFEB overexpression may enhance toxicity depending on the cell type. However, in cells that have a more efficient lysosomal exocytosis process and Cu levels are

properly handled, TFEB overexpression decreases oxidative stress induced by moderate Cu exposure (1-10 μM Cu for 1 hour). At prolonged Cu exposure (16 hours) at 100 μM Cu, TFEB overexpression was not cytoprotective (Figure 19B). Thus, when studying the therapeutic effect of TFEB expression, it is crucial to analyze how various cells respond to TFEB overexpression, especially when oxidative stress is involved.

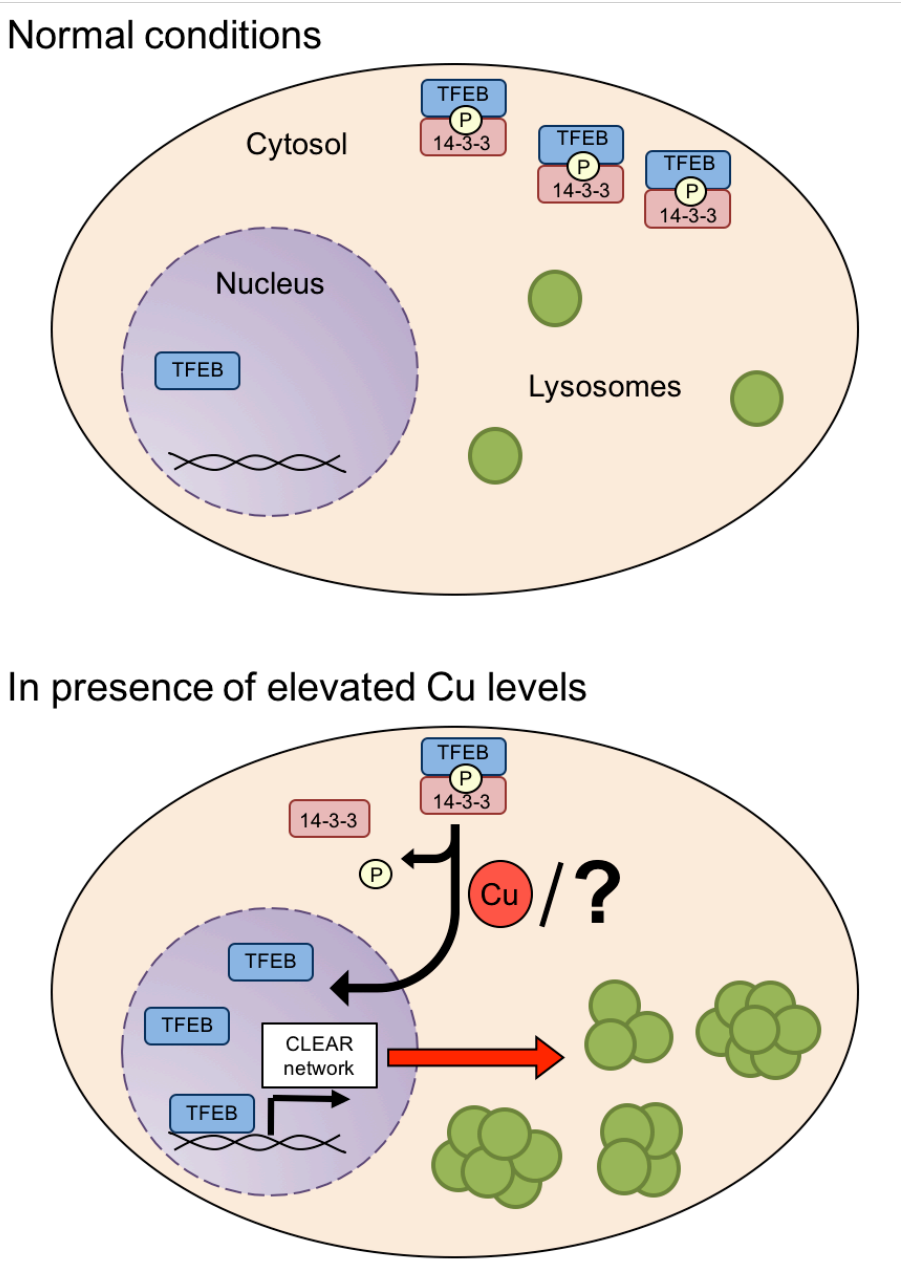

Figure 24. TFEB activation in response to copper

Under normal conditions (top panel), the majority of TFEB is phosphorylated and sequestered in the cytoplasm by interacting with 14-3-3 proteins. In the presence of Cu (bottom panel), TFEB is dephosphorylated and translocated to the nucleus, inducing the expression of CLEAR network genes. Lysosomal biogenesis is increased and clustering of lysosomes (green) is observed in response to Cu. The mechanism by which Cu activates TFEB remains unknown.

5.2 COPPER HAS A DUAL EFFECT ON LYSOSOMAL EXOCYTOSIS

The data presented in Chapter 4 indicate that short exposure to Cu (1 hour) stimulates lysosomal exocytosis in order to avoid Cu-induced toxicity (Figure 20). On the contrary, prolonged exposure to Cu (24 hours) inhibits lysosomal exocytosis (Figure 19E, Chapter 3). Together, the data show that stimulation of lysosomal exocytosis by Cu is an important mechanism of Cu homeostasis, in line with recently published data [22]. Prolonged exposure to Cu may result in inhibition of lysosomal function, likely as a consequence of increased oxidative stress. Future studies should focus on understanding what other aspects of lysosomal function are impaired by Cu. As discussed in Chapter 3, Cu induced the aggregation of lysosomes, suggesting that Cu affects transport and movement of lysosomes. This, in turn, could result in inhibition of lysosomal exocytosis. Therefore, it is important to address the effect of Cu on lysosomal transport towards the plasma membrane. Figure 25 summarizes the dual effect of Cu on lysosomal exocytosis.

In addition, my data show that Cu-stimulated lysosomal exocytosis requires both the lysosomal SNARE VAMP7 and extracellular Ca (Figure 21). Since Cu was unable to stimulate lysosomal exocytosis in the absence of extracellular Ca (Figure 21C), it is possible that Cu stimulates a Ca-dependent step of the exocytosis process. More studies are needed to establish the exact mechanism by which Cu stimulates lysosomal exocytosis. A recent study suggests that Cu-induced ATP7B translocation to the lysosome is responsible for the activation of lysosomal exocytosis [22]; however my data shows that, although lysosomal exocytosis was reduced, the stimulatory effect of Cu was not eliminated by ATP7B knockdown (Figure 22C). This indicates that Cu is able to stimulate lysosomal exocytosis for outside the lysosomal lumen. Future studies

should focus on understanding how Cu stimulates lysosomal exocytosis from the cytoplasm. Specifically, studied should address if Cu affects lipid composition of membranes, resulting in increased lysosomal exocytosis or if Cu affects SNARE interaction and function.

Importantly, my research shows that there is a relationship between oxidative stress and Cu excretion through lysosomal exocytosis. When VAMP7 was depleted from cells, therefore lysosomal exocytosis was inhibited, Cu-induced oxidative stress was higher than in control cells (Figure 19D). In addition, inhibition of Cu loading into lysosomes by ATP7B knockdown also resulted in increased Cu-induced oxidative stress (Figure 22B). Furthermore, induction of oxidative stress with TBHP decreased lysosomal exocytosis (Figure D). Together, these data suggest that lysosomal exocytosis is essential to avoid Cu-induced oxidative stress.

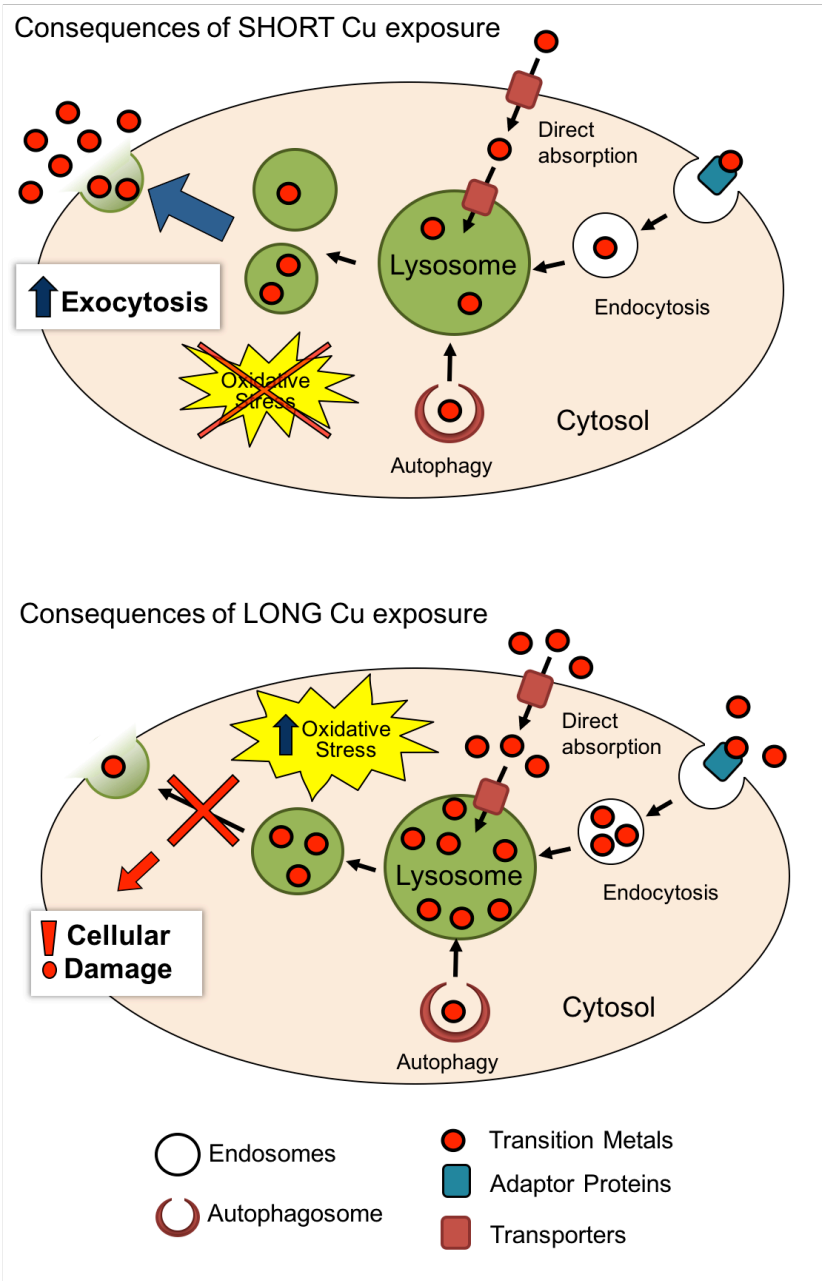

Figure 25. Model of the dual effect of copper on lysosomal exocytosis

Short exposure to Cu (1 hour) stimulates lysosomal exocytosis in order to excrete Cu and avoid Cu-induced oxidative stress (top panel). On the other hand, long exposure to Cu (24 hours) inhibits lysosomal exocytosis, resulting in increased Cu-induced oxidative stress and cell damage (bottom panel).

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