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Review

The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism ☆

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ABSTRACT

Mitochondria play a key role in iron metabolism in that they synthesize heme, assemble iron–sulfur (Fe/S) proteins, and participate in cellular iron regulation. Here, we review the latter two topics and their intimate connection. The mitochondrial Fe/S cluster (ISC) assembly machinery consists of 17 proteins that operate in three major steps of the maturation process. First, the cysteine desulfurase complex Nfs1–Isd11 as the sulfur donor cooperates with ferredoxin–ferredoxin reductase acting as an electron transfer chain, and frataxin to synthesize an [2Fe–2S] cluster on the scaffold protein Isu1. Second, the cluster is released from Isu1 and transferred toward apoproteins with the help of a dedicated Hsp70 chaperone system and the glutaredoxin Grx5. Finally, various specialized ISC components assist in the generation of [4Fe–4S] clusters and cluster insertion into specific target apoproteins. Functional defects of the core ISC assembly machinery are signaled to cytosolic or nuclear iron regulatory systems resulting in increased cellular iron acquisition and mitochondrial iron accumulation. In fungi, regulation is achieved by iron-responsive transcription factors controlling the expression of genes involved in iron uptake and intracellular distribution. They are assisted by cytosolic multidomain glutaredoxins which use a bound Fe/S cluster as iron sensor and additionally perform an essential role in intracellular iron delivery to target metalloproteins. In mammalian cells, the iron regulatory proteins IRP1, an Fe/S protein, and IRP2 act in a post-transcriptional fashion to adjust the cellular needs for iron. Thus, Fe/S protein biogenesis and cellular iron metabolism are tightly linked to coordinate iron supply and utilization. This article is part of a Special Issue entitled: Cell Biology of Metals.

1. Introduction

Mitochondria are long known as the powerhouses of the cell converting the energy of carbohydrates into the synthesis of ATP by oxidative phosphorylation. Recently, novel important roles have been assigned to mitochondria such as a crucial function in apoptosis, a dynamic communication with the endoplasmic reticulum, and the pathway of iron–sulfur (Fe/S) protein biogenesis. Mitochondria not only assemble their own set of Fe/S proteins, but are crucially involved in the biogenesis of Fe/S proteins located in the cytosol and nucleus. In fact, it is the assembly of these extra-mitochondrial Fe/S proteins that explains why this process is indispensable for cell viability in virtually all eukaryotes. None of the mitochondrial Fe/S proteins such as aconitase or the respiratory complexes would per se explain the essential character of Fe/S protein biosynthesis in, e.g., the yeast *Saccharomyces cerevisiae*. The role of mitochondria (and related organelles; see below) in extra-mitochondrial Fe/S protein biogenesis can therefore be viewed as their minimal function. Cytosolic and nuclear Fe/S proteins with indispensable functions for cell viability include the Fe/S ABC protein Rli1 (ABCE1) which participates in ribosome assembly and ribosome recycling during termination of polypeptide synthesis [1,2]. Other examples may include the ATP-dependent DNA helicases such as Rad3, XPD, FANCJ, and RTEL1 which are involved in DNA damage repair and telomere maintenance [3]. Recently added prominent examples of essential, nuclear Fe/S proteins are the eukaryotic replicative DNA polymerases which contain an Fe/S cluster in their C-terminal domain [4,5]. The metal cofactor in these proteins appears to be necessary for the efficient interaction of the polymerase catalytic subunits with their accessory proteins during DNA replication.

The fact that the viability of eukaryotes essentially depends on organellar Fe/S protein biogenesis is most impressively documented by the discovery of mitosomes. These double membrane-bounded organelles of protists are derived from mitochondria and have been functionally reduced during evolution. Thereby, mitosomes have lost most of the well-known functions of classical mitochondria

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such as heme synthesis, citric acid cycle, oxidative phosphorylation, fatty acid oxidation and mitochondrial gene expression [6–8]. However, mitosomes still contain all of the key components needed for the maturation of Fe/S proteins [9,10]. Since mitosomes are not known to contain any functionally important Fe/S proteins, one may speculate that their main task may be the synthesis of extra-mitosomal Fe/S proteins.

The efficiency of synthesizing Fe/S clusters in mitochondria is intimately linked to cellular iron homeostasis, simply because iron is a substrate of the process. Conversely, failure to assemble mitochondrial Fe/S proteins, e.g., due to defects in the biogenesis components, results in increased cellular iron acquisition and eventually mitochondrial iron overload [11,12]. This shows that cells use the efficiency in synthesizing mitochondrial Fe/S proteins as a device to regulate iron homeostasis and maintain proper intracellular levels of this essential heavy metal [13]. Conspicuously, with the notable exception of red blood cells, mitochondrial iron accumulation is not seen upon defects in heme biosynthesis which also needs the steady supply of iron [14]. Thus, mitochondrial Fe/S protein biogenesis performs an additional role as a sensor for the regulation of cellular iron acquisition and intracellular iron distribution. This function is unique and is conserved from yeast to man.

Whereas the process of Fe/S protein biogenesis in mitochondria is highly conserved in virtually all eukaryotes, the mechanisms and regulation of iron homeostasis differ fundamentally in fungi and mammalian cells (see other articles in this BBA issue). Fungi like the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* employ a transcriptional regulatory mechanism with iron-responsive transcription factors that control the expression of multiple genes involved in iron uptake, distribution and utilization. In contrast, mammalian cells use a post-transcriptional mechanism involving two iron regulatory proteins (IRP) which determine the translational efficiency of a few proteins involved in iron uptake, distribution and storage [15,16]. IRP1 contains an Fe/S cluster which dissociates upon iron scarcity allowing the apoprotein to bind to iron-responsive elements (IRE) of certain mRNAs of iron-regulated proteins. IRP2 also binds such IREs under low iron condition and is degraded under iron-replete conditions by a ubiquitin–proteasome-mediated mechanism. Despite the fundamental difference of the fungal and mammalian systems for iron regulation, both are severely influenced by the efficiency of mitochondrial Fe/S protein biogenesis thus linking the efficiency of cellular iron uptake to its intracellular consumption during the generation of cellular Fe/S proteins.

This review provides an overview on the molecular mechanisms of mitochondrial Fe/S protein biogenesis and its impact on cellular iron regulation. We will highlight the links between these important processes, and discuss their relevance for human disease. Our summary will mainly be focused on findings since 2006, when the first series on 'Cell Biology of Metals' was published [17]. For more detailed descriptions of earlier studies, we refer the reader to comprehensive reviews on Fe/S protein biogenesis in eukaryotes [18–27] and iron homeostasis [28–33]. Likewise, these and further excellent summaries can be used as a comprehensive introduction into the structure and function of Fe/S clusters and proteins and the functional role of iron-binding proteins. Plant systems will not be discussed here but have recently been reviewed elsewhere [34,35]. Unless stated otherwise, we will use the protein names defined for *S. cerevisiae* to avoid confusion of the readers. The alternative names of the Fe/S protein biogenesis components in mammalian cells and the related proteins of the bacterial ISC system are listed in Table 1.

2. General overview on Fe/S protein biogenesis in eukaryotes

The process of mitochondrial Fe/S protein biogenesis has been discovered in the late phase of the last century, and is supported by the so-called ISC assembly machinery. Its discovery took advantage of similar machinery encoded by the *isc* operons of numerous bacteria [36–38]. Work during the last dozen years has shown that not only the ISC components but also the mechanisms of Fe/S cluster synthesis and insertion into target apoproteins are highly similar in bacteria and mitochondria, the eukaryotic endosymbionts of bacterial origin. The process of Fe/S protein generation can currently be subdivided into three major steps (Fig. 1). First, a [2Fe–2S] cluster is synthesized de novo on a scaffold protein termed Isu1. This requires the sulfur donor Nfs1–Isd11, the electron transfer chain comprised of NAD(P)H – ferredoxin reductase – ferredoxin, and frataxin as a potential iron donor or regulator of this synthesis step. In the second step, the Fe/S cluster is released from Isu1 by binding of a dedicated ATP-dependent Hsp70 chaperone system to Isu1 which labilizes Fe/S cluster association (Figs. 1 and 2). The Fe/S cluster may transiently be taken over by a monothiol glutaredoxin coordinating the Fe/S cluster together with the tripeptide glutathione (GSH) and finally be handed over to apoproteins. This third step is assisted by several ISC targeting factors that on the one hand facilitate the formation of [4Fe–4S] clusters and on the other hand are specific for the maturation of certain Fe/S proteins. The first two steps are required for maturation of all mitochondrial Fe/S proteins, for cytosolic and nuclear Fe/S protein biogenesis, and for transcriptional iron regulation. Consequently, these components are termed 'core ISC proteins' (Fig. 1).

The formation of Fe/S proteins in the cytosol and nucleus of yeast and mammalian cells depends on the core mitochondrial ISC assembly machinery. Depletion of, e.g., Nfs1–Isd11, ferredoxin or the chaperones results in a simultaneous defect of Fe/S cluster insertion into cytosolic and nuclear target apoproteins such as Rli1, Leu1, Ntg2 or Rad3 [4,11,39]. Apparently, the core ISC assembly machinery synthesizes a component that is exported to the cytosol and utilized by the cytosolic Fe/S protein assembly (CIA) machinery [27] (Fig. 1). The export reaction is supported by the ABC transporter Atm1 of the mitochondrial inner membrane, yet the identification of the transported compound has remained unresolved. Since Nfs1 as a sulfur donor is required inside mitochondria in both yeast and mammalian cells to support extra-mitochondrial Fe/S cluster synthesis, it is likely that the exported compound contains the sulfur moiety to be incorporated into the Fe/S clusters [11,40,41]. Depletion of Atm1 generates a similar phenotype as that of components of the core ISC assembly machinery, namely a defect in cytosolic-nuclear Fe/S proteins and a mitochondrial iron accumulation, yet during Atm1 deficiency mitochondrial Fe/S proteins are matured normally. Therefore, it seems likely that Atm1 not only plays a role in cytosolic-nuclear Fe/S protein biogenesis, but also makes the connection to cellular iron regulation by exporting a sensor molecule which attenuates the nuclear transcriptional regulatory system (Figs. 1 and 3). Whether the same or similar molecules are responsible for the two processes awaits the identification of the transported species. Two additional components are implicated in both cytosolic-nuclear Fe/S protein biogenesis and cellular iron regulation. Depletion of the sulfhydryl oxidase Erv1 and the tripeptide glutathione (GSH) phenocopies the effects of a functional deficiency in Atm1 suggesting that they assist the ABC transporter in its function, and together they form the ISC export machinery [39,42].

The CIA machinery currently consists of seven known compounds [18,27]. The CIA components do not show any sequence similarity to the ISC components, and their depletion does not have any detectable effects on the mitochondrial assembly of Fe/S proteins. Nevertheless, the basic mechanisms of Fe/S cluster synthesis and insertion into apoproteins appear to follow similar biosynthetic rules. In a first reaction the Fe/S cluster is synthesized on the hetero-tetramer of the P-loop NTPases Cfd1–Nbp35 serving as a scaffold [43–45]. This reaction requires the core components of the mitochondrial ISC assembly machinery including Nfs1–Isd11 as a sulfur donor. An electron transfer chain consisting of NADPH, the diflavin reductase Tah18 and the Fe/S protein Dre2 is needed for stable insertion of the Fe/S clusters into Nbp35 [46,47], but the mechanism and exact molecular function of the electron transfer chain are still unknown. In addition,

Table 1
Components of the ISC assembly machinery in mitochondria of non-plant eukaryotes.

Yeast name	Mammalian name	Full name	Bacterial relative	Function	Fe/S cluster type
Core ISC assembly components					
Nfs1	NFS1	Cysteine desulfurase	IscS	Sulfur donor	–
Isd11	ISD11	ISC biogenesis desulfurase-interacting protein of 11 kDa	–	Stabilizes Nfs1	–
Arh1	FDXR	Ferredoxin reductase	–	Ferredoxin-$NADP^+$ reductase	–
Yah1	FDX2	Ferredoxin	Fdx	Electron transport	[2Fe–2S]
Yfh1	FXN	Frataxin	CyaY	Iron donor, allosteric regulator of Nfs1?	–
Isu1, Isu2[a]	ISCU	Scaffold protein	IscU	Scaffold protein for Fe/S cluster synthesis	[2Fe–2S]
Ssq1[a], Ssc1	GRP75	Mitochondrial Hsp70 chaperone	Hsc66	Fe/S cluster transfer	–
Jac1	HSC20	Mitochondrial Hsp70 cochaperone	Hsc20	Fe/S cluster transfer	–
Mge1	GRPE-L1/2	Mitochondrial GrpE	–	Nucleotide exchange	–
Grx5	GLRX5	Glutaredoxin 5	GrxD	Fe/S cluster transfer	Bridging [2Fe–2S], GSH
ISC targeting components					
Isa1, Isa2	ISCA1, ISCA2	A-type ISC assembly proteins	IscA	Biogenesis of [4Fe–4S] clusters	–
Iba57	IBA57	Isa-interacting protein		Biogenesis of [4Fe–4S] clusters	–
Nfu1	NFU1	Scaffold protein	C-terminal domain of NifU, NfuA	Maturation of lipoate synthase and SDH	[4Fe–4S]
–	IND1 (NUBPL)	Iron–sulfur protein required for NADH-dehydrogenase	–	Maturation of respiratory complex I	[4Fe–4S]
Aim1 ?	BOLA3	Potential glutaredoxin-interacting protein	BolA ?	Maturation of lipoate synthase and SDH	–

[a]Present only in *S. cerevisiae* and few other yeasts; ?, Function unclear or not yet analyzed.

the monothiol glutaredoxins Grx3–Grx4 are required for cluster synthesis on Dre2. Since these glutaredoxins function in iron delivery in the eukaryotic cytosol and of mitochondria [48], their depletion elicits broad effects on virtually all iron-requiring proteins including those in mitochondria (Fig. 3). Consequently, despite their involvement in cytosolic-nuclear Fe/S protein biogenesis Grx3–Grx4 are not considered to be CIA proteins. In a second step, the Fe/S cluster is transferred from the Cfd1–Nbp35 scaffold to target apoproteins, a reaction requiring the CIA proteins Nar1, Cia1 and possibly Cia2 [49–51]. The molecular mechanisms of CIA protein functions are still poorly defined, but the system is conserved in virtually all eukaryotes.

In yeast the CIA machinery has no direct impact on iron homeostasis suggesting that CIA-dependent Fe/S proteins are not involved in this regulatory step [47,49] (Fig. 3). This contrasts the situation in mammals where depletion of Nbp35 and Nar1 strongly impairs the assembly of Fe/S clusters on IRP1, and consequently has an impact on the synthesis of iron-regulated proteins such as ferritin and transferrin receptor [52,53]. This major difference between yeast and human cells is a logic consequence of the fundamentally different molecular mechanisms of iron regulation in fungi and mammals as outlined below.

3. The molecular function of the ISC assembly machinery in the maturation of mitochondrial Fe/S proteins

In this chapter, we summarize the current knowledge on Fe/S protein biogenesis in mitochondria with an emphasis on the description of the molecular role and functional interaction of the participating ISC components. The chapter is divided into the three major biosynthetic steps currently known to underlie the maturation of mitochondrial Fe/S proteins, namely the de novo synthesis of an Fe/S cluster on the Isu1 scaffold protein, the dislocation of the cluster from Isu1, and its transfer to specific ISC targeting factors which deliver the Fe/S cluster to and facilitate its insertion into apoproteins. In addition, we provide a brief summary on the role of the ISC assembly protein frataxin whose function is highly debated.

3.1. De novo synthesis of the Fe/S cluster on the scaffold protein Isu1 by complex formation with the cysteine desulfurase Nfs1–Isd11

The de novo synthesis of Fe/S clusters in mitochondria requires the interplay of six different ISC proteins (Fig. 1). Synthesis has been shown to occur on Isu1 (in yeast also on the functionally redundant Isu2; on ISCU in mammalian cells; Table 1) [12,54–56]. This protein is highly conserved from bacteria to man and contains three conserved cysteine residues all of which are crucial for synthesis of the Fe/S cluster. Isu1 tightly interacts with the cysteine desulfurase complex Nfs1–Isd11 which releases the sulfur moiety from cysteine to generate alanine and a persulfide on a conserved Cys residue on Nfs1. The reaction mechanism of the pyridoxal phosphate-dependent cysteine desulfurase has been worked out for bacterial IscS proteins [57,58]. The sulfur is then transferred to Isu1 likely converting a Cys residue of Isu1 to a persulfide. Structural analysis of the apoform of the bacterial IscS–IscU complex provided the first insights into the 3D arrangement of the two proteins [59,60]. The dimeric IscS binds two IscU molecules at opposite sides. It is therefore likely that two largely independent synthesis reactions occur on each of the two IscU proteins. A recently published crystal structure of the holoform of the IscS–IscU dimer provided the first exciting clues of how the Fe/S cluster may be synthesized and transiently coordinated [61]. The structure shows an intermediate in which the [2Fe–2S] cluster is coordinated by the three Cys residues of IscU and, unexpectedly, the active-site Cys residue 321 of IscS. To entertain this coordinative function this Cys residue has to undergo a large 1.4 nm movement from the pyridoxal phosphate-binding active site of IscS where persulfide formation initially occurs. Likely, this oscillatory movement can be repeated to supply the second sulfur moiety (S^0) without the need for dissociation of the IscU–IscS complex.

How the persulfidic sulfur (S^0) is reduced to a sulfide (S^{2-}) present in the cluster and what may be needed to transfer the iron ions to Isu1 are still unknown. The electron transfer chain comprised of NAD(P)H, the ferredoxin reductase Arh1, and ferredoxin (Yah1 in yeast and Fdx2 in mammals) is required for the synthesis of the Fe/S cluster on Isu1 [56,62–65]. Hence, it has been speculated that this chain may support this reduction step. However, no specific interaction partner of Yah1 in the ISC assembly machinery has been described hitherto. Ferredoxin is the only known essential Fe/S protein in mitochondria and binds a [2Fe–2S] cluster. All other mitochondrial Fe/S proteins are either not essential (such as aconitase) or bind their cluster only transiently (Isu1). Interestingly, Yah1 requires the core ISC assembly machinery for its own maturation [66], i.e. it is both a component and a target of the ISC assembly system. Additionally, the protein has functions in heme A and coenzyme Q biosynthesis [67,68]. These three functions fully explain the essential character of

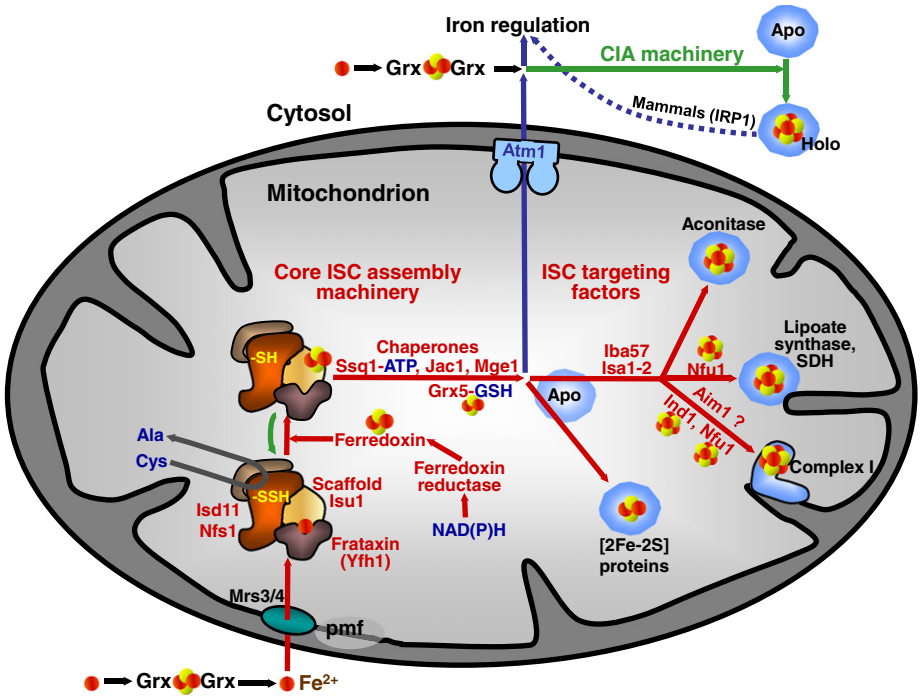

Fig. 1. A current model of Fe/S protein biogenesis in mitochondria. Mitochondria import iron (red circle) from the cytosol involving monothiol glutaredoxins (Grx) as iron donors and the inner membrane carriers Mrs3–Mrs4 which use the proton motive force (pmf) as a driving source for membrane transport. The biogenesis of mitochondrial Fe/S proteins is accomplished by the ISC assembly machinery in three major steps. First, the [2Fe–2S] cluster is synthesized on the scaffold protein Isu1, a step which requires the cysteine desulfurase complex Nfs1–Isd11 as a sulfur (yellow circle) donor releasing sulfur from cysteine via persulfide intermediates (−SSH). This step further requires frataxin (yeast Yfh1) that undergoes an iron-dependent interaction with Isu1 and may serve as an iron donor and/or an allosteric regulator of the desulfurase enzyme. An electron transfer chain consisting of NAD(P)H, ferredoxin reductase (Arh1) and ferredoxin (Yah1) is needed for Fe/S cluster assembly on Isu1. In the second step, the Isu1-bound Fe/S cluster is labilized by functional involvement of a dedicated chaperone system comprising the ATP-dependent Hsp70 chaperone Ssq1, its co-chaperone Jac1, and the nucleotide exchange factor Mge1 (see Fig. 2 for details). The monothiol glutaredoxin Grx5 then helps to transfer the Fe/S cluster toward apoproteins, presumably via transient binding of the Fe/S cluster in a glutathione-containing complex (GSH). The mentioned proteins are involved in the biogenesis of all mitochondrial Fe/S proteins, and are thus termed the core ISC assembly components. In a third step, specialized ISC targeting components catalyze the generation of [4Fe–4S] clusters by involving Isa1–Isa2–Iba57 proteins, and they assist the insertion of Fe/S clusters into specific apoproteins. For instance, Nfu1 is required for efficient assembly of lipoate synthase and respiratory complex II (SDH), while Ind1 is specific for complex I. Both proteins transiently bind the [4Fe–4S] cluster which may be transferred to the respective target apoproteins. The role of the BolA protein Aim1 is still hypothetical. The core ISC assembly components are crucially required for cytosolic-nuclear Fe/S protein biogenesis that is catalyzed by the cytosolic Fe/S protein assembly (CIA) machinery and for cellular iron regulation (see Fig. 3 for details). In mammals iron regulation involves the cytosolic Fe/S protein IRP1, providing a tight link between mitochondrial Fe/S protein biogenesis and iron homeostasis.

Yah1 and in turn of Arh1. Finally, frataxin (yeast Yfh1) has been shown to be required for Fe/S cluster assembly on Isu1 [56]. The protein has been implicated to function as an iron chaperone to supply Isu1 with this metal. As outlined below, frataxin binds to both Nfs–Isd11 and Isu1 [69], and has high-affinity binding sites for iron [26,70]. The precise binding site of frataxin on the Nfs1–Isd11–Isu1 complex is not known, but has been modeled based on biophysical studies [59,60]. Nevertheless, it remains unclear how iron is channeled into the site of synthesis of the Fe/S cluster on Isu1 and in which order sulfur and iron are provided (see also below).

In eukaryotes the function of the cysteine desulfurase Nfs1 depends on its binding partner Isd11 [71,72], a member of the LYR family of proteins which show low sequence conservation and have roles in complex III assembly and are subunits of complex I [73]. No bacterial homolog of eukaryotic Isd11 has been found in the bacterial ISC assembly machinery [74]. In the absence of Isd11, purified Nfs1 can function as a cysteine desulfurase releasing sulfide in the presence of the reductant dithiothreitol (DTT) [40,71,72,75]. However, in vivo no Fe/S cluster can be formed on Isu1 in the absence of a functional Isd11 showing that the physiological cysteine desulfurase in eukaryotes is a complex of Nfs1 and Isd11. Nevertheless, sulfide can be produced by purified Nfs1 without Isd11 in the presence of DTT, a reaction that has been used to assemble Fe/S clusters on apoproteins in vitro [75,76]. It should be mentioned though that the sulfide production by Nfs1 likely is of no physiological relevance, since in vivo a persulfide is the productive reaction intermediate for Fe/S cluster formation.

Human Isd11 has been shown to be required for mitochondrial and cytosolic Fe/S protein biogenesis by using RNA interference (RNAi)-mediated depletion of Isd11 [77,78]. While Shan et al. report a mitochondrial localization of Isd11, the work of Shi et al. suggests an additional nuclear version of this protein, consistent with the presence of Nfs1 in the nucleus [79,80]. The molecular function of nuclear Nfs1–Isd11 is unknown to date, since nuclear Nfs1 was not found to be of importance for Fe/S protein biogenesis [41]. Isd11 is also present in organisms such as Microsporidia hosting mitosomes or Trichomonads hosting hydrogenosomes instead of classical mitochondria [74]. This documents the importance of Isd11 for eukaryotic Nfs1 cysteine desulfurase function.

The important role of the electron transfer chain comprised by NAD(P)H, ferredoxin reductase and ferredoxin has been first established in yeast (see above), but recently its conservation has been confirmed in human cells [64,65]. The human genome encodes two ferredoxins. Adrenodoxin (now termed Fdx1) has long been known to function in steroid production in cooperation with mitochondrial cytochromes P_{450} [81,82]. The recently characterized Fdx2 shows a 43% sequence identity to Fdx1. Despite the high sequence similarity, our work has shown that these proteins fulfill different, highly specific functions in human mitochondria [64]. RNAi-mediated depletion of Fdx2, but not that of Fdx1 in HeLa cells elicited a strong defect in mitochondrial and cytosolic Fe/S protein biogenesis. In support of this notion, only human Fdx2 but not Fdx1 could functionally replace yeast Yah1 in Fe/S protein biogenesis [64]. When isolated Fdx2 was tested for activity as an

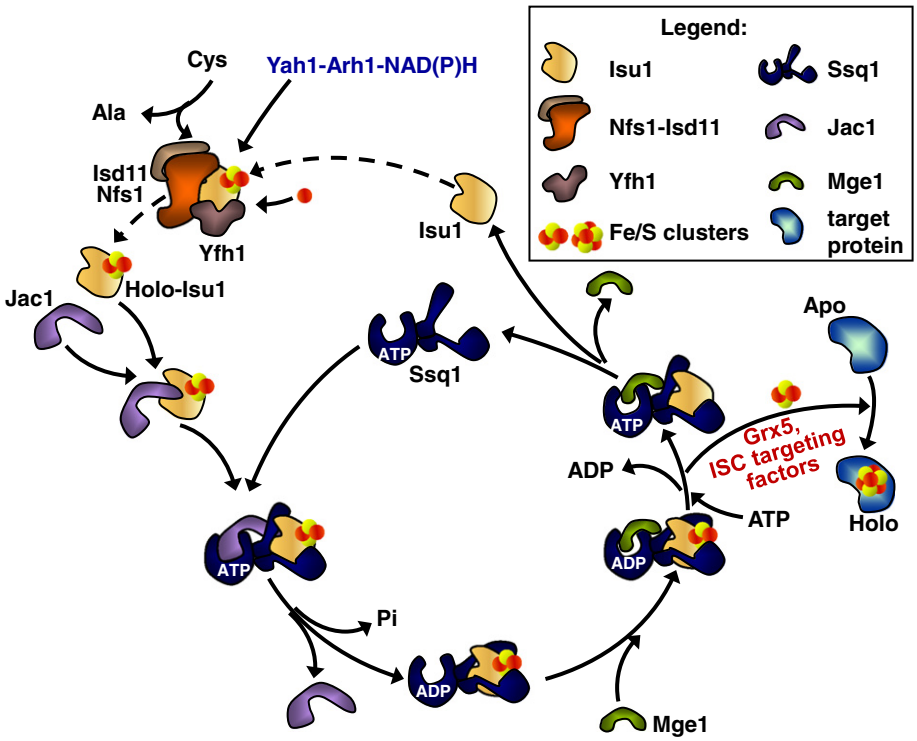

Fig. 2. The working cycle of the dedicated chaperone system of mitochondrial Fe/S protein biogenesis. The working cycle of the ISC chaperone system is similar to that of Hsp70 chaperones in protein folding [118]. After synthesis of the [2Fe–2S] cluster on the scaffold protein Isu1 (see Fig. 1) the co-chaperone Jac1 recruits holo-Isu1 and delivers it to the ATP-bound form of the Hsp70 chaperone Ssq1. ATP hydrolysis triggers a conformational change of the peptide binding domain of Ssq1 thus creating a tight binding interaction with the LPPVK motif of Isu1. In turn, this is believed to induce a conformational change on Isu1 and may weaken the binding of the Fe/S cluster to Isu1. Eventually, this results in Fe/S cluster transfer from Isu1 to Grx5 and late-acting ISC targeting factors (Fig. 1). Concomitantly, ADP is exchanged for ATP by the exchange factor Mge1 which triggers a conformational change of the peptide binding domain of Ssq1 from the closed to an open state thus leading to disassembly of the Ssq1–Isu1 complex. The reaction cycle can then resume with the binding of a new holo-Isu1–Jac1 complex to Ssq1–ATP.

electron donor for steroid oxidation, no physiologically relevant enzyme activity compared to Fdx1 was detected. Apparently, the two human ferredoxins fulfill distinct functions that do not overlap. This specific function is nicely supported by the almost exclusive expression of Fdx1 in adrenal gland and kidney cells [64], i.e. the major site of steroid hormone production [64]. In contrast, a recent study suggested that Fdx1 might also perform a function in Fe/S protein biogenesis [65]. While RNAi depletion of Fdx1 for 6 days did not elicit any defects in Fe/S proteins, longer treatment was effective. The authors therefore concluded that also Fdx1 may have a role in Fe/S protein biogenesis. However, the depletion efficiency of Fdx1 did not further increase upon prolonged RNAi treatment, and was comparable to that reported in our study. Moreover, it was not explained why Fdx2 which still should be functional in Fdx1-depleted cells does not support Fe/S protein assembly. Notably, overexpression of Fdx1 did not complement the Fe/S-related defects arising upon depletion of Fdx2 [64]. Conspicuously, the levels of Fdx2 in Fdx1-depleted cells were not tested in Ref. [65] by immunostaining, and, no functional complementation was performed to show the specificity of the effects. In vitro reconstitution of the function of ferredoxin in Fe/S protein biogenesis might help to clarify the specificities of Fdx1 and Fdx2 in this process.

In keeping with the important function of the Isu1 scaffold protein, mutations in human ISCU cause a genetic disease. A point mutation in the *ISCU* gene results in a muscle-specific splicing defect and low levels of ISCU protein in affected muscle cells. This causes a myopathy with exercise intolerance [83,84]. The mutation, heterozygously combined with a missense point mutation leading to a truncated ISCU protein, resulted in a more severe, progressive phenotype with cardiomyopathy [85]. Mice homozygous for deletion of the *ISCU* gene show early embryonic lethality [86]. On the basis of the important function of ISCU and its indispensable function for life, it is surprising how comparatively weak the phenotype of the affected patients is. Affected tissues showed an iron accumulation [83,85,86], consistent with the phenotypical effects resulting from a defective mitochondrial Fe/S protein biogenesis in yeast and human cells [12,55]. In other cell types the splicing abnormality resulting from the *ISCU* mutation was observed at lower efficiencies showing the muscle-specific phenomenon of the splicing variation [86,87]. Recently, the splicing factor RBM39 and the RNA binding protein IGF2BP1 were shown to shift the splicing ratio toward the incorrectly spliced form [88]. This finding may eventually explain the tissue specificity of this phenotype.

3.2. The role of frataxin in the de novo Fe/S cluster synthesis on Isu1 in mitochondria

Frataxin (yeast Yfh1) is deficient in the neurodegenerative disease Friedreich's ataxia, and in the last decade numerous studies have been performed to clarify its molecular function. The work on frataxin pathology and function has been reviewed intensely (see, e.g., Refs. [25,26,89,90]). Here, we concentrate on a potential scenario how the protein may functionally contribute to the process of Fe/S protein biogenesis. To date, no clear consensus has been reached on the mechanistic role of this protein, even though it is well accepted that the primary function of this protein both in yeast and human cells is in Fe/S protein biogenesis. This is supported by earlier direct functional studies depleting the protein in yeast and mammalian cells [91–94], by physical interactions of frataxin with Nfs1, Isd11 and Isu1 [69,77,95,96], and by recent in vitro studies showing that frataxin is required for Nfs1–Isd11 desulfurase function during in vitro Fe/S cluster synthesis on the Isu1 scaffold protein [97].

Frataxin deficiency leads to a defect in Fe/S cluster synthesis on Isu1 suggesting that the protein acts early in the pathway [56] (Fig. 1). In contrast to the other early-acting ISC components (see

above) yeast frataxin is not essential for viability, whereas humans with only 30% of frataxin compared to healthy individuals develop Friedreich's ataxia and eventually die. In mice, deletion of the *FRDA* gene is embryonically lethal [91]. The bacterial homolog of frataxin, CyaY, is not contained within the *isc* operon, and no severe phenotype is obvious upon its deletion in *Escherichia coli* [98]. This indicates that at least in yeast and bacteria the function of frataxin can bypassed without the loss of cell viability. The non-essential function of yeast Yfh1 was recently supported by the identification of a point mutation in Isu1 as a suppressor of the growth defect of yfh1Δ yeast cells [99]. The mutation close to one of the conserved Cys residues of Isu1 almost fully restores Fe/S protein biogenesis and normalizes mitochondrial iron levels. The non-essential character of yeast and bacterial frataxin shows that this ISC assembly component can improve the efficiency of Fe/S cluster synthesis but is not absolutely necessary.

What might be the molecular function of frataxin in Fe/S cluster synthesis? Several lines of evidence suggest that frataxin may serve as the iron donor for this reaction. The idea that frataxin performs a direct role in iron metabolism came from the iron accumulation phenotype of cells lacking yeast Yfh1 or mammalian frataxin, and from the finding that isolated frataxin may bind iron at an acidic ridge with reasonable affinity [26]. An unambiguous in vivo confirmation for the iron binding has so far not been obtained. However, Yfh1 was reported to interact with Nfs1–Isu1 in an iron-stimulated fashion [69,77], but this property is not reproduced with mouse proteins recombinantly expressed in *E. coli* [96]. However, an iron-dependent interaction between purified Isu1 and frataxin has been reported [100,101] (Fig. 1). A direct transfer of frataxin-bound iron to acceptor proteins has been documented [100], but the physiological relevance of these biochemical observations remains unclear, the more so as frataxin-bound iron can also be used for heme formation [102,103], even though the protein is dispensable for this function in vivo [93,104]. Another, yet indirect observation that frataxin might have an iron-related function, came from studies overproducing the iron storage protein ferritin in human mitochondria deficient in frataxin. The severe phenotypes of frataxin-deficient cells were ameliorated by mitochondrial ferritin [105]. The relevance of this observation for the mechanistic function of frataxin remains unclear. Consistent with a physiological role for iron binding to frataxin, the simultaneous mutation of some amino acid residues at an acid α-helical ridge of frataxin creates a phenotype similar to a complete functional loss of frataxin, i.e. an Fe/S protein defect, an induction of the iron regulon and a mitochondrial iron accumulation [106,107]. The iron binding affinity is weak (micromolar range) and the coordination chemistry is somewhat unusual in that iron binding occurs on the protein surface at acidic amino acid residues rather than histidine and cysteine residues [26,108]. *Bacillus subtilis* contains a structural relative of CyaY termed YdhG which recently has been shown to be involved in cellular iron metabolism [109,110]. Deletion of its gene is associated with severe phenotypes including a decrease in cellular aconitase activity. While these data may provide a hint for the physiological relevance of the frataxin–iron connection, one has to keep in mind that *B. subtilis* does not contain the ISC assembly but rather the SUF system for Fe/S protein assembly [38,111,112]. A regulatory interaction of YdhG and SufS, the cysteine desulfurase of the SUF system, remains to be documented.

Recent elegant biochemical work by the group of Barondeau has provided an alternative suggestion for frataxin function which is not necessarily mutually exclusive with the idea of frataxin being an iron donor [97]. In a reconstituted system of de novo Fe/S cluster formation it was found that frataxin was necessary for stimulating the activity of cysteine desulfurase. When human Nfs1–Isd11 was incubated with frataxin and Isu1 in the presence of cysteine, sulfide production was

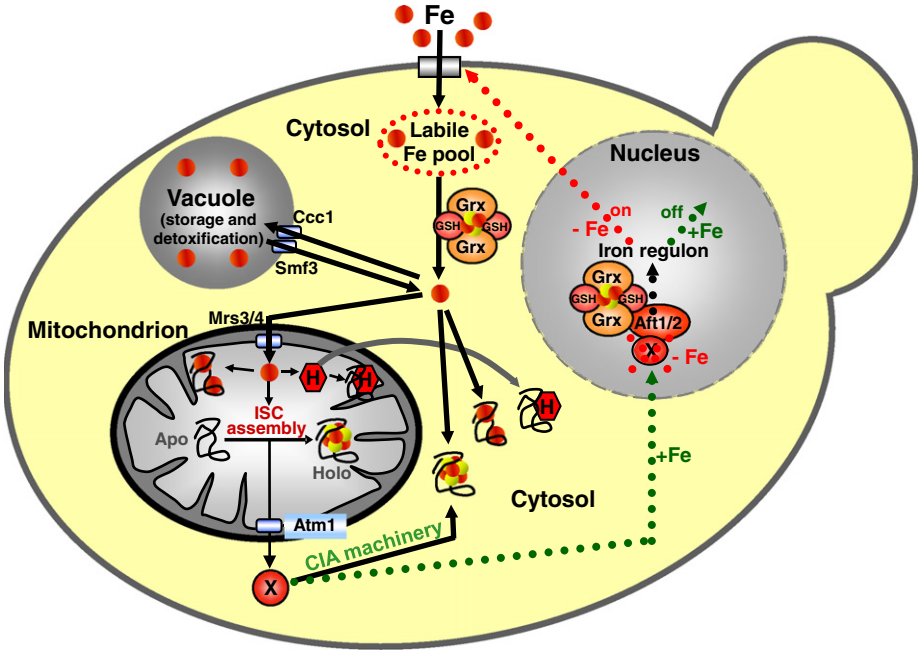

Fig. 3. A model for intracellular iron trafficking and sensing in *S. cerevisiae*. Iron acquired from the environment through plasma membrane iron or siderophore transporters enters the cytosol, where it binds to diverse low molecular mass compounds. From this "labile iron pool" the iron is removed by multi-domain monothiol glutaredoxins Grx3–Grx4 (Grx) which bind a bridging, glutathione-coordinated [2Fe–2S] cluster (red and yellow circles). Grx facilitate the delivery of iron to cytosolic iron-dependent enzymes such as various Fe/S proteins, iron binding proteins and to various intracellular compartments. Mitochondria import iron by the carriers Mrs3–Mrs4 and use it for Fe/S and di-iron proteins and for heme (H) synthesis. Vacuoles are a storage and detoxification compartment that import iron via Ccc1 and export it via Smf3. In the absence of Grx or its bound Fe/S cluster, iron accumulates in the cytosol but is not biologically available. The Grx-bound Fe/S cluster functions as a sensor for the iron-responsive transcription factor Aft1 (and possibly Aft2) signaling the status of the cytosolic iron pool. In addition, Aft1 responds to the levels of a mitochondria-supplied molecule (X) that transmits the iron status of mitochondria. This molecule is produced by the mitochondrial ISC assembly machinery, exported by the ABC transporter Atm1, and also required for the maturation of cytosolic-nuclear Fe/S proteins by the CIA machinery. In the presence of X or the holo form of Grx (+Fe), Aft1 dissociates from the promoters and is exported into the cytosol. In the absence of either X or the Fe/S cluster on Grx (−Fe), Aft1 constitutively activates transcription of multiple genes involved in cellular iron uptake, the so-called iron regulon. Abbreviation: GSH, glutathione.

largely increased. The addition of iron further improved sulfide generation indicating that only the complete system of Nfs1–Isd11, Isu1, frataxin, cysteine and iron allowed the desulfurase to be fully active. This observation is compatible with frataxin being a regulator of the cysteine desulfurase permitting toxic sulfide production only when all components needed for Fe/S cluster synthesis are present, i.e. the Isu1-bound Fe/S cluster can be formed. Hence, frataxin was proposed to be an allosteric switch. These findings are relevant for Friedreich's ataxia, as frataxin variants carrying patient mutations are less effective in stimulating the desulfurase activity, in some cases because binding of frataxin to the Nfs1–Isd1 and Isu1 complex is decreased [113,114].

In a similar in vitro reconstitution system with bacterial IscS desulfurase and IscU scaffold proteins the frataxin homolog CyaY had opposite effects, i.e. inhibited rather than stimulated the desulfurase activity [115]. The physiological meaning of this finding is difficult to reconcile because neither deletion nor overproduction of CyaY in *E. coli* or Salmonella has a substantial effect on growth and the efficiency of Fe/S protein formation in vivo [98,116]. However, when combined with other lesions of genes possibly involved in Fe/S protein biogenesis, substantial effects on various Fe/S protein activities can be observed providing a first in vivo hint that CyaY might contribute to this process in bacteria as a non-essential component. The puzzle of stimulating and inhibitory effects of eukaryotic and bacterial frataxin, respectively, was recently addressed in vitro [117]. By mixing the relevant bacterial and mitochondrial ISC components it was seen that surprisingly the desulfurase and not frataxin is responsible for the stimulatory or inhibitory effects. Thus, it was concluded that frataxin in general serves a regulatory role which was changed in evolution from an inhibitory effect of the frataxin–desulfurase interaction in bacteria to a stimulatory function in eukaryotes. Together, these observations point to a role of frataxin as an allosteric regulator of cysteine desulfurases.

3.3. Dislocation of the Fe/S cluster from the Isu1 scaffold protein to target apoproteins by a dedicated chaperone system and a glutaredoxin

After the initial phase of Fe/S cluster synthesis on Isu1, the cluster has to be released from the scaffold, transferred to apoproteins and inserted into the polypeptide chain. Recent studies have shown that this is a more complex reaction than initially thought and involves at least ten different ISC assembly proteins. The entire process can currently be subdivided into two major steps, i) the release and transfer of the Isu1-bound Fe/S cluster to intermediate proteins that transiently bind the cluster, and ii) the apoprotein-specific Fe/S cluster insertion into the polypeptide chain (Fig. 1). The first step is executed by the dedicated chaperone system comprising Ssq1, Jac1, and Mge1 as well as the monothiol glutaredoxin Grx5. They belong to the core ISC assembly system since these ISC proteins are generally required for biogenesis of all mitochondrial Fe/S proteins. The remainder proteins are termed ISC targeting factors as they exhibit substrate specificity for subsets of Fe/S proteins. We will first discuss the function of the chaperones and Grx5 in some detail, before we explain the roles of the ISC targeting factors terminating the assembly process.

Depletion of the ISC components Ssq1, Jac1 and Grx5 leads to an accumulation of iron, possibly in the form of an Fe/S cluster, on Isu1 [56]. This suggests that these proteins are not required for Fe/S cluster synthesis on Isu1, but rather for efficient dissociation of the cluster to finally become inserted into apoproteins (Fig. 1). The conclusion that the ATP-dependent Hsp70 chaperone Ssq1 and its co-chaperone Jac1 are not involved in the de novo synthesis of the Fe/S cluster on Isu1 was later confirmed in vitro using purified proteins [76]. The working cycle of mitochondrial Ssq1–Jac1 and their bacterial orthologs Hsc66–Hsc20 has been worked out in the laboratories of Craig–Marszalek and Vickery, respectively. The mechanism turned out to be similar to that of other well-studied Hsp70 (DnaK) and Hsp40 (DnaJ) chaperone systems [118], yet only in mitochondria the reaction cycle additionally requires the nucleotide exchange factor Mge1 to efficiently replace ADP for ATP at the Hsp70 (Fig. 2). An atypical feature of the ISC-specific mechanism is the substrate specificity of Ssq1–Hsc66. Instead of binding to hydrophobic stretches of unfolded proteins, the peptide binding domain of Hsp70 recognizes the conserved LPPVK sequence of Isu1 (or bacterial IscU). This is believed to induce a conformational change in Isu1–IscU that labilizes the binding of the bound [2Fe–2S] cluster and prepares it for its dissociation. Direct confirmation of this idea was obtained for the bacterial proteins in spectroscopic studies to demonstrate the acceleration of Fe/S cluster transfer from IscU to target apoproteins [119,120]. In the mitochondrial system this Fe/S cluster transfer reaction can occur efficiently without further assistance in vitro, but is fully dependent on the Hsp70 chaperones in vivo [56]. The lability of Fe/S cluster binding to mitochondrial Isu1 so far precluded a more detailed analysis of the role of the eukaryotic chaperones in Fe/S cluster transfer from Isu1 to acceptor proteins in a purified system.

The co-chaperone Jac1 (mammalian HSC20; Table 1) helps recruiting Isu1 in binding to Hsp70 (Fig. 2). Binding of Jac1 to Isu1 presumably occurs in its Fe/S cluster-bound form, and then engages contacts with the ATP-bound form of Ssq1. Interaction of the Hsp70 with Isu1 triggers its ATPase, and in the ADP-bound form the Isu1 scaffold is stably associated. In this configuration the Fe/S cluster may be labilized and transferred toward apoproteins. This partial reaction is accompanied by the Mge1-assisted exchange of ADP for ATP which then triggers the dissociation of Isu1 from Ssq1. In its ATP-bound form the Hsp70 is ready for the next cycle of Jac1–Isu1 (or Hsc20–IscU) binding.

The specialized chaperone Ssq1 is present only in a few fungal species, and with high preference binds to its cognate peptide LPPVK of Isu1 rather than other typical Hsp70 target peptides [121]. In all other eukaryotes the generic mitochondrial Hsp70 (fungal Ssc1 or mammalian GRP75; Table 1) performs this function in Fe/S cluster biogenesis in addition to its essential roles in mitochondrial protein import and folding. A thorough evolutionary analysis revealed that Ssq1 arose in the fungal lineage by gene duplication, and hence it is not orthologous to bacterial Hsc66, even though these proteins are mechanistically highly similar [121]. Another analysis revealed that the co-chaperone Jac1 co-evolved with Ssq1, the specialized Hsp70 arising from a gene duplication [122]. All Ssq1-containing fungi encode a slightly shorter form of Jac1 that has optimized the interaction with Ssq1. In general, the co-chaperone Jac1 confers the specificity for the recognition of the ISC protein Isu1 in eukaryotes lacking Ssq1. The binding surface of Jac1 for Isu1 association has recently been mapped and found of importance for cell viability documenting the physiological relevance of the recruiting of Isu1 by Jac1 for efficient Hsp70 binding [123].

The conserved function of HSC20 in human cells was recently reported [124,125]. Aside from the expected impairment of mitochondrial and cytosolic Fe/S proteins, depletion of HSC20 by RNAi technology caused a massive alteration in cellular iron metabolism (see below). This is explained by the general role of the mitochondrial ISC machinery in cytosolic Fe/S protein assembly including IRP1. A mechanistically unexplained iron-dependent interaction of Jac1 with frataxin was reported in one study, but the functional relevance remains to be determined [125]. Human HSC20 can functionally replace yeast Jac1 and interacts with Isu1 and GRP75, the cognate human mitochondrial Hsp70 [124] (Fig. 2). The functional role of GRP75 has not been studied yet. Since the protein is also involved in mitochondrial preprotein import and folding, the effects of its depletion are expected to be complex. Nevertheless, one can safely assume that the role of the Hsp70 chaperone co-chaperone system in mitochondrial Fe/S maturation is conserved.

Another core component with a role in mitochondrial Fe/S protein maturation is the monothiol glutaredoxin Grx5. Deletion of its gene in yeast is not lethal, unlike that of most other core ISC assembly genes [126]. Grx5-depleted cells show a severe oxidative-stress phenotype, a condition which is known to lead to severe damage of Fe/S clusters

such as that of aconitase. Strikingly similar phenotypes are observed upon deletion of *SOD2*, encoding the mitochondrial matrix superoxide dismutase. Therefore, assuming a direct role of Grx5 in Fe/S protein biogenesis from defective Fe/S protein activities may not be fully convincing and conclusive. The possibly best arguments for a direct participation of Grx5 in this process are i) the accumulation of Fe/S clusters on Isu1 and ii) the iron overload of mitochondria in the absence of Grx5, similar to what is seen for depletion of Ssq1 and Jac1 [56,126]. Moreover, a recent study supported the function of Grx5 as an ISC assembly component showing that the Fe/S cluster accumulation on Isu1 is also observed under anaerobic conditions (M.A. Uzarska et al. unpublished). Grx5-depleted cells show almost wild-type growth under these conditions and have only a weak Fe/S protein deficiency suggesting that Grx5 is involved in a step after Fe/S cluster formation on Isu1, may be efficiently bypassed under these conditions, and may be particularly important under aerobic conditions.

What might be the exact molecular function of Grx5? Monothiol glutaredoxins are conserved in evolution and known to bind a glutathione-coordinated [2Fe–2S] cluster [127–129] (Fig. 1). Formation of this cluster depends on the core ISC assembly machinery including Isu1 and the chaperones (M.A. Uzarska et al. unpublished). Hence, it seems likely that this Fe/S cluster is first assembled on Isu1 and then transferred to Grx5 by the assistance of the chaperones. The transient character of Fe/S cluster binding and the ability to transfer it to target apoproteins have been taken to suggest that Grx5 assists Fe/S protein maturation by serving as a transient Fe/S cluster binding site before the cluster is inserted into apoproteins [128]. No in vivo confirmation for this hypothesis has been obtained so far. The dependence of both [4Fe–4S] and [2Fe–2S] proteins on Grx5 function indicates that it is a (non-essential) part of the core ISC assembly machinery required for all mitochondrial Fe/S proteins.

Functional inactivation of relatives of Grx5 in higher eukaryotes is associated with severe effects and displays characteristic phenotypes. Morpholino-mediated depletion of Grx5 in Zebrafish leads to a severe hematological phenotype [130]. This was experimentally attributed to the role of the mitochondrial ISC assembly machinery in the assembly of the cytosolic Fe/S protein IRP1 which regulates, among other proteins, the expression of erythroid-specific δ-aminolevulinate synthase (ALAS2). In the absence of Fe/S cluster formation on IRP1, the apoprotein binds to iron-responsive elements (IREs) in the mRNA of ALAS2 and represses its translation thus adjusting the efficiency of heme synthesis to the available amounts of iron. Strikingly, morpholino-mediated depletion of IRP1 relieved the hematological phenotype showing that Grx5 is only indirectly responsible for this heme deficiency phenotype. In humans a mutation in Grx5 is associated with sideroblastic microcytic anemia [131]. The affected patient showed an iron overload and ringed sideroblasts (i.e. iron-loaded mitochondria; [132]) suggesting that the functional defect of this human ISC component has a severe effect on cytosolic iron regulation. This is indicated by an increase of transferrin receptor and a decrease of both ferritin and ALAS2, thus mimicking the Zebrafish phenotype. The phenotypes of the Grx5 patient were recently reproduced in a cell culture system in which Grx5 was depleted by RNAi [133]. Grx5-depleted cells were defective in mitochondrial and cytosolic Fe/S proteins, and showed mitochondrial iron overload and a deregulation of cellular iron homeostasis, i.e. typical signs of an ISC assembly defect. Collectively, the previous reports on Grx5 show that the protein is a core ISC assembly component involved in the transfer of the Fe/S cluster from Isu1 to later stages of the maturation process, but its precise function remains to be clarified.

3.4. The specific role of ISC targeting components in the synthesis of [4Fe–4S] clusters and the assembly of different subsets of apoproteins

The remaining ISC components appear to perform a more specific role in Fe/S protein biogenesis. Conspicuously, they are not required for the biogenesis of mitochondrial [2Fe–2S] proteins suggesting that the core ISC components discussed above are sufficient for the maturation of these proteins (Fig. 1). The generation of [4Fe–4S] clusters critically depends on the A-type ISC proteins Isa1 and Isa2 and the interacting protein Iba57 in both yeast and human cells [66,134–136]. Their depletion is associated with highly similar phenotypes including a lack of all mitochondrial [4Fe–4S] proteins and eventually a loss of mitochondrial DNA, probably as a result of the impairment of mitochondrial aconitase and lipoic acid synthase. None of the mitochondrial [4Fe–4S] proteins performs an essential function for yeast cell viability, fitting well to the non-essential phenotype of *ISA* and *IBA57* gene deletions. In Trypanosomes the Isa proteins are essential for viability in the procyclic stage but not in the blood stream stage [137]. Isa1 and Isa2 perform a non-redundant role and are known to function as a hetero-oligomer, because they cannot functionally replace each other. However, any of the close bacterial relatives, IscA, SufA and ErpA, can complement yeast Isa1, but not Isa2 mutants [66]. The Isa proteins have been shown to interact with Grx5 [126,138], but the functional implication of this finding is unknown.

Cytosolic Fe/S proteins can also be affected by Isa protein depletion both in yeast and human cells, at least under certain growth conditions, but this has recently been shown to likely be an indirect consequence of the severe phenotype of Isa protein depletion in mitochondria. For instance, the indirect character of the Isa protein influence on the cytosol is seen from the lack of an induction of the iron regulon in yeast and from hardly any increase of mitochondrial iron upon their functional impairment [66,137]. The most likely scenario is that the oxidative stress prevailing in Isa- and Iba57-depleted cells leads to destruction of the Fe/S clusters of sensitive proteins such as cytosolic yeast Leu1 and human IRP1. These particular proteins contain a [4Fe–4S] cluster that is coordinated by three rather than four amino acid ligands and hence are more sensitive to damage.

In human cells RNAi depletion of the Isa and Iba57 proteins creates an eye-catching morphological phenotype on mitochondria [136]. They are at least threefold enlarged and lack cristae membranes. This is likely caused by the defective biogenesis of the Fe/S-cluster-containing respiratory complexes I and II and further phenotypic effects arising from the lack of important mitochondrial enzymes such as aconitase and lipoate synthase. Strikingly, the human [2Fe–2S] cluster-containing enzyme ferrochelatase was functional under Isa and Iba57 deficiency and the heme content was not significantly changed indicating that also in human cells the Isa and Iba57 proteins are specifically involved in the maturation of [4Fe–4S] proteins [136].

The precise molecular function of the A-type ISC proteins and of Iba57 is unknown to date. A recent study in yeast showed that both Isa1 and Isa2 bind Fe in vivo [66]. Similar findings were made for human Isa1 after expression and purification from *E. coli* [139]. Since sulfide was not found associated with these proteins, it was concluded that the Isa-bound Fe is not part of an Fe/S cluster [66]. In the absence of yeast Iba57 or upon deletion of the major yeast mitochondrial Fe/S protein aconitase, the Isa proteins show increased iron binding suggesting that Iba57 may be involved in the displacement of the iron and potentially in its use for the synthesis of [4Fe–4S] clusters [66]. The presence of iron in mitochondrial Isa proteins matches a few reports on *E. coli* IscA which have found iron bound to this protein [140]. It was speculated that the IscA-bound iron may be used for assembly of the Fe/S cluster on IscU. This mechanistic pathway can be excluded in yeast, since Isa1–Isa2 are not required for Fe/S cluster loading of Isu1 [66]. Vice versa, iron binding to Isa1–Isa2 does not require the function of Isu1 providing a strong criterion that the iron is not part of the Fe/S cluster. These findings suggest that the Isa and Iba57 proteins act late in Fe/S protein biogenesis facilitating the generation of a [4Fe–4S] cluster from the [2Fe–2S] clusters originally assembled on Isu1. In contrast, other groups have provided convincing evidence that the bacterial A-type ISC protein SufA binds

an [2Fe–2S] cluster in vivo [141]. Since this cofactor can easily be inserted into purified Fe/S apoproteins, bacterial A-type proteins are believed to function as Fe/S cluster transfer proteins (also termed carrier proteins) that mediate the delivery of Fe/S clusters from their site of synthesis on a scaffold protein to its final destination [142]. The presence of the [2Fe–2S] cluster on both A-type proteins and Isu1 may suggest a simple mechanism for the generation of [4Fe–4S] clusters, namely the fusion of these two [2Fe–2S] clusters. Such a reaction has been observed for two IscU-bound [2Fe–2S] clusters in vitro [143,144]. It remains unclear which of the two iron binding states may be physiologically relevant for the A-type ISC protein-catalyzed [4Fe–4S] cluster formation. Currently, it is not even excluded that the A-type ISC proteins support even diverse mechanisms. In conclusion, the Isa and Iba57 proteins are essential biogenesis factors for virtually all mitochondrial [4Fe–4S] proteins. How the Isa and Iba57 might operate in molecular terms, what the precise function of the Isa-bound iron may be, and what the contribution of the Iba57-bound tetrahydrofolate cofactor [145] may be will require further studies.

The function of the ISC protein Nfu1 was long unknown because deletion of its gene in yeast is not associated with major effects [12]. Mutant cells show a slight growth defect on non-fermentable carbon sources only, and tested Fe/S proteins are affected only mildly. Double deletion of *NFU1* and *ISU1* was associated with more pronounced effects documenting that the Nfu1 protein can be linked to mitochondrial ISC protein biogenesis. A role in this process had previously been suggested from the sequence similarity of a 70 amino acid residue long domain of Nfu1 and the C-terminus of bacterial NifU, a multi-domain protein functioning as a scaffold for Fe/S cluster synthesis during nitrogenase maturation (Table 1). NifU also encodes a portion with homology to Isu1 at its N-terminus [146]. Mechanistic insight into Nfu1 function and the stage of involvement in the Fe/S pathway came from patients harboring mutations in the Nfu1 gene [147,148]. Affected individuals were born without evident symptoms, but soon after birth they displayed signs of severe developmental retardation, brain abnormalities and pulmonary hypertension, eventually leading to death between about 3 months [147] to around one year [148] after birth. Genetic analysis showed a non-sense mutation creating a premature stop codon or a G→C point mutation leading to a Gly to Cys exchange, respectively, in these patients. The biochemical investigation showed no evident abnormalities in the Fe/S protein aconitase, but striking defects in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, a hyperglycinemia and an increase in organic ketoacids. All these phenotypes point to a defect in the Fe/S protein lipoate synthase, an enzyme required for synthesis of lipoic acid, a cofactor required for the four mitochondrial enzymes pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain ketoacid dehydrogenase and the glycine cleavage system [149]. This evident phenotype was not discovered earlier, since lipoate synthase is not routinely assayed during Fe/S protein biogenesis studies. RNAi-depletion of Nfu1 in human cell culture revealed striking defects in the activity of pyruvate and α-ketoglutarate dehydrogenases and low levels of lipoic acid attached to the E2 subunits of these enzymes and to the glycine cleavage protein H [147,148]. Additionally, a severe complex II (succinate dehydrogenase, SDH) defect was detected, and, in the Canadian study, also a respiratory complex I defect. The less severe phenotype of the Spanish patients is easily explained by a residual function of the mutated Nfu1 protein seen in complementation studies in yeast [148], while the Canadian individuals contain no Nfu1. From these data one can assign a role to Nfu1 in the assembly of complex Fe/S proteins (respiratory complexes I and II and lipoate synthase) containing eight, three and two Fe/S clusters, respectively.

Where in the ISC assembly pathway might Nfu1 act? Earlier studies had proposed that human Nfu1 may act as a scaffold protein alternatively to Isu1, because Nfu1 can assemble a labile [4Fe–4S] cluster in vitro and pass it on to apoproteins [150]. Plant homologs are residing in plastids and mitochondria [151,152]. A plastid-localized homolog termed Nfu2 was shown to be required for photosystem I and ferredoxin maturation, but not for other Fe/S proteins such as Rieske Fe/S protein and glutamate synthase [151,152]. These observations and the human patient phenotype (see above) thus pointed to some Fe/S target protein specificity for Nfu1 function. In striking contrast, when human Isu1 was depleted by RNAi technology, virtually all mitochondrial and cytosolic Fe/S proteins were severely affected [148]. Strong evidence for a potential function of Nfu1 consecutively to Isu1 was obtained from yeast studies. While wild-type Nfu1 did not show any detectable iron binding in vivo, a Nfu1 variant carrying the patient mutation did. This iron association was dependent on Nfs1 function suggesting that the bound iron is part of an Fe/S cluster. Importantly, Isu1 depletion abolished Fe/S cluster association on mutant Nfu1 showing that the Isu1-bound Fe/S cluster may be transferred to Nfu1 where it is possibly only transiently bound before cluster delivery to specific target proteins. These results suggest a target-specific function of Nfu1 in the transfer of Fe/S clusters from Isu1 to dedicated Fe/S proteins such as lipoate synthase and SDH (Fig. 1). Hence, Nfu1 qualifies as a specific ISC targeting factor which transiently binds [4Fe–4S] clusters and hands them over to its target proteins. Conspicuously, Fe/S cluster association to Nfu1 in vivo occurred independently of the Isa proteins, an observation that may indicate that Nfu1 can facilitate the generation of [4Fe–4S] clusters on its own [148]. While the site of action of Nfu1 late in the ISC pathway became clear, further insights are needed to unravel its precise molecular function in the maturation of complex Fe/S proteins.

A phenotype similar to that of Nfu1 patients was reported for individuals with a mutation in the gene *BOLA3*, encoding a mitochondrial protein of the BolA protein family [147]. Patients and cultured cells in which BOLA3 (yeast homolog Aim1) was depleted by RNAi show defects in lipoic acid-containing proteins and in respiratory complexes I and II (Fig. 1). Based on the interaction of the BolA-like yeast cytosolic protein Fra2 with monothiol glutaredoxins (see below) and the bacterial BolA with glutaredoxin GrxD [153] it may be speculated that BOLA3 interacts with Grx5. No proof for this idea and no information on the precise molecular role of BOLA3 and its putative yeast relative Aim1 have been published to date (Table 1).

A specific role in Fe/S protein biogenesis has been described for the mitochondrial P-loop NTPase Ind1. Deficiency of this protein in the yeast *Yarrowia lipolytica* or in human cells specifically affected respiratory complex I assembly, while other mitochondrial Fe/S proteins were not or only indirectly affected [154,155] (Fig. 1). The protein is similar in sequence to the two scaffold proteins of the CIA machinery Cfd1 and Nbp35, and shares about 40% identical amino acid residues with these proteins. Cfd1–Nbp35 form a hetero-tetramer and bind a bridging [4Fe–4S] cluster which is transferred to apoproteins with the help of further CIA proteins [44,53,156]. Similarly, Ind1 can bind the [4Fe–4S] cluster at two conserved Cys residues at the C-terminus, presumably also in a bridging coordination as Cfd1–Nbp35. Fe/S cluster assembly on Ind1 depends on Nfs1 and Isu1 suggesting that its cluster is received from Isu1 [154]. This makes it unlikely that Ind1 serves as a general scaffold protein like Cfd1–Nbp35. Rather, Ind1 may be a specific ISC targeting factor transiently binding the Fe/S cluster and inserting it into at least one of the six [4Fe–4S] cluster binding sites of complex I. In that sense Ind1 may play a similar role as Nfu1 discussed above. How Ind1 may function in molecular terms remains to be determined.

Depletion of human IND1 (also called NUBPL; Table 1) causes morphological changes of mitochondria, with enlarged organelles that have lost their cristae membranes [155]. As a result of the complex I deficiency, the respiratory supercomplexes are rearranged. The appearance of a subcomplex corresponding to part of the membrane-spanning arm of complex I fits well the proposed assembly function of Fe/S clusters in the matrix-exposed electron transfer arm of complex I. Human IND1 has recently been identified in a high-throughput sequencing screen searching for mutations causing mitochondrial complex I deficiency [157]. Affected patients presented with mitochondrial encephalomyopathy. The decreased complex I activity in patient fibroblast could be restored to

almost wild-type levels by introducing the wild-type DNA of *IND1* confirming that the mutations in the *IND1* gene are responsible for the complex I defect and the disease phenotypes.

The common feature of Nfu1 and Ind1, both binding [4Fe–4S] clusters, may be that they accept this cluster from earlier components of the ISC assembly machinery and transfer it to specific target proteins such as lipoate synthase and complex II (Nfu1) or complex I (Ind1), proteins which appear to be specific targets of both ISC factors. How the assembly of the Fe/S cluster into the polypeptide chain takes place, how the coordinating cysteine residues are shielded before Fe/S cluster arrival, and whether the ISC targeting factors physically bind to their target proteins, remain to be clarified.

4. The role of the mitochondrial ISC assembly system in the regulation of cellular iron homeostasis

In this chapter we discuss in detail the critical role of the ISC assembly and export systems for maintaining cellular iron homeostasis. We first address the potential causes for the mitochondrial iron overload observed during ISC defects. We then summarize the current knowledge of the mechanisms of cellular iron regulation in fungi followed by a discussion of the rather different pathways in higher eukaryotes. Finally, we review the dual function of cytosolic multidomain monothiol glutaredoxins in cellular iron uptake regulation and intracellular iron delivery.

4.1. Mitochondrial iron overload—a pathologic event induced by mitochondrial ISC assembly defects

Research in recent years has shown that the mitochondrial ISC assembly and export systems serve as key regulators of cellular iron homeostasis in eukaryotes, as their status has a critical influence on cellular uptake of iron, and its intracellular distribution and utilization [20,31]. In *S. cerevisiae* and higher eukaryotes alike, defects in the mitochondrial Fe/S cluster assembly and export systems are associated with a substantial accumulation of iron within mitochondria [18]. In fact, for several key components of the mitochondrial ISC assembly and export systems this phenotype was described before their primary function in cellular Fe/S cluster maturation was identified [158–160]. In fungi, mitochondrial iron accumulation may be an un-physiological event as the vacuole serves as the natural iron reservoir [32,161]. In addition, certain filamentous fungi utilize intracellular siderophores for iron storage and intracellular distribution [162–164]. Nevertheless, increased iron flux into mitochondria might be used in times of iron shortage during the synthesis of Fe/S proteins. In higher eukaryotes, bulk intracellular iron is stored mainly in cytosolic ferritin and to a much lesser extent in the mitochondrial version of ferritin which is believed to function mainly in protection against iron-mediated oxidative stress [105,165–168]. *S. cerevisiae* cells with defects in the mitochondrial ISC systems accumulate mitochondrial iron in form of ferric (Fe^{3+}) phosphate nanoparticles that are detectable as electron-dense precipitates in electron micrographs [169–171]. These precipitates dissolve upon reduction and are not observed in cells cultivated under anaerobic conditions, despite the fact that severe cellular ISC assembly defects remain in the absence of oxygen. In wild-type cells, there is no evidence for the presence of higher amounts of ferric iron, indicating that these nanoparticles likely are an un-physiological dead-end rather than a hyper-accumulation of a physiologically relevant form of iron. In Friedreich's ataxia patients, most of the mitochondrial iron is present in the form of poorly organized ferrihydrite that is only partially associated with mitochondrial ferritin [165].

In *S. cerevisiae* with artificially increased iron uptake systems by constitutive expression of the iron-responsive transcription factor Aft1 (see below), iron accumulates mainly as a soluble mononuclear ferric iron species that is localized outside mitochondria, most likely in the vacuole [171,172]. Upon supplementation with additional iron, a fraction accumulates as mitochondrial nanoparticles similar to those found in ISC mutants while the majority of iron is exported into the vacuole [171]. Hence, the induction of cellular iron uptake systems alone is insufficient to cause the mitochondrial iron accumulation observed in ISC assembly mutants. Based on mouse models of Friedreich's ataxia, mitochondrial iron accumulation in ISC mutants was suggested to be caused by increased expression of the mammalian mitochondrial iron importer mitoferrin 2, which facilitates mitochondrial iron influx [173]. In *S. cerevisiae* the mitoferrin ortholog Mrs4 is induced upon iron limitation and upon defects in the mitochondrial ISC assembly and export systems (Figs. 1 and 3) [172,174,175]. Deletion of *MRS4* and its paralog *MRS3* cures mitochondrial iron accumulation in yeast cells in which the frataxin gene is deleted [176]. In addition, overproduction of the vacuolar divalent metal transporter Ccc1 that exports cytosolic iron into the vacuole also prevents mitochondrial iron accumulation in ISC mutants [177]. The sum of these data strongly suggests that mitochondria import iron from the cytosol in a reaction mediated by Mrs3–Mrs4 or mitoferrin (Figs. 1 and 3). Nevertheless, whether elevated Mrs-mitoferrin levels are indeed the cause of mitochondrial iron accumulation in cells with defective mitochondrial ISC systems remains to be demonstrated. The fact that the overproduction of Mrs4 in *S. cerevisiae* or mitoferrin in fruit fly causes an improved maturation of extra-mitochondrial Fe/S proteins rather than mitochondrial iron overload may argue against this idea [178,179].

4.2. Role of the mitochondrial ISC systems in the regulation of cellular iron homeostasis in fungi

S. cerevisiae cells depleted of members of the mitochondrial ISC assembly and export systems display a strong transcriptional remodeling with a substantial overlap to the transcriptional response during iron deprivation [171,173,174,180]. In these cells, virtually all iron-dependent cellular pathways are deregulated in the same direction as in iron-deprived cells. Most prominently, this response includes the induction of the yeast iron regulon, a set of some 40 genes encoding proteins with functions in reductive and siderophore-mediated cellular iron uptake and in intracellular iron distribution to various compartments [31,32] (Fig. 3). Their expression is under the control of the iron-responsive transcription factors Aft1 and Aft2 [181–184]. Both transcription factors have overlapping functions, but Aft1 preferentially controls the expression of iron uptake genes, while Aft2 is more responsible for the expression of genes involved in intracellular iron distribution [185]. *S. cerevisiae* Aft1 and Aft2 belong to the class of eukaryotic WRKY and GCM1 zinc finger proteins that are restricted to a small group of ascomycete yeasts including *Candida glabrata* and *Kluyveromyces lactis* [186,187]. Despite the fact that Aft1 is among the best-studied iron-responsive transcription factors in eukaryotes, its molecular mode of iron-sensing remains unknown. Aft1 shuttles between the cytosol and nucleus in an iron-responsive manner and acts as a transcriptional activator under iron-limiting conditions [188,189]. Nuclear export upon iron sufficiency is mediated by an iron-dependent interaction with the specialized nuclear exportin Msn5 [190].

In cells with defects in mitochondrial ISC assembly and export systems, Aft1 and Aft2 are constitutively activated [20,171]. Since the impairment of mitochondrial Fe/S cluster formation is not associated with the depletion of cytosolic iron levels, it has been proposed that Aft1 and Aft2 require a signal molecule that is produced and exported by the mitochondrial ISC machineries and is essential for proper iron sensing [191,192]. The nature of this molecule is unknown, but it is crucial for the deactivation of Aft1 and Aft2 under iron sufficiency. Most likely, this signal molecule is identical to the molecule exported by the mitochondrial inner membrane ABC transporter Atm1 that is required for the maturation of extra-mitochondrial Fe/S proteins. Consistent with this conclusion, defects in the specialized ISC targeting

components such as Isa and Iba57 proteins, that are dedicated to the assembly of mitochondrial [4Fe–4S] proteins, do not elicit any signs of a deregulated iron homeostasis [66,134]. The impact of the mitochondrial ISC systems on the regulation of cellular iron homeostasis seen in *S. cerevisiae* is conserved in other fungi [193,194]. This is remarkable as most fungi utilize iron-responsive transcription factors that are structurally unrelated to Aft1 and Aft2 from *S. cerevisiae* (see below).

S. cerevisiae with defects in mitochondrial ISC system show a massive transcriptional down-regulation of genes encoding proteins of the mitochondrial respiratory chain and the citric acid cycle together with a transcriptional remodeling of biosynthetic pathways that involve iron-dependent enzymes [171,174,180]. A similar response is seen in iron-deprived cells, suggesting that yeast adapts to iron deprivation by minimizing dispensable iron-dependent processes in order to liberate and spare iron for more essential tasks [31,195]. In *S. cerevisiae*, iron sparing is achieved by a combination of post-transcriptional mRNA degradation and transcriptional regulation via iron-responsive small molecules (Fig. 4). Post-transcriptional mRNA degradation is mediated by two conserved tandem zinc finger-containing mRNA-binding proteins, Cth1 and Cth2 [195–198]. Cth1 and Cth2 bind to specific AU-rich elements within the 3′-untranslated region on mRNAs of many iron responsive genes and promote mRNA degradation at cytoplasmic P-bodies under iron-limiting conditions. The *CTH2* gene, a member of the yeast iron regulon, is induced under iron-limiting conditions by Aft1 [185,199]. Virtually all genes that are repressed upon iron deprivation display an aberrant expression in cells lacking *CTH1* and *CTH2*, demonstrating the global significance of this process [195].

Transcriptional regulation via iron-responsive small molecules operates in most pathways involving iron-dependent enzymes [200]. In *S. cerevisiae*, the transcription of structural genes of metabolic pathways frequently depends on a single key regulatory intermediate that functions as a co-activator of a dominant transcription factor [201] (Fig. 4). For pathways involving iron-dependent enzymes, the level of this regulatory intermediate may be regulated by the enzymatic activity of iron-dependent enzymes of the particular pathway. Hence, upon iron limitation or upon defects in cellular Fe/S protein maturation or heme synthesis, the levels of these regulatory metabolites decline resulting in decreased transcription. For instance, transcription of leucine biosynthesis genes by the transcription factor Leu3 responds to the co-activating metabolite of leucine biosynthesis, α-isopropylmalate (α-IPM). IPM synthesis requires the upstream function of the mitochondrial Fe/S protein Ilv3 [201]. Further, the transcription of a large number of genes involved in respiration is directly responsive to cellular heme levels [202]. Cells with defects in the mitochondrial ISC systems are also heme-deficient, due to an indirect inhibition of ferrochelatase, the last enzyme in the biosynthesis of heme [103]. Hence, the expression of respiratory genes is low in these cells [171,200]. The iron-responsive expression of *CCC1* encoding the divalent metal transporter involved in iron import into the vacuole (Fig. 3) is controlled by both transcriptional and post-transcriptional mechanisms in *S. cerevisiae* [203,204]. Under conditions of cellular iron overload, *CCC1* is induced by the bZIP transcription factor Yap5 that is essential for survival under high iron conditions (Fig. 5A). Upon iron limitation, Yap5 is inactivated by an unknown mechanism resulting in

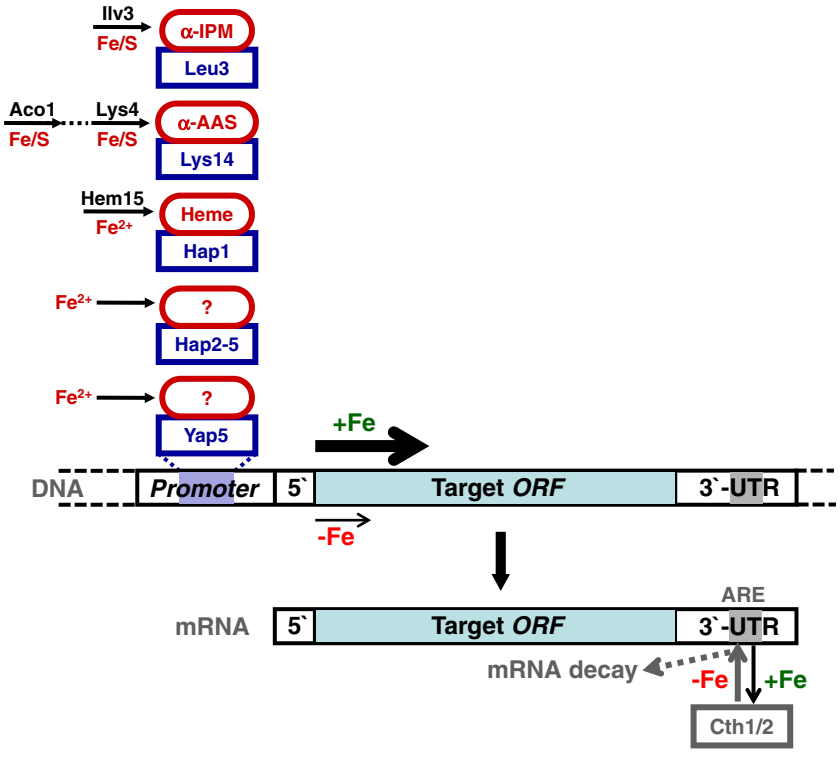

Fig. 4. Dual regulation of iron-responsive gene expression in *S. cerevisiae*. Top: Gene expression for a number of iron-dependent metabolic pathways is regulated by transcriptional activators (blue rectangles) that are co-activated by iron-dependent molecules (red ovals). During iron limitation the levels of these molecules, usually metabolites, decline resulting in a diminished expression of target genes. For instance, expression of leucine biosynthesis genes by Leu3 depends on the co-activator α-isopropylmalate (α-IPM) that requires the mitochondrial Fe/S protein Ilv3 for its synthesis. Only under iron sufficiency high levels of α-IPM are produced and leucine biosynthesis genes are expressed. Transcription of lysine biosynthesis genes by Lys14 makes use of the inducer α-aminoadipate semialdehyde (α-AAS) that requires the mitochondrial Fe/S proteins Aco1 and Lys4 for its synthesis. Activation of genes involved in respiration is controlled by the heme-activated transcription factor Hap1 and the Hap2–5 complex. Heme synthesis involves the iron-consuming ferrochelatase Hem15. The mode of the iron-dependent activation of Hap2–5 is unresolved. The molecule involved in activating Yap5 that controls the expression of *CCC1* encoding the vacuolar divalent metal transporter (see Fig. 3) is also unknown. Bottom: Iron-responsive post-transcriptional mRNA decay is triggered by the RNA binding proteins Cth1 and Cth2. Under iron-limiting conditions they bind to AU-rich elements (ARE) in the 3′-untranslated region (UTR) of mRNAs of certain iron-regulated genes and induce their degradation. Transcriptional (top) and post-transcriptional (bottom) regulatory mechanisms work in parallel on several genes of iron-dependent pathways that show a diminished expression under iron-limiting conditions. Abbreviations: ORF, open reading frame.

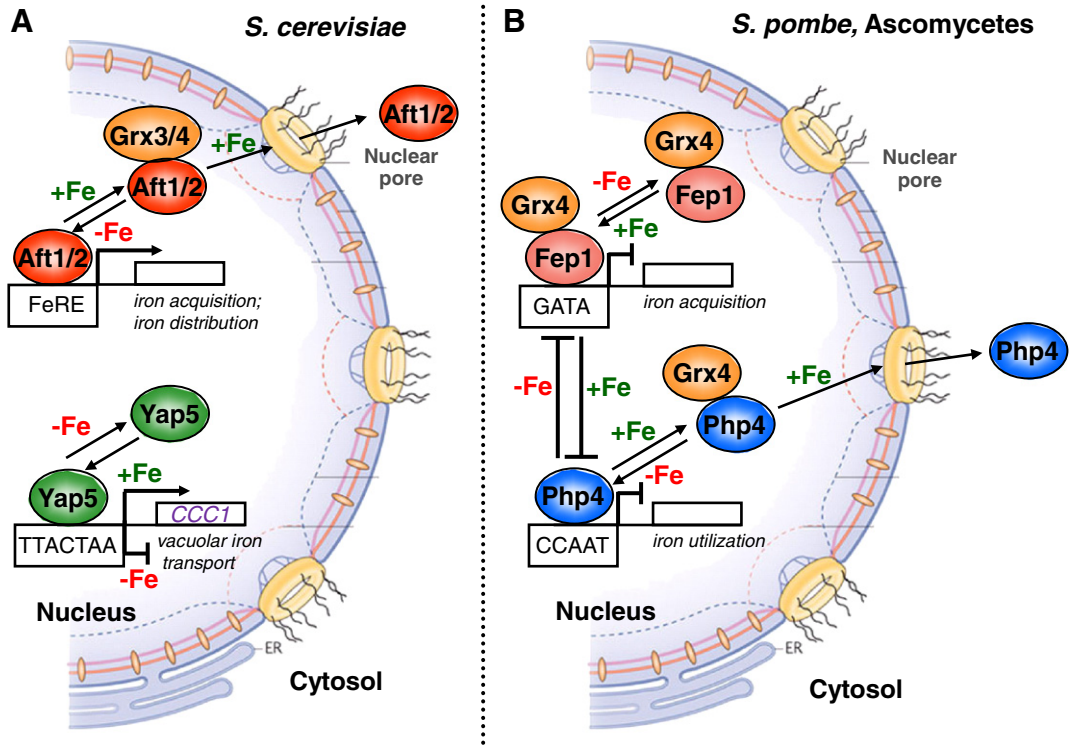

Fig. 5. Diverse mechanisms of transcriptional regulation of iron metabolism in fungi. A. The regulation of cellular iron homeostasis in *S. cerevisiae* involves transcriptional activators. Genes encoding proteins with a function in cellular iron acquisition and intracellular iron distribution are induced upon iron limitation by the iron-responsive transcriptional activators Aft1 and Aft2. Aft1–Aft2 shuttle between the cytosol and nucleus in an iron-responsive manner and interact with the cytosolic monothiol glutaredoxins Grx3–Grx4 that are involved in their inactivation (see also Fig. 3). Under high iron conditions the transcriptional activator Yap5 induces the expression of some genes including *CCC1* encoding the vacuolar iron importer. Its mode of iron sensing and the process of inactivation are largely unknown. B. In the fission yeast *Schizosaccharomyces pombe* and in ascomycete fungi, genes involved in reductive and siderophore-mediated iron acquisition are repressed upon iron sufficiency by the iron-responsive repressors Fep1 (*S. pombe*) or SreA (ascomycetes; not shown). Transcription of genes involved in intracellular iron utilization is attenuated upon iron limitation by the repressors Php4 (*S. pombe*) or HapX (ascomycetes; not shown). Fep1 also represses Php4 under iron sufficiency, and Php4 mutually represses Fep1 under iron deficiency. Both repressors bind to their target promoters in an iron-dependent manner and interact with the monothiol multidomain glutaredoxin Grx4 that is involved in their inactivation. Furthermore, Php4 is exported to the cytosol under iron sufficiency.

decreased expression of *CCC1*. Moreover, the levels of *CCC1* mRNA are diminished by Cth1–Cth2-dependent mRNA decay [195] (Fig. 4).

4.3. Role of the mitochondrial ISC systems in the post-transcriptional regulation of cellular iron homeostasis in vertebrates

Defects in the mitochondrial ISC assembly and export systems are associated with a dysregulated iron homeostasis in fungi, fruit fly, zebrafish, mice and man [23,30,125,172,205]. The generality of this link indicates that the role of the mitochondrial ISC assembly and export systems as critical regulators of cellular iron homeostasis is conserved in eukaryotes, despite the fact that these organisms differ largely in the mechanisms of cellular iron uptake and in the mode of regulation of cellular iron metabolism. In vertebrates iron is administered to tissue cells through the plasma iron transport protein transferrin (Tf) [206,207]. Tf binds to transferrin receptor-1 (TfR1) on the cell membrane of iron-consuming cells and is internalized by receptor-mediated endocytosis. Iron released from Tf is reduced by endosomal ferric reductases and transported into the cytosol via the divalent metal transporter DMT1. Iron is then used for various cellular processes, and excess iron is deposited within the storage protein ferritin. Cellular iron levels are post-transcriptionally controlled by iron regulatory proteins IRP1 and IRP2 [15,31,166,206,207]. IRP1 is a cytosolic Fe/S protein with homology to mitochondrial aconitase. Upon iron deprivation, IPR1 is converted from an active Fe/S enzyme to its iron-regulatory apoform that binds to IRE [15,166]. IRP2 is degraded under high iron concentrations in a ubiquitin–proteasome-dependent pathway. In iron-deficient cells, IRP1 and IRP2 bind to iron-responsive elements (IRE) located in the 3′- or 5′-untranslated regions of mRNA transcripts of proteins involved in iron metabolism, such as TfR1, ferritin H and L chains, or DMT1. IRP binding to IRE either stabilizes the mRNA against degradation or inhibits translation by blocking ribosome scanning, respectively. The resulting changes in the level of IRP-regulated proteins increase cellular iron uptake (through TfR1) and decrease intracellular iron storage (in ferritin).

The fact that iron regulation involves the Fe/S protein IRP1 explains how the mitochondrial ISC assembly and export systems affect the regulation of cellular iron homeostasis in vertebrates (Fig. 1). The depletion of components of the mitochondrial ISC assembly and export systems by RNAi techniques in cell culture models consistently causes the activation of IRP1 binding to IRE and the consequent up-regulation of TfR1-mediated cellular iron uptake and the down-regulation of ferritin [55,64,77,93,208,209]. The same is seen in tissues from patients with defects in mitochondrial ISC assembly or ISC export defects [82,130,210] or from corresponding conditional knockout mice [90,172,211]. In contrast to *S. cerevisiae*, defects in mammalian members of the cytosolic CIA system also cause a deregulated iron homeostasis, e.g., in cultivated human cells [53,171]. In fruit fly, frataxin deficiency results in deregulated cellular iron homeostasis, increased susceptibility to iron toxicity, and deregulated ferritin expression in adults [205]. In zebrafish, the mutant "shiraz" that harbors a defect in the mitochondrial ISC assembly member Grx5 develops hypochromic anemia, a blood-specific phenotype resulting from a defect in hemoglobin production [129]. This anemic phenotype that is also caused by Grx5 deficiency in human erythroblasts [132] is a result of an impaired maturation of the Fe/S cofactor on IRP1. The resulting constitutive activation of apo-IRP1 blocks the translation of the mRNA for erythroid ALAS2, the pace maker enzyme of heme biosynthesis. The molecular causes of the erythropoiesis

defects in the dedicated mammalian ISC targeting factors Isa1–Isa2 and Iba57 have so far not been identified [212].

Heart tissues of conditional frataxin knockout mice display a transcriptional down-regulation of key molecules involved in Fe/S cluster biosynthesis synthesis, several enzymes involved in heme synthesis, and mitochondrial ferritin [172,213]. This repression of iron-dependent mitochondrial pathways is reminiscent of the response of *S. cerevisiae* to ISC defects or iron limitation. The mechanisms underlying this adaptation in vertebrates are largely unknown. Taken together, in eukaryotes, defects in the mitochondrial ISC assembly and export systems induce a remodeling of cellular iron homeostasis that is remarkably similar to that caused by iron limitation. This observation strongly suggests that the mitochondrial ISC assembly and export systems function as central regulators that communicate the status of mitochondrial iron availability to the cytosolic iron-regulatory systems, regardless of whether these utilize transcriptional or post-transcriptional mechanisms for regulation. Notably, iron regulation in plants seems to follow fundamentally different routes as deletion of the Atm1 homolog does not elicit conspicuous changes in intracellular iron levels [214].

4.4. Monothiol glutaredoxins: Fe/S proteins with central functions in fungal iron metabolism

Fe/S protein maturation and cellular iron homeostasis are intimately linked to cellular redox balance and oxidative stress protection [215]. Reactive oxygen species may cause defects in Fe/S proteins, especially aconitase-type Fe/S proteins such as IRP1 [216,217]. Mitochondrial ISC assembly systems may also be targets of oxidative stress, which may impair the function of the respiratory chain or cause Fenton chemistry by inducing cellular iron overload [218–221]. In addition, as discussed above, two classical redox control molecules play central roles in cellular Fe/S protein biogenesis, GSH and the mitochondrial monothiol glutaredoxin Grx5. In addition to Grx5, most eukaryotes harbor additional monothiol glutaredoxins (Grx) in the cytosol. These have recently been characterized as important players in cellular iron metabolism in fungi [48,222].

Grx are small glutathione-disulfide-oxidoreductases that belong to the large thioredoxin fold family [222–225]. Cytosolic monothiol Grx are fusion proteins consisting of a thioredoxin domain and one to three C-terminal glutaredoxin domains [126,225]. Despite the designation 'cytosolic monothiol Grx' a significant amount of these proteins resides in the nucleus. Monothiol Grx with only one active-site cysteine residue rarely catalyze glutathionylation and deglutathionylation of target proteins. *S. cerevisiae* cells with low levels of the cytosolic monothiol glutaredoxins Grx3 and Grx4 show hardly any signs of oxidative damage, indicating that they are not involved in protection against oxidative stress or the maintenance of cellular redox balance. In yeasts, this function is preferentially executed by the classical cytosolic dithiol Grx [215,224,226]. Monothiol Grx are capable of binding a [2Fe–2S] cofactor in vitro that bridges two Grx monomers. The Fe/S cluster is coordinated by the sulfur atom of the active-site cysteine residue and two non-covalently bound glutathione molecules [128,222,225,227,228]. Fe/S cluster binding has been demonstrated in vitro for several dithiol Grx with non-canonical active-site motifs such as human Grx2 [222,225,229]. However, Fe/S cluster binding in the native host cell has been demonstrated so far only for the cytosolic multidomain monothiol Grx proteins from fungi and humans [48,230,231].

The first hint for an involvement of cytosolic monothiol Grx in cellular iron metabolism of eukaryotes came through the observation of a direct physical interaction of the functionally redundant yeast Grx3 and Grx4 with the iron-responsive transcription factor Aft1 [31,232,233] (Fig. 3). In cells with low levels of Grx3–Grx4, Aft1 is retained in the nucleus in a constitutively active form indicating that these monothiol Grx proteins are essential for nuclear export of Aft1 in response to iron sufficiency (Fig. 5A). Mutations that affect Fe/S cluster binding on yeast Grx3–Grx4 cause a similar phenotype, indicating that the Fe/S cofactor of monothiol Grx is essential for proper iron sensing by Aft1 (and possibly Aft2) [48,230]. Aft1 additionally interacts with the protease-related protein Fra1 and the cytosolic BolA protein Fra2 that both have an effect on the regulation of Aft1 [234]. Fra2 forms complexes with Grx4 in vitro that are held together by a bridging and GSH-coordinated [2Fe–2S] cluster [235]. The physiological significance of this heteromeric Fra2–Grx4 Fe/S complex is unknown. In conclusion, Aft1 and Aft2 from *S. cerevisiae* receive two independent regulatory inputs that signal the status of cellular iron metabolism (Fig. 3). The first is provided by the mitochondrial signal molecule X that is exported by Atm1 and transmits the mitochondrial iron status to the nuclear gene expression apparatus. The second is provided by the Grx3–Grx4-bound Fe/S cluster and integrates the cytosolic iron status into the overall regulatory outcome.

Remarkably, the regulatory role of cytosolic monothiol Grx in cellular iron homeostasis is functionally conserved in fungi that utilize iron regulatory systems that differ from the Aft1–Aft2 transcriptional activator system of *S. cerevisiae*. Most fungi utilize a combination of two iron-responsive transcriptional repressors that act under high or low iron conditions, respectively (Fig. 5B). In the fission yeast *S. pombe*, genes involved in reductive and siderophore-mediated iron uptake are repressed upon iron sufficiency by the iron-responsive transcription factor Fep1 (SreA in ascomycetes), while genes involved in intracellular iron utilization are repressed upon iron limitation by Php4 (HapX in ascomycetes) that interacts with the Hap complex [236,237] (Fig. 5B). In ascomycetes, both repressors mutually decrease the expression of the respective other. Similar to *S. cerevisiae* Aft1, Php4 shuttles between the nucleus and the cytosol in an iron-dependent manner, and both Fep1 and Php4 interact with the monothiol glutaredoxin Grx4. In cells lacking Grx4, both Fep1 and Php4 are retained in the nucleus and remain constitutively active, effectively causing repression of their respective target genes [238–240]. Whether the regulatory function of cytosolic monothiol Grx in cellular iron homeostasis is also conserved in ascomycete fungi that utilize the iron-responsive repressors HapX and SreA remains to be explored.

In addition to their regulatory role the cytosolic monothiol Grx execute a central function in intracellular iron delivery. In *S. cerevisiae*, low levels of Grx3–Grx4 induce the immediate loss of function of virtually all cellular iron-dependent pathways [48]. This effect is caused by a general failure in the insertion of iron into iron-dependent proteins, despite the fact that Grx3–Grx4-depleted cells accumulate large amounts of intracellular iron. A similar generalized defect of iron-containing proteins is not observed in cells with a constitutively activated iron uptake [171]. Pathways affected in Grx3–Grx4-depleted cells include those containing heme- and Fe/S cluster-enzymes, mono-iron and di-iron proteins, and the biosynthesis of heme and Fe/S clusters (Fig. 3). Several of the affected iron-dependent proteins are essential for growth, explaining why Grx3–Grx4 are indispensable for viability of some strain backgrounds of *S. cerevisiae*. As discussed above, depletion of yeast Grx3–Grx4 influences mitochondrial iron uptake, resulting in diminished mitochondrial iron levels and synthesis defects of Fe/S clusters and heme [48]. The failure to incorporate the bridging, GSH-coordinated [2Fe–2S] cluster into Grx3–Grx4, e.g., by mutation of the coordinating Cys residue of the Grx, elicits the same severe phenotype as that observed in cells lacking Grx3–Grx4 entirely. Hence, the Fe/S cofactor on the cytosolic monothiol Grx is essential for iron handling in the cytosol, defining a new function of this highly conserved, ubiquitous class of eukaryotic redox control proteins. Iron accumulated in Grx3–Grx4-depleted cells is soluble, indicating that the labile iron pool is inflated in the absence of Grx3–Grx4. This suggests that the cytosolic monothiol Grx may mediate the passage of iron from the labile iron pool to target proteins, a reaction which apparently does not occur spontaneously (Fig. 3). Whether the function of the cytosolic monothiol Grx in intracellular iron trafficking as identified in *S. cerevisiae* is conserved in higher eukaryotes is currently unknown. If cytosolic Grx play a conserved role in cytosolic iron handling, the protein may assist the iron chaperones

PCBP1 and BCBP2 in loading iron into their target proteins ferritin and HIF prolyl hydroxylase [241,242].

5. Conclusions and perspectives

The last decade has led to the discovery and initial cell biological and biochemical characterization of the mitochondrial ISC assembly machinery which is essential not only for the biogenesis of mitochondrial but also of cytosolic and nuclear Fe/S proteins. The mechanism of Fe/S protein maturation can now be separated into three distinct major steps and the function of many of the associated components can be envisioned. However, we still lack a fundamental biochemical understanding of many aspects of the process at molecular resolution. Therefore, we believe that, after the initial phase of discovery of the ISC components and the coarse definition of their function, we are entering a new phase which will lead to precise molecular understanding of the partial reactions of the process. This will require the biochemical reconstitution of the various steps of maturation, associated with repeated verification of the findings by in vivo studies to assure the physiological relevance of the suggested molecular mechanisms defined with purified components. Undoubtedly, these biochemical efforts must be supported by and will benefit from both 3D structural information of the ISC components and kinetic tracing of the dynamic interactions between each other and with apoproteins. Another exciting area of future research will be the clarification of the role of mitochondria in cytosolic-nuclear Fe/S protein maturation and the molecular dissection of the CIA machinery. The recently described relevance of Fe/S protein biogenesis for nuclear DNA maintenance and genomic stability makes discovery in this fascinating field of metal biology even more exciting and rewarding [243].

A striking observation that has been made concomitantly with the discovery of the ISC assembly machinery was the intimate link of this process to cellular iron homeostasis. We now can appreciate that virtually all eukaryotic cells use the efficiency of mitochondria to synthesize Fe/S proteins as one important device to regulate cellular iron uptake and intracellular iron distribution. The generality of this link is surprising because the iron regulatory processes are not conserved in lower and higher eukaryotes, in contrast to the strict conservation of the mitochondrial ISC assembly machinery from yeast to man. Different organisms use rather diverse machinery and mechanisms to adjust the iron acquisition to cellular needs and lifestyles, e.g., in various ecological niches. Nevertheless, virtually all eukaryotic organisms appear to respond to the ISC assembly machinery for maintenance of proper iron homeostasis. Only a dynamic and tight crosstalk of ISC assembly and iron regulation will eventually lead to a balanced homeostasis of this essential trace element and its derived Fe/S and heme cofactors. Future cellular studies combined with in vitro investigations of the participating functional components will lead to a better understanding of the physiological mechanisms governing the intertwining of these two processes and will reveal the organism-specific solutions.

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