Function and biogenesis of iron–sulphur proteins

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Iron–sulphur (Fe–S) clusters have long been recognized as essential and versatile cofactors of proteins involved in catalysis, electron transport and sensing of ambient conditions. Despite the relative simplicity of Fe–S clusters in terms of structure and composition, their synthesis and assembly into apoproteins is a highly complex and coordinated process in living cells. Different biogenesis machineries in both bacteria and eukaryotes have been discovered that assist Fe–S-protein maturation according to uniform biosynthetic principles. The importance of Fe–S proteins for life is documented by an increasing number of diseases linked to these components and their biogenesis.

Many proteins depend on cofactors for their function. Typically, these low-molecular-mass compounds bind to well-defined and evolutionarily conserved sequence motifs either covalently or non-covalently. Organic cofactors include various nucleotides (such as flavin mononucleotide and flavin adenine dinucleotide), vitamins (biotin, pantothenate and folate) and metal–organic compounds (haem and molybdenum cofactor). Common inorganic cofactors include various metal ions (Mg^{2+}, Zn^{2+}, Mn^{2+}, $Cu^{1+/2+}$, $Fe^{2+/3+}$ and so on), which usually bind to mononuclear binding sites. The oldest and most versatile inorganic cofactors are probably Fe–S clusters, which can participate in electron transfer, catalysis and regulatory processes[1]. The chemically simplest Fe–S clusters are the rhombic [2Fe–2S] and the cubane [4Fe–4S] types, which contain iron ($Fe^{2+/3+}$) and sulphide (S^{2-}). Fe–S clusters are usually integrated into proteins through coordination of the iron ions by cysteine or histidine residues, yet alternative ligands (Asp, Arg, Ser, CO, CN^- and so on) are known, particularly in more complex Fe–S clusters. Several different structural folds have been recognized to coordinate these simple Fe–S clusters[2]. Nevertheless, it has remained difficult to predict the presence of Fe–S clusters from protein sequences. More complex Fe–S clusters, often containing other metal ions, are present in, for example, nitrogenase or hydrogenase enzymes, but are not discussed here (see pages 814 and 839).

Fe–S clusters were discovered in the early 1960s by purifying enzymes with characteristic electron paramagnetic resonance signals. Some of the first Fe–S proteins to be discovered include plant and bacterial ferredoxins and respiratory complexes I–III of bacteria and mitochondria. In the late 1960s, chemists and biochemists devised chemical reconstitution protocols to assemble Fe–S clusters into apoproteins *in vitro*, leading to the view that these cofactors can assemble spontaneously on proteins[3]. However, genetic, biochemical and cell-biological studies in the 1990s provided ample evidence that the maturation of Fe–S proteins in living cells is a catalysed process rather than a spontaneous one. In striking contrast to the chemical simplicity of Fe–S clusters, their biosynthesis *in vivo* appears to be a rather complex and coordinated reaction[4–13]. In the past decade, numerous biogenesis components were identified, and the first insights into the mechanisms of biogenesis were obtained. In this Review, I summarize our current knowledge on the biogenesis of Fe–S proteins in bacteria and eukaryotes, and emphasize the importance of Fe–S proteins for essential physiological pathways in living cells. First, I briefly address the properties of various Fe–S clusters and the biological functions of Fe–S proteins.

Functions of Fe–S proteins depend on cofactor properties

The most common function of Fe–S clusters, electron transfer, is based on the propensity of Fe to formally switch between oxidative states +2 and +3 (ref. 1). Within a given proteinaceous surrounding, Fe–S clusters can adopt redox potentials from −500 mV to +300 mV (ref. 2). Thus, Fe–S clusters can serve as excellent donors and acceptors of electrons in a variety of biological reactions. Examples are bacterial and mitochondrial respiratory complexes I–III, photosystem I, ferredoxins and hydrogenases. Another well-studied function of Fe–S clusters is in enzyme catalysis, a classic example being aconitase, in which a non-protein-coordinated Fe at one edge of a [4Fe–4S] cluster serves as a Lewis acid to assist H_2O abstraction from citrate (the substrate), which is converted to isocitrate. Other special cases are the radical *S*-adenosyl-L-methionine (SAM) enzymes biotin synthase and lipoate synthase, which bind two Fe–S clusters each. It is believed that one of these clusters is disassembled during the formation of the products biotin and lipoic acid, respectively, thus serving as a sulphur donor[14]. Numerous other catalytic functions are known for bacterial and eukaryotic Fe–S enzymes involved in metabolism[2]. However, in many cases the precise role of the Fe–S cluster is still unclear, and it is therefore possible that in some proteins the Fe–S cluster simply plays a structural role. This may be true for the recently discovered Fe–S clusters in adenosine triphosphate (ATP)-dependent DNA helicases involved in nucleotide excision repair (Rad3, XPD (also known as ERCC2), FANCJ (BRIP1) and so on)[15]. Likewise, the precise role of the two [4Fe–4S] clusters in the ABC protein Rli1 (ABCE1 in humans) involved in ribosome biogenesis and function is unknown, despite the availability of a crystal structure of the holoprotein[16,17].

A third general role of Fe–S clusters is in sensing environmental or intracellular conditions to regulate gene expression (see page 823). Examples are the bacterial transcription factors FNR, IscR and SoxR, which sense O_2, Fe–S clusters and superoxide/NO, respectively. The switch between the activating and repressed states depends on the presence or absence of an Fe–S cluster (FNR or IscR) or the redox state of the [2Fe–2S] cluster present in SoxR[18]. A classical example for post-transcriptional regulation of gene expression by Fe–S clusters is provided by the mammalian

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cytosolic iron regulatory protein 1 (IRP1, also known as ACO1). Under iron-replete conditions, IRP1 holds a [4Fe–4S] cluster and functions as an aconitase. When the protein loses its labile cluster under iron deprivation, it can bind to stem-loop structures (termed iron-responsive elements (IREs)) in certain messenger RNAs of proteins involved in iron uptake, storage and distribution in the cell[19–22]. Binding of apo-IRP1 to 5′-located IREs blocks translation by inhibiting ribosome scanning to the start AUG codon, whereas association with 3′-located IREs protects mRNAs from nucleolytic degradation, leading to increased translation.

Dedicated machineries for maturation of Fe–S proteins

The maturation of bacterial Fe–S proteins has been intensely studied in *Escherichia coli* and the azototrophic (nitrogen-fixing) *Azotobacter vinelandii*. Studies have identified three different systems for the biogenesis of bacterial Fe–S proteins: the NIF system, for specific maturation of nitrogenase in azototrophic bacteria; and the ISC assembly and SUF systems, for the generation of housekeeping Fe–S proteins under normal and oxidative-stress conditions, respectively[4,9,12,13]. During evolution, the latter two machineries were transferred by endosymbiosis to eukaryotes containing Fe–S proteins in mitochondria, cytosol and the nucleus. Mitochondria have retained components homologous to those of the bacterial ISC assembly system[6,8], whereas plastids host proteins of the SUF machinery[5,11]. Assembly of Fe–S proteins in the eukaryotic cytosol and nuclei requires the assistance of both the mitochondrial ISC assembly machinery and a mitochondrial ISC export system[23]. Additionally, maturation of these Fe–S proteins is dependent on the essential cytosolic Fe–S-protein assembly (CIA) machinery, which is present in virtually all eukaryotes[6,8]. The yeast *Saccharomyces cerevisiae* has served as an excellent model organism with which to establish the first details of the complex biosynthesis pathways in eukaryotes. Recent investigations in human cell culture and other model systems have demonstrated that the entire process is highly conserved from yeast to human (see below).

Despite the obvious differences among the various biogenesis systems found in bacteria and in eukaryotes, general biosynthetic principles seem to underlie the *in vivo* synthesis of Fe–S clusters and their assembly into apoproteins (explained in detail in Box 1). In the following, I briefly summarize our current knowledge of the components of the various assembly systems and describe their respective mechanistic functions. The special assembly pathways of nitrogenases, used by the NIF system, and hydrogenases, used by dedicated maturation systems, will not be addressed here. The reader is referred to recent reviews[24–26].

The ISC assembly systems in bacteria and mitochondria

The experimental study of Fe–S-protein biogenesis was boosted by the identification of the bacterial *isc* operon[27]. This discovery not only aided work on bacterial Fe–S-protein assembly, but also influenced the first attempts to identify biogenesis proteins in eukaryotes. The evolutionary relationship between bacteria and mitochondria led to the identification and functional characterization of several mitochondrial proteins homologous to the bacterial ISC system[23,28]. The striking similarities between the bacterial and mitochondrial ISC components and the underlying assembly mechanisms justify a comparative discussion of these related systems (Table 1).

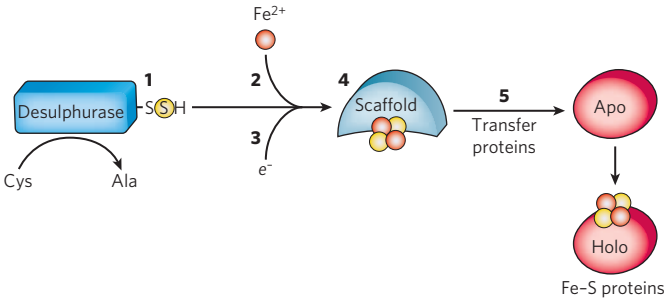
As explained in Box 1, biosynthesis of Fe–S proteins can be separated into two main steps. In the ISC systems, an Fe–S cluster is initially and transiently assembled on the scaffold proteins IscU (bacteria) and Isu1 (mitochondria), which contain three conserved Fe–S-cluster-coordinating cysteine residues[29–31] (Figs 1 and 2). Then the Fe–S cluster is transferred from Isu1/IscU to recipient apoproteins for incorporation into the Fe–S apoprotein by coordination with specific amino-acid residues[32,33]. The first reaction, Fe–S-cluster assembly on Isu1/IscU, critically depends on the function of a cysteine desulphurase as a sulphur donor (Box 1). In bacteria, this reaction is performed by IscS, which is highly similar to the founding member of this protein family, NifS, involved in nitrogenase maturation[34] (Fig. 1). The crystal structures of several desulphurases are known and show a dimeric two-domain protein, with one domain harbouring the pyridoxal-phosphate-binding site and a smaller domain

Box 1 | Biosynthetic principles of Fe–S-protein biogenesis

Several systems for the biogenesis of Fe–S proteins have been identified in bacteria and in various cell compartments of eukaryotes, namely the bacterial NIF system, the bacterial and mitochondrial ISC assembly machineries, the bacterial and plastid SUF systems and the eukaryotic CIA machinery for cytosolic and nuclear Fe–S proteins. These different machineries may follow common biosynthetic rules. The overall biogenesis process can be split into two main steps: the *de novo* assembly of an Fe–S cluster on a scaffold protein, and the transfer of the Fe–S cluster from the scaffold to target apoproteins and its subsequent assembly into the polypeptide chain. Each of these steps involves the participation of several proteins and cofactors, which execute specific biosynthetic partial reactions. These reactions may define general principles that underlie all known biosynthetic systems, but for some systems the partial reactions remain to be identified.

The bold numbers in the figure correspond to the following steps.

(1) Sulphur donor. A cysteine desulphurase (termed NifS, IscS or SufS in bacteria and Nfs1–Isd11 in mitochondria) releases the sulphur required for Fe–S-cluster formation from cysteine to produce alanine. As an intermediate, a persulphide is formed on a conserved cysteine residue of the enzyme. This persulphide may then be transferred to conserved cysteine residues of helper proteins (SufE) or directly to scaffold proteins (see below).



(2) Iron donor. Iron is unlikely to be free in solution. To guarantee its accurate delivery to scaffold proteins, specific iron donors may be needed. Such a donor function is performed by bacterial (CyaY) and mitochondrial (Yfh1) ISC components, which bind iron, the desulphurase and the scaffold protein Isu1/IscU.

(3) Electron transfer. Electrons are needed for the reduction of S^0 (present in cysteine) to sulphide (S^{2-}, present in Fe–S clusters). An electron transfer function may be provided by ferredoxin reductase and ferredoxin of the ISC assembly machineries and by the central, ferredoxin-like domain of NifU in the NIF system.

(4) Scaffold proteins. These proteins serve as a platform for the *de novo* biosynthesis of an Fe–S cluster. They contain conserved cysteine residues and bind an Fe–S cluster in a labile manner, meaning that this cluster can be transferred to target proteins and stably integrated. The most highly conserved scaffolds are bacterial IscU and SufU, and eukaryotic Isu1. Other scaffolds include bacterial NifU, plastid NFU proteins and bacterial IscA and SufA.

(5) Cluster transfer proteins. Specific factors are involved in transferring the labile Fe–S clusters bound to the scaffolds to apoproteins, which are converted from the apo form to the holo form. And these transfer proteins may have three roles: to induce dissociation of the scaffold-bound Fe–S cluster; to guarantee its accurate and specific transfer to bona fide Fe–S apoproteins; and to promote correct assembly of the Fe–S cluster at the proper acceptor sites. In many cases, these cluster transfer proteins can be bypassed in experiments *in vitro* but are essential in living cells. Examples are the mitochondrial Ssq1 and Jac1 and bacterial HscA and HscB chaperones of the ISC assembly systems, and Nar1 and Cia1 of the CIA machinery.

Table 1 | Compilation of central components involved in Fe–S-protein biogenesis

Function	Bacterial NIF	Bacterial SUF	Bacterial ISC	Mitochondrial ISC	Eukaryotic CIA
Cysteine desulphurase, sulphur donor	NifS	SufS–SufE	IscS	Nfs1–Isd11	Mitochondrial Nfs1–Isd11
U-type scaffold for Fe–S-cluster assembly	NifU (amino-terminal domain)	SufU	IscU	Isu1	–
A-type scaffold for Fe–S-cluster assembly	$IscA^{Nif}$	SufA	IscA, ErpA	Isa1, Isa2, Iba57?	–
NFU-type scaffold for Fe–S-cluster assembly	NifU (carboxy-terminal domain)	–	NfuA	Nfu1 (also present in plastids)	–
Scaffold for Fe–S-cluster assembly	–	SufB	–	–	–
P-loop NTPase scaffold for Fe–S-cluster assembly	–	–	–	Ind1?	Cfd1–Nbp35
Electron transfer	NifU (middle domain)	–	Fdx	Yah1–Arh1	–
Iron donor	–	–	CyaY	Yfh1 (frataxin)	–
Transfer of Fe–S cluster from scaffold to target proteins	–	SufC?	HscA, HscB	Ssq1, Jac1, Mge1, Grx5	Nar1, Cia1

P-loop NTPase, phosphate-binding-loop nucleoside triphosphatase.

containing the active-site cysteine that transiently carries the sulphur released from free cysteine as a persulphide[35,36].

In mitochondria, the cysteine desulphurase comprises a complex consisting of the IscS-like desulphurase Nfs1 and the 11-kDa protein Isd11 (Fig. 2; refs 37, 38). Although isolated Nfs1 contains the enzymatic activity as a cysteine desulphurase and releases sulphide from cysteine *in vitro*, the Nfs1–Isd11 complex is the functional entity for sulphur transfer from Nfs1 to Isu1 *in vivo*. This reaction is aided by direct interaction between Nfs1 and Isu1 (IscS and IscU in bacteria).

On binding of iron to Isu1/IscU, the Fe–S cluster is formed by an unknown mechanism[4,12]. The iron-binding protein frataxin (Yfh1 in yeast and CyaY in bacteria) is believed to function as an iron donor (Box 1) by undergoing an iron-stimulated interaction with Isu1–Nfs1 (refs 39–42). An alternative view recently suggested by *in vitro* studies is that CyaY functions as an iron-dependent regulator of the biosynthesis reaction by inhibiting IscS[43]. Fe–S-cluster assembly on Isu1 further depends on electron transfer from the [2Fe–2S] ferredoxin Yah1 (Fdx in bacteria), which receives its electrons from the mitochondrial ferredoxin reductase Arh1 and NADH[30] (Fig. 1). It is likely that the electron flow is needed for reduction of the sulphan sulphur (S^0) present in cysteine to the sulphide (S^{2-}) present in Fe–S clusters, but this remains to be verified experimentally. An additional electron requirement was suggested for the fusion of two [2Fe–2S] clusters to a [4Fe–4S] cluster by reductive coupling[32,33].

The second main step of biogenesis formally comprises the release of the Fe–S cluster from Isu1/IscU, cluster transfer to apoproteins and its assembly into the apoprotein. However, these three partial reactions have not been separated experimentally so far. The overall process is specifically assisted by a dedicated chaperone system comprising the Hsp70 ATPase Ssq1 and the DnaJ-like co-chaperone Jac1 (respectively HscA and HscB in bacteria). In mitochondria, the nucleotide exchange factor Mge1 is also required (Fig. 2), whereas in bacteria the related GrpE seems to be dispensable owing to the lability of adenosine diphosphate bound to HscA[7,44]. Ssq1/HscA undergoes an ATP-hydrolysis-dependent, highly specific interaction with the LPPVK motif of Isu1/IscU[45,46]. This complex formation and the involvement of Jac1/HscB is thought to induce a structural change in Isu1/IscU, thereby labilizing Fe–S-cluster binding and, thus, facilitating cluster dissociation and transfer to apoproteins[47–49]. An ancillary, non-essential role in Fe–S-cluster transfer from Isu1 to apoproteins is performed by the mitochondrial monothiol glutaredoxin Grx5, yet its precise function is unknown[30]. The plant Grx5 proteins were suggested to serve as scaffolds for the formation of [2Fe–2S] clusters[50].

The aforementioned ISC proteins are required for generation of all mitochondrial Fe–S proteins, but some biogenesis components perform a more specific function. The interacting mitochondrial proteins Isa1, Isa2 and Iba57 (Table 1) are specifically involved in the maturation of a subset of Fe–S proteins, that is, members of the aconitase superfamily

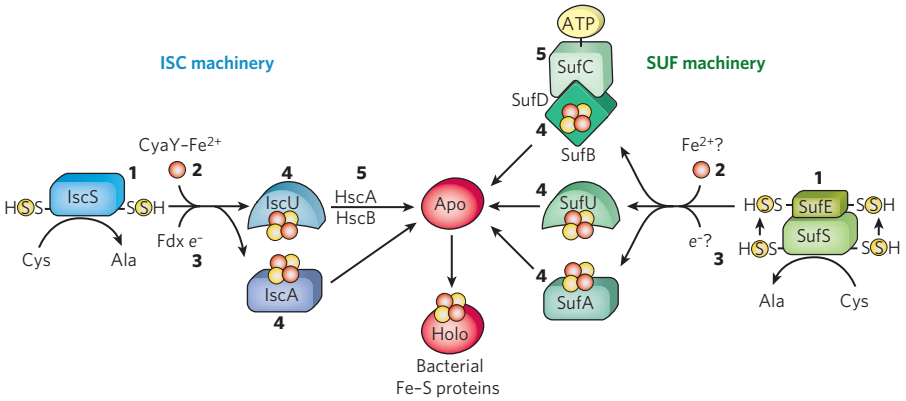


Figure 1 | A model for Fe–S-protein biogenesis in bacteria: the ISC and SUF machineries. For the maturation of housekeeping Fe–S proteins (to become the holo form), bacteria use one of two biogenesis systems or both systems in parallel. The ISC machinery (left) consists of the cysteine desulphurase IscS, which liberates sulphur (yellow circle) from cysteine, generating an IscS-bound persulphide (-SSH) on a conserved cysteine residue. A transient Fe–S cluster is formed on the scaffold proteins IscU and/or IscA. *De novo* Fe–S-cluster synthesis involves the transfer of iron (red circle) from the iron-binding protein CyaY (and other, unknown, factors). The [2Fe–2S] ferredoxin (Fdx) possibly serves to reduce the sulphur in cysteine to sulphide. The transiently bound Fe–S cluster is then transferred from the scaffolds to apoproteins (Apo) for coordination with specific residues (usually cysteine or histidine). Transfer from IscU is aided by the dedicated chaperone system HscA–HscB, which binds to a conserved motif of IscU (Leu-Pro-Pro-Val-Lys). Whereas the ISC machinery is functional under normal conditions in *Escherichia coli*, the SUF machinery (right) is active under oxidative-stress and iron-limiting conditions. A SUF system is also functional in the plastids of plants. Biogenesis is initiated by the cysteine desulphurase SufS, which functions comparably to IscS except that the sulphur is first transferred to a conserved cysteine residue of SufE and bound as a persulphide. Putative iron and electron donors in this system are still unknown. Three components might be scaffolds for *de novo* Fe–S-cluster assembly: SufU and SufA, which show similarities to IscU and IscA, respectively; and SufB, which forms a stable complex with SufC–SufD. SufC is an ATPase and may facilitate Fe–S-cluster dissociation from SufB and subsequent transfer to apoproteins. The bold numbers refer to the biogenesis steps defined in Box 1.

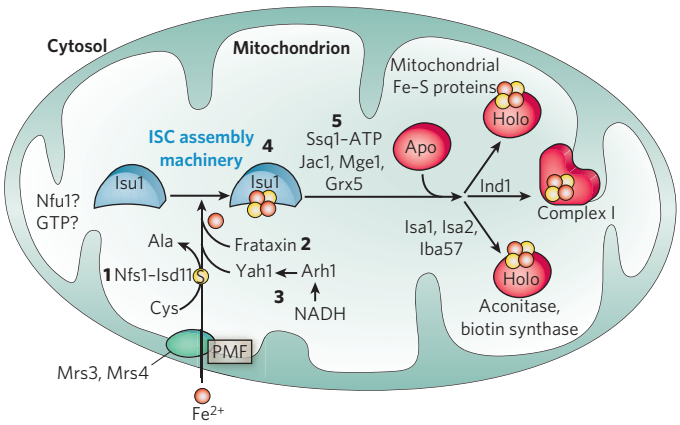


Figure 2 | A model for Fe–S-protein assembly in mitochondria. Mitochondria import ferrous iron (Fe^{2+}) from the cytosol in a membrane-potential-dependent manner (with the proton-motive force (PMF) as a source of energy). Importation is aided by the inner membrane carriers Mrs3 and Mrs4 (known as mitoferrin in mammals). The maturation of mitochondrial Fe–S proteins (to the holo form) starts with sulphur liberation from cysteine by the cysteine desulphurase complex Nfs1–Isd11. The synthesis of a transiently bound Fe–S cluster on the scaffold protein Isu1 (and Isu2 in yeast) further depends on the iron-binding protein frataxin (Yfh1 in yeast) as an iron donor and the electron-transport chain consisting of NADH, ferredoxin reductase (Arh1) and ferredoxin (Yah1), which possibly provides electrons for the reduction of sulphur to sulphide. The release of the Fe–S cluster from Isu1, and its transfer and incorporation into recipient apoproteins (Apo) are facilitated by the ATP-dependent Hsp70 chaperone Ssq1, the DnaJ-like co-chaperone Jac1, the nucleotide-exchange factor Mge1 and the monothiol glutaredoxin Grx5. Proteins of the aconitase family and radical SAM proteins such as biotin synthase specifically need Isa1, Isa2 and Iba57 in addition for maturation of their Fe–S clusters. Assembly of respiratory complex I also requires the P-loop NTPase Ind1. The roles of Nfu1 and GTP are still unclear[28,100]. The bold numbers refer to the biogenesis steps defined in Box 1.

and radical SAM proteins[51] (Fig. 2). Depletion of these proteins results in corresponding enzyme defects and auxotrophies. Similarly, a deficiency of the Isa-protein-related IscA in bacteria, in conjunction with the homologous SufA (see below; Table 1), affects the assembly of the [4Fe–4S] proteins aconitase and dihydroxy-acid dehydratase, whereas the maturation of some [2Fe–2S] proteins such as ferredoxin is unaltered[52]. The third bacterial member of this protein class, ErpA (Table 1), is essential for growth and involved in the maturation of an Fe–S protein of isoprenoid biosynthesis[53]. Several members of the Isa1/IscA protein family (Table 1) were shown *in vitro* to bind an Fe–S cluster by means of three conserved cysteine residues in two motifs characterizing these proteins[4,9,12,13]. SufA binds a [2Fe–2S] cluster *in vivo* that can be transferred to both [2Fe–2S] and [4Fe–4S] proteins *in vitro*[54]. Together, these observations may support the view that the Isa1/IscA proteins function as alternative scaffolds for a subset of Fe–S proteins (Fig. 1). However, the relative specificity of the Isu1/IscU and Isa1/IscA scaffolds and their functional cooperation will require further scrutiny *in vivo* to test the physiological relevance of this proposal, particularly because IscA was also shown to bind mononuclear iron[4].

The mitochondrial P-loop NTPase Ind1 is important for the assembly of respiratory complex I (ref. 55) (Fig. 2). On the basis of its homology with the cytosolic scaffold-protein complex Cfd1–Nbp35 (see below; Table 1), it was proposed that Ind1 serves as a specific scaffold or transfer protein for the assembly of the eight Fe–S clusters into complex I. Consistent with this idea, Ind1 was shown to assemble a labile Fe–S cluster that can be passed on to apoproteins *in vitro*.

The SUF machinery in bacteria and plastids

Deletion of the *isc* operon from *E. coli* is not associated with a major phenotype. Cell viability is affected only when the SUF biogenesis system is simultaneously inactivated[56,57]. The *suf* genes are organized in an operon that is induced under iron-limiting and oxidative-stress conditions[9,56] (Table 1). Gene expression from the *isc* and *suf* operons is coordinately regulated by the Fe–S proteins IscR and SufR, which function as transcriptional repressors of their respective operons[9]. During iron deficiency or oxidative stress, the apo form of IscR additionally activates the *suf* operon. Thereby, both proteins link the efficiency of Fe–S-protein maturation to the extent of gene expression of the two operons.

Components of the SUF machinery are found in a variety of prokaryotes, including Archaea and photosynthetic bacteria. The various SUF components fulfil some of the biosynthetic conditions of Fe–S-protein biogenesis (Box 1). A complex of SufS and SufE serves as the cysteine desulphurase (Fig. 1), in which SufS acts similarly to bacterial IscS or NifS and mitochondrial Nfs1–Isd11, but functions mechanistically distinctly[58–60]. SufE stimulates SufS activity more than tenfold and allows the cysteine-bound persulphide intermediate on SufS to be transferred to a conserved cysteine residue on SufE, from where it is passed on to scaffold proteins[61,62]. Unexpectedly, SufE has a structure similar to the IscU-type scaffold proteins, but it is not known to function as one[63]. A specific iron donor and an electron requirement (Box 1) in the SUF system are not yet known, but corresponding steps are probably also involved in this pathway. Several SUF proteins may provide a scaffold function for *de novo* Fe–S-cluster synthesis, but their relative importance and specificity remain to be clarified (Fig. 1). SufA was discussed above as a functional IscA homologue. SufB contains several conserved cysteine residues that can assemble an Fe–S cluster[9,12]. SufC is an ATPase that is stimulated 100-fold by complex formation with SufB–SufD[58,64]. Hence, SufC is a likely candidate for a transfer protein facilitating Fe–S-cluster delivery from SufB to target proteins (Box 1). Some bacteria contain an IscU-related protein termed SufU that may or may not be encoded in the *suf* operon[65,66]. Notably, SufU differs from Isu1/IscU in that it lacks the HscA binding sequence LPPVK of IscU.

SUF proteins are also present in plastids, reiterating that this biosynthetic system seems to be less sensitive to high oxygen concentrations. The functionality of plastid SufS, SufE and SufA has been confirmed by *in vitro* experiments or bacterial complementation studies, but direct experimental evidence for their biogenesis function *in planta* is usually more difficult to achieve[11,67]. It should be mentioned in this context that in plastids the SUF proteins may not be the only proteins to support Fe–S-protein biogenesis[11]. An important role, possibly as scaffold proteins, is performed by NFU1, NFU2 and NFU3 (also known as Cnfu1, Cnfu2 and Cnfu3), which have homologues in photosynthetic bacteria. NFU proteins show sequence similarity in a 60-residue segment to the C-terminal domain of NifU in bacteria and a similar segment present in Nfu1 in yeast, the function of which is unknown (Fig. 2). In particular, plastid NFU2 has been examined in more detail and shown to function as a scaffold that can assemble a [2Fe–2S] cluster *in vitro* and transfer it to apoferredoxin[68]. The cnfu2 mutant plants show a dwarf phenotype with faint pale-green leaves and a deficiency in photosystem I and ferredoxins documenting the important role of NFU2 in Fe–S-protein assembly.

Biogenesis of cytosolic and nuclear Fe–S proteins

Fe–S-protein maturation in both the cytosol and the nucleus strictly depends on the function of the mitochondrial ISC assembly machinery[23,69] (Fig. 3), but the molecular details of this dependence remain to be defined. In human cell culture, small amounts of some ISC proteins have been found in the cytosol[10,70,71]. A function for the cytosolic human homologue of Isu1 in *de novo* assembly of cytosolic Fe–S proteins could not be shown, but the protein may play a role in Fe–S-cluster repair after oxidative damage or iron deprivation. Likewise, cytosolic human Nfs1 does not support Fe–S-protein assembly in the cytosol in the absence of mitochondrial Nfs1 (ref. 72). The mitochondria-localized ISC assembly machinery is suggested to produce a (still unknown) component (X in Fig. 3) that is exported from the mitochondrial matrix to the cytosol, where it performs an essential function in the maturation process. Because, in particular, Nfs1 is required inside mitochondria to participate in cytosolic and nuclear Fe–S-protein biogenesis in both yeast and human cells, compound X is predicted to be a sulphur-containing

moiety[23,72]. Whether iron is also exported, possibly as part of a pre-assembled Fe–S cluster, or joins from the cytosol, is currently unknown. The export reaction is accomplished by the ABC transporter Atm1 (ABCB7 in humans) of the mitochondrial inner membrane[23,73,74].

Another required component of the export reaction is the sulphydryl oxidase Erv1, located in the intermembrane space. This enzyme has also been shown to catalyse the formation of disulphide bridges in the intermembrane space during Mia40-dependent protein import into the intermembrane space[75], and thus performs a dual function. Strikingly, depletion of GSH in yeast shows a similar phenotype as the downregulation of Atm1 or Erv1, that is, defective cytosolic Fe–S-protein biogenesis and increased iron uptake in the cell and mitochondria (see below), whereas the assembly of mitochondrial Fe–S proteins is unaffected. Hence, Atm1, Erv1 and GSH have been described as the 'ISC export machinery' (Fig. 3).

Maturation of cytosolic and nuclear Fe–S proteins crucially involves the cytosolic Fe–S-protein assembly (CIA) machinery, which comprises five known proteins (Table 1). According to recent *in vivo* and *in vitro* studies, this process can be subdivided into two main partial reactions[76] (Fig. 3). First, an Fe–S cluster is transiently assembled on the P-loop NTPases Cfd1 and Nbp35 (refs 77, 78), which form a heterotetrameric complex and serve as a scaffold (Box 1). As mentioned above, this step essentially requires the mitochondrial ISC machineries. From Cfd1–Nbp35, the Fe–S cluster is transferred to apoproteins, a step that requires the CIA proteins Nar1 and Cia1. Cfd1 and Nbp35 take part in the maturation of Nar1 by assisting the assembly of two Fe–S clusters on this iron-only hydrogenase-like protein (Fig. 3). Thus, Nar1 is both a target and a component of the CIA machinery, creating a 'chicken-and-egg' situation for its maturation process. Nar1 holoprotein assists Fe–S-cluster transfer to target apoproteins by interacting with Cia1, a WD40 repeat protein that serves as a docking platform for binding Nar1 (ref. 79). Recently, another CIA component, termed Dre2, has been identified but its precise molecular function is currently unknown[80]. The protein coordinates Fe–S clusters, and is probably both a target and a component of the CIA machinery, similar to Nar1. A crucial function of the human homologues of Nar1 and Nbp35 in cytosolic Fe–S-protein biogenesis has been experimentally verified in cultured cells using RNA-interference technology to deplete these proteins to critical levels[81,82].

The essentiality of Fe–S-protein biogenesis in eukaryotes

Most functional studies of eukaryotic Fe–S-protein biogenesis have been performed in yeast and practically all of the ISC and CIA components have been first discovered and characterized in this model system. Nevertheless, the process seems to be highly conserved in virtually all eukaryotes. This notion has now been documented in functional studies in human cell culture, mouse, zebrafish and *Drosophila* models for central components of the ISC and CIA machineries, such as Nfs1, Isu1, frataxin, Grx5, Atm1, Nbp35 and Nar1 (ref. 8). It is likely that the remaining components also have functions similar to those in yeast. Nevertheless, gene duplications of some of the ISC and CIA genes in vertebrates indicate that the process may be more complex in such eukaryotes or that the additional components perform other (non-Fe–S-related) tasks.

Most of the ISC and CIA components are essential for the viability of yeast and human cells[71,72,83]. In fact, Fe–S-protein biogenesis is the only known function of mitochondria that is indispensable for the viability of yeast cells. In comparison, yeast mutants in oxidative phosphorylation result in a respiratory growth defect, but they can grow on glucose-containing media. Because yeast mitochondria are not known to contain essential Fe–S proteins (apart from the ferredoxin Yah1, which is involved in Fe–S-protein biogenesis), the essential character of the mitochondrial ISC assembly machinery is explained by its role in the maturation of extra-mitochondrial Fe–S proteins. The first-known example of an essential cytosolic–nuclear Fe–S protein is the ABC protein Rli1, an Fe–S component involved in ribosome assembly and export from the nucleus[16]. Maturation of its Fe–S clusters strictly depends on the two mitochondrial ISC systems and the CIA machinery providing a tight link between mitochondrial function and cytosolic protein translation. Recently, two other essential (nuclear) Fe–S proteins were identified, one (Rad3) with a function in nucleotide excision repair and the other (Pri2) with a function in RNA primer synthesis for DNA replication[15,84]. It seems probable that their maturation requires mitochondria, possibly

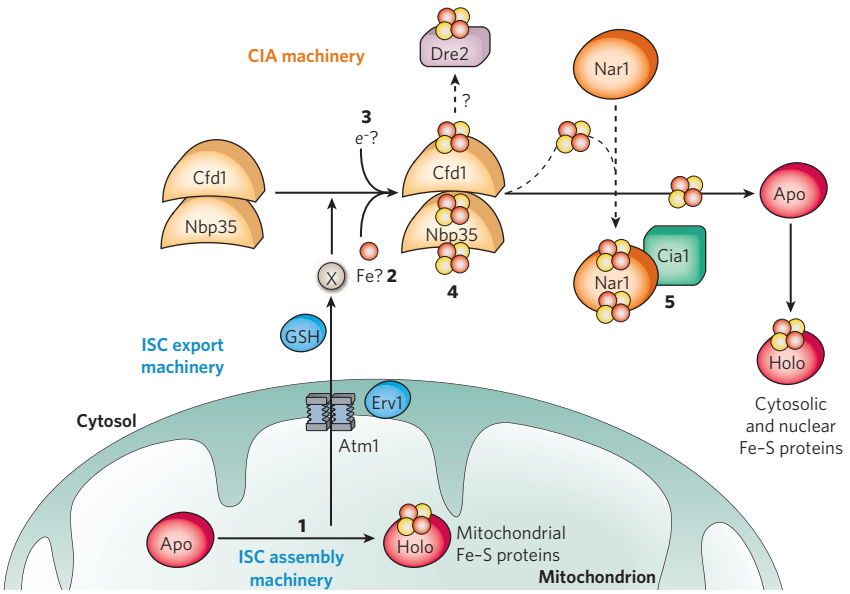


Figure 3 | The roles of mitochondria and the CIA machinery in Fe–S-protein biogenesis in the cytosol and nucleus of eukaryotes. Cytosolic and nuclear Fe–S-protein biogenesis requires both the mitochondrial ISC assembly and export machineries. The ABC transporter Atm1 of the mitochondrial inner membrane exports an unknown compound (X) to the cytosol for use in Fe–S-protein assembly, and is assisted by the tripeptide glutathione (GSH) and the intermembrane-space sulphydryl oxidase Erv1, which introduces disulphide bridges into substrates. In the cytosol, the components of the CIA machinery catalyse Fe–S-protein maturation in two main steps. First, Fe–S clusters are assembled on the P-loop NTPase complex Cfd1–Nbp35. The source of iron and the potential need for electron transfer remain to be elucidated. The Fe–S clusters bound to Cfd1–Nbp35 are labile, and, aided by the iron-only hydrogenase-like protein Nar1 and the WD40-repeat protein Cia1, can be transferred to cytosolic and nuclear apoproteins (Apo). The site of involvement of the Fe–S-cluster-containing CIA protein Dre2 is still unknown. Maturation of the two Fe–S clusters on Nar1 (and presumably on Dre2) depends on mitochondria and Cfd1–Nbp35 function. The bold numbers refer to the biogenesis steps defined in Box 1.

Table 2 | Diseases related to Fe–S proteins and their biogenesis

Human protein	Yeast homologue	Function	Associated disease
Biogenesis components			
Mitoferrin	Mrs3, Mrs4	Mitochondrial carrier; putative iron transporter	Erythropoietic protoporphyria[90]
ISCU	Isu1	Mitochondrial scaffold	Myopathy with exercise intolerance[91,92]
Frataxin	Yfh1	Iron donor for Isu1 scaffold	Friedreich's ataxia[98]
GLRX5	Grx5	Monothiol glutaredoxin; transfer of Fe–S clusters from Isu1 to apoproteins	Microcytic anaemia[89]
ADR	Arh1	Adrenodoxin reductase; electron transfer from NADH to adrenodoxin; also involved in steroid hormone biosynthesis	Tumour suppressor
ABCB7	Atm1	ABC transporter, ISC export machinery component	X-linked sideroblastic anaemia and cerebellar ataxia[99]
Fe–S proteins			
Complex I	n.p.	Complex I of respiratory chain (NADH:ubiquinone oxidoreductase)	Various mitochondrial diseases such as LHON, MELAS, Leigh syndrome
Complex II	Complex II (Sdh1, Sdh2, Sdh3, Sdh4)	Complex II of respiratory chain (succinate dehydrogenase)	Tumour suppressor
XPD	Rad3	Fe–S protein involved in nucleotide excision repair	Xeroderma pigmentosum, Cockayne syndrome, trichothiodystrophy
FANCJ	Rad3	Fe–S protein involved in nucleotide excision repair	Fanconi anaemia
MUTYH	Ntg2	Fe–S protein (glycosylase) involved in DNA repair	Colon cancer

n.p., not present in *S. cerevisiae*.

linking these organelles to other fundamental processes of life. This indicates that mitochondria and the process of Fe–S-protein maturation have been maintained in evolution to mature extra-mitochondrial Fe–S proteins with an essential function in gene expression. This intimate connection between the endosymbiontic host and the bacterial ancestor of mitochondria may have been decisive for the maintenance of these organelles in eukaryotes.

The crucial role of mitochondria in Fe–S-protein biogenesis has raised the pressing question of how 'amitochondriate' organisms lacking classical mitochondria (such as *Giardia*, Microsporidia and *Entamoeba*) assemble their Fe–S proteins. Genomic information on several of these organisms has provided evidence for the presence of homologues of the ISC assembly and CIA machineries, whereas genes for classical functions of mitochondria, such as respiration, haem biosynthesis or the citric-acid cycle, are missing. Cell-biological studies of several amitochondriate species have shown that the organisms harbour small double-membrane-bounded organelles, termed mitosomes, that contain homologues of the mitochondrial chaperones Hsp60 and Hsp70 as marker proteins[85,86]. It is now accepted that mitosomes descended from classical mitochondria by reductive evolution, that is, by successively losing functions that became dispensable owing to the usually intracellular lifestyle of these parasites. Localization and functional studies have shown that mitosomes from *Giardia* and Microsporidia in fact contain a functional set of ISC assembly proteins[87,88]. Fe–S-protein biogenesis therefore seems to be the remnant function of these organelles, explaining their maintenance in these species. This, however, does not readily clarify why the process of Fe–S-protein biogenesis still depends on mitosomes, that is, why the pathway still is compartmentalized and apparently cannot normally be taken over by the cytosol.

The central importance of Fe–S-protein biogenesis in mammals is impressively documented in numerous diseases associated with defects in Fe–S-protein biogenesis components or Fe–S proteins (Table 2). Depletion of frataxin, the putative iron donor for Fe–S-cluster formation on Isu1, causes the neurodegenerative disease Friedreich's ataxia, which is associated with Fe–S-protein activity defects and iron accumulation. Defects in the glutaredoxin Grx5 and in mitoferrin (Fig. 2) are associated with diseases exhibiting mainly haematological phenotypes, namely microcytic anaemia and erythropoietic protoporphyria, respectively[89,90]. This surprising connection between Fe–S-protein biogenesis and haematopoiesis was experimentally explained by the fact that Fe–S-protein biogenesis is crucial for the maturation of IRP1, which regulates the synthesis of several proteins involved in iron uptake, storage and utilization (see above). One such example is the erythroid δ-aminolaevulinate synthase (ALAS2), catalysing the committed step of haem biosynthesis in mitochondria. Translation of this protein in the cytosol is largely decreased when iron is scarce or Fe–S-protein biogenesis is hampered, intimately integrating the efficiency of haem and Fe–S-protein biosynthesis, the two major iron-consuming processes in the cell. An additional connection between haem and Fe–S-protein biosynthesis may be the presence of an Fe–S cluster in mammalian ferrochelatase, the final enzyme of haem biosynthesis. A splicing defect in the *ISCU* gene in humans results in a myopathy with exercise intolerance and intracellular iron overload[91,92]. Some Fe–S proteins appear to be connected to human disease (Table 2). Succinate dehydrogenase (complex II of the respiratory chain) and adrenodoxin reductase (an ISC member, but not an Fe–S protein) have been described as tumour suppressors[8]. Mutations in the putative human Fe–S proteins XPD and FANCJ cause multiple disease phenotypes including xeroderma pigmentosum and Fanconi anaemia[8].

Impact of Fe–S-protein biogenesis on other cellular processes

Defects in Fe–S-protein biogenesis in both bacteria and eukaryotes have a severe impact on several other cellular processes. On the one hand, this connection is explained by the function of particular Fe–S proteins in pathways such as amino-acid biosynthesis, the citric-acid cycle, respiration, cofactor biosyntheses (haem, biotin, lipoic acid and molybdenum cofactor) and gene expression (DNA replication, DNA repair and translation). On the other hand, some ISC components perform a second (moonlighting) function in other cellular processes. For instance, thio modification of transfer RNAs requires IscS in bacteria and mitochondrial Nfs1–Isd11 as a sulphur donor[8,93,94]. Thio and other modifications of tRNAs depend on the function of Fe–S proteins both in bacteria (for example, MiaB) and eukaryotes (for example, Elp3). Furthermore, the mitochondrial [2Fe–2S] ferredoxin Yah1, together with its reductase Arh1 and Cox15, is required for the hydroxylation of protohaem for the synthesis of haem A of cytochrome oxidase. The mammalian homologue of Yah1, adrenodoxin, plays a long-known role in steroid biosynthesis.

An intimate connection exists between Fe–S-protein biogenesis in mitochondria and iron regulation in the cell. The mechanisms of this regulatory pathway are fundamentally different in yeast and mammalian cells, yet the input (iron requirement for Fe–S-protein biogenesis) and the outcome (iron-uptake regulation) of the regulatory chain are similar. In yeast, iron uptake and intracellular distribution are mainly regulated by a transcriptional mechanism involving the transcription factors Aft1 and Aft2 (ref. 95). In the absence of iron, Aft1 and Aft2 translocate to the nucleus, where they activate genes of the iron regulon including iron

transporters, siderophore transporters and proteins for intracellular iron distribution. Low iron concentrations inside mitochondria or defects in mitochondrial Fe–S-protein biogenesis (that is, ISC protein deficiencies) activate Aft1–Aft2 and lead to a concomitant iron accumulation in mitochondria. Thus, mitochondria have a crucial role in regulating cellular iron homeostasis. The regulatory mechanism includes both the ISC assembly and ISC export systems, suggesting that the same or a similar molecule as that exported by Atm1 to the CIA machinery may communicate the iron status of the mitochondrial matrix to Aft1 and Aft2 (ref. 96). Strikingly, the yeast CIA proteins have no major role in iron sensing and regulation, refuting the idea that a canonical cytosolic or nuclear Fe–S protein may be responsible for the regulatory function in iron homeostasis.

In mammals, cellular iron regulation occurs predominantly at the post-transcriptional level and involves both the Fe–S protein IRP1 (see above) and iron-regulatory protein 2, which has no Fe–S cluster yet is degraded under iron-replete conditions[19–21]. Insertion of the Fe–S cluster into IRP1 has been shown to require mitochondrial Nfs1, Isu1, frataxin, Grx5, ABCB7, Nbp35 and Nar1 (refs 71–74,81,82,89,97). Hence, in mammals, both the ISC and the CIA machineries are critical for the regulation of cellular iron uptake, creating the notable difference from yeast, in which the CIA machinery is not needed.

Outlook

Despite the impressive progress in research on Fe–S-protein biogenesis during the past decade, exciting years of further exploration are ahead of us, ranging from basic structural, biochemical and cell-biological research to physiology and molecular medicine. Only some of the most pressing problems to be solved in future research can be mentioned here. The solving of three-dimensional structures of the various ISC and CIA proteins, in particular of the holo forms of different Fe–S scaffolds, will facilitate the elucidation of the molecular mechanisms of *de novo* Fe–S-cluster assembly in living cells. It will be crucial to combine *in vitro* and *in vivo* approaches to verify the physiological relevance of findings with isolated proteins. The specificity of, and differing needs for, the various scaffold proteins has to be clarified to understand their individual roles. The mechanisms of Fe–S-cluster transfer from the scaffold proteins must be dissected into partial steps, and the precise roles of the transfer-assisting proteins will have to be understood. It will be important to elucidate the conversion of [2Fe–2S] clusters to [4Fe–4S] clusters and define the function of participating biogenesis components.

Novel biogenesis components, in particular the iron and electron donors of the SUF and CIA machineries, will be identified and their function characterized. A particularly pressing question is the isolation and chemical characterization of the substrate exported from mitochondria to assist extra-mitochondrial Fe–S-protein biogenesis. This achievement will crucially depend on the development of an *in vitro* reconstitution protocol for the export process. It is possible that new moonlighting functions of Fe–S-protein biogenesis components will be identified. In that respect, it will be interesting to clarify the function of the nuclear form of Nfs1. Advances in isolating mitosomes will make it possible to test their suggested function in cellular Fe–S-protein biogenesis experimentally. Finally, new diseases associated with this fundamental process of life will allow us to understand the physiological consequences of Fe–S-protein defects. It is clear that thorough molecular insights into the mechanisms of Fe–S-protein biogenesis are prerequisite for the future development of therapeutic strategies in the treatment of Fe–S diseases. ■

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