Special topic

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Metal cofactors trafficking and assembly in the cell: a molecular view

https://doi.org/10.1515/pac-2018-0720

Abstract: Metal ions are essential cofactors required by the proteome of organisms from any kingdom of life to correctly exert their functions. Dedicated cellular import, transport and homeostasis systems assure that the needed metal ion is correctly delivered and inserted into the target proteins and avoid the presence of free metal ions in the cell, preventing oxidative damaging. Among metal ions, in eukaryotic organisms copper and iron are required by proteins involved in absolutely essential functions, such as respiration, oxidative stress protection, catalysis, gene expression regulation. Copper and iron binding proteins are localized in essentially all cellular compartments. Copper is physiologically present mainly as individual metal ion. Iron can be present both as individual metal ion or as part of cofactors, such as hemes and iron-sulfur (Fe-S) clusters. Both metal ions are characterized by the ability to cycle between different oxidation states, which enable them to catalyze redox reactions and to participate in electron transfer processes. Here we describe in detail the main processes responsible for the trafficking of copper and iron sulfur clusters, with particular interest for the structural aspects of the maturation of copper and iron-sulfur-binding proteins.

Keywords: CIA machinery; copper; Distinguished Women in Chemistry and Chemical Engineering; iron-sulfur proteins biogenesis; ISC machinery; metal homeostasis; metal transport; metallochaperones.

Introduction

A large share of the proteome of organisms from any kingdom of life require metal ions to correctly perform their function. To be functionally active, however, the proteins need to recruit the correct metal as soon as they are synthesized. For this purpose, organisms have developed transport and homeostasis systems, which both assure that each protein receives the needed metal ion and prevent not suitable metal ions to occupy metal binding sites in the newly produced proteins. These systems tightly control the trafficking of metal ions in the cell, from their import to the final destinations.

This is achieved by always having the metal ions bound to a protein and also by binding and buffering by low molecular weight ligands, such as glutathione or amino acids, thus preventing the presence of free metal ions in the cell and allowing the transfer from one protein to another only through very selective molecular recognition pathways.

In this minireview we describe and analyze some of these processes, responsible for trafficking copper and iron sulfur clusters. The detailed description of the molecules involved, of their metal binding mode, and of their interaction patterns allowed us to have a complete, atomic level, understanding of the processes within the cellular context.

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Copper trafficking in human cells

Copper is an essential metal ion for eukaryotic organisms even if it is present in relatively low amount ($1–2\times10^{-3}$ g/kg of adult human) and is bound to a relatively low number of proteins (<100 in humans) [1–4]. Despite its low abundance it is involved in absolutely essential functions, such as respiration, oxidative stress protection, catalysis [5–9]. Copper binding proteins are localized in essentially all cellular compartments, from the cytoplasm, to mitochondria, to the Golgi system.

Inside the cells, copper is present in its +1 oxidation state. Copper proteins functions, and the related processes, are based on the copper ability to cycle between +1 and +2 oxidation states, and therefore copper catalyzes redox reactions as well as it is involved in electron transfer processes.

Copper ions are characterized by the highest affinity among the first-row transition metal ions towards a coordination binding site [10]. This implies that the metal binding sites in metalloproteins would preferentially bind copper which is even able to displace the physiological ion, thus impairing the protein function [11]. To control this behavior and to overcome its deleterious effects, cells developed processes which prevent copper to be free in the cell, and which assure the highest selectivity and highest specificity in its delivery to those proteins which require it to function [12–19]. In eukaryotic cells, copper is imported by specific plasma membrane-embedded proteins, CTRs, which allow copper to pass through the membrane and to be coordinated on a binding site exposed on the internal side of the plasma membrane [20–22]. Here copper is coordinated by transporting proteins, one specific for each transfer pathway and each final receiving protein [9, 13, 19, 23–27]. The transfer processes occur only through associative mechanisms: a specific transporter protein interacts, through a very selective recognition pattern, with a specific receiving protein being the sharing of the metal ion ligands an essential requirement [28–30]. Indeed, while the interacting surface on the two proteins is selective for unique molecular recognition, the two partner proteins do not interact each other in absence of the metal ion. They do interact only through the shared coordination of the metal ion with ligands from both partner proteins [28]. This is indeed beneficial and essential for the physiological process, as the two partners do not need to interact when in the metal-free form while they have to interact when they are metal loaded.

The copper binding site in transporting proteins is usually on the surface and solvent exposed as the coordinating residues of the receiving protein have also to coordinate the copper ion in the intermediate complex [28, 31]. Copper (I) prefers a tri-coordinated geometry [2, 14], and this is usually accomplished by

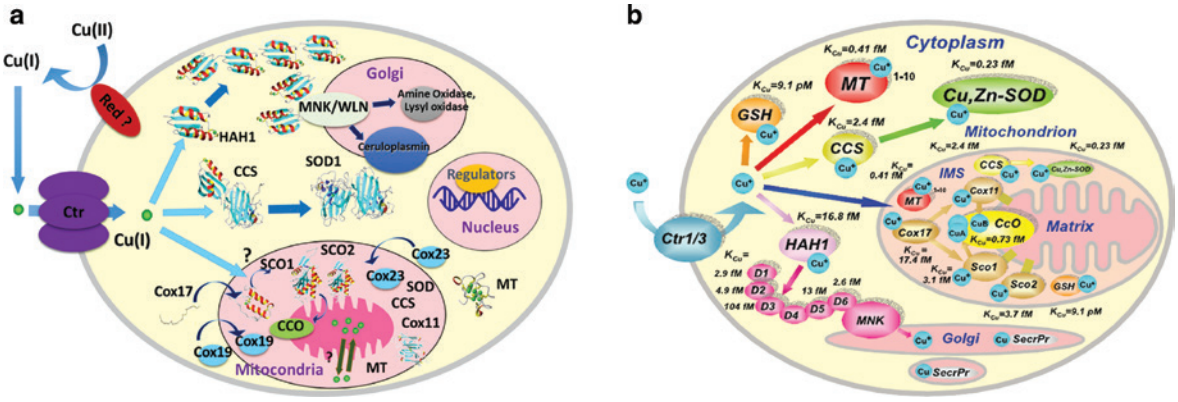

Fig. 1: Copper transport and homeostasis in human cells. Schematic representation of copper homeostasis and trafficking. In (a) we have highlighted the proteins (represented as ribbon) whose structures were solved by our group. Copper enters the cell via CTRs proteins and is directed to intracellular sites by copper chaperones, one specific for each transfer pathway and each final receiving protein. Copper is transported to the mitochondrial inner membrane via Cox11. Atx1 delivers copper to the trans-Golgi network where it is packed into vesicles by ATPase7A/B and bound to ceruloplasmin for excretion. CCS chaperons copper for use in Cu/Zn-SODs. The direction of the transfer is determined by the gradient in affinity for copper (b).

two cysteines and by a third ligand, which should be easily displaced by the incoming donor atoms of the receiving protein [28]. Usually the third ligand is a glutathione (GSH) molecule as in the case of ATX1-type transporters [32–35], or a histidine as in SCO1 [36] and SCO2 [37]. The direction of the transfer is determined by thermodynamic factors: the gradient in affinity for copper defines which is the donating and which is the receiving protein [38]. This drives the coordination of copper to the site of the receiving protein, eventually inducing release of the transporting protein from the complex.

A number of pathways have been characterized in detail in terms of structural properties and interaction patterns, as well as thermodynamics properties (Fig. 1). We here describe a few examples.

Maturation of SOD1

An essential process in aerobic organisms is the scavenger of reactive oxygen species (ROS), which can be highly toxic for the cells. One of the key enzymes, essential for the removal of radical species in eukaryotes, is Cu,Zn-superoxide dismutase 1 (SOD1) [39]. This enzyme is homodimeric, with each subunit binding a zinc ion and a copper ion and featuring a completely conserved, highly stable, disulfide bond [40, 41]. The zinc ion has a structural role, stabilizing a conformation suitable to receive the copper ion and preventing protein aggregation, a process easily occurring in the metal-free protein [42–44]. The copper ion catalyzes the dismutation of the superoxide radical ($O^{\bullet}_{2}{}^{-}$), which can be formed as by-product during respiration. In this dismutation process copper ions cycle between the +1 and +2 oxidation states, transforming $O^{\bullet}_{2}{}^{-}$ in H_2O_2 and in O_2, respectively, in a "ping-pong" mechanism [45, 46].

Copper ion is therefore essential for the catalytic function of this enzyme; however no free copper is available in the cell but is transferred and delivered to SOD1 by a specific copper transporting protein, i.e. a copper chaperone, CCS (copper chaperone of SOD1) [14, 47, 48]. This protein is formed by three domains, the first binds a copper (I) ion through a CXXC motif, the second recognizes the SOD subunits and the third is responsible for the SOD1 disulfide bond formation [49].

The transfer process occurs through protein-protein interactions between one zinc-loaded SOD1 domain and the second domain of CCS. In this conformation the first domain of CCS can interact with the SOD1 copper binding site and transfer its copper ion. Simultaneously, the third domain of CCS, which is unstructured and also contains two cysteines which are oxidized in the active form, receives two electrons from two cysteines of SOD1, which then form the disulfide bond important for structural stability [49].

These processes have been characterized and understood in detail through extensive work in vitro, by X-ray and NMR structural characterization and by biophysical studies, and in living human cells, through in cell NMR. The latter approach allowed us the structural characterization of proteins at atomic resolution directly in living cells [50]. From this type of characterization, we have learned that, when human cells (HEK 293T cells in this specific case) are growing without addition of zinc, SOD1 is produced in essentially only its metal-free, reduced monomeric state [51]. When zinc is added to the culture medium, SOD1 acquires a zinc ion per subunit and dimerizes. It is relevant to note that two metal binding sites (i.e. for zinc and for copper) have similar affinities for zinc and indeed in vitro both sites can be occupied by zinc ions. Zinc binding promotes protein dimerization, where however the subunits are still in the reduced state. Addition of also copper ions to the grown media induces only very partial copper binding and disulfide bond formation.

These studies are performed by slightly overexpressing the protein of interest for sensitivity reasons as well as for increasing selectivity of the labeling [52]. In these conditions the proteins partner of that under investigation are at lower than physiological level and the status of the studied protein can be altered and/or its maturation process affected.

This is the case of SOD1 which is at expression levels higher than the physiological ones of the CCS protein partner. When the latter protein is overexpressed at similar levels as those of SOD1, cells are able to uptake copper and CCS can insert copper to SOD1. Simultaneously, also the SOD1 disulfide bond is fully formed thus leading to the fully mature, stable state of SOD1 [51].

This detailed structural characterization in cells was particularly meaningful when we applied it to SOD mutants related to familial Amyotrophic lateral sclerosis (ALS) [53]. It has been proposed for a member of

familial ALS mutants that their toxicity relies on the inability to reach a mature, structurally stable form remaining essentially in a metal free form, which is prone to aggregate [54]. Through in cell NMR studies, we have learned that the behavior with respect to zinc binding is striking different in vitro with respect to what occurs in living cells. While in vitro the investigated mutants bind zinc normally and achieve a folded, stable structure, in living cells they do not acquire zinc and take a partially unstructured form which is unable to reach a folded state [53]. This unstructured form could be a precursor of the species which then aggregate.

For the correct fold of the SOD1 ALS-related mutants, CCS is needed which, when expressed at levels similar to those of SOD1, acts as molecular chaperone, guiding SOD1 mutants to the correct fold and zinc uptake [55].

Copper incorporation in CCO

A number of copper proteins is also present in mitochondria, where they need to acquire the metal ion after their import and folding [56]. One of the processes with absolute requirement for copper in order to function is respiration where, in the last step of the chain, complex IV or cytochrome C oxidase (CCO) needs three copper ions (in two subunits) for reducing molecular oxygen to water [57].

In humans, CCO is constituted by 13 subunits, with subunits 1 and 2, the most conserved in all CCOs, harboring one and two copper ions, respectively [58, 59].

As for the cytoplasmic proteins, also for these systems specific copper chaperones are required in order to acquire the functionally essential copper ions. In these CCO subunits copper is incorporated in the IMS through two sequential transfer processes. Copper (I) ions, acquired from a still unknown mechanism from the matrix, are initially bound to Cox17. The latter is a small, soluble protein with a helix-coil-helix folding where the two amphipathic helices are hold together and stabilized by two disulfide bonds [60]. The protein further contains a third cysteine pair which has a higher reduction potential so to be stabilized in the reduced state and therefore to be able to bind a copper (I) ion [60–62].

Copper-loaded Cox17 is able to interact and transfer the copper ion to both SCO1 and SCO2 and to Cox11 [63–65]. The first two proteins are responsible for delivering copper to the Cu_A site of subunit 2 of CCO [66], while Cox11 incorporate copper in subunit 1 of CCO [57]. For this latter process, no detailed characterization nor structural studies are available for eukaryotic organisms, as no lab up to now has been able to clone and produce the protein, with structure available only for bacterial proteins [67]. On the contrary, detailed structural and functional studies are available for the maturation of the Cu_A site in subunit 2. The Cu_A site binds two copper ions through two cysteine residues bridging the two copper ions. The two ions have different coordination spheres, completed by one histidine and one methionine one ion and only a histidine the other.

SCO1 and SCO2 are homologous proteins which feature a thioredoxin fold [68] and which have a copper binding site formed by two cysteines and a histidine [36, 37, 63]. Both proteins are dimeric in eukaryotic cells where they are anchored to the external mitochondrial matrix membrane thus protruding in the IMS. They both bind copper with similar affinity, higher than that of Cox17, thus determining the direction of copper transfer [38]. However, the two proteins have a quite different functional role.

In order to characterize the transfer steps, the recipient proteins in the metal-free form need to be made to interact with the copper-loaded transporting proteins. However, the eukaryotic subunits are absolutely unstable when lack the metal ions. To overcome this limitation, we produced a chimera protein where, over the scaffold of the protein from *Thermus thermophilus*, which is very stable, we engineered the copper binding sites with all the regions in their surrounding with residues of the human protein sequence [69]. The chimera protein binds two copper ions and, in its oxidized form, displays the typical spectroscopic signatures of mixed-valence CuA sites [70]. The chimera protein is absolutely selective for the human partner proteins, while lacks the ability to interact with any of the partners from *T. thermophilus* (Fig. 2). We showed that SCO1 is the copper transferring protein, incorporating two copper ions in the Cu_A site of subunit 2, while SCO2 is able, through its copper-bound ion, to reduce the cysteines of the Cu_A site in case they get oxidized [69]. This can occur during respiration because of the oxygen flow or in case of oxidative stress [71].

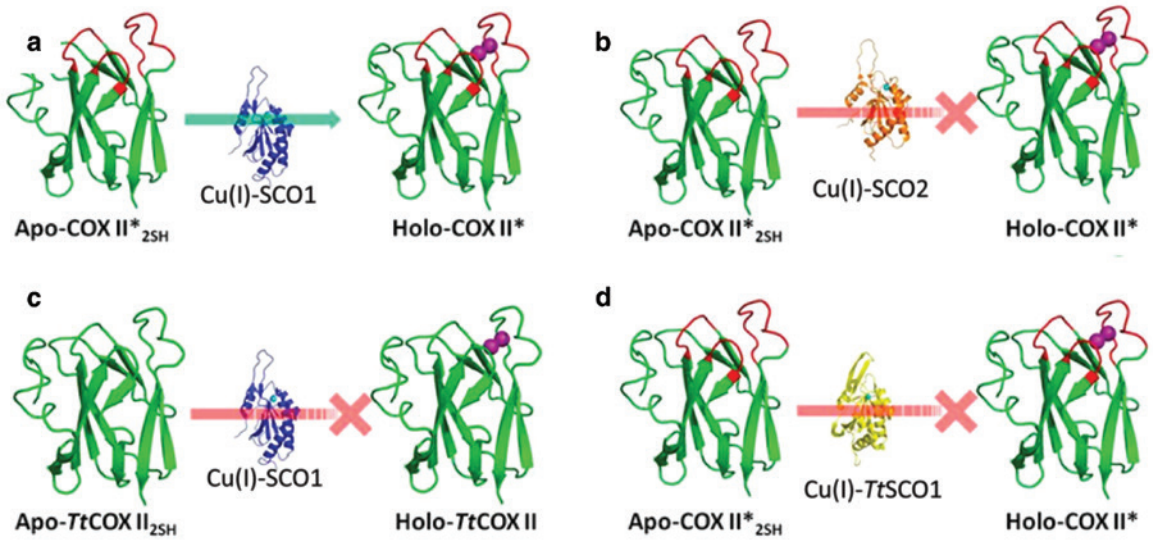

Fig. 2: Assembly of the Cu_A site of human COXII by human SCO1. A chimeric COX II* was obtained by engineering the copper binding loop of the human protein (represented in red in the model) over a scaffold of the *T. thermophilus* oxidase (represented in green), and its ability to receive Cu(I) ions from human SCO1 and SCO2 proteins was investigated by NMR spectroscopy. Green arrow indicates the stoichiometric transfer of two Cu(I) ions from SCO1 to COX II* (a), and the red lines indicate unsuccessful Cu(I) transfer assays. Cu(I) transfer does not occur from human Cu(I)-SCO2 to COX II* (b). Transfer from Cu(I)-SCO1 to *T. thermophilus* COX II (c) nor *T. thermophilus* Cu(I)-SCO1 to the chimeric apo COX II* (d) does not occur, indicating complete selectivity from molecular recognition [67].

Copper import to Golgi through two Cu-ATPases

The Golgi organelle requires copper in order to mature a number of copper enzymes. Its membrane harbors two P-type ATPases which are responsible for copper import as well as its excretion, ATPase7A and ATPase7B. They are also called Menkes and Wilson proteins from the diseases originating from ATPase7A and 7B mutations which determine lack or overload of copper, respectively.

Both are integral membrane proteins, having eight transmembrane helices and six soluble domains each of them able to bind a copper (I) ion. The latter is coordinated by two cysteines in the "classical" CXXC binding motif with the third position occupied by a GSH molecule. These domains have similar, even if not identical, affinity for copper and only some of them can be copper loaded. They receive the copper ion from the soluble cytosolic protein ATX1 (or HAH1 in humans) which shares the same fold (i.e. the immunoglobulin fold) as that of each individually folded domain and the same copper binding, CXXC motif.

In mammalian cells copper import is mainly performed by the high affinity copper transporter CTR1. However, alternative routes for copper loading have been observed in cells lacking CTR1 [72]. Once ATX1 has acquired the copper ion, it is able to interact with the ATPases metal binding domains through a copper-mediated protein-protein interaction. Copper ion in ATX1 is coordinated, in addition to the two cysteines of the conserved motif, by another ligand, most likely a GSH molecule.

ATX1 interacts with one or more of the copper binding domains of ATPase7A or 7B, through a well defined surface which specifically recognizes the partner proteins [73]. Indeed, when copper ATX1 is presented to other ATPases which binds different metal ions, such as those binding zinc ion, it is unable to transfer the metal cargo as there is no interaction between the two proteins [74]. Mutations on the surface of the receiving protein in such a way to optimize the recognition of ATX1 make the copper transfer possible even to sites which require a different metal ion [75].

In the ATX1-ATPases protein complexes, the copper transfer occurs by displacing the exogenous ligand from the transporting protein and the copper coordination by the ligands of the receiving protein. The higher affinity of the latter drives the transfer of the metal ion and the subsequent release of transporter [28].

Iron-sulfur cluster biogenesis and trafficking in human cells

Iron, as well as copper, is an essential metal ion for eukaryotic organisms, the iron proteome being larger than the copper one [76–78]. Iron is physiologically present either as individual metal ion, or as part of cofactors, such as hemes and iron-sulfur (Fe-S) clusters. The last are ancient and versatile inorganic cofactors, which are present in all kingdoms of life, and which are involved in a variety of essential life processes, thanks to their structural and chemical versatility. Common Fe-S clusters are the rhomboid [2Fe-2S], the cuboidal [3Fe-4S], and the cubane [4Fe-4S] clusters, all containing iron ions ($Fe^{2+/3+}$) and inorganic sulfide (S^{2-}) (Fig. 3) [79]. More complex clusters, harboring also other metal ions, such as molybdenum, vanadium and nickel, have been found in some enzymes, such as hydrogenase and nitrogenase [79]. The binding of the Fe-S clusters to the polypeptide chain occurs, in the vast majority of cases, through the coordination of the $Fe^{2+/3+}$ ions, by thiol groups provided by cysteine residues and/or by the imidazolic nitrogen of histidine residues. However, also other donor groups, provided by aspartic acid, serine, and arginine residues, or CO, CN^-, and GSH molecules, can act as iron ligands. In eukaryotes, Fe-S proteins are present in the mitochondrion (either inside or associated with the outer mitochondrial membrane), in the endoplasmic reticulum, in the cytosol, and in the nucleus and perform a multiplicity of functions, participating in several metabolic reactions, in electron transport, in DNA repair and in gene expression regulation, acting as sensors for environmental or intracellular conditions [80, 81].

A common function of Fe-S clusters is electron transfer, which is based on the propensity of iron to switch between +2 and +3 oxidation states. Redox potentials of Fe-S clusters range from –500 mV to +300 mV, depending upon the proteinaceous surrounding [82–86], thus enabling Fe-S clusters to act both as electron donors and as acceptors in a variety of biological reactions [79]. Electron transfer reactions are often performed by systems where several Fe-S clusters are placed close to each other forming a "chain" through which electrons flow toward the final target(s). An example of electron transfer chain is the mitochondrial respiratory complexes I, which contains eight Fe-S clusters [87].

A second important function of Fe-S proteins is enzymatic catalysis, based on the ability of the Fe-S clusters to directly bind the substrate, to act as a Lewis acid and to transfer sulfur atoms [79, 88]. An example of a Fe-S enzyme is the citric acid cycle enzyme aconitase, which belongs to the dehydratases family. The solvent exposed [4Fe-4S] cluster in aconitase directly participates in the binding of citrate (the substrate), and acts as a Lewis acid in the conversion of citrate to isocitrate, through the extraction of a molecule of H_2O. A second family of important Fe-S enzymes is represented by S-adenosyl-L-methionine (SAM)-dependent enzymes, such as lipoic acid synthase (LIAS), responsible for the synthesis of lipoate [89], and biotin synthase, which is responsible for biotin synthesis in lower organisms [90], each of the two enzymes binding two [4Fe-4S] clusters, one of which acts as a sulfur donor.

As mentioned, Fe-S proteins are involved also in gene expression regulation [91]. The cytosolic bifunctional enzyme iron regulatory protein 1 (IRP1), also known as cytosolic aconitase (ACO1), for example, plays

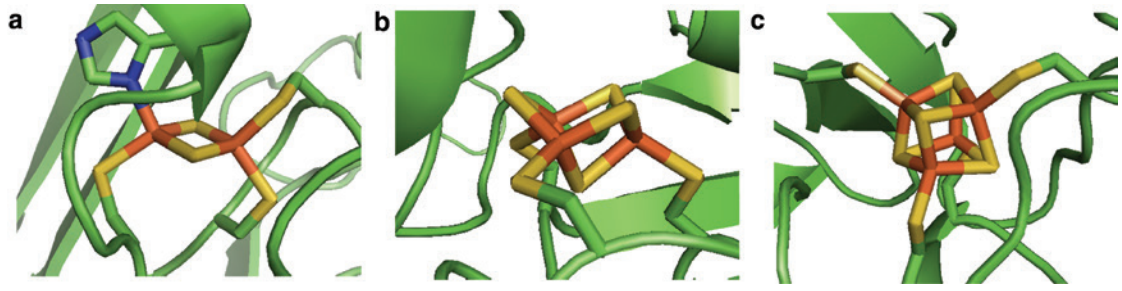

Fig. 3: Most common types of Fe-S clusters and their ligands found in human proteins. Schematic representation of (a) rhomboid [2Fe-2S] cluster, coordinated by three cysteines and one histidine as in NEET proteins (PDB code 3EW0), (b) cuboidal [3Fe-4S] coordinated by three cysteines (PDB code 4AAY), and (c) cubane [4Fe-4S] cluster coordinated by four cysteines (PDB code 1HRQ). Sulfur, iron and nitrogen atoms colored in yellow, orange and blue, respectively.

an essential role in the regulation of cytosolic iron homeostasis. Driven by environmental or intracellular conditions, such enzyme is able to switch between an holo state, where IRP1 binds a [4Fe-4S] cluster and acts as an aconitase (this occurring under iron-replete conditions), and an apo state, where the protein, once lost its labile cluster (this occurring under iron deprivation) is able to specifically bind to iron-responsive elements (IREs) in mRNAs of proteins involved in iron uptake, storage and distribution in the cell [92, 93].

The biogenesis of Fe-S clusters in vivo is a highly conserved, multistep process, based on a sequence of protein-protein interactions and conformational changes, involving several dedicated multimeric complexes, which ensure that the Fe-S clusters, which might be redox-sensitive and labile, are correctly assembled and then delivered to specific target apoproteins, remaining protected during these processes. The pathways have been extensively investigated in bacteria, plants, yeast and mammals [94–96]. Although many of the steps are common to all kingdoms of life, in eukaryotes the overall process appears to be more complex, due to the distribution of Fe-S proteins in distinct subcellular compartments, such as mitochondria, plastids, cytosol and nuclei [97, 98].

In eukaryotes two distinct machineries define two different pathways for the maturation of Fe-S proteins in mitochondria and in cytosol, respectively. The mitochondrial iron-sulfur cluster (ISC) assembly machinery synthesizes de novo, and then incorporates, the Fe-S clusters into the correct mitochondrial proteins [99], whereas the maturation of cytosolic and nuclear Fe-S proteins relies on the cytosolic iron-sulfur assembly (CIA) machinery [100], which, however, to be fully functional requires the ISC machinery to be efficiently working [101].

Human mitochondrial ISC assembly machinery

The ISC-mediated assembly of Fe-S clusters was first described in bacteria [95], where a single operon contains all the genes encoding for the proteins involved in Fe-S clusters assembly and trafficking. In *Azotobacter vinelandii* and *Escherichia coli*, the Isc operon encodes for seven proteins, the regulator IscR, the pyridoxal-5′-phosphate-dependent cysteine desulfurase IscS, the scaffold protein IscU, the A-type carrier protein IscA, the DnaK-like chaperone HscA and the DnaJ-like co-chaperone HscB, and finally the electron donor ferredoxin [88]. In eukaryotes the ISC machinery, which shares many similarities with the bacterial system, is localized in mitochondria. In addition to their function in Fe-S proteins maturation, is also involved in the regulation of cellular iron homeostasis [102].

In human mitochondria, the number of [2Fe-2S] cluster proteins is very close to that of [4Fe-4S] cluster proteins [76]. In both cases, maturation of mitochondrial Fe-S proteins starts with the de novo assembly of a [2Fe-2S] cluster on the scaffold protein ISCU2 (Fig. 4). This assembly requires ferrous ions (Fe^{2+}), which are imported into the mitochondria by specific intermembrane transporters, and sulfide ions (S^{2-}). The latter are produced by the conversion of cysteines into alanines. Such process is catalyzed by the mitochondrial pyridoxal-5′-phosphate-dependent cysteine desulfurase NFS1, which transiently binds the released sulfur atom on a conserved cysteine residue, by forming a persulfide intermediate [103, 104]. NFS1 activity relies on the formation of a heterocomplex with the protein ISD11 (also known as LYRM4), which prevents NFS1 self-aggregation [105–107], and the mitochondrial acyl carrier protein (ACP), which interacts with ISD11 [108, 109]. Although not necessary for the NFS1 activity, human frataxin (FXN) enhances NFS1 desulfurase activity and favors persulfide formation, upon interaction with the NFS1-ISD11-ACP complex [110, 111]. Such complex successively interacts with the scaffold protein ISCU2 and the persulfide group is transferred from NFS1 to ISCU2, where the [2Fe-2S] cluster is finally assembled [112, 113].

In addition to Fe^{2+} and S^{2-}, the assembly of the [2Fe-2S] cluster requires electrons. Using yeast as a model system, we showed that electrons are supplied by a mitochondrial electron transfer chain formed by ferredoxin reductase (FDXR) and [2Fe-2S]-ferredoxin ([2Fe-2S]-FDX) [114], which was then shown to act with the same mechanism also in humans [109]. In this system electrons are transferred from NADPH to FDXR, and then to [2Fe-2S]-FDX. Human mitochondria possess two ferredoxins, FDX1 and FDX2, which can interact directly with the NFS1-ISD11-ACP complex, in both their oxidized and reduced states [109]. However, FDX2 is more efficient in providing the two electrons to form the [2Fe-2S] cluster on ISCU2 [109, 115].

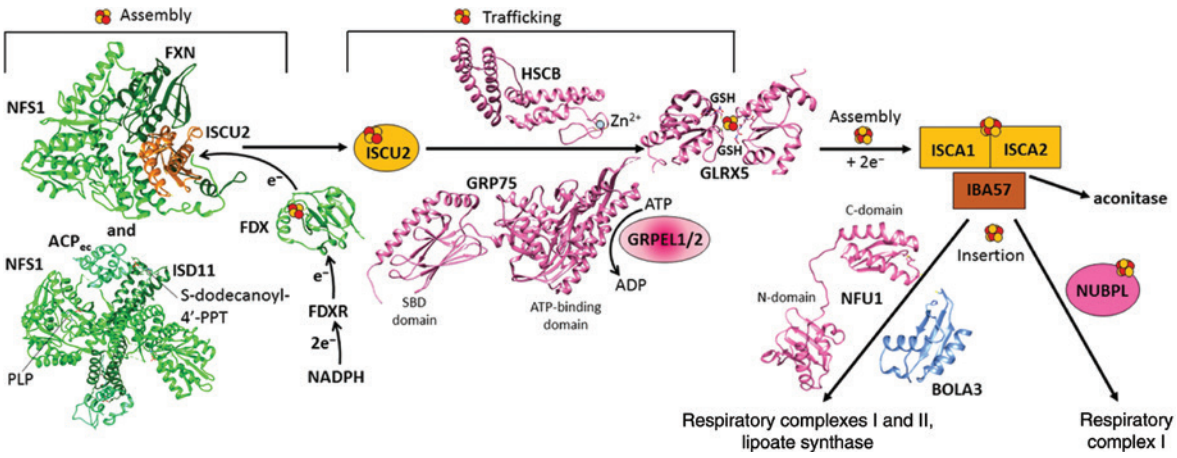

Fig. 4: Scheme of the human mitochondrial Fe-S protein assembly pathway. The [2Fe-2S] cluster is de novo assembled on the scaffold complex composed of NFS1, ISD11, ACP, FXN and ISCU2. The process requires electrons, which are provided by the electron transfer chain NADPH-FDXR-FDX. The cluster is then transferred from ISCU2 to GLRX5, with the assistance of chaperons. Finally, two [2Fe-2S] clusters are donated by GLRX5 and converted into a [4Fe-4S] cluster on the ISCA1-ISCA2 complex, which can be transferred to the final targets, with the assistance of other proteins such as NUBPL and NFU1. Protein color code: yellow, scaffold proteins; green, proteins involved in electron transfer and sulfide production; magenta, proteins involved in Fe-S cluster trafficking and insertion/assembly processes; brown, protein with unknown function [92]. Adapted by permission of The Royal Society of Chemistry.

In the current working model, the de novo assembled [2Fe-2S] cluster is transferred from ISCU2 to the mitochondrial monothiol glutaredoxin GLRX5 (Fig. 4). The process is mediated by the formation of a complex between ISCU2 and the ATP-binding chaperone GRP75, its co-chaperone HSCB and the nucleotide exchange factors GRPEL1/2 [116]. The formation of such complex starts with the interaction of [2Fe-2S]-ISCU2 with the co-chaperone HSCB, which induces a conformational change on ISCA2 favoring its interaction with GRP75 [117, 118]. At the same time GRPEL1/2 induces ADP dissociation from GRP75 [119].

GLRX5 has been proposed to act as a mitochondrial metallochaperone, delivering [2Fe-2S] clusters to specific mitochondrial targeting proteins and to intermediate scaffold proteins, responsible for the maturation of mitochondrial [4Fe-4S] cluster-binding proteins [120]. Through a detailed in vitro NMR characterization, we have shown that human GLRX5 is monomeric in solution and can bind a [2Fe-2S] cluster upon dimerization. The $[2Fe\text{-}2S]^{2+}$ cluster is coordinated by a cysteine residue contained in a conserved CGFS motif of each GLRX5 monomer, and by the thiol moieties of two GSH molecules [121]. We also found that GSH in solution can bind to the [2Fe-2S] cluster of GLRX5 in two different modes, generating two distinct [2Fe-2S]-GLRX5 species in equilibrium with each other [121]. Among them, only one can efficiently transfer the [2Fe-2S] cluster to two A-type Fe-S assembly proteins ISCA1 and ISCA2 [120, 121]. We have characterized at a molecular level the [2Fe-2S] cluster transfer from GRX5 to both ISCA1 and ISCA2, and we showed that it occurs through a specific protein-protein recognition mechanism, which suggests that the structural plasticity of the dimeric state of [2Fe-2S]-GLRX5 is crucial for its activity [121]. We found that human ISCA1 is present in solution as an equilibrium between a monomer and a homodimer, while human ISCA2 is homodimeric [120]. Both ISCAs contain three conserved cysteine residues in a CX_nCGC sequence motif. The two cysteines located in the unstructured and flexible C-terminal tail of the ISCA proteins are directly involved in the extraction of the [2Fe-2S] clusters from GLRX5, while the third conserved cysteine plays a role in promoting the extraction of the second [2Fe-2S] cluster from another GLRX5 molecule, which is then reductively coupled to the first one, to form a [4Fe-4S] cluster [120]. Although we have shown that both ISCA1 and ISCA2 can receive a [2Fe-2S] cluster from [2Fe-2S]-GLRX5 in vitro, the generation of [4Fe-4S] clusters needs the interaction between ISCA1 and ISCA2, and the formation of a heterodimeric complex [115, 120]. We have recently showed that free copper (I) can compromise the mitochondrial [4Fe-4S] cluster assembly process both by occupying the Fe-S cluster binding site of

ISCA1/2 proteins and GLRX5, and by displacing the Fe-S cluster bound either to GLRX5 or to ISCA1/2 proteins [122]. This event is likely overcome in vivo through a tight regulation of the mitochondrial cellular concentration of available copper (I), and of the copper trafficking and [4Fe-4S] cluster assembly pathways.

A third protein, the iron-sulfur cluster assembly factor for biotin synthase- and aconitase-like mitochondrial proteins, named IBA57, has been proposed to have a role in the mitochondrial [4Fe-4S] clusters assembly [115, 123], and has been shown to interact in vivo with ISCA2, but its function is still poorly understood.

The [4Fe-4S] cluster assembled on the ISCA1-ISCA2 scaffold complex is then inserted into mitochondrial targets. In some cases, this process is mediated by other ISC components, which can bind the [4Fe-4S] cluster and facilitate its insertion into specific [4Fe-4S] cluster proteins. NUBPL and NFU1 are two well-known ISC targeting factors [124–126]. NUBPL acts in the maturation of the human respiratory complex I [124], whereas NFU1 is involved in the maturation of the subunits of respiratory complexes I and II and of the lipoic acid synthase LIAS [126, 127].

Our work recently allowed us to classify as ISC machinery components two further human proteins, i.e. BOLA1 and BOLA3, based on their ability to interact with human GLRX5 [128]. BOLA1 and BOLA3 belong to the highly conserved BOLA proteins family. Their yeast orthologs function as ISC assembly factors, facilitating the insertion of [4Fe-4S] clusters into specific mitochondrial targets, such as lipoate synthase and succinate dehydrogenase [128, 129]. In humans, the functional role of BOLA1 and BOLA3 proteins is still unclear, but we have carefully characterized their interaction with GLRX5 [128, 130]. We showed that both BOLA1 and BOLA3 can form a heterodimeric complex with GLRX5 in vitro in both apo and [2Fe-2S]-cluster bound states. BOLA1-GLRX5 complex coordinates a reduced, Rieske-type $[2Fe\text{-}2S]^{+}$ cluster, while BOLA3-GLRX5 complex binds, with a lower affinity than the BOLA1-GLRX5 complex, an oxidized, ferredoxin-like $[2Fe\text{-}2S]^{2+}$ cluster [130]. Moreover, the solution structures of BOLA1 and BOLA3 showed that they have a similar fold, but local structural differences in the regions containing conserved sequence patterns, comprising cluster ligands [130]. The different structural and redox properties observed for the two [2Fe-2S] BOLAs-GRX5 complexes as well as their different stability suggested that they could have a diverse molecular function.

Cytosolic and nuclear Fe-S proteins maturation: the CIA machinery

A sequence-based bioinformatic analysis of the human genome pointed out that, in cytosol, the [4Fe-4S] clusters binding proteins are almost twice those that bind [2Fe-2S] clusters, and that nuclear Fe-S proteins bind almost exclusively [4Fe-4S] clusters, with a ratio of [4Fe-4S]/[2Fe-2S] cluster-binding proteins nearly 5.0:1.4 [76].

In the human CIA machinery, 13 dedicated, highly conserved proteins are responsible for the synthesis, trafficking, and insertion of clusters into cytosolic and nuclear Fe-S proteins (Fig. 5) [76, 131, 132].

[4Fe-4S] clusters are assembled de novo by the two P-loop NTPase proteins NUBP1 and NUBP2 (orthologues of yeast NBP35 and CFD1), localized in the cytosol and in the nucleus. Co-immunoprecipitation experiments showed that the two proteins interact in vivo, by forming an heterocomplex, and likely cooperate in the same molecular process of the CIA machinery [133]. Both NUBP1 and NUBP2 have four cysteine residues at the C termini, in the $Cx_{18}Cx_2Cx_2C$ motif in NUBP2 which is only partially conserved in the NUBP1 family. In addition to the C-terminal cysteine rich motif, NUBP1 also displays a conserved $Cx_{13}Cx_2Cx_5C$ motif at the N terminus. The latter motif tightly binds a [4Fe-4S] cluster in NUBP1 and in its yeast homolog NBP35, while the C-terminal cysteine-rich motif binds a labile [4Fe-4S] cluster in vitro and in vivo [134, 135]. The yeast heterocomplex might perform a scaffold function in the early stage of the CIA machinery, by assembling a [4Fe-4S] cluster on the C-terminal motif [136, 137], with a still elusive mechanism, and the same function for the NUBP1-NUBP2 complex is likely conserved also in humans. Indeed, depletion of NUBP1 in HeLa cells dramatically reduced the efficiency of cells in assembling cytosolic and nuclear Fe-S proteins [133].

Assembly of the [4Fe-4S] cluster on the NUBP1-NUBP2 heterocomplex requires Fe^{2+} and sulfide ions, and electrons. In the current working model, an X-S unknown compound, containing inorganic sulfur, produced

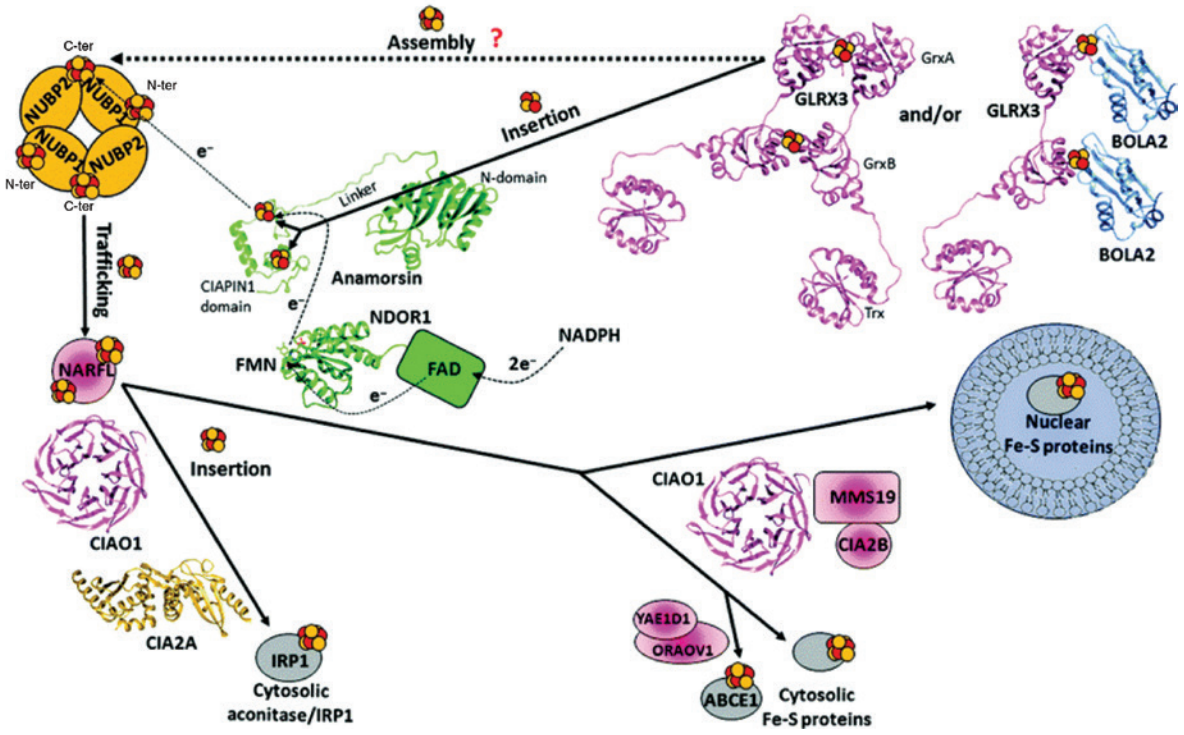

Fig. 5: Scheme of the human cytosolic Fe-S protein assembly pathway. In the early stage of the CIA machinery a [4Fe-4S] cluster is assembled on the tetrameric NUBP1-NUBP2 complex, likely with a reductive coupling of two [2Fe-2S] clusters. The two electrons necessary for the process are likely provided by the NADPH-NDOR1-anamorsin electron transfer chain. Anamorsin, in turn, receives its active [2Fe-2S] cluster by the holo forms of the GLRX3 and/or GLRX3-BOLA2 complex. The de novo assembled [4Fe-4S] cluster is transferred to NARFL, for its subsequent delivering to cytosolic and nuclear target proteins. The latter process is assisted by the CIA targeting complex, formed by CIAO1-MMS19-CIA2B. Protein color code: yellow, scaffold proteins; green, proteins involved in electron transfer and sulfide production; magenta, proteins involved in Fe-S cluster trafficking and insertion/assembly processes; brown, protein with unknown function [92]. Reproduced by permission of The Royal Society of Chemistry.

in mitochondria, is exported to cytosol through a specific trans-membrane ISC export machinery. Electrons are likely supplied to the NUBP1-NUBP2 complex by the NADPH-NDOR1-anamorsin CIA electron transfer chain, similarly to the mitochondrial NADPH-FDX-FDXR electron transfer chain [114, 138]. Indeed, it was shown in plants the occurrence of a specific, although weak, interaction between anamorsin and NUBP1 homologous [138]. In the NADPH-NDOR1-anamorsin chain, a well characterized protein-protein complex is formed between diflavin reductase NDOR1 and anamorsin. The former protein has two domains, one binding FMN, with the folding typically found in FMN-binding domains of diflavin reductases [139], and the second one binding FAD and NADPH. Anamorsin has two domains as well, a N-terminal domain, with a well-defined structure, resembling a typical *S*-adenosylmethionine (SAM)-dependent methyltransferase fold [140, 141], but with no SAM cofactor binding properties, and a largely unstructured C-terminal cytokine-induced apoptosis inhibitor 1 (CIAPIN1) domain [141], which contains two highly conserved CX_8CX_2CXC and $CX_2CX_7CX_2C$ motifs. The first motif in vitro binds a [2Fe-2S] cluster and the second motif can bind either a [2Fe-2S] or a [4Fe-4S] cluster, depending on the preparation of the protein sample [142–144]. A flexible linker of 51 residues connects the two anamorsin domains and directly and specifically interacts with the FMN-binding domain of NDOR1, leading to the formation of a stable complex between the two proteins, both in vitro [139] and in vivo [145]. In this electron transfer chain, electrons are transferred within NDOR1 from NADPH to FAD, and then to FMN [146]. By using NMR spectroscopy we have characterized the last steps of the electron transfer process, describing in details how one electron is selectively transferred from the hydroquinone state of the FMN of NDOR1 to the oxidized $[2Fe\text{-}2S]^{2+}$ cluster bound to the first motif of anamorsin, and then from $[2Fe\text{-}2S]^{+}$-anamorsin to possible final targets [142, 147].

The maturation of anamorsin depends on a third early stage CIA component, the cytosolic monothiol glutaredoxin 3 (GLRX3) [148–150], which can act as a [2Fe-2S] cluster trafficking protein in cytosol, in analogy to GLRX5 in mitochondria [96]. Human GLRX3 consists of an N-terminal thioredoxin (Trx) domain and two monothiol glutaredoxin (Grx) domains, the last two containing each a CGFS motif [151], which enable the protein to bind two [2Fe-2S] clusters upon dimerization [152, 153]. In addition to homodimerization, GLRX3 forms a heterocomplex with the cytosolic protein BOLA2 [149, 150, 152]. A detailed, atomic level characterization allowed us to show that each Grx domain of GLRX3 binds a BOLA2 molecule, forming a heterotrimeric complex in vitro [149]. This complex can bind two $[2Fe\text{-}2S]^{2+}$ clusters, each bridged between BOLA2 and a Grx domain of GLRX3, which are more stable to oxidation than the bridged clusters in the GLRX3 homodimer [152, 154].

Moreover, we have shown how, in vitro, the maturation of anamorsin occurs via the formation of a specific protein-protein complex between the Trx domain of GLRX3 and the N-terminal domain of anamorsin. Such interaction is essential to enable the transfer of two [2Fe-2S] clusters from either GLRX3 or GLRX3-BOLA2 complex to the two cluster-binding motifs of CIAPIN1 domain of anamorsin [148, 149]. This mechanism was confirmed by in vivo studies which showed that maturation of anamorsin occurs through the formation of a complex between [2Fe-2S] GLRX3-BOLA2 and the anamorsin-NDOR1 complex [150].

In conclusion, GLRX3, both as a homodimer and as a heterotrimeric complex with BOLA2, might function as a [2Fe-2S] chaperone in the cytosol, inserting $[2Fe\text{-}2S]^{2+}$ clusters into cytosolic [2Fe-2S] target proteins, such as Anamorsin, or into the NUBP1-NUBP2 heterotetrameric complex to assemble a $[4Fe\text{-}4S]^{2+}$ cluster [96].

Once the labile [4Fe-4S] cluster is assembled on the NUBP1-NUBP2 scaffold complex, it needs to be transferred to cytosolic target proteins. Several CIA machinery components are involved in this late stage of Fe-S cluster protein biogenesis. The principal components of this phase are the ternary CIAO1-CIA2B-MMS19 targeting complex, and the NARFL protein (also named IOP1, Iron-Only hydrogenase-like Protein) [155–157]. The first complex is responsible for the maturation of most of the nuclear and cytosolic Fe-S proteins [100]. In such complex, MMS19 directly interacts with CIA2B, preventing its proteosomal degradation, and CIA2B mediates, in turn, the interaction between MMS19 and CIAO1 [157]. NARFL protein interacts both with the ternary complex [158] and with NUBP2 [159]. Interaction studies have shown that NARFL also interacts with CIA2A protein, another CIA machinery component, homologous to CIA2B, that, as CIA2B does, can interact with CIAO1 [160, 161]. Very recently we have characterized in vitro the interaction between CIA2A and CIAO1, which leads to the formation of a heterotrimeric [4Fe-4S] cluster-loaded $CIA2A_2$-CIAO1 complex, which can transfer the [4Fe-4S] cluster to IRP1, thus producing the active form of the enzyme, i.e. aconitase [161].

Finally, the CIAO1-CIA2B-MMS19 CIA targeting complex interacts with the complex formed by two recently identified CIA machinery components, ORAOV1 and YAE1D1 [162]. The ORAOV1-YAE1D1 complex acts as an adaptor, connecting the CIAO1-CIA2B-MMS19 CIA targeting complex with a specific [4Fe-4S] target protein, a cytosolic ABCE1 protein [162, 163].

Conclusions

In this mini-review we have presented and summarized some of the key cellular processes for metal trafficking and metal cofactor biogenesis. Metal cofactors, such as copper ions and Fe-S clusters, are required in a number of essential metal-binding proteins involved in many enzymatic reactions and physiological processes. Perturbations of the process of metals homeostasis or of their trafficking often lead to pathological states. The essential role of metal cofactors and the absolute importance of their functions have made them a high impact topic of many studies in recent years. In particular, atomic resolution investigation of the trafficking processes of copper both in vitro and in living cells and of the Fe-S clusters assembly and insertion processes into mitochondrial and cytosolic proteins in human cells, has greatly advanced our knowledge in the copper and Fe-S clusters cofactors biology. We have here discussed some of our latest results, framing them within the current knowledge as reported in literature. We therefore think that this mini-review provides a general, complete picture of these processes.

Acknowledgements: A large part of our work here presented and reviewed has been supported by Instruct-ERIC, a Landmark ESFRI Research Infrastructure, and by iNEXT and by other projects, previously funded by EC.

Article note: A special collection of invited papers by recipients of the IUPAC Distinguished Women in Chemistry and Chemical Engineering Awards.

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