

Iron and Copper in Mitochondrial Diseases

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Transition metals are frequently used as cofactors for enzymes and oxygen-carrying proteins that take advantage of their propensity to gain and lose single electrons. Metals are particularly important in mitochondria, where they play essential roles in the production of ATP and detoxification of reactive oxygen species. At the same time, transition metals (particularly Fe and Cu) can promote the formation of harmful radicals, necessitating meticulous control of metal concentration and subcellular compartmentalization. We summarize our current understanding of Fe and Cu in mammalian mitochondrial biology and discuss human diseases associated with aberrations in mitochondrial metal homeostasis.

Roles of Metals in Normal Mitochondrial Metabolism

Iron is the dominant metal in mitochondrial metabolism, but copper has important roles (Figure 1). Fe-protoporphyrin (heme), Fe-S clusters, and Cu are essential components of the mitochondrial inner membrane complexes constituting the electron transport chain. Three of the four complexes of the electron transport chain are physically associated (complexes I, III, and IV) and provide most of the proton motive force (for review, see Winge, 2012). Complex I (NADH dehydrogenase) contains eight Fe-S clusters involved in the transfer of electrons from reduced flavin mononucleotide (FMN_{H2}) to ubiquinone (for review, see Mimaki et al., 2012). Complex II (succinate dehydrogenase) contains a heme *b* prosthetic group in its anchor domain, which is essential for the structural integrity and function of the complex. Electrons abstracted from dehydrogenation of succinate are channeled through three Fe-S clusters ([2Fe-2S], [4Fe-4S], and [3Fe-4S]) to reduce ubiquinone to ubiquinol. Succinate dehydrogenase also participates in the tricarboxylic acid (TCA) cycle, catalyzing the oxidation of succinate to fumarate coupled to the reduction of ubiquinone (for review, see Rutter et al., 2010). Complex III (ubiquinol: cytochrome *c* oxidoreductase or cytochrome *bc1* complex) catalyzes the reduction of cytochrome *c* by oxidation of ubiquinol. Complex III contains a cytochrome *b* subunit with two heme moieties, a cytochrome *c*₁ subunit with one heme, and a Rieske protein subunit (UQCRC1) with a [2Fe-2S] cluster. The heme moieties and the Fe-S clusters participate in electron transfer. One electron from each ubiquinol is transferred through the [2Fe-2S] Rieske protein and cytochrome *c*₁ to reduce soluble cytochrome *c*. The second electron passes from one heme to the other of the cytochrome *b* moiety as part of the Q cycle, resulting in proton translocation across the inner membrane (for review, see Covian and Trumppower, 2008). Complex IV (cytochrome *c* oxidase) mediates the final step in the electron transport chain, catalyzing the reduction of oxygen to water. It contains two heme *a* moieties and two Cu centers, all of which participate in the electron transfer process. Cytochrome *c* is oxidized, leading to electron passage through the binuclear Cu center to one heme moiety, then to a heme-copper center to reduce oxygen (for review, see Yoshikawa et al., 2011).

Some soluble mitochondrial enzymes also require metals. Heme is a cofactor for sulfite oxidase, important for metabolism of sulfur-containing amino acids. The TCA cycle enzyme aconitase, which catalyzes the conversion of citrate to *cis*-aconitate, contains a [4Fe-4S] cluster in its active site. Cu is found in Cu-Zn superoxide dismutase (SOD1), which partially localizes to the mitochondrial intermembrane space. A distinct enzyme, Mn superoxide dismutase (SOD2), is found in the mitochondrial matrix.

Mitochondria as a Site of Fe Cofactor Assembly

Mitochondria host complex biosynthetic pathways to produce heme and Fe-S clusters for their own use and for use in other cellular compartments.

Heme Biosynthesis

Most mitochondrial Fe is used to produce heme, an important prosthetic group in a variety of cellular proteins, including electron transport chain complex subunits, hemoglobin, myoglobin, and cytochromes. There are several physiologically relevant forms of heme that differ in functional groups attached to the protoporphyrin ring. Heme biosynthesis is a highly conserved process dependent upon enzymatic steps both in the mitochondria and cytosol (Figure 2). Although the enzymatic reactions are well understood, much remains to be learned about transport of heme intermediates into and out of the mitochondrion. Heme synthesis begins with the formation of aminolevulinic acid (ALA) from glycine and succinyl-CoA, catalyzed by aminolevulinic acid synthase (ALAS) in the mitochondrial matrix. Mammals have two forms of ALAS: ALAS1, expressed ubiquitously, and ALAS2, restricted to erythroid cells. ALA is exported to the cytosol to be converted into porphobilinogen, hydroxymethylbilane, uroporphyrinogen III, and finally coproporphyrinogen III through a series of enzymatic reactions. Coproporphyrinogen III is transferred into the mitochondrial intermembrane space where it is modified to form protoporphyrin IX. ABCB6 has been proposed to mediate the translocation of coproporphyrinogen III across the mitochondrial outer membrane (Krishnamurthy et al., 2006), but human patients lacking ABCB6 have no evidence of anemia or other clinical abnormalities (Helias et al., 2012), making this function unlikely. Fe is

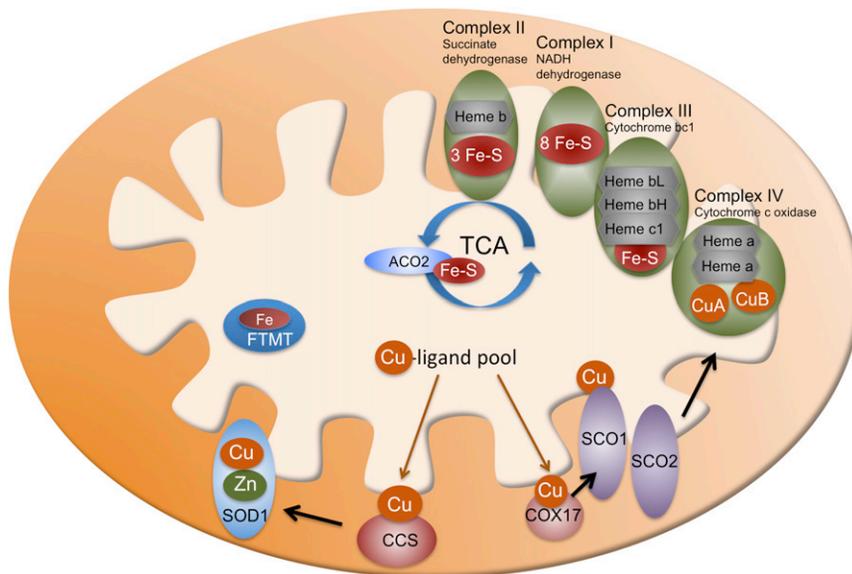


Figure 1. Mitochondrial Proteins Containing Fe and Cu

Complexes I–IV of the electron transport chain require Fe-S clusters, heme moieties, and Cu centers to function. Complex II functions both in electron transport and also in the tricarboxylic acid (TCA) cycle. Complexes I, III, and IV copurify (not depicted here). Mitochondrial aconitase (ACO2), another TCA cycle enzyme, contains an Fe-S cluster. Complex IV acquires Cu from a copper-ligand pool in the mitochondrial matrix through the action of cytochrome c oxidase assembly factors COX17, SCO1, and SCO2. Cu-Zn superoxide dismutase (SOD1) contains Cu provided by the Cu chaperone CCS. Mitochondrial ferritin (FTMT) can store Fe.

(Wingert et al., 2005). Fe-S cluster assembly also requires an electron procured from NADH via FDX1, and FDX2 proteins (Shi et al., 2012). Fe-S clusters are released from the scaffold and transferred through a chaperone

inserted into protoporphyrin IX by ferrochelatase (FECH) to produce heme.

Newly synthesized heme is delivered to various polypeptides in the cytosol, mitochondria, endoplasmic reticulum, and peroxisomes. Although several possible mechanisms have been described for its transport, it is still uncertain how heme leaves mitochondria to be incorporated into apo-hemoproteins elsewhere.

Iron-Sulfur Cluster Biosynthesis and Assembly of Fe-S Proteins

Fe-S clusters, which are typically coordinated to cysteine or histidine residues in proteins, serve as cofactors for molecules involved in electron transfer, catalysis, redox reactions, ribosome assembly, DNA damage repair, telomere maintenance, DNA replication, environmental sensing, and other processes (for review, see Lill et al., 2012). They are useful and versatile because Fe and S atoms in the clusters can accept and donate electrons. Fe-S cluster-containing proteins have important roles in mitochondria, but they are also found in the cytosol (e.g., iron regulatory protein 1, described later) and the nucleus (e.g., endonuclease III, involved in base excision repair). The biosynthesis and insertion of Fe-S clusters into extramitochondrial apo-proteins depends on both mitochondrial and the cytosolic machinery. Fe-S cluster biosynthesis has been intensively studied in bacteria and yeast (for review, see Lill et al., 2012), but the mammalian process and nomenclature are summarized here (for review, see Rouault, 2012).

Fe-S clusters are transiently assembled on a scaffold (ISCU or NFS1) in the mitochondrial matrix (Figure 3). A complex of NFS1 and ISD11 provides sulfur appropriated from cysteine (Adam et al., 2006; Wiedemann et al., 2006). The Fe-binding protein frataxin (FXN) effects a conformational change in ISCU-NFS1 that promotes cluster formation (Schmucker et al., 2011; Tsai and Barondeau, 2010). Glutathione has been postulated to be an Fe source (Hider and Kong, 2011). Fe-glutathione binds to glutaredoxins, and one of these, grx5, has been shown to be important in mitochondrial Fe-S cluster biosynthesis in the zebrafish

process to apo-proteins to form new Fe-S proteins. The maturation of aconitase and radical SAM enzymes (e.g., biotin synthase) requires a complex of A-type scaffold proteins (Gelling et al., 2008).

Fe-S cluster precursors must be exported to the cytosolic Fe-S cluster assembly machinery for incorporation into cytoplasmic and nuclear apo-proteins. ABCB7 is a transmembrane transporter important for cytoplasmic Fe-S cluster formation, but its substrate is unknown (Bekri et al., 2000). A distinct ATP binding cassette protein, ABCB8, has also been implicated in Fe-S cluster maturation by a severe phenotype in mice lacking ABCB8 in the heart (Ichikawa et al., 2012). Deficiency of ABCB8 impairs cytosolic but not mitochondrial Fe-S formation. While it has been suggested to function as a mitochondrial iron exporter because decreased expression leads to mitochondrial iron accumulation, the precise function of ABCB8 remains unknown.

Cytoplasmic/Mitochondrial Homeostasis

The inner mitochondrial membrane is impermeable to metal ions without the aid of dedicated transport systems. Homeostasis must be maintained in cytoplasmic and mitochondrial metal distribution to avoid toxicity associated with metal-induced reactive oxygen species (ROS) formation. Additionally, mitochondria must have mechanisms to ensure that cellular metal uptake is sufficient to meet their needs. These molecular functions are partially understood.

Fe

Iron can enter cells through direct plasma membrane uptake or through release from endosomes formed through receptor-mediated endocytosis of Fe-transferrin bound to cell surface transferrin receptor (TFRC) (for review, see Andrews, 2008). Divalent metal transporter 1 (SLC11A2) is important for both plasma membrane and endosomal transport, though there is evidence that other molecules can substitute. An unrelated molecule, ferroportin (SLC40A1), is the only known protein that can transport iron out of mammalian cells.

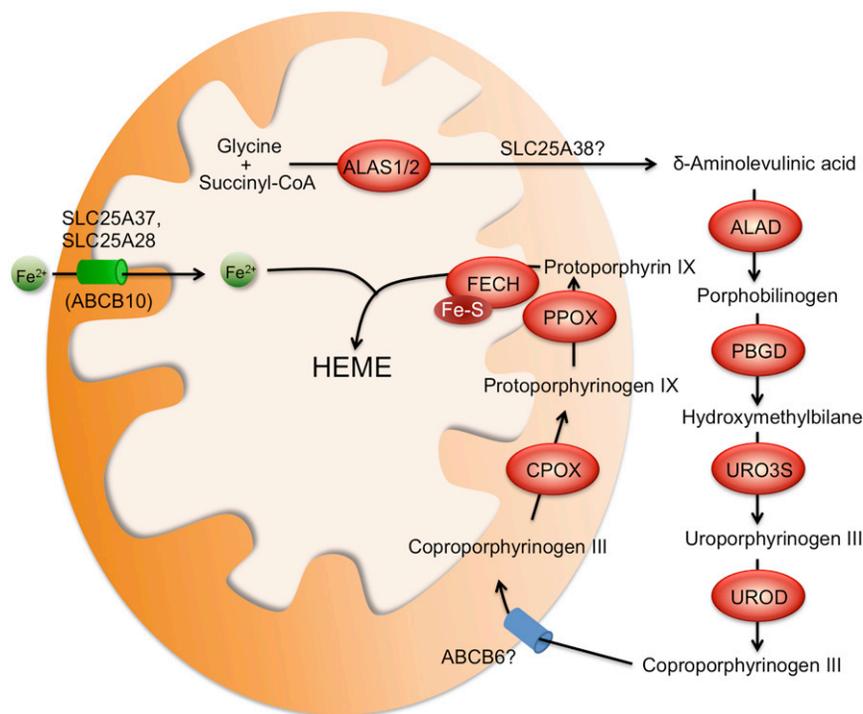


Figure 2. Mammalian Heme Biosynthesis

Heme biosynthesis begins and ends in the mitochondrion, requiring four mitochondrial enzymes (δ -aminolevulinic synthase, ALAS1/2; coproporphyrinogen III oxidase, CPOX; protoporphyrinogen oxidase, PPOX; and ferrochelatase, FECH) and four cytoplasmic enzymes (ALAD, aminolevulinic dehydratase; PBGD, porphobilinogen deaminase; uroporphyrinogen III synthase, URO3S; and uroporphyrinogen decarboxylase, UROD). The process is initiated by ALAS, which catalyzes the production of aminolevulinic acid from glycine and succinyl coA. Mitoferrin proteins (SLC25A37 and SLC25A28) transport Fe across the inner mitochondrial membrane to make it available for insertion into protoporphyrin IX by FECH.

across the inner mitochondrial membrane in erythroid and nonerythroid cells by mitoferrins 1 and 2 (SLC25A37 and SLC25A28), respectively (Paradkar et al., 2009; Shaw et al., 2006), which are homologous to the yeast mitochondrial Fe transporters Mrs3/4p (Foury and Roganti, 2002; Mühlhoff et al., 2003). An accessory protein, ABCB10, has been shown to stabilize SLC25A37 during hemoglobinization of erythroid cells, to

Cellular Fe homeostasis is maintained by iron regulatory proteins (IRP1 and IRP2) that recognize iron response elements (IREs) located in untranslated portions of a subset of mRNAs involved in Fe homeostasis (for review, see Hentze et al., 2010; Rouault, 2006). These include mRNAs encoding TFRC, SLC11A2, SLC40A1, and subunits of the iron storage protein ferritin (FTL and FTH). IRPs act to increase or decrease translation, depending on the location of IREs within the mRNA. When cellular Fe is depleted, binding of IRPs to IREs promotes the synthesis of TFRC and SLC11A2 and inhibits production of SLC40A1 and ferritin, thus increasing cytoplasmic Fe availability. Conversely, when cellular Fe is abundant, IRPs are inactivated, Fe import is restricted, storage is increased, and export is potentiated. The two IRPs are regulated through distinct mechanisms: IRP1 is inactivated by incorporation of a [4Fe-4S] cluster (reviewed by Volz, 2008), whereas IRP2 is targeted for degradation by a ubiquitin ligase complex that senses Fe through FBXL5 (Salahudeen et al., 2009; Vashisht et al., 2009).

Transition metals change oxidation state readily and are generally bound to proteins to attenuate their reactivity. Although protein chaperones for Cu have been characterized in great detail (see below), cytoplasmic Fe chaperone proteins were not identified until recently. Poly (rC)-binding proteins 1 and 2 (PCBP1 and PCBP2) have been shown to deliver Fe to ferritin and to a prolyl hydroxylase that regulates hypoxia-inducible factor 1 (Nandal et al., 2011; Shi et al., 2008). PCBPs have many homologs, and it is possible that related proteins will similarly turn out to serve as Fe chaperones.

The mitochondrial outer membrane has 2–3 nm pores through which small molecules can pass freely, but the inner mitochondrial membrane is not permeable to metals. Fe is transferred

ensure the high flux of Fe needed for heme biosynthesis (Chen et al., 2009). No transporters mediating export of mitochondrial Fe have been definitively identified. It may be that Fe only leaves the mitochondrion in the form of heme or Fe-S clusters.

Within the mitochondrion Fe has several possible fates: it can be used to synthesize heme and Fe-S clusters, or incorporated into mitochondrial ferritin (FTMT; Levi et al., 2001). The normal function of FTMT is not well understood. Although it is implicated in sideroblastic anemias (see below), mice lacking Ftmt have no apparent phenotype (Bartnikas et al., 2010). Overexpression of FTMT sequesters mitochondrial Fe, making it unavailable for use (Nie et al., 2005).

Failure of Fe-S cluster biosynthesis alters Fe balance in mammalian cells (for review, see Lill et al., 2012). Fe-S clusters act as a switch for IRP1, transforming the IRE-binding protein into a cytoplasmic aconitase (Rouault, 2006). When Fe-S cluster biosynthesis is disrupted, IRP1 is constitutively active, causing cellular Fe to increase. In parallel, heme biosynthesis is inhibited because active IRP1 inhibits the translation of ALAS2 and because FECH, the last enzyme in heme biosynthesis, requires a [2Fe-2S] cluster for activity (Wu et al., 2001). The complex interplay between Fe-S and heme biosynthesis was demonstrated in vivo in zebrafish lacking grx5, a glutaredoxin necessary for Fe-S cluster production (Wingert et al., 2005).

Studies of yeast cells lacking FXN and other components of the Fe-S cluster biogenesis machinery provide insight into cytoplasmic/mitochondrial Fe homeostasis (Babcock et al., 1997; Lill et al., 1999; Rouault and Tong, 2005). The mutant yeast cells are unable to carry out oxidative phosphorylation, and they activate a high-affinity Fe uptake system on the plasma membrane. Total cellular Fe increases, but the Fe accumulates in mitochondria

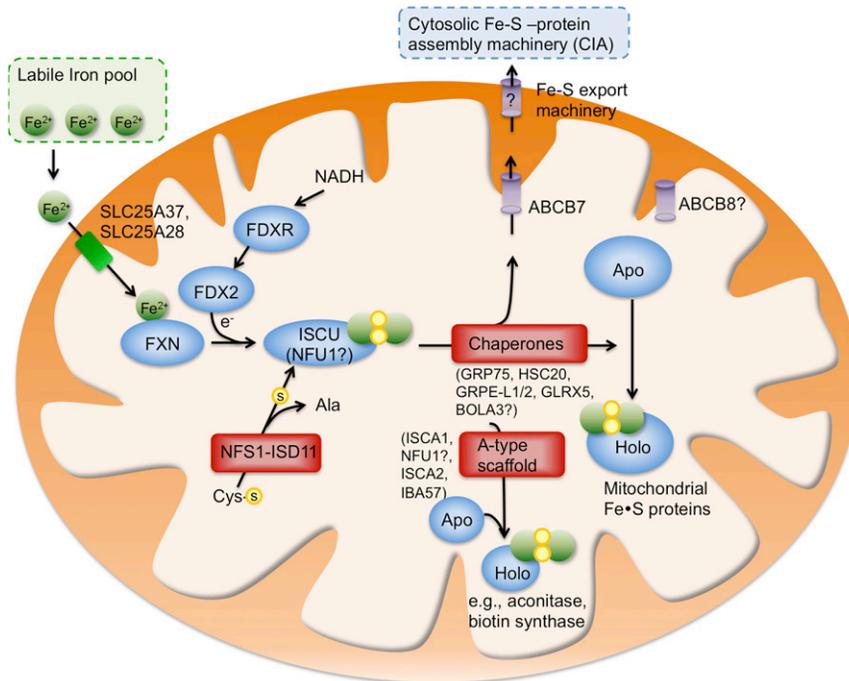


Figure 3. Mammalian Fe-S Cluster Biosynthesis

Fe-S cluster biosynthesis is a complex process that also begins in the mitochondria (see text). Mitoferrin proteins (SLC25A37 and SLC25A28) are necessary for Fe procurement. Sulfur is derived by enzymatic conversion of cysteine to alanine.

Cu

Similar to Fe, there are relatively few proteins known to function as transmembrane Cu transporters (for review, see Wang et al., 2011). There is one confirmed cellular importer, CTR1 (SLC31A1), which has a close homolog, CTR2 (SLC31A2), of uncertain function. Reduction of copper to Cu^+ is required prior to transport. There are two cellular Cu exporters, ATP7A and ATP7B, both located predominantly in the *trans*-Golgi network. ATP7A can relocate to the plasma membrane for cellular Cu export; ATP7B traffics to vesicles and, among other functions, mediates excretion of Cu into bile. The subcellular

at the expense of the cytoplasm. Consistent with markedly increased mitochondrial Fe, the yeast show increased sensitivity to oxidative stress.

Fe-S cluster biogenesis may not be as severely affected in patients with Friedreich's ataxia or animals carrying *FXN* mutations. Mitochondrial Fe accumulation has been reported (Lamarque et al., 1980; Puccio et al., 2001), but others suggest that it is an inconsistent and late finding (Bayot et al., 2011). However, *Fxn*-deficient mouse cells do show altered expression of genes that favor Fe uptake and mitochondrial import over Fe storage and export (Huang et al., 2009).

Cardiolipin is a phospholipid that is found predominantly in the inner mitochondrial membrane (Schlame et al., 2000). Defects in cardiolipin synthesis in human patients lead to Barth Syndrome, characterized by cardiac and skeletal myopathies and other abnormalities. Recently it was shown that disruption of cardiolipin synthesis in yeast leads to a defect in Fe-S cluster biogenesis, most likely due to a failure of mitochondrial import or processing of proteins involved in Fe-S production (Patil et al., 2012). Similar to other defects in Fe-S cluster synthesis, this results in induction of iron uptake genes and mitochondrial iron accumulation.

When heme biosynthesis is interrupted by treatment with succinylacetone to inhibit ALAS (the first enzyme of heme biosynthesis), cellular Fe uptake is increased and Fe accumulates in mitochondria (Ponka et al., 1982).

Taken together, these observations have two important implications that may be generalizable: (1) that failure of Fe cofactor biosynthesis triggers excess mitochondrial Fe accumulation and (2) that mitochondrial Fe needs are communicated to increase plasma membrane Fe transport. It is an open question whether other types of mitochondrial dysfunction also lead to mitochondrial Fe accumulation.

trafficking of CTR1, ATP7A, and ATP7B responds to cellular copper levels.

There is very little, if any, free Cu within cells: Cu is bound to proteins or liganded to glutathione or other small molecules. The problem of making Cu available for cuproenzymes is solved through the use of Cu chaperones, which bind Cu tightly and deliver it to target proteins. In mammals, the chaperones are ATOX1, COX17, and CCS, which deliver Cu to ATP7A/ATP7B, cytochrome c oxidase and SOD1, respectively (for review, see Nevitt et al., 2012). Most SOD1 is cytoplasmic, receiving Cu from CCS without entering the mitochondrion. However, a small fraction of SOD1 resides in mitochondria, predominantly in the mitochondrial intermembrane space. Unmetallated CCS and COX17 are imported into the mitochondrial intermembrane space through a GFER/MIA40-mediated disulfide relay mechanism (for review, see Herrmann and Riemer, 2012). The entry of SOD1 into this compartment, as well as its maturation, metalation, and retention, is controlled by CCS (for review, see Kawamata and Manfredi, 2010). The subcellular localization of CCS, and as a consequence, the subcellular localization of SOD1, is modulated by oxygen concentration; high oxygen concentration inhibits import into the mitochondrion (Kawamata and Manfredi, 2008).

COX17 is found both in the cytoplasm and on the inner mitochondrial membrane, but translocation is not necessary for its function, implying that it can receive Cu without having to leave the mitochondrion (Maxfield et al., 2004). The mitochondrial source of metal for both CCS and COX17 is a liganded pool of Cu in the matrix. This pool influences the overall Cu status of the cell, but the signals responsible have not been identified (for review, see Leary et al., 2009b). The ligand is not a protein, but its identity is unknown. It is thought that apo-ligand in the cytoplasm binds Cu and easily crosses the outer membrane

and then crosses the inner membrane through a protein-mediated transport system that is not well understood. Cu-ligand is retained in the matrix until its Cu is mobilized for use by COX17 or CCS.

COX17, SCO1, and SCO2 are involved in Cu incorporation into cytochrome *c* oxidase (Complex IV). Partially oxidized COX17 in the intermembrane space hands off Cu and two electrons to oxidized SCO1 (Banci et al., 2008). A homologous protein, SCO2, has a distinct role. It appears to oxidize Cu-coordinating cysteines in SCO1 to transfer metal for maturation of cytochrome oxidase, though the mechanism is not fully understood (Leary et al., 2009a).

Cells deficient in SCO1 or SCO2 have cytoplasmic Cu deficiency but normal mitochondrial Cu content, suggesting the existence of homeostatic mechanisms governing Cu distribution (Dodani et al., 2011). SCO1 and SCO2 also appear to modulate cellular Cu efflux through mechanisms not yet understood (Leary et al., 2009a).

Diseases Associated with Failure of Mitochondrial Metal Homeostasis

Mitochondria are found in all mammalian cells, but they are particularly abundant in the heart, muscles, and nervous system, where they support the energy demands of muscle contraction and neuronal function. Mitochondria also play a critical role in hemoglobin production in erythroblasts. Accordingly, erythroid precursors, muscle cells, and neurons are particularly vulnerable to diseases associated with failure of metal homeostasis and consequent mitochondrial dysfunction. Inborn errors that affect mitochondrial metal metabolism commonly cause pathology in more than one of these major target tissues. Furthermore, the brain is particularly susceptible to increased oxidative stress and oxidative damage because of its high rate of oxygen consumption and relatively poor ability to deal with ROS.

Red Blood Cell Disorders

As discussed earlier, disorders that impair production of heme and Fe-S clusters trigger homeostatic mechanisms that result in mitochondrial Fe accumulation. Because red blood cells require far more heme than other mammalian cells, erythropoiesis is particularly sensitive to these defects. The phenotype is sideroblastic anemia: Fe-laden mitochondria in bone marrow erythroid precursors and decreased production of circulating erythrocytes, which may appear Fe deficient. These diseases typically cause increased intestinal Fe absorption, highlighting another level of regulation: communication of erythroid needs to signal alterations in total body Fe homeostasis.

A priori, the fact that there are many steps and many enzymes involved in heme and Fe-S cluster biosynthesis (Figures 2 and 3) suggests that sideroblastic anemias will be a heterogeneous set of disorders due to mutations in a variety of genes. This has turned out to be the case, and there are still some inherited sideroblastic anemias for which the cause has not yet been identified (Fleming, 2011). Interestingly, most defects in heme biosynthetic enzymes do not cause sideroblastic anemia, but rather result in porphyrias: diseases associated with the accumulation of porphyrin intermediates. Although mitochondrial iron accumulation has been reported in the heart, sideroblasts have not been observed in patients with Friedreich's ataxia (see below).

The most common form of congenital sideroblastic anemia results from mutations in ALAS2, the rate-limiting, erythroid-specific form of the first enzyme in heme biosynthesis (Cotter et al., 1992). Because the *ALAS2* gene is on the X chromosome, this disorder is far more common in males than females. Total loss of ALAS2 function is probably incompatible with life, and accordingly, only missense mutations have been described. ALAS2 uses pyridoxine (vitamin B6) as a cofactor, and many patients benefit from treatment with pharmacological doses of the vitamin, distinguishing this form of sideroblastic anemia from other congenital forms. No mutations in ALAS1, the ubiquitous form of the enzyme, have been described in humans.

A second form of X-linked sideroblastic anemia results from mutations in ABCB7, a mitochondrial transporter important in Fe-S cluster formation (Allikmets et al., 1999). The anemia is milder than in patients with ALAS2 mutations, but accompanied by ataxia and other neurological manifestations, indicating an important but as yet undefined role for ABCB7 in the brain. Recently, ABCB7 was implicated in the pathogenesis of acquired refractory anemia with ringed sideroblasts (Nikpour et al., 2012).

Autosomal recessive sideroblastic anemia can also result from mutations in another gene involved in Fe-S cluster biosynthesis, *GLRX5* (Camaschella et al., 2007). These patients have no manifestations other than anemia, possibly because erythroid cells are uniquely sensitive to perturbations in IRP1 activity resulting from loss of its Fe-S cluster (Ye et al., 2010). Autosomal recessive sideroblastic anemia is seen in patients with mutations in SLC25A38, an inner mitochondrial membrane protein with similarity to amino acid transporters, which is predominantly expressed in hematopoietic cells (Guernsey et al., 2009). Although it is clear that SLC25A38 is important for heme biosynthesis, its biological function is unknown. It may be a mitochondrial importer for glycine, a transporter for 5-aminolevulinic acid, or perhaps a bifunctional carrier that accomplishes both tasks. No nonhematopoietic homolog of SLC25A38 has been identified, but presumably another protein carries out an analogous function in other cell types.

Recently, Shah and colleagues discovered that a zebrafish mutant with hypochromic, microcytic red blood cells carried a mutation in *atpif1a* resulting in decreased mRNA levels (Shah et al., 2012). This gene is one of two zebrafish orthologs for *ATPIF1*, encoding an inner mitochondrial membrane protein that modulates the function of the F_1F_0 -ATP synthase. Although there was no previously described link between *ATPIF1* and heme biosynthesis, the mutant zebrafish were unable to produce adequate levels of heme for normal erythroid differentiation. The defect was specific to erythroid cells, presumably because a homolog, *atpif1b*, compensates in other cell types, and the phenotype was replicated in murine cells made deficient in *Atpif1*. *Atpif1*-deficient murine erythroid cells had depletion of cellular ATP, increased mitochondrial membrane potential, increased mitochondrial pH, and increased mitochondrial Fe (Shah et al., 2012). However, in spite of normal amounts of mitochondrial protoporphyrin IX, heme was not produced effectively due to a defect in FECH activity at elevated pH. Pharmacological agents that lowered mitochondrial membrane potential restored heme biosynthesis, as did expression of yeast FECH, which lacks an Fe-S cluster. Their results indicated that, although

Fe-S cluster synthesis was not perturbed, the [2Fe-2S] cluster in vertebrate FECH is sensitive to mitochondrial changes attributable to deficiency of *ATPIF1*. Although mutations in *ATPIF1* have not yet been found in human patients, erythroid-restricted loss of function of its gene product would be predicted to be a cause of sideroblastic anemia.

Autosomal recessive sideroblastic anemia accompanied by myopathy and lactic acidosis (MLASA disorders) can be caused by mutations in nuclear genes that are important for overall mitochondrial function, including tyrosyl tRNA synthetase-2 (*YARS2*; Riley et al., 2010) and pseudouridine synthase (*PUS1*; Bykhovskaya et al., 2004). This phenotype reinforces the concept that defects in mitochondrial function can alter cytoplasmic/mitochondrial Fe homeostasis and lead to mitochondrial Fe loading.

The mitochondrial genome encodes respiratory chain subunits, ribosomal RNAs, and tRNAs, and mitochondrial DNA deletions cause pleiotropic abnormalities. Pearson Marrow Pancreas Syndrome, characterized by sideroblastic anemia, pancytopenia, and abnormalities of the exocrine pancreas, results from mitochondrial DNA deletions that may come about during replication (Rötig et al., 1991). Sideroblastic anemia can also be caused by toxins and toxic drug effects, as well as deficiencies of thiamine and Cu.

Cardiomyopathy

Systemic Fe overload results from mutations in hemochromatosis disease genes, from increased Fe absorption secondary to anemias characterized by ineffective erythropoiesis or from hypertransfusion of red blood cells. In each of these conditions, excess Fe accumulates in cardiac myocytes and can cause cardiomyopathy through promotion of hydroxyl radical formation, Fe-mediated damage to mitochondrial DNA, and disruption of the mitochondrial respiratory chain.

Deficiencies of Fe (Petering et al., 1990) and Cu (Klevay, 2000) have also been associated with mitochondrial dysfunction and cardiomyopathy in animals, and Fe deficiency has been associated with cardiomyopathy in humans (Hegde et al., 2006). However, because Cu deficiency leads to Fe deficiency through insufficiency of ferroxidases needed for Fe transport, it can be difficult to tease out how nutritional Cu deficiency results in specific mitochondrial defects. This has been clarified by targeted inactivation of the Cu transporter *Ctr1* in the mouse heart. The mutant animals develop severe cardiomyopathy with abnormal mitochondria and decreased activity of Cu-containing cytochrome *c* oxidase (Kim et al., 2010).

Friedreich's ataxia, caused by decreased expression of active FXN, is characterized by progressive ataxia and sensory loss due to neurodegeneration of dorsal root ganglia, cardiomyopathy, and sometimes diabetes mellitus. While neurological impairment may be the most prominent finding, hypertrophic cardiomyopathy is the most common cause of death. Cardiac mitochondrial iron accumulation was observed in a small series many years before FXN was identified (Lamarche et al., 1980). As discussed earlier, insufficient expression of this Fe-S cluster pathway protein leads to mitochondrial failure, and dysfunctional mitochondria proliferate at the expense of muscle fibers (for review, see Payne and Wagner, 2012).

Cardiomyopathy is also a prominent feature of fatal infantile cardioencephalopathy, due to cytochrome *c* oxidase deficiency resulting from mutations in the mitochondrial Cu chaperone

protein SCO2 (Papadopoulou et al., 1999). Interestingly, most or all patients identified to date have at least one allele carrying an E140K mutation (Leary et al., 2007), which disrupts a salt bridge close to the CXXC Cu binding motif. Although there is a profound loss of motor neurons resulting in hypotonia and weakness, death is typically caused by heart failure. Cu content of the heart is markedly reduced as a result of increased Cu efflux (Leary et al., 2007). SCO1 is a close homolog of SCO2, but mutations in SCO1 cause a different disorder, with hepatic failure and death soon after birth (Valnot et al., 2000). Accordingly, SCO proteins have distinct roles in cytochrome *c* oxidase maturation (Leary et al., 2009a).

Cardiomyopathy is a severe consequence of inactivation of the gene encoding Mn-dependent, mitochondrial SOD2 gene in mice (Li et al., 1995). The mutant animals are anemic, with metabolic acidosis and lipid accumulation in the liver and muscle, and death before the end of the second week of life. Activity of two Fe-S-requiring enzymes, succinate dehydrogenase (complex II) and aconitase, is markedly reduced, suggesting that inadequate defense against nondismutated O₂⁻ in mitochondria leads to destruction of Fe-S clusters, compromising the electron transport chain and the TCA cycle. The heart may be less able to deal with oxidative stress than other organs.

Skeletal Myopathy

Abnormalities of mitochondrial metal homeostasis can also cause disorders of skeletal muscle, with or without abnormalities in other tissues. In addition to disorders described earlier, myopathies not accompanied by sideroblastic anemia are caused by mutations in several genes responsible for Fe-S cluster biogenesis. Deficiency of the scaffold protein ISCU, due to a splicing error, causes autosomal recessive myopathy with mitochondrial Fe overload (Mochel et al., 2008; Olsson et al., 2008). Interestingly, the aberrant splicing event appears to be restricted to skeletal muscle; normal splicing is favored in cardiomyocytes, hepatocytes, and very likely in erythroid precursors (Crooks et al., 2012; Nordin et al., 2011).

Global Mitochondrial Dysfunction

Four infants in two unrelated families were reported to have lactic acidosis and multisystem mitochondrial pathology leading to death in the first year of life (Seyda et al., 2001). This disorder, termed multiple mitochondrial dysfunctions syndrome, showed linkage to chromosome 2p14-2p13. Surprisingly, although the phenotypes were similar, the two families carried deleterious mutations in different genes, *NFU1* and *BOLA3*, located 5 Mbp apart (Cameron et al., 2011). Other groups identified mutations in *NFU1* (Navarro-Sastre et al., 2011) and *BOLA3* (Haack et al., 2012) in distinct families with phenotypic similarity.

NFU1 is known to be important in Fe-S cluster biogenesis and has been postulated to act as a scaffold in a pathway parallel to the ISCU scaffold (Figure 3; Tong et al., 2003). A severe defect in Fe-S cluster synthesis would explain the dramatic clinical presentation and deficiencies in mitochondrial electron transport complexes observed in these patients (Cameron et al., 2011; Navarro-Sastre et al., 2011). In contrast, the role of *BOLA3* was not previously known, although homologs have been reported to act as reductases interacting with a glutaredoxin (Huynen et al., 2005) and to affect iron homeostasis in yeast (Li et al., 2011). Taken together with clinical and biochemical phenotypes similar to those observed in a patient with *NFU1* mutation, a likely

role for BOLA3 is collaboration with GLXR5 as a chaperone in Fe-S synthesis.

There are many rare genetic disorders, e.g., Leigh syndrome, resulting from defects in proteins involved in the formation and/or function of the mitochondrial respiratory chain (for review, see Wong, 2012). Although all tissues should be affected, these early-onset disorders typically have neurodegeneration as the most prominent feature and, sometimes, cardiomyopathy and muscle disease. A subset have mutations in proteins containing or involved in insertion of heme (e.g., COX10 [Antonicka et al., 2003], COX15 [Bugiani et al., 2005], and SURF1 [Bundschuh et al., 2009]) or Fe-S clusters (e.g., NDUFV1 [Vilain et al., 2012], NDUFS3 [Bénil et al., 2004], NUBPL [Calvo et al., 2010], and NDUFS8 [Loeffen et al., 1998]).

Neurodegenerative Diseases

The brain needs far more energy than other organs, necessitating optimal ATP production by neurons. Many neurodegenerative diseases are associated with both mitochondrial dysfunction and metal deposition in the vicinity of brain lesions, but in general the pathogenesis is unclear. Several key proteins associated with neurodegenerative diseases have been reported to have intimate connections to transition metals. Amyloid precursor protein (APP) was reported to have an intrinsic ferroxidase activity (Duce et al., 2010), although other investigators have not observed this (Ebrahimi et al., 2012). Alpha-synuclein (SNCA) and prion protein (PRNP) assemble into metal-containing aggregates. Although APP, SNCA, and PRNP are not known to be directly involved in mitochondrial metal metabolism, mitochondrial quality and quality control are strongly implicated in the neurodegenerative diseases with which they are associated.

Neurodegeneration with brain Fe accumulation (NBIA) disorders are distinguished by characteristic Fe accumulation in the basal ganglia visualized by magnetic resonance imaging and obvious brown (Fe) discoloration of the brain on autopsy. The gene most commonly mutated in NBIA, pantothenate kinase 2 (PANK2), encodes an enzyme important for mitochondrial coenzyme A biosynthesis (Zhou et al., 2001). Other genes mutated in NBIA patients include a novel mitochondrial protein, C19orf12, possibly also involved in coenzyme A metabolism (Hartig et al., 2011).

Neurodegeneration with impaired mitochondrial function in motor neurons has been reported in *Ireb2*^{-/-} mice lacking IRP2 (Jeong et al., 2011). Abnormal cellular Fe homeostasis results in decreased complex I and II activity in the mitochondria of lower motor neurons, apparently because of inappropriate regulation resulting in decreased expression of Tfrc and increased storage of iron in ferritin. Accordingly, the phenotype was ameliorated by a genetic maneuver to decrease ferritin production. Taken together, these results suggest that cellular Fe deficiency can also cause neuronal degeneration.

The gene encoding Cu-Zn SOD1 is mutated in a subgroup of patients with familial amyotrophic lateral sclerosis (ALS), and mitochondrial dysfunction is a common feature of both genetic and sporadic forms of the disease. SOD1 mutations are considered to be gain-of-function, and the mutant protein preferentially accumulates in the mitochondrial intermembrane space. Various models for toxic gain-of-function mutations have been proposed, involving increased production of ROS and Ca²⁺ dysre-

gulation (for review, see Faes and Callewaert, 2011), but the pathophysiology is still unresolved.

Much remains to be learned about metals, mitochondria, and neurodegenerative diseases. There is substantial overlap in clinical presentation and pathologic features among these disorders, but there is still uncertainty about cause and effect. Does mitochondrial dysfunction lead to metal accumulation, or does metal accumulation lead to ROS production and mitochondrial dysfunction? Are protein-metal aggregates causing mitochondrial dysfunction and neurodegeneration, or are they a marker of neurodegeneration, or both?

Conclusions

The relationship between metals and mitochondria has been studied for decades, but much remains to be learned. Our current understanding relies heavily on elegant studies in yeast and on inborn errors of metabolism in human patients. Fully elucidating the interaction between metals and mitochondria in disease will require new approaches, but it will undoubtedly enrich our understanding of mitochondrial biology.

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