5. Metal homeostasis in plant mitochondria

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5.1 Introduction

Most scientists describe a "transition metal" as any element in the *d-block* (groups 3 to 12) of the periodic table. Based on their chemical properties, transition metals are essential in many biochemical processes. They are central for life because of their ability: i) to undergo oxidation-reduction (redox) state changes under biological conditions; and ii) to establish several stable coordinative bonds to electron pair donor atoms of organic ligands (such as proteins) in a defined geometry. Indeed, several metal ions participate in multiple roles in protein structure and function such as catalysis, electron transfer, ligand binding and structural integrity, defining the so-called metalloproteins which represent about one third of all structurally characterized proteins (Finney and O'Halloran 2003). According to the Irving-Williams series, metal ions bind to organic ligands, such as those in a metal-binding site of a metalloprotein, with different affinities (Nieboer and Richardson 1980; Fraústo da Silva and Williams 2001). In addition, thanks to the different chemical properties of metals (i.e. different redox potential, coordination geometry, charge and thermodynamic and kinetic properties of ligand exchange), each metal ion plays a specific chemical function in the cell (Krämer and Clemens 2006 and references therein).

The preeminent role of metals in living organisms results from the complex and long evolution of life. Before the rise of O_2 concentration in the atmosphere and in water availability, the primitive oceans were saturated with several metal ions such as iron (Fe^{2+}), manganese (Mn^{2+})or molybdenum (Mo^{6+}), among others (Bekker et al., 2010; Hong Enriquez and Do, 2012). Fe^{2+} was the most common metal in this anoxic environment. When the Earth's atmosphere became oxygenic, the bioavailability of a number of transition metal ions for living organisms radically changed with either a decreased or an increased bioavailability of some metals. The case of Fe and copper (Cu) represents a nice example: O_2 by reacting with soluble Fe^{2+} in the water of ancient seas gave rise to layers of insoluble iron oxides (Fe^{3+}) (Haber and Weiss, 1932) whereas, at the same time, Cu became more soluble when reacting with O_2 by shifting from Cu^{1+} to Cu^{2+} (Bekker et al., 2010). The dramatic changes in O_2 concentration and metal bioavailability led the early forms of life to

avoid, adapt, detoxify or use metal ions in different ways. It markedly influenced the selection of inorganic nutrients to perform many essential biochemical functions (Fraústo da Silva and Williams, 2001).

Metal ions can also be highly toxic to cells because of these same chemical properties that make them essential for living organisms. Indeed, metal imbalance (excess or deficiency) may result in uncontrolled reactions triggering oxidative stress or the replacement of endogenous metals from binding sites (Stohs and Bagchi, 1995; Goyer, 1997). Metal homeostasis must therefore be tightly regulated maintaining the concentration of metal ions within physiological limits in all organisms.

Within the metal homeostasis network, metal ions generally undergo three types of processes: i) transport across biomembranes mediated by metal transport proteins, ii) chelation by low-molecular-weight chelator molecules, and iii) controlled binding to specific proteins. Interestingly, some of the molecular components mediating these processes are highly conserved across organisms both functionally and structurally. At the cellular level, the plant metal homeostasis network involves the modification of the solubility of extracellular metal ions by electron transfer, chelation or acidification of the apoplast, the uptake of metal ions, the chelation and/or the trafficking within the cell, delivery into cellular compartments and organelles, and storage or efflux of metal ions under excess conditions (Clemens et al., 2002).

Mitochondria are organelles with a high metal ion demand due to the involvement of metallic cofactors in electron transport chains and other proteins essential for its key metabolic activity. The most important metals present in plant mitochondria are Fe, zinc (Zn), Cu, Mn, Mo and cobalt (Co), which constitute the mitochondrial metallome. Indeed, the molar ratio of these metals in isolated mitochondria of *Arabidopsis thaliana* has been determined as 26:8:6:1 for Fe:Zn:Cu:Mn, respectively, while Co and Mo are present in traces (Tan et al., 2010; Nouet et al., 2011). Hence, approximately 75% of the mitochondrial metallome in *A. thaliana* is represented by redox-cycling metals (Fe and Cu) (Figure 1).

Structurally, mitochondria are extremely complex and possess several compartments. The simultaneous presence of both an outer membrane (OM) and an inner membrane (IM) defines additional compartments such as an inter-membrane space (IMS), between OM and IM, and the matrix, the volume enclosed in the IM. Considering that the OM represents a non-selective barrier for metal import into mitochondria (Duncan et al., 2013), the IMS, IM and matrix are sites of specific biochemical processes and thereby are characterized by the presence of specific metalloproteins and/or metal transporters. Consequently, a specific ultrastructural distribution of metals within the mitochondria can be expected. Fractionation of mitochondria from *A. thaliana* plants into soluble and membrane components revealed that Fe and Cu are mainly present in the integral membrane proteome, while Mn is evenly distributed between the soluble and integral membrane compartments (Tan et al., 2010) (Figure 1).

In this chapter, the current knowledge concerning the mitochondrial metal homeostasis in plants will be discussed.

5.2. Iron

Iron plays crucial roles in cells. Plants have evolved specific and complex mechanisms to acquire Fe from the environment. The plant Fe uptake mechanisms have been reviewed in several recent papers (Palmer and Guerinot 2009; Kobayashi and Nischizawa 2012; Thomine and Vert 2013).

The chemical properties of Fe, which exists in the two interchangeable oxidation states, Fe^{2+} (ferrous form with six *d* electrons) and Fe^{3+} (ferric form with five *d* electrons) make Fe a useful cofactor for the numerous electron transfer reactions that take place in mitochondria. However, as a redox-active element, Fe is potentially toxic and thereby its intracellular concentration must to be tightly regulated. In fact, Fe is the most abundant transition metal in mitochondria where it is required in great amounts to support the respiratory chain activity as well as iron-sulphur (Fe-S) cluster biogenesis, one of the major Fe utilization pathways in the cell. In plants, one respiratory

unit (comprising Complexes I to IV) needs 41 Fe atoms to work: 20 for Complex I, 9 for Complex II, 10 for Complex III, 2 for Complex IV, 2 for alternative oxidases (AOX) (Table 1) (Vigani et al., 2009). Therefore, understanding plant Fe homeostasis in mitochondria is a topical issue (Vigani et al,. 2013a). Recently, important studies have shed light on the machinery involved in mitochondrial Fe uptake, storage, trafficking and utilization.

5.2.1 Heme and Fe-S clusters

In mitochondria, Fe plays its biological function essentially as enzyme cofactor and it is mainly bound to ligands, i.e. Fe-heme groups and Fe-S clusters, which are components of the mitochondrial respiratory complexes (Table 1). Moreover, in addition to being a major user of these ligands, mitochondria also host important steps of heme and Fe-S cluster biogenesis.

Heme is a highly stable structure where Fe is coordinated in a tetrapyrrole ring. In the heme group, Fe can bind oxygen (e.g. in haemoglobin and myoglobin) or water to catalyze hydroxylations and other reactions (as in cytochrome P450 enzymes). Heme can occur as free heme, but it is highly insoluble and its concentration inside the cell is extremely low (Hamza and Dailey, 2012). Although heme biosynthesis is known to be localised in plastids, recent reports have indicated that the final step in its biosynthesis, i.e. the insertion of Fe into the tetrapyrrole ring by ferrochelatase (FC), may also take place in mitochondria (see also below section 5.2.2 and Tanaka and Tanaka 2007; Mochizuki et al., 2010).

The combination of $Fe^{2+/3+}$ with S^{2-} determines the formation of Fe-S clusters in which the S^{2-} does not undergo redox transitions, and the *d* electrons of Fe become delocalized (Balk and Schaedler, 2014 and references therein). Commonly, Fe-S clusters show rhombic (Fe_2S_2) and cubane (Fe_4S_4) conformations (Beinert et al., 1997; Zhang et al., 2012) but several other types of Fe-S clusters are known. Within Fe-S complexes, Fe ions cycle between Fe^{2+} (reduced) or Fe^{3+}(oxidized) states, thus playing an essential function in electron transport chains. Unlike

quinones and flavins, Fe-S clusters generally undergo redox reactions without releasing or acquiring protons (Balk and Schaedler, 2014).

Fe-S cluster assembly takes place in different cellular compartments and mitochondria have a crucial role for the assembly of Fe-S clusters required for both mitochondrial and cytosolic proteins (Balk and Schaedler 2014). In fact, the components for Fe-S cluster assembly belong to three systems in plants, namely the SUF (sulphur mobilization), ISC (iron-sulphur cluster) and CIA (cytosolic iron-sulphur cluster assembly) machineries for plastidial, mitochondrial and cytosolic/nuclear Fe-S proteins, respectively (Lill and Mühlenhoff, 2008), which have distinct evolutionary origins. The detailed pathways have been reviewed recently (Balk and Schaedler 2014). The SUF and/or ISC machineries are found in most living organisms, while the CIA machinery is specific to eukaryotes (Couturier et al., 2013). In the ISC machinery of budding yeast (*Saccharomyces cerevisiae*), two cysteine desulphurases, NFS1 and ISD11, mobilize sulphur from cysteine. Frataxin (FH, see section 5.2.2) then promotes the interaction with the ISU scaffold proteins favouring sulphur transfer reactions (Couturier et al., 2013) and the subsequent binding with Fe. After Fe-S assembly on scaffold proteins, carrier proteins mediate cluster insertion into target apoproteins (Balk and Schaedler 2014). In eukaryotes, an ISC export system connecting mitochondrial and cytosolic machineries is present. Indeed, mitochondrial Fe-S cluster biosynthesis provides important precursors for the CIA machinery (Balk and Schadler, 2014). A mitochondrial transporter mediating the efflux of these precursors has been identified in yeast, plant and mammals: the ATP-binding cassette transporter of the mitochondria (ATM) (Csere et al., 1998; Kispal et al., 1999; Bernard et al., 2009). In yeast, ATM1 is localized in the inner membrane of mitochondria and the plant ATM3 and human ABCB7 are functional orthologues of ATM1, since they can complement the phenotype of the yeast *Δatm1* mutant (Csere et al., 1998; Kushnir et al., 2001; Shaedler et al., 2014). First evidence suggested that ATMs might transport Fe-S cluster intermediates (Kispal et al., 1997). However, more recent findings indicate that the plant ATM3 seems to be implicated in several processes such as Fe-S cluster biosynthesis, heavy metal

resistance and molybdenum cofactor (Moco) biosynthesis (Kim et al., 2006 and Teschner et al., 2010). Recently, it has been determined that ATM3 in *A. thaliana* and ATM1 in yeast are involved in the export of glutathione polysulfide from mitochondria, which provides the persulfide required for both Fe-S cluster assembly and Moco biosynthesis (see section 5.6) in the cytosol (Schaedler et al., 2014) (Figure 2). The export of glutathione polysulfide by ATM proteins possibly explains their function in heavy metal resistance reported both in *Chlamydomonas reinhardtii* (Hanikenne et al., 2001; Hanikenne et al., 2005) and in *A. thaliana* (Kim et al., 2006).

5.2.2 Fe binding proteins

As stated above, Fe homeostasis in mitochondria must be tightly regulated, which requires specific proteins. Accordingly, the presence of Fe chaperones and chelators in mitochondria have been observed (Flatmark and Romslo, 1975). Mitochondrial ferritin and frataxin (FH) have been implicated in Fe storage and control of Fe homeostasis in the mitochondrial matrix (Babcock et al., 1997; Corsi et al., 2002) (Figure 2).

Plant ferritins are conserved proteins that oligomerize into 24-mers to form a hollow sphere and are essential for Fe homeostasis. Ferritin can bind approximately 4,000 Fe^{3+} atoms per 24-mer molecule (Carrondo, 2003) sequestrating Fe in excess and thereby preventing oxidative damage (Zhao et al., 2002; Arosio et al., 2009; Ravet et al., 2009). Fe sequestration occurs by a ferroxidase activity which oxidizes Fe^{2+} and stores it within the ferritin core in the form of hydrous ferric oxides along with phosphates (Arosio et al., 2009). In contrast, the mechanism of Fe release from ferritins is not very well understood. In animals, Fe release seems to require iron chelators or proteolytic degradation of ferritin proteins (Briat et al., 2010). Contrarily to the usual cytosolic localization in animals, plant ferritins are primarily localized in plastids. However, there is evidence that ferritins also localise to mitochondria both in animal cells (Levi et al., 2001) and in plant cells (Zancani et al., 2004; Tarantino et al., 2010).

A. thaliana possesses four ferritin (FER1–4) proteins, all of which are known to be localized to chloroplasts (Jain and Connolly, 2014 and references therein). However, *AtFER4* loss-of-function (*atfer4* mutants) results in an increase in mitochondrial Fe levels, in damaged mitochondrial electron transport chain components, and in a diminished O_2 consumption rate. These observations indicate that *AtFER4* might be involved in mitochondrial Fe homeostasis (Tarantino et al., 2010). Recently, the presence of a functional ferritin as a Fe storage protein in cucumber mitochondria has been observed (Vigani et al., 2013b).

FH is a conserved mitochondrial protein found in several organisms including plants (Busi et al., 2006). In human, FH deficiency causes an autosomal recessive cardio-neurodegenerative disease known as Friedreich's ataxia (Campuzano et al., 1996). The first FH homolog identified in a photosynthetic organism was the *A. thaliana* AtFH (Busi et al., 2004). *AtFH* is essential for plant growth as the loss-of-function (*atfh*) mutants exhibit an embryo lethal phenotype (Vazzola et al., 2007). The oxidative stress observed in *atfh* mutants is accompanied by an increase in nitric oxide (NO) production. In addition to its important role as signalling molecule, NO is also a potent antioxidant, which protects the cell by scavenging peroxide radicals (Beligni and Lamattina, 1999) and by inducing the expression of ferritin genes to sequester free Fe in excess (Murgia et al., 2002; Martin et al., 2009). It has been observed that *atfh* mutants show reduced activity of two Fe–S cluster containing enzymes, mitochondrial aconitase and succinate dehydrogenase, while the activity of non-Fe–S containing enzymes such as malate dehydrogenase is not altered. This indicates that FH likely plays a role in Fe–S cluster biogenesis and/or in Fe–S moiety assembly with mitochondrial proteins in *A. thaliana* (see also above, section 5.2.1). Indeed, it was shown that AtFH plays an instrumental role in Fe–S cluster biogenesis in plant mitochondria (Turowski et al., 2012). AtFH interacts with a cysteine desulphurase (AtNFS1), which supplies S to the Fe-S cluster assembly machinery and modulates its kinetic properties (Turowski et al., 2012).
Interestingly, FH seems to also participate in heme biosynthesis. In fact, the expression of several genes involved in this process (e.g. FC2, HEMA1, GSA1) is down regulated and the content of total

heme is decreased in *atfh* mutant plants (Maliandi et al., 2011). It was shown in human that FH interacts with, and delivers Fe to, ferrochelatase (FC) during the last step of mitochondrial heme biosynthesis (Yoon and Cowan, 2004). However, there is no evidence concerning such interaction in plant mitochondria. Overall, FH seems to play important roles in the protection against oxidative stress and in the biogenesis of Fe–S cluster and heme-containing proteins (Maliandi et al., 2011).

5.2.3 Fe transport

Mitochondrial Fe transporters are conserved proteins belonging to the mitochondrial carrier family (MCF; Wiesenberger et al., 1991; Metzendorf et al., 2009; Bashir et al., 2011a,b). The MCF proteins localize to the mitochondrial inner membrane where they transport solute (e.g., ketoacids, nucleotides, aminoacids, etc.) into the mitochondrial matrix (Kunji and Robinson, 2006). The first mitochondrial iron transporters (MRS3 and MRS4) identified were discovered in yeast (Waldherr et al., 1993; Foury and Roganti, 2002). Mitochondrial iron transporters (named Mitoferrins) have also been identified and characterized in other organisms such as zebrafish, human, and Drosophila (Shaw et al., 2006; Metzendorf et al., 2009; Paradkar et al., 2009). In *A. thaliana*, the MitoFerrin-Like1 (*AtMfl1*) gene has been studied as a candidate gene involved in Fe transport into plant mitochondria, based on its protein sequence homology with the *Danio rerio* (zebrafish) mitoferrin2 (MFRN2) (Tarantino et al., 2011). However, the authors showed that *AtMfl1* is actually involved in Fe trafficking in chloroplasts (Tarantino et al., 2011). The first identification and characterization of a Mitochondrial Iron Transporter (MIT) was obtained with rice (Bashir et al., 2011b). *MIT* is an essential gene for rice and *mit* knockdown mutants (*mit2*) exhibit a slow growth phenotype and a reduced chlorophyll concentration. Moreover, the *mit2* mutation leads to a mislocalization of Fe in the cell. Indeed, in *mit2* plants, the mitochondrial Fe concentration is decreased while the total Fe concentration is increased when compared to wild-type plants (Bashir et al., 2011b). These data, together with the upregulation of the vacuolar iron transporter1 (*VIT1*) gene (Kim et al., 2006) in the mutant, suggested that excess cytosolic Fe may be directed toward vacuoles. Interestingly, the

fact that *mit* loss-of-function lines show altered chlorophyll concentration and altered ferritin expression supports the idea of crosstalk between mitochondrial and chloroplastic Fe homeostasis. Beside the decreased mitochondrial Fe transport, the *mit2* mutation affects Fe-S cluster assembly, in agreement with previous observations in other organisms. In yeast and mammals, the loss of mitochondrial Fe transport affects heme and Fe–S cluster synthesis (Zhang et al., 2005; Shaw et al., 2006; Zhang et al., 2006). In rice, partial loss of MIT results in a decrease in total and mitochondrial aconitase activities, indicating that Fe–S cluster synthesis is affected both at the mitochondrial and cytosolic levels. Altogether, the MIT protein seems to act as a high affinity Fe uptake system in plant mitochondria by analogy to the yeast MRS3/4 homologous transporters that are thought to serve as high affinity ferrous ion transporters which are essential in the absence of other low affinity mitochondrial iron transporters (Froschauer et al., 2009) (Figure 2).

However, it has been hypothesized that, before entering the mitochondria, Fe^{3+} must be reduced to Fe^{2+} by a putative ferric reductase localized on the inner mitochondrial membrane. Reduction of the ferric to the ferrous form at the root surface is mediated by metalloreductase enzymes belonging to the Ferric Reductase Oxidase (FRO) family (Jeong and Connolly, 2009). Several members of this family have been identified in *A. thaliana* and they function as metalloreductases primarily involved in the reduction of Fe, and to a lesser extent also of other metals such as Cu. It is well known that FRO2 is localised on the plasma membrane and is responsible for the reduction of Fe^{3+} to Fe^{2+} at the root surface for the Fe uptake from the soil (Robinson et al., 1999). In contrast, the other FRO family members are less well studied and their characterization has started more recently (Figure 2).

Jeong et al. (2008) demonstrated that the *A. thaliana* FRO7 localises in the chloroplast and performs the reduction of Fe^{3+} to Fe^{2+} at the surface of the chloroplast for subsequent uptake into the organelle. FRO3 and FRO8 are predicted to localize to mitochondria (Jeong and Connolly, 2009). However, there is no evidence to date that these proteins function in mitochondrial Fe metabolism (Jeong and Connolly, 2009). Indeed, whereas *FRO3* shows expression throughout

seedlings, expression of *FRO8* is restricted to shoots during senescence (Mukherjee et al., 2006; Jeong and Connolly, 2009) suggesting that the FRO3 and FRO8 may be involved in reducing Fe^{3+} at different stages of development. Interestingly, a proteomic study identified FRO8 in the mitochondrial membrane (Heazlewood et al., 2004). Despite these few evidences, the exact role of FRO3 and FRO8 still remains to be clarified. In rice, the two identified members of the FRO family do not seem to localize to the mitochondria, suggesting the presence of a non-reductive Fe uptake pathway in mitochondria of grass plants (Victoria et al., 2012; Jain and Connolly, 2014).

5.3. Copper

Similarly to Fe, Cu can also be present in two oxidation states, Cu(I) and Cu(II), in plant cells and therefore Cu-binding proteins can participate in electron transfer reactions. At the same time, these redox properties also make Cu toxic when present in excess via ROS (Reactive Oxygen Species) production. Therefore, as for Fe, free Cu levels must be precisely regulated in the cell in order to limit the damage produced by Cu excess as well as to avoid the effects of Cu deficiency (Burkhead et al., 2009; Ravet and Pilon 2013; Garcia et al., 2014)

In plants, Cu uptake from the soil is well characterised and it is mediated by transporters belonging to the COPT (Copper Transporter) and ZIP (Zrt- Irt-like Proteins) families (Puig and Peñarrubia, 2009; Pilon, 2011). However, mitochondrial Cu import mechanisms remain unknown in plants (Figure 3), while transporters involved in Cu delivery to several cellular compartments, such as chloroplasts, vacuoles, endoplasmic reticulum and the trans-Golgi network have been identified (reviewed in Puig and Peñarrubia, 2009). Recently, a member of the mitochondrial carrier family, PIC2, has been suggested to be involved in Cu import to mitochondria in yeast (Vest et al., 2013). It is not known if there is a PIC2 homologue in plants.

Cu is associated with several metalloproteins playing essential functions both within mitochondria and in the whole cell. In mitochondria, Cu is part of Complex IV (also named cytochrome *c* oxidase, or COX) of the respiratory chain and thereby involved in energy

transduction (Figure 3). In fact, most efforts aiming at a better understanding of Cu homeostasis in plant mitochondria have been dedicated to deciphering the COX biosynthesis mechanisms. Both Fe and Cu are important cofactors for COX activity (Figure 3 and Table 1). COX contains two spectrally distinct heme species (heme *a* and heme a_3), together with at least two Cu atoms known as Cu_A and Cu_B. The COX1 subunit coordinates a binuclear metal center Cu_B-heme a_3, whereas COX2 chelates the bivalent Cu_A (Garcia et al., 2014 and references therein).

Insertion of Cu into COX is an intricate process that requires the participation of several mitochondrial proteins (Herrmann and Funes, 2005; Robinson and Winge, 2010). Many of these proteins act as metallochaperones that function in the delivery and insertion of metals, particularly Cu, in the right location in the target protein. Among them, the Synthesis of Cytochrome *c* Oxidase (SCO) protein family is involved in copper insertion into COX, particularly into the COX2 subunit (Banci et al., 2011). Furthermore COX17, COX11 and SCO1 proteins, acting as Cu chaperones and delivering the metal to COX, have been identified in yeast and mammals (Robinson and Winge, 2010). While COX17 is a small soluble protein from the IMS (Palumma et al., 2004; Voronova et al., 2007), SCO1 and COX11 are bound to the inner membrane and both proteins bind Cu through conserved cysteines (Carr et al., 2002; Balatri et al., 2003; Horng et al., 2005; Banci et al., 2011). An additional Cu chaperone, named COX19, has been identified in human cells (Leary et al., 2013). However, COX19 seems to be localized both in mitochondria and the cytosol. Therefore, it has been suggested that COX19 might be not related to the Cu transport into mitochondria (Leary et al., 2013).

Proteins similar to COX assembly factors are present in plants. Two genes (*AtCOX17-1* and *AtCOX17-2*) that encode putative COX17 homologues as well as two other genes, *AtCOX19-1* and *AtCOX19-2*, which encode putative homologues of yeast COX19, were identified in *A. thaliana* (Wintz and Vulpe, 2002; Attallah et al., 2007a, b). Moreover, COX17 is required for full COX activity in the green alga *C. reinhardtii* (Remacle et al., 2010). It has been recently hypothesized that AtCOX17 and AtCOX19 may act in signalling pathways related to Cu and redox homeostasis

in mitochondria, suggesting that these proteins may be considered as candidates to act as both transducers of redox changes in the IMS and sensors to detect cellular Cu (Garcia et al., 2014). Moreover, two proteins similar to SCO1, named Homologs of the yeast Copper Chaperones SCO1, HCC1 and HCC2, are present in the *A. thaliana* genome (Attallah et al., 2011; Steinebrunner et al., 2011). Knocking-out *HCC1* in *A. thaliana* causes embryo development arrest and a decrease in COX activity suggesting that HCC1 proteins act as COX assembly factors in plants (Attallah et al., 2011; Steinebrunner et al., 2011). It is likely that HCC1 delivers Cu to the COX catalytic center (Steinebrunner et al., 2014). In contrast, *hcc2* knock-out plants display no changes in COX activity. It has recently been observed that HCC2 seems to be involved directly or indirectly in UV-B-stress response in plants (Steinebrunner et al., 2014). A gene encoding a putative homologue of COX11 is also present in the *A. thaliana* genome (Welchen and Gonzalez, 2005) although the function of this protein still remains unknown.

5.4. Zinc

Zinc is an essential element in all organisms because of several unique features of its chemistry (Berg and Shi 1996; Fraústo da Silva and Williams 2001):

- unlike other transition metal elements from the first row of the periodic table, the Zn ion (Zn^{2+}) contains a full *d* orbital (d10), thus, does not participate in redox reactions, but does act as a Lewis acid by accepting a pair of electrons. Zn therefore exhibits high binding affinity for soft bases (i.e sulphide ligands) as well as for hard bases (i.e. amino and carboxylate ligands (Williams 1987).
- Zn occurs in a single oxidation state [Zn(II)] in the cell, avoiding any risk of initiating free radical reactions. Indeed, Zn is comparably safe in the vicinity of sensitive macromolecules, in particular DNA in the nucleus.
- Zn is a highly effective cofactor since its coordination geometry is highly flexible (Fraústo de Silva and Williams, 1991). For this reason, Zn is an important structural component of

small protein motifs, named Zinc fingers, characterized by the coordination of one or more Zn ions in order to stabilize protein structures. Zinc finger motifs are known to bind several target ligands, e.g. DNA.

Thanks to these different properties, Zn can play different roles when interacting with proteins: (i) catalytic, where Zn ions directly participate in the reaction (e.g. carbonic anhydrase); (ii) co-catalytic, where Zn plays a catalytic role together with several metal ions that interact with each others in a co-catalytic Zn site (e.g. alkaline phosphatase); (iii) structural, where Zn ions stabilize the tertiary structure of the enzyme in a similar way to the disulphide bonds (e.g. DNA-binding proteins; alcohol dehydrogenase) (Vallee and Auld 1990; Escudero-Almanza et al., 2012).

In most organisms, Zn is acquired either from the environment or from the diet by specific membrane transport proteins, sometimes operating in conjunction with chelators (Eide 2006; Palmer and Guerinot 2009). In addition, transporters of divalent metal cations (e.g. the iron transporter IRT1) often exhibit broad substrate specificity, so that a deficiency in Cu, Fe or manganese (Mn) may result in enhanced uptake and accumulation of toxic amounts of Zn (Palmer and Guerinot 2009, Thomine and Vert 2013; Socha and Guerinot, 2014). Note that no mitochondrial Zn uptake system has been identified so far (Figure 4).

In plants, Zn-dependent processes are located in all cellular compartments, including mitochondria (Heazlewood et al., 2004). To date, the requirement for Zn inside plant mitochondria has been linked to its role in important processes such as the degradation of mitochondrial presequences (Table 1) (Moberg et al., 2003; Tan et al., 2010). Once the cytosolic polyribosomes have synthesized nuclear-encoded mitochondrial proteins, these proteins must be imported post-translationally following the information of their mitochondrial pre-sequences (Neupert and Brunner, 2002). It has been suggested that mitochondrial presequences are potentially harmful to the integrity and function of mitochondria because they can dissipate the membrane potential and uncouple respiration (Glaser and Cumsky, 1990; Nicolay et al., 1994). The activation of proteases is therefore required to remove and degrade presequence peptides (Figure 4).

In mitochondria, several proteases have been identified, including the membrane metalloproteases. Among them, Zn-dependent metalloproteases (Zn-MPs) have a great importance (Moberg et al., 2003). Zn-MPs harbour an inverted Zn-binding motif and are classified in the pitrilysin subfamily. However, Zn-MPs are not exclusive to mitochondria since it was shown that they are responsible for the degradation of not only mitochondrial but also chloroplastic transit peptides. Indeed, the Zn-MP was found to be dually targeted and imported to both organelles in *A. thaliana* plants (Moberg et al., 2003) (Figure 4). Moreover, Zn represents an essential cofactor for several other mitochondria enzymes in yeast, including Adh3, Adh4, and Leu9 proteins, which are localised in the matrix, and Sod1 and Hot13 which are localised in the intermembrane space (Masecke et al., 2008).

Zn also has additional functions in mitochondria: it is required by the COX4 subunit of cytochrome *c* oxidase (Coyne et al., 2007), is involved in small TIM (Translocase of the Inner Membrane) folding (Ceh-Pavia et al., 2013) and is a prosthetic group for enzymes involved in RNA editing in mitochondria (Krämer and Clemens 2006). Considering the numerous Zn metalloenzymes within mitochondria, it has been suggested that a significant bioavailable Zn(II) pool must exist for the metallation of these molecules upon their import. Indeed, it has been demonstrated recently in yeast that a bioavailable pool of Zn(II) exists within the mitochondrial matrix that appears to be critical for mitochondrial function (Atkinson et al., 2010). The physiological significance of such a matrix Zn(II) pool may be related either to a detoxification mechanism for the metal or to a Zn(II) reservoir for metallation of Zn-binding proteins within the matrix (Atkinson et al., 2010).

5.5. Manganese

Similar to the other transition metals, such as Fe and Cu, Mn can exist in various oxidation states. The availability of Mn to plants depends on its oxidation state, as the oxidized forms Mn(III) and Mn(IV) are not bioavailable (Clarkson, 1988; Rengel, 2000). It is the Mn(II) reduced form that

is transported (as the divalent cation Mn^{2+}) into the cell. The concentration of Mn^{2+} in the soil solution can vary markedly, depending on the soil solution pH.

Mn is an essential nutrient in most organisms, because of two aspects: i) it exerts an activating role on enzymes or ii) it serves as a catalytically active metal.

Several enzymes are activated by Mn, including malic enzyme, isocitrate dehydrogenase, PEP carboxykinase, and phenylalanine ammonia lyase (Marschner 1995). Proteins belonging to this group are involved in the shikimic acid pathway and subsequent pathways leading to the formation of aromatic amino acids, lignins, flavonoids, and the phytohormone indole acetic acid (i.e. auxin). Mn activation has also been reported for enzymes involved in nitrogen metabolism (glutamine synthetase, arginase), gibberellic acid biosynthesis, RNA synthesis (RNA polymerase), and fatty acid biosynthesis.

Mn ions have a catalytic role in the oxalate oxidase (Requena and Bornemann, 1999) and in the catalytic centre for light-induced water oxidation in photosystem II (Goussias et al., 2002; Barber, 2003; Nickelsen and Rengstl, 2013) as well as in the Mn-containing superoxide dismutase (MnSOD) which is an important enzyme protecting the cell from damaging effects of ROS (Kliebenstein et al., 1998).

Despite its importance, Mn is required by plants in relatively small amounts, yet the capacity for Mn uptake greatly exceeds this requirement (Clarkson, 1988). Mn can be toxic to plant growth and to overcome such toxicity plant, may activate the conversion of the metal to a metabolically inactive compound (i.e., Mn^{2+}-chelate complex), or sequester Mn^{2+} ions in the vacuole. Indeed, Mn^{2+} accumulates predominantly in the vacuole and to some extent in chloroplasts, but can also be associated with the cell wall fraction (McCain & Markley, 1989; Quiquampoix et al., 1993; González & Lynch, 1999). However, Mn is also required in other organelles. For instance, MnSOD has been identified in peroxisomes and mitochondria, and a variety of Golgi-localized enzymes, such as glycosyltransferases, require Mn (Marschner, 1995).

In *A. thaliana*, the main Mn transporter for uptake from the soil by the roots is NRAMP1,

which is crucial for plant growth under Mn deficiency (Cailliatte et al., 2010). In addition, MTP11 transports Mn in the Golgi and plays a role in Mn tolerance in *A. thaliana* (Peiter et al., 2007, Delhaize et al., 2007). Another MTP protein is involved in Mn transport into the plant vacuole (Migocka et al., 2014). More generally, Mn is transported by members of several metal transporter families in plants, which often display broad specificity for several divalent cations, such as Fe, Cu, Cd, Co, Ca (Socha and Guerinot, 2014). This includes among others NRAMPs (Natural Resistance Associated Macrophage Protein), MTPs (Metal Tolerance Proteins) or ZIPs. In *Medicago truncatula*, a mitochondrial localization has been predicted for a putative Mn transporter, MtZIP4 (Lopez-Millan et al., 2004). However, no Mn-specific mitochondrial transporter has been identified so far in plants.

In plant mitochondria, Mn is mainly present as cofactor for the MnSOD which is the typical SOD isoform localised in mitochondria (Table 1, Figure 5). In yeast, Mn is essential for the activation of the MnSOD (SOD2) in mitochondria: SOD2 is completely activated only when the Mn has been inserted into the apoprotein (Archibald, 2003). Three proteins involved in the delivery of Mn to SOD2 have been identified in yeast: SMF1, SMF2, and MTM1. As NRAMP1 (see above), SMF1 and SMF2 are metal transporters belonging to the NRAMP family (Chen et al., 1999; Luk and Culotta 2001). SMF1 is localized to the plasma membrane (Portnoy et al., 2000) and it is the limiting factor for Mn uptake and trafficking in the cell (Chen et al., 1999). SMF2 is localized to intracellular vesicles and is an extremely important transporter for the Mn-requiring enzyme sugar transferase (STase) present in the Golgi and for SOD2 present in mitochondria (Cohen et al., 2000; Luk and Culotta 2001).

Deleting the *MTM1* gene inactivates the MnSOD, suggesting that MTM1 is required for proper insertion of Mn into SOD2 in the mitochondria (Luk et al., 2003). During maturation, apo-SOD2 is sent to the mitochondrial matrix where Mn is inserted during folding. MTM1 may act either in the import of Mn, or as a chaperone for Mn insertion into SOD. However, the Mn concentration within *mtm1*-defective mitochondria is elevated compared to WT, ruling out the

possibility that MTM1 imports Mn (Luk et al., 2003). Additionally, the *mtm1* yeast mutant exhibits both a loss of SOD2 enzymatic activity but also an accumulation of iron in the mitochondria. This iron overload may preclude proper insertion of the appropriate Mn ion cofactor and thereby explain the loss of SOD2 activity (Luk et al., 2003; Yang et al., 2006).

The *AtMTM1*-encoded protein is targeted to mitochondria in *A. thaliana* cells, functions similarly to MTM1 in activating SOD2 in yeast mitochondria, and may be involved in activation of MnSOD1 in *A. thaliana* (Su et al., 2007).

5.6. Trace metals in plant mitochondria

The first report characterizing the mitochondrial metallome of plants revealed the presence of traces of other transition metals in the mitochondria: Molybdenum (Mo) and Cobalt (Co). Very little information about a possible role of Co in plant mitochondria is available so far (Tan et al., 2010). Co is considered a beneficial nutrient for plants (Marschner 1995) and is an essential component of several enzymes and co-enzymes. It has been shown that Co deficiency affects growth and metabolism of plants, to different degrees, depending on the concentration and status of Co in the rhizosphere and soil. In plant mitochondria, Co^{2+} may substitute for other metals in the activation of some enzymes such as NAD-malic enzyme and succinyl-CoA ligase (Palmer and Wedding 1966; Macrae, 1971) but it is not known whether there is an *in vivo* requirement for trace amounts of Co for plant respiratory metabolism. More generally, the interaction of Co with other metals mainly depends on the concentrations of each metal in the environment. For example, high levels of Co^{2+} induce Fe deficiency in plants and suppress uptake of Cd by roots (Palit et al., 1994; Gàl et al., 2008). It also interacts synergistically with Zn, Cr, and Sn (Palit et al., 1994). Ni overcomes the inhibitory effect of Co on protonemal growth in mosses, thus indicating an antagonistic relationship (Palit et al., 1994).

Mo is an essential metal for at least four enzymes in plants, all involved in crucial metabolic pathways: nitrate reductase (nitrogen assimilation), sulfite oxidase (sulfite detoxification), aldehyde

oxidase (abscisic acid biosynthesis) and xanthine dehydrogenase (purine degradation). In cells, Mo is present as molibdate which alone does not have any biological activity. However, when molybdate is incorporated in a pterin group, it leads to the formation of the Moco cofactor which is used by the four enzymes mentioned above. The first committed step in molybdopterin biosynthesis has recently been shown to occur in mitochondria. Indeed, the synthesis of cyclic pyranopterin monophosphate (cPMP), a precursor for the Moco formation, takes place in mitochondria. This is consistent with the fact that mitochondria act as a control point in regulating whole plant Mo content (Schwartz and Mendel 2006; Mendel, 2007) (Figure 2). Mo and Fe homeostasis are interconnected in plant mitochondria. Indeed, cPMP synthesis in mitochondria is carried out by the CXN2 and CXN3 enzymes, which are Fe-S-containing proteins (Mendel, 2007). In addition, mitochondrial Fe and Mo homeostasis seem to rely on a common mitochondrial export system involving ATM proteins (see section 5.2.1).

Interestingly, two Mo transporters, named MOT1 and MOT2, have been identified in *A. thaliana* (Tomatsu et al., 2007; Baxter et al., 2008; Gasber et al., 2011). MOT transporters belong to the sulfate transporter superfamily, and can transport Mo when expressed in yeast. In fact, sulfate and molybdate share a high degree of similarity as they possess a double negative charge (SO_4^{2-}, MoO_4^{2-}), are similar in size, and have tetrahedral structures. It was thus proposed that molybdate import and distribution are facilitated by sulfate transporters or related systems. While MOT2 seems to be localised to the tonoplast membrane (Gasber et al., 2011), two reports on the MOT1 transporter suggest a different sub-cellular localization of the protein: Tomatsu et al. (2007) primarily showed an endomembrane localization whereas Baxter et al. (2008) reported a mitochondrial localization. In these two reports, the localization of MOT1 was determined using a GFP fusion construct, either at the N- (Tomatsu et al., 2007) or C-terminus (Baxter et al., 2008) of MOT1, respectively. A N-terminal fusion may block the predicted mitochondrial localization signal, and result in mislocalizing the GFP::MOT1 fusion protein.

In addition, Baxter et al. (2008) speculate that (i) considering that characterized members of the sulfate transporter superfamily are SO_4^{-2}/H^+ co-transporters, MOT1 may transport MoO_4^{-2} from the acidic mitochondrial intermembrane space to either the cytoplasm or the matrix; (ii) MOT1 regulates whole plant Mo accumulation at the level of the mitochondria in the root. Nevertheless, the reason for the presence of a Mo transporter, and thereby the need of Mo by mitochondria, still remains enigmatic. So far, no Mo-containing mitochondrial protein has been identified in plants. In contrast, a new Mo-containing protein localised on the external mitochondrial membrane has been identified recently in animal cells (Havemeyer et al., 2011). This protein is named Mitochondrial Amidoxime Reducing Component (mARC) and it catalyses the reduction of N-hydrolised compounds. Such a protein has also been identified in the green alga *Chlamydomonas* and, in the *A. thaliana* genome, the presence of two sequences homologous to mARC has been observed (Chamizo-Ampudia et al., 2011; Llamas et al., 2011; Krompholz et al., 2012). Therefore, mARC protein is also likely present in plant cells, even if the specific function of this protein is not yet known (Bittner, 2014).

5.7. Metallome perturbation within mitochondria

Plant cells have evolved several strategies to adapt their metabolism to different metal concentrations and availability. These include the regulation of metal uptake, trafficking and allocation. As stated above, metals are indispensable for mitochondria. However, metal ions can also be highly toxic to the cell and specifically detrimental to organelle function. Therefore, mitochondria require specific mechanisms to limit damage mediated by a metal homeostasis imbalance. Considering the great importance of metals for mitochondrial function, as well as the importance of mitochondria for the cell, understanding acclimation mechanisms to metal perturbation in mitochondria is of high priority.

The presence of free (redox-active) metal cations can initiate or propagate oxidative stress and it is known that mitochondrial metabolism accounts for a large part of the total ROS generation

in cells, even in photosynthetic tissues (Noctor et al., 2007). Under normal conditions, the electron transport chain and ATP synthesis are tightly coupled. However, biotic or abiotic stress conditions may cause an over-reduction of electron carriers causing electron leakage from the system. These electrons possess sufficient free energy to reduce directly molecular O_2 producing ROS such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Sweetlove et al., 2007). Complex I and III of the respiratory chain are the main sites of ROS production in the mitochondria (Møller, 2001) where the formed superoxide anions are dismutated to H_2O_2. The uncharged H_2O_2 can react with reduced Fe^{2+} and Cu^{+} in the mitochondria to produce highly toxic hydroxyl radicals ($OH^{\cdot}$) causing oxidative damage to mitochondrial proteins (Sweetlove et al., 2007).

One of the best described mechanisms of metal-linked damage is metal-catalyzed oxidation (MCO) of proteins. This process involves the oxidation of metal-binding proteins that can be more susceptible to damages (Stadtman, 1990). One of the major consequences of MCO is the irreversible formation of reactive carbonyls on amino acid side chains, with Arg, Lys, Pro and His as the more susceptible to MCO (Stadtman, 1990).

It has been recently demonstrated that the metal content of mitochondria is dynamic and changes during oxidative stress. In *A. thaliana* mitochondria, the induction of oxidative stress by H_2O_2 treatment leads to a 40% decrease in Cu content both in the soluble and integral membrane protein fractions as well as a 40% to 50% reduction of Fe and Mn contents in the soluble fraction. The loss of Cu suggests damage to soluble mitochondrial cuproproteins and the membrane-bound electron transport chain, whereas the loss of Fe and Mn suggests that H_2O_2 treatment damaged the matrix metalloproteins such as Fe-S containing aconitase and MnSOD (Tan et al., 2010). Interestingly, treatment of mitochondria with drugs that induce ROS production, Antimycin A or menadione, led to the identification of drug-specific effects related to their modes of action. Antimycin A is an inhibitor of complex III that ultimately leads to superoxide production within mitochondria (Maxwell et al., 1999), while menadione induces broader superoxide production from cellular membranes (Hollensworth et al., 2000). Both inhibitors lead to an accumulation of Fe in the

mitochondria and a decrease of Cu in the integral membrane fraction. The decrease in the membrane-bound Cu may be linked to the decrease of COX complexes, which are the major Cu user in mitochondria. In contrast, the accumulation of Fe may be explained by considering that the Antimycin A treatment induces ferroproteins (such as cytochrome *c* and AOX), which may substitute for the loss of COX (Yu et al., 2001). Furthermore, aconitase is known to release ferrous ions when oxidatively damaged (Verniquet et al., 1991; Brazzolotto et al., 1999).

Tan et al. (2010), using immobilized affinity chromatography with different metals, identified mitochondrial proteins undergoing metal binding and which are thereby susceptible to MCO under oxidative stress. They also recovered several subunits of mitochondrial respiratory complexes indicating that these mitochondrial proteins are able to bind different metals, which places them as targets of MCO reactions as well.

To avoid and/or overcome ROS production, mitochondria possess an antioxidant defence system characterized by both antioxidant enzymes and energy-dissipating systems. The *A. thaliana* genome encodes eight SOD genes, comprising all three types of isoenzymes, Fe-, Mn- and Cu/Zn-SOD. These isoforms are ubiquitously found in plants and differ by their subcellular localization (Kliebenstein et al., 1998; del Rio et al., 2003). Plant mitochondria possess a highly conserved MnSOD (see above) and FeSOD (Gutteridge and Halliwell, 2000). The H_2O_2 produced as catalytic by-product of the mitochondrial SODs is further reduced to water by peroxidases (i.e type II peroxiredoxin, Finkemeiner et al., 2005), ascorbate peroxidase (Chew et al., 2003) and glutathione peroxidase (Navrot et al., 2006).

Plant mitochondria contain energy-dissipating systems able to regulate the mitochondrial membrane potential and thereby reduce mitochondrial ROS production resulting from electron transport chain over-reduction. Indeed, the plant respiratory chain contains several additional proteins that enable electron transport without generation of a proton gradient, or allow dissipation of the proton gradient without synthesis of ATP. The proteins determining such bypass systems are

the alternative oxidase, uncoupling proteins and alternative NAD(P)H dehydrogenases (Vigani, 2012 and references therein).

The involvement of such bypass systems upon metal perturbation may be explained considering what has been observed in mitochondria of Fe-deficient plants. Under Fe deficiency, plant mitochondria undergo complex metabolic reprogramming. These processes have been studied in detail using cucumber root as a model. Generally, a low concentration of Fe affects mitochondrial metabolism by decreasing respiratory chain activity. In particular, because Complex I and II have the highest requirement for Fe among respiratory complexes, their activities are strongly affected by Fe deficiency. In contrast, complexes III, IV and ATP synthase (Complex V) only show a moderate decrease in activity under Fe-deficiency. Alternative NAD(P)H dehydrogenase activities strongly increase, bypassing the defective activity of Complex I (Vigani et al., 2009; Higa et al., 2010; Vigani and Zocchi 2010). This allows a sustained electron flux ensuring that both glycolysis and TCA cycle continue to operate under Fe deficiency. Moreover, two enzymes belonging to the Krebs cycle are Fe-containing proteins: succinate dehydrogenase (complex II) and aconitase. Any specific effect of Fe deficiency on the TCA cycle activity has not yet been demonstrated, but it has been suggested that, under Fe deficiency, the TCA cycle may shift from a circular to a linear mode, bypassing the Fe-dependent enzymatic steps, which are limiting factors when Fe is present in low concentrations (Vigani, 2012). However, it has been observed that the flexibility of both mitochondrial and cellular metabolism allows the cell to respond efficiently to this nutritional stress. The effect of Fe deficiency on plant mitochondria represents a clear example of how metal deficiency may alter the function of this organelle and have knock-on effects on whole cell metabolism.

5.8. Conclusion

Metals are indispensable for the cell. In particularly, Fe, Cu, Zn and Mn are essential for mitochondrial function. Mitochondria are responsible for the control of both the cellular redox

status and the synthesis of metal cofactors (as in the case of Fe-S cluster and Moco), thus contributing to cellular metal homeostasis. Therefore, a detailed understanding of the mechanisms regulating mitochondrial metal homeostasis is of high priority. Despite the great progress that has been made, our knowledge about mitochondrial metal homeostasis still remains limited. In the last decade, efforts have been made in deciphering mitochondrial Fe and Cu homeostasis, but very little is known about Zn and Mn. Furthermore, the role of Mo inside mitochondria should be clarified, since Mo and Fe homeostasis are strictly interconnected. Understanding the interactions between metal homeostatic mechanisms inside mitochondria would also clarify the role of mitochondria in the regulation of cellular metal homeostasis. Additionally, the mitochondrial metallome should be characterized separately in root and leaf tissues. Indeed, in leaf mesophyll, where mitochondria and chloroplasts compete for the same metals, such as Fe, regulation of the mitochondrial metallome composition in response to environmental stress may differ from that in roots.

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Figure legends

Figure 1. Ultrastructural distribution of transition metals in plant mitochondria. The metallome of plant mitochondria mainly consists of Fe, Zn, Cu, Mn, Mo and Co. The molar ratio of these metals in isolated mitochondria of *Arabidopsis thaliana* has been determined as 26:8:6:1 for Fe:Zn:Cu:Mn respectively, while Co and Mo are present in traces. Thus, approximately 75% of the mitochondrial metallome in *A. thaliana* is represented by the redox-cycling metals (Fe and Cu) (Tan et al., 2010). The distribution of these metals in mitochondria is shown in the figure, with the number of symbols for each metal being representative of the relative abundance of each metal in each mitochondrial compartment. Fe is mainly present in the IM and in the matrix, as an essential element for heme and Fe-S cluster cofactors that are required by respiratory complexes and aconitase. Zn is required for presequence cleavage occurring during protein import into the IMS and it is an important cofactor for Zn-protease enzymes. Cu is required for the complex IV (COX) activity and Mn is required for the MnSOD (superoxide dismutase) activity in the matrix. *Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.*

Figure 2. Representation of mitochondrial iron (Fe) homeostasis. Recent data suggest that mitochondrial Fe uptake is mediated by an identified transporter (MIT) and a putative ferric reductase (FRO3) localized at the IM. Once inside mitochondria, Fe is required for Fe-S cluster assembly. Fe-S clusters are necessary for (i) respiratory chain complexes, (ii) aconitase of the tricarboxylic acid cycle and (iii) the enzymes CXN2 and CXN3 which are responsible for the synthesis of the Moco (Molybdenum Cofactor) precursor, cPMP (cyclic pyranopterin monophosphate). cPMP is exported by the transporter ATM3 to the cytosol to complete Moco biosynthesis. Recently, it has been observed that ATM3 is also able to export from mitochondria to the cytosol a sulphur-containing metabolite (identified as GS-S-SG) required for the cytosolic Fe-S cluster assembly machinery. To assist Fe homeostasis, two proteins are present in mitochondria: ferritins (Fer) that are able to store excess Fe avoiding oxidative stress and Frataxin (FH) which acts as chaperone for the Fe-S cluster assembly pathway. *Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.*

Figure 3. Representation of mitochondrial Copper (Cu) homeostasis. Mitochondrial Cu uptake is mediated by an unknown process (?). Observations suggest that a ferric reductase localized to the IM may be involved in a reduction reaction of Cu prior to uptake, but direct evidence is lacking. Considering the presence of Cu chaperones in the IMS (i.e. COX17, COX19), in the matrix, and in the IM (i.e. COX11, HCC1), Cu may be delivered to COX subunits from different mitochondrial compartments. *Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.*

Figure 4. Representation of mitochondrial Zinc (Zn) homeostasis. Mitochondrial Zn uptake mechanisms remain unknown so far. It has been demonstrated that Zn activates the protein import system at the IMS level and that it is required for metalloprotease activity in the matrix. Zn-proteases cleave the mitochondrial transit peptides allowing correct folding of imported proteins. *Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.*

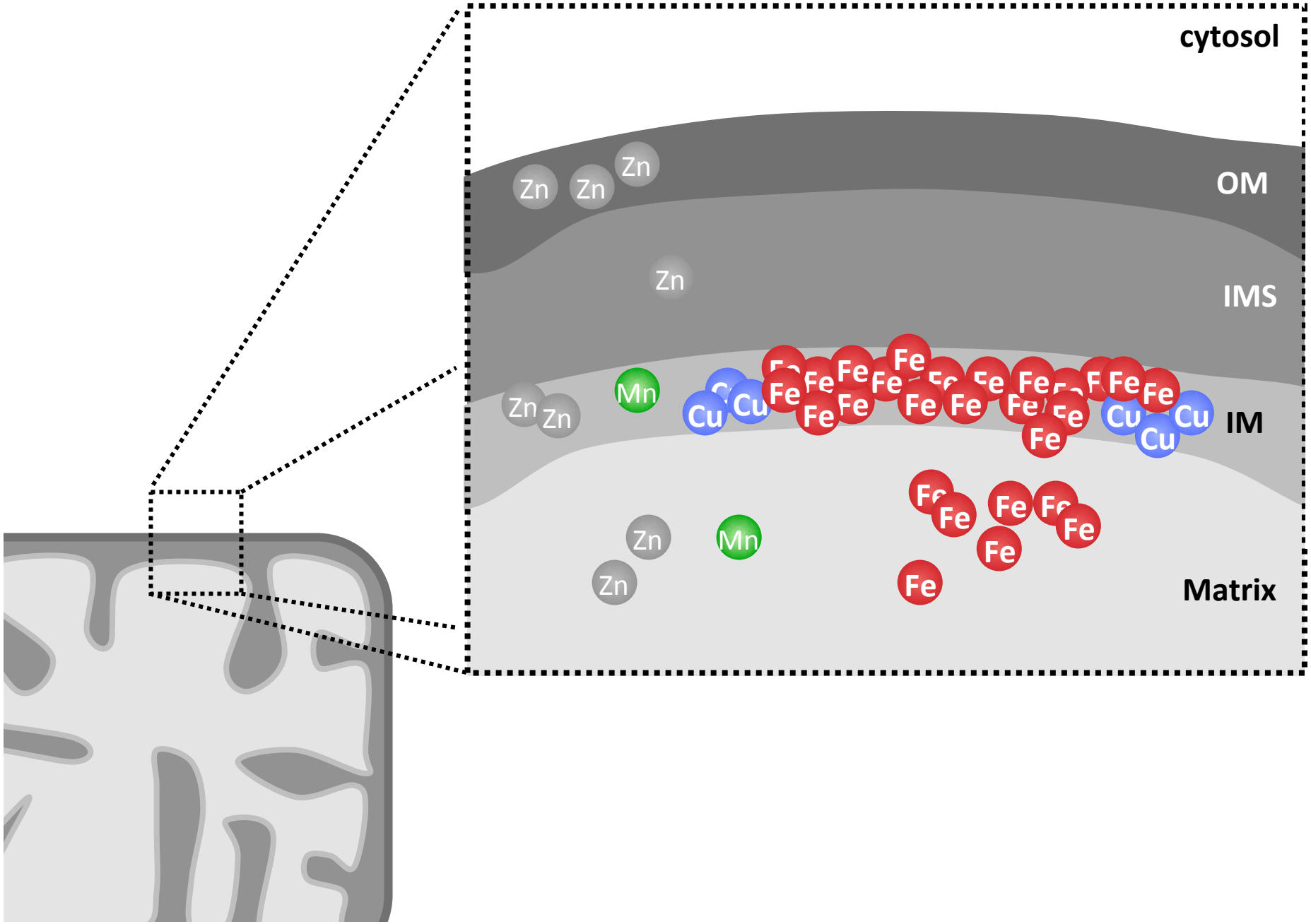
Figure 5. Representation of mitochondrial Manganese (Mn) homeostasis. Mn is essentially required as cofactor for the activity of the Mn isoform of superoxide dismutase (MnSOD) and thereby it is mainly involved in the mitochondrial responses to oxidative stress. No specific Mn transporter for mitochondrial uptake has been identified so far. *Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.*

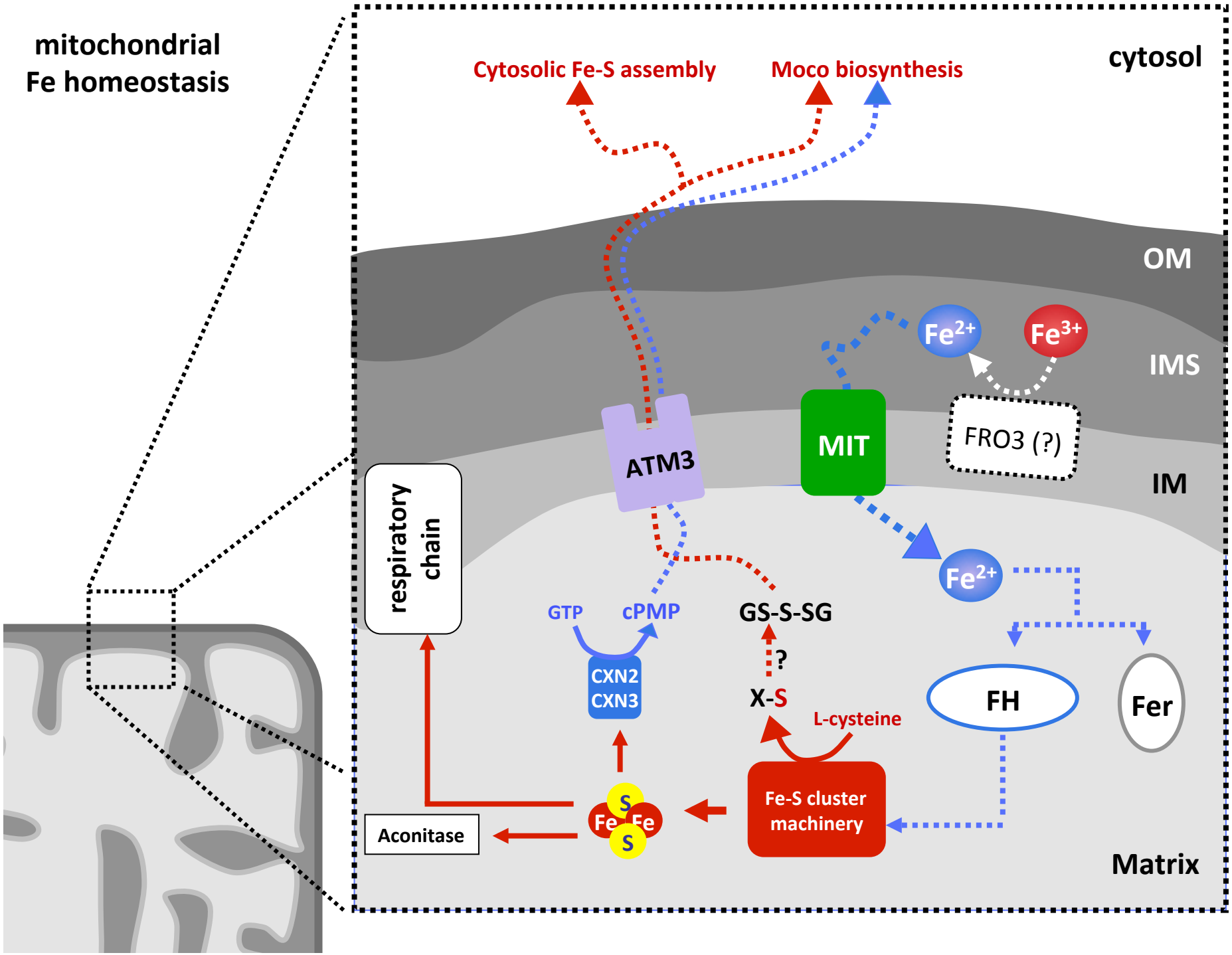
Table 1. Major metal-using processes in plant mitochondria.

		Redox activities		**Catalytic and structural functions**	**Catalytic functions**
Mitochondrial processes	**Enzymes**	**Fe**	**Cu**	**Zn**	**Mn**
Respiratory chain activity	**Complex I**	2[2Fe-2S] 4[4Fe-4S]	-	-	-
	ComplexII	[2Fe-2S]; [3Fe-4S]; [4Fe-4S]; Fe-heme(cyt*b*)	-	-	-
	Complex III	3Fe-heme (cytb_{562}; cytb_{566}; cytc_1) [2Fe-2S]	-	-	-
	AOX	2 Fe atoms	-	-	-
	Complex IV (COX)	2 Fe-heme (cyt*a*; cyta_3)	2 Cu atoms	COX4	-
Krebs cycle	**aconitase**	[4Fe-4S]	-	-	-
ROS scavenging	**SOD**	FeSOD	-	-	MnSOD
Protein import		-	-	TOM and TIM protein	-
Proteolysis		-	-	Zn-metalloproteases	-

Abbreviations: AOX, Alternative Oxidase; COX, Cytc Oxydase; ROS, Reactive Oxygen Species; SOD, Superoxide dismutase; TOM, Translocase of the Outer Membrane; TIM, Translocase of the Inner Membrane.

Ultrastructural distribution of metal in mitochondria




mitochondrial
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Fe3+
respiratory chain
GTP
cPMP
CXN2
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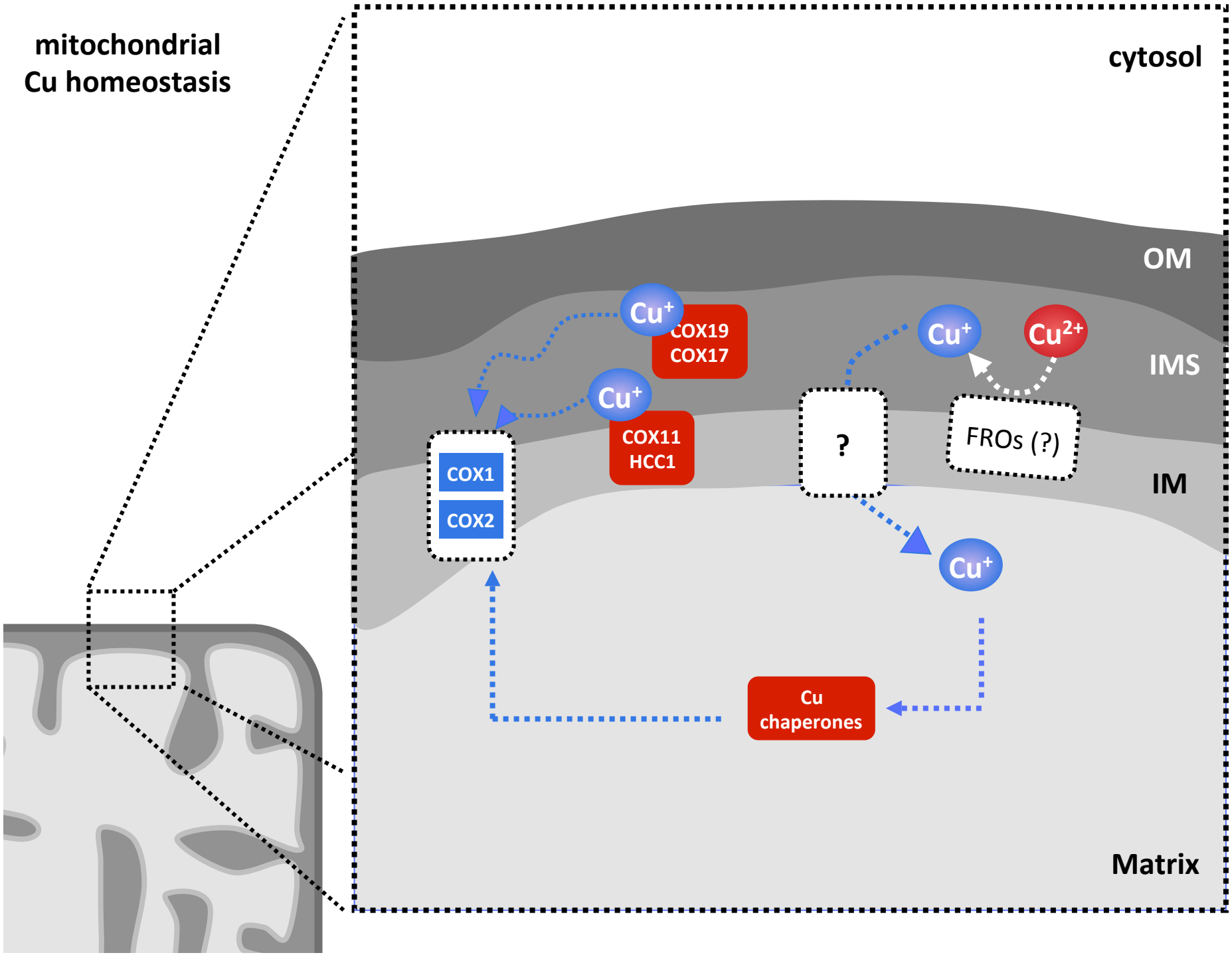
mitochondrial
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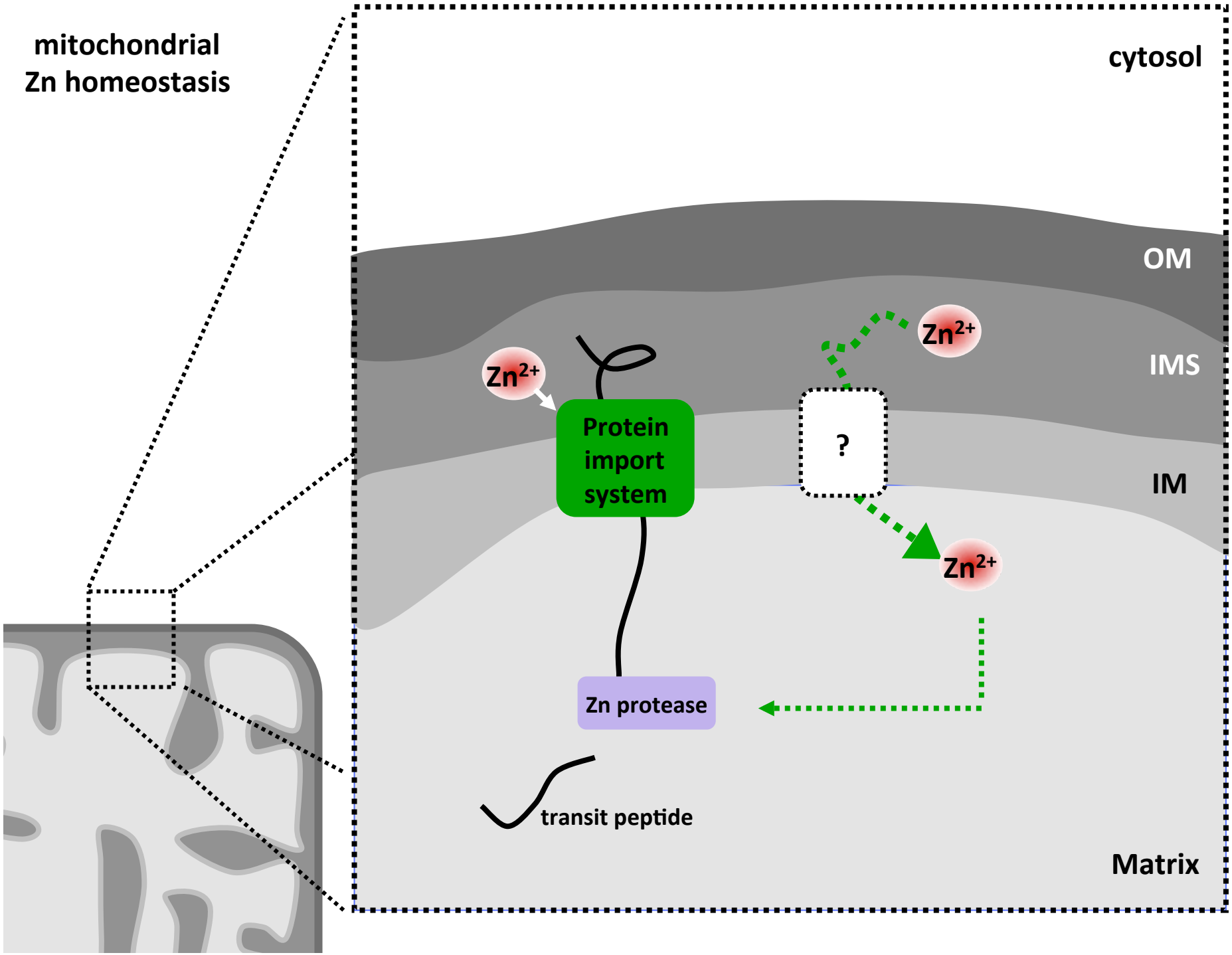
Cu2+
FROs (?)
Cu+
Cu
chaperones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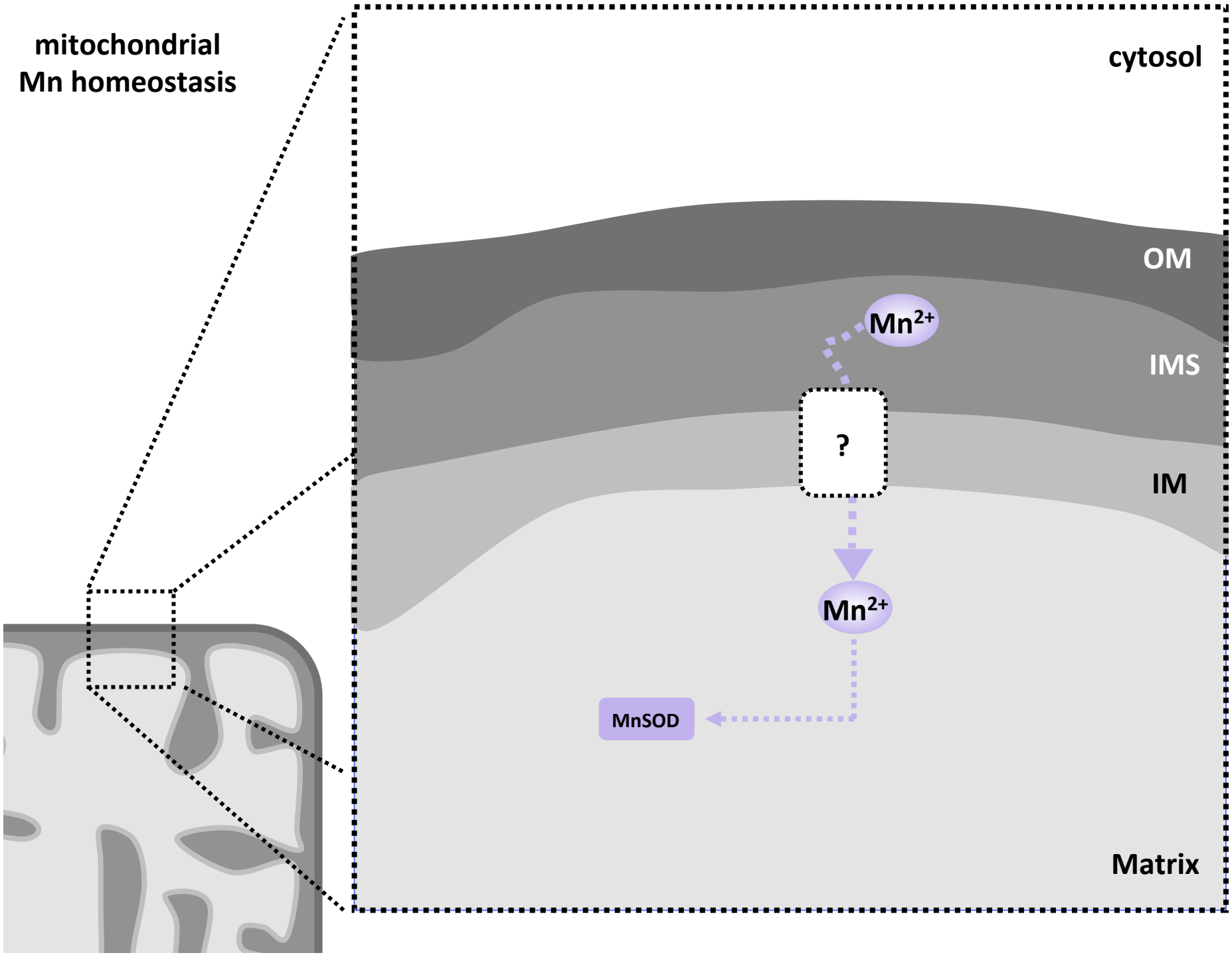
mitochondrial
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