## PERSPECTIVE

# A comprehensive mechanistic model of iron metabolism in *Saccharomyces cerevisiae*†

Paul A. Lindahl (ORCID)

The ironome of budding yeast (*circa* 2019) consists of approximately 139 proteins and 5 nonproteinaceous species. These proteins were grouped according to location in the cell, type of iron center(s), and cellular function. The resulting 27 groups were used, along with an additional 13 nonprotein components, to develop a mesoscale mechanistic model that describes the import, trafficking, metallation, and regulation of iron within growing yeast cells. The model was designed to be simultaneously mutually autocatalytic and mutually autoinhibitory – a property called autocatinhibitory that should be most realistic for simulating cellular biochemical processes. The model was assessed at the systems' level. General conclusions are presented, including a new perspective on understanding regulatory mechanisms in cellular systems. Some unsettled issues are described. This model, once fully developed, has the potential to mimic the phenotype (at a coarse-grain level) of all iron-related genetic mutations in this simple and well-studied eukaryote.

## Introduction

Anyone who has contemplated developing a biochemical model describing the mechanism of virtually any process in a cell soon realizes the practical impossibility of the task. Cellular processes are enormously complicated and modeling them at the biochemical level requires huge amounts of kinetic and mechanistic information much of which is unavailable. I discovered this when considering how to model iron metabolism in budding yeast, *S. cerevisiae*. A popular alternative is to collect and organize existing metabolic/stoichiometric data into genome-scale bioinformatic models.[1,2] A recent stoichiometric model of the yeast metabolic network, including iron metabolism, involves 963 genes.[3] Using bioinformatics methods, the ironome in human cells was found to involve 398 genes; 48% encode heme proteins, 35% encode mononuclear Fe and Fe–O–Fe proteins, and 17% encode iron–sulfur clusters (ISCs).[4] Such models are related to those I seek, but my objective goes beyond a statistical analysis; it is to obtain *mechanistic* insight at the systems' level analogous to that gained by viewing a busy metropolis from an altitude of 30 000 ft.

Here I develop a model using published data relevant to iron metabolism in yeast. I focused on iron because of my background in this area, as well as the critical importance of this transition metal ion for all eukaryotic cells. Iron is found in the mitochondrial respiratory complexes used to generate cellular energy, as well as in enzymes that replicate and repair DNA, synthesize lipids, proteins, amino acids, and nucleotides.

By manually searching the literature, I identified 139 proteins and 5 nonprotein iron species in *S. cerevisiae* that are intimately involved in iron metabolism – the *ironome circa* 2019 (Table S1, ESI†). For each species, gathered information included the type of iron center, cellular location, and physiological role. I then organized the proteins into 27 groups and constructed the model (Table S2, ESI†). Grouping simplified the ironome and made it amenable for modeling. Thirteen nonproteinaceous components were added to complete the so-called "40-component" model. The model was designed to be mutually autocatalytic and autoinhibitory – a combination coined *autocatinhibitory*. I expand on this modeling principle below.

## 1. The iron proteome

### Cell wall and plasma membrane

Under normal aerobic conditions, environmental iron is in the Fe$^{III}$ oxidation state, whereas in anaerobic environments, it is generally Fe$^{II}$. Iron is imported into yeast *via* three pathways. In the nonreductive Fe$^{III}$ pathway, Fe$^{III}$ ions bind to foreign siderophore chelators in the environment and are imported into the cell *via* receptors **Arn1**, **Arn2**, **Arn3** and **Arn4**.[5,6] Cell-wall mannoproteins **Fit1**, **Fit2**, and **Fit3** promote uptake of Arn-associated siderophore-ferric chelators.[5–9] When exponentially growing yeast cells shift to stationary phase, the cell wall accumulates iron in a magnetically-ordered Fe$^{III}$ form.[10] Under iron-deficient conditions, expression of these receptors

*Departments of Chemistry and of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-3255, USA. E-mail: Lindahl@chem.tamu.edu*

and mannoproteins is induced by the Aft1/2-dependent iron regulon (see below).

The reductive $Fe^{III}$ import pathway involves proteins **Fet3**, **Ftr1**, and **Fre1**. Environmental $Fe^{III}$ is reduced to $Fe^{II}$ by the NADPH-dependent ferrireductase Fre1 and is then reoxidized by the $O_2$-dependent multicopper oxidase Fet3.[11–14] Ftr1 is a permease which translocates the resulting $Fe^{III}$ ions (channelled from Fet3) into the cytosol where they are reduced to the $Fe^{II}$ state.[5,15–18] The characteristics of the resulting cytosolic $Fe^{II}$ species have not been established, but the model described below assumes a low-molecular mass (LMM) species called $Fe^{II}_{cyt}$. The only iron-containing protein composing the high-affinity import system is Fre1, which contains two interacting heme b centers.[5] This "bis-heme" motif is probably also present in other metalloreductases including **Fre2**, **Fre3**, **Fre4**, **Fre5**, **Fre7**, and **Fre8**. The $Fe^{II}$ import pathway involves permeases **Fet4** and **Smf1**. Fet4 expression increases under hypoxic conditions whereas Smf1 expression is not $O_2$-sensitive.[19–21]

**Cytosol**

The Cytosolic Iron–sulfur Assembly (CIA) proteins in the cytosol assemble $[Fe_4S_4]$ clusters and install them to various apo-protein targets. The "early" CIA complex is composed of **Cfd1** and **Nbp35** both of which are P-loop NTPases in the MinD family. An $[Fe_4S_4]$ cluster assembles at the interface of a Cfd1 homodimer[20] (or Cfd1:Nbp35 heterodimer) analogous to the $[Fe_4S_4]$ cluster that bridges the subunits of the iron protein of nitrogenase – another MinD family protein.[22] Nbp35 also binds an $[Fe_4S_4]$ cluster at its dimer interface. These clusters are labile and one or both of them transfer to downstream targets. Nbp35 subunits bind an additional permanent $[Fe_4S_4]$ cluster.[23–25]

The iron and sulfur substrates used to assemble clusters on the early CIA complex are also unknown. Lill and coworkers have suggested a sulfur-containing substrate called X–S that is related to glutathione persulfide.[26] Balk and coworkers suggested that X–S is glutathione polysulfide.[27] Both groups propose that X–S is exported from mitochondria through the inner membrane (IM) transporter Atm1 (see below). One implication of this is that the iron substrate used for ISC assembly would originate from the cytosol – probably from $Fe^{II}_{cyt}$. In contrast, Li and Cowan hypothesize that X–S is an $[Fe_2S_2]$ cluster with four glutathiones coordinated as exoligands.[28] The structure of the binding site on Atm1 suggests that X–S is related to glutathione, though other molecules are also possible.[27] Pain and coworkers discovered a 500–1000 Da sulfur-containing specie that is exported from intact mitochondria in an Atm1-dependent fashion.[29] They subsequently reported that mitochondria also export an Fe–S intermediate through Atm1 and presented evidence that the iron in the intermediate originates from mitochondria;[30] it's exciting to consider that this might be Cowan's $[Fe_2S_2]$ cluster.

**Dre2** binds the early CIA complex and catalyzes the transfer of electrons from NADPH to the complex during CIA-dependent $[Fe_4S_4]$ assembly. Physically associated with Dre2 is the flavoprotein **Tah18**.[31–33] This complex catalyzes the transfer of electrons from NADPH (which is bound to Tah18) to an

undetermined substrate during CIA-dependent $[Fe_4S_4]$ assembly. Although the mechanism of $[Fe_4S_4]$ cluster assembly on the CIA complex is unestablished, a popular assumption is that two $[Fe_2S_2]^{2+}$ clusters bind to each subunit of Cfd1 (and/or Nbp35) and are reductively coupled to form a bridging $[Fe_4S_4]^{2+}$ cluster; the simplified reaction would be $2[Fe_2S_2]^{2+} + 2e^- \rightarrow [Fe_4S_4]^{2+}$. The required electrons in this reaction would be delivered by Tah18 and Dre2. Dre2 reportedly contains 1 $[Fe_2S_2]$ cluster and 1 $[Fe_4S_4]$ cluster, though there is some uncertainty.[34] Dre2 clusters originate from either Grx3 homodimers or Grx3:Bol2 heterodimers (see below).[23] Metallation of Dre2 does not involve the CIA machinery, which only installs $[Fe_4S_4]$ clusters, implying either that Dre2 exclusively contains $[Fe_2S_2]$ clusters or that it contains a CIA-independent $[Fe_4S_4]$ cluster.

The "late" CIA complex consists of proteins **Cia1**, **Cia2**, and **Mms19**; none contains an iron center. This complex transfers $[Fe_4S_4]$ clusters from the early CIA complex to the apo forms of various target proteins in the cytosol. **Nar1** links the early CIA complex to the late complex; it contains a permanent $[Fe_4S_4]$ cluster and a second cluster which is surface-exposed and unstable.[31–33]

Cytosolic proteins **Lto1** and **Yae1** adapt the late CIA complex such that it can install $[Fe_4S_4]$ clusters into **Rli1**, a multifunctional protein that helps export ribosomes from the nucleus and is essential for other processes related to protein biosynthesis.[34]

Like Rli1, **Dph1**, **Dph2**, **Dph3**, and **Dph4** are also involved in protein biosynthesis, albeit indirectly. Dph1 and Dph2 are radical-SAM enzymes that contain $[Fe_4S_4]$ clusters. They form a heterodimeric complex that modifies a histidine residue on Translation Elongation Factor 2.[35] Dph3 coordinates an $Fe^{II}$ ion and uses it to donate electrons to SAM clusters in the Dph1:Dph2 complex.[35–37] Dph3 functions with **Cbr1**, a cytochrome $b_5$ reductase that helps link the metabolic state of the cell to protein translation. Dph4 is a J-protein cochaperone that reversibly binds an $Fe^{II}$ ion at a cysteine-rich site.[38] Dph4 associates with the large ribosomal subunit and also functions in ribosome biogenesis.[39] The $[Fe_4S_4]$ clusters in these proteins are likely metallated by the CIA.

**Elp3** is the catalytic subunit (histone acetyl-transferase) of the elongator complex of RNA polymerase II which helps the enzyme switch from initiation to elongation mode; it also contains a radical-SAM $[Fe_4S_4]$ cluster.[40–46] The elongator complex also modifies wobble uridines in tRNAs without which mRNA translation would be inefficient.

The CIA also installs $[Fe_4S_4]$ clusters into a**Leu1**, a**Glt1**, and a**Met5** (the prefix *a* refers to apo-forms), cytosolic proteins involved in amino acid biosynthesis. Leu1 (isopropylmalate isomerase) catalyzes the synthesis of branched-chain amino acids leucine, isoleucine, and valine.[47] Glt1 catalyzes the synthesis of glutamate.[48,49] Met5 (the β subunit of sulfite reductase) catalyzes the reduction of sulfite to hydrogen sulfide thereby allowing synthesis of cysteine and methionine.[50–52] Met5 contains an $[Fe_4S_4]$ cluster linked through a bridging cysteinate to a siroheme ring. Sirohemes are like hemes but require an enzyme (**Met8**) other than ferrocheletase to install $Fe^{II}$.[53,54] The source of the installed $Fe^{II}$ ion is unknown but the model below assumes it to be $Fe^{II}_{cyt}$.

Other cytosolic iron proteins are involved in nucleic acid biochemistry. The $[Fe_4S_4]$-containing **Ade4** (glutamine phosphoribosylpyrophosphate amidotransferase) is involved in purine biosynthesis.[55,56] The $O_2$-dependent $Fe^{II}$-containing **Bna1** (3-hydroxyanthranilate 3,4-dioxygenase) helps synthesize pyrimidine nucleotides.[57] **Tyw1** is a radical-SAM $[Fe_4S_4]$ protein that helps synthesize wybutosine-modified tRNA, a modification that increases the accuracy of protein synthesis. Tyw1 expression is stimulated by Yap5 under high-iron conditions (see below).[58,59]

**Rnr2** is the small $R_2$ subunit of ribonucleotide reductase. It helps convert ribonucleotides into their deoxy counterparts; each subunit contains an [Fe–O–Fe] center.[60–62] The iron-free $R_1$ subunit resides in the cytosol whereas $R_2$ toggles between the cytosol and the nucleus to control activity – which is an exclusive property of the $(R_1)_2(R_2)_2$ heterotetrameric complex. Under healthy iron-replete conditions, $R_2$ is mainly in the nucleus whereas under iron-deficiency or other forms of stress, it shifts to the cytosol and stimulates activity. This behavior reveals the critical need for the cell to express this iron-containing enzyme under iron-deficient conditions when so many other iron-containing proteins are degraded so as to economize cellular iron. Assembly of the $R_2$ [Fe–O–Fe] centers does not involve the CIA[63,64] but the assembled center is probably reduced by NADPH *via* Dre2 and Tah18.[60] Ribonucleotide reductase and other [Fe–O–Fe] proteins are thought to be metallated by Grx3/4 proteins;[64–66] however, in our model they are metallated by $Fe^{II}_{cyt}$ (see below).

Also in the cytosol are **Grx3** and **Grx4**, monothiol glutaredoxins that form dimers with bridging $[Fe_2S_2]$ clusters.[67] Clusters are bound to each subunit through a single cysteine residue; the other external coordinating ligand is the sulfur of glutathione. Grx3/4 are involved in a signaling pathway that leads from mitochondria to the nucleus. This pathway controls expression of the *iron regulon* genes (see below). **Bol2** forms a heterodimer with Grx3 or Grx4 which also contains a bridging $[Fe_2S_2]$ cluster with glutathione coordination. In fact, most Grx3 in the cell is bound to Bol2 as a heterodimer.[65,66] Eventually, the bridging $[Fe_2S_2]$ cluster is passed to Aft1/2, transcription factors that control expression of the iron regulon. Grx4 binds to Aft1/2 promoting dissociation from promotors and repressing expression of the iron regulon. The $[Fe_2S_2]$ cluster on Grx3/4 is likely transferred to Dre2[65,66] whereas the cluster on Bol2 may transfer to the CIA for reductive coupling.[66–68]

The cytosol also contains heme proteins, many of which are involved in diminishing reactive oxygen species (ROS). **Ctt1** is a heme-b-containing catalase that catalyzes the disproportionation reaction $2H_2O_2 \rightarrow O_2 + 2H_2O$.[69,70] **Cta1** is another heme-b-containing catalase (but located in peroxisomes) that catalyzes the same reaction, degrading $H_2O_2$ that has been generated during fatty acid β-oxidation.[71] **Yhb1** is flavohemoglobin, also called NO dioxygenase. This heme-b-containing enzyme is located in the cytosol (and mitochondrial IMS) when cells grow aerobically, but is located in the mitochondrial matrix under anoxic conditions. It catalyzes the $O_2$-dependent oxidation reaction $NO + O_2 + e^- \rightarrow NO_3^-$ and is part of the cell's detoxification system.[72] Yhb1 works with catalase to maintain low levels of NO and peroxynitrite, both of which can be toxic.[73–76] The last heme-containing protein that helps diminish ROS is **Ccp1**, cytochrome *c* peroxidase. This mitochondrial IMS enzyme catalyzes the reduction of peroxide $H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$, using electrons from cytochrome *c*.

## Nucleus

The CIA installs $[Fe_4S_4]$ clusters into numerous nuclear proteins, *all* of which help replicate and repair DNA. **Ntg2** is an endonuclease III and a DNA glycosolase that is involved in base-excision repair; it excises oxidatively-damaged pyrimidine bases.[34,77–84] **Dna2** is a nuclease and helicase. It helps mature Okazaki fragments and maintain telomeres.[60,85–87] **Rad3** is a helicase involved in DNA excision repair and transcription.[34,88–91] **Chl1** is a helicase involved in sister chromatid cohesion and heterochromatin organization.[34] **Pri2** is the large subunit of the heterodimeric DNA primase which catalyzes the synthesis of short RNA primers that help initiate DNA replication.[92,93] It also helps DNA polymerase α (**Pol1-α**), another $[Fe_4S_4]$-containing nuclear enzyme, transition from RNA to DNA synthesis.[34,92,94–102] The catalytic subunit of DNA polymerase II ε A (**Pol2**) appears to contain an $[Fe_4S_4]$ cluster (there is some uncertainty).[32,103,104] The catalytic subunit of $[Fe_4S_4]$-containing DNA polymerase δ (**Pol3**) takes over synthesis from Pol1-α to complete each Okazaki fragment;[105] it primarily synthesizes the lagging strand.[34,99] $[Fe_4S_4]$-containing **Rev3**, the catalytic subunit of DNA polymerase ζ,[99] possesses $5' \rightarrow 3'$ polymerase activity[106] and is involved in the post-replication repair of DNA.

**Tpa1** is the only iron-containing DNA-repair enzyme that does not contain an $[Fe_4S_4]$ cluster. This 2-oxoglutarate-dependent $Fe^{II}$-containing dioxygenase catalyzes the oxidative demethylation of DNA during the repair of methyl-base lesions.[107] It also hydroxylates a proline on a ribosomal protein. Tpa1 helps terminate translation efficiently by degrading mRNA and interacting with translation release factors and the poly(A)-binding protein.

The remaining iron-associated proteins in the nucleus are all involved in regulation. **Aft1** and **Aft2** are transcription factors that regulate expression of the iron regulon. They toggle between the nucleus and cytosol according to whether an $[Fe_2S_2]$ cluster is bound. This depends, in turn, on the ISC activity in mitochondria. When that activity is high, Aft1/2 are metallated (by Grx3/4, Bol2 complexes) which promotes their translocation to the cytosol and halts expression of iron regulon genes. When mitochondrial ISC activity is low, Aft1/2 in their apo-forms shift into the nucleus and stimulates iron regulon expression.[6,108–113] Aft1/2 controls expression of genes encoding Fet3, Fet4, Fet5, Hmx1, Ftr1, Fth1, Fre1, Fre2, Fre3, Fre4, Fre5, Fre6, Arn1, Arn2, Arn3, Arn4, Fit1, Fit2, Fit3, Isu1, Smf3, Mrs4, Grx4, Cth1, and Cth2.[111,114]

Although the last two proteins on this list, paralogs **Cth1** and **Cth2**, do not bind iron, they are intimately involved in iron metabolism. Cth1/2 promote decay of mRNA transcripts that encode nonessential iron-containing proteins associated with the TCA cycle (Sdh4), the mitochondrial electron transport chain (ETC), heme biogenesis (Hem15), and heme utilization

(Ccp1).[115] The decay of these targets during iron deficiency allows the cell to economize iron by shifting to fermentation which does not require iron. Cth1/2 bind mRNA transcripts in the nucleus and shuttle them to the cytosol for degradation.[116] Cth2 also promotes decay of an inhibitor of ribonucleotide reductase, which stimulates ribonucleotide reductase activity.[62,115,117] Cth2 also promotes decay of its own mRNA; this autoinhibition allows respiration to recover quickly if and when iron-sufficiency is reestablished.

Yap5 promotes transcription of Ccc1, Grx4, and Tyw1 genes.[58,118,119] Ccc1 is an iron-importer on the vacuolar membrane (see below), Grx4 is involved in the Aft1/2 signal-transduction pathway (see above), and Tyw1 increases the efficiency of protein synthesis. Under iron-replete conditions, Yap5 binds two $[Fe_2S_2]$ clusters and undergoes a conformational change that activates transcription.[120] Activation involves two Yap5 monomers forming a dimer that opens and closes like a clam in response to changing intracellular iron levels.[58,118–122]

When cells switch from fermentation to respiration, heme levels rise, and this stimulates Hap1 expression (and expression of the Hap2/3/4/5 complex). Hap1 is a nuclear transcription factor that binds heme b; this stimulates expression of heme-associated genes[123] including those involved in respiration (Cyc1, Cyc7, Cyt1, and Cyb2), the synthesis of ergosterol, and the expression of superoxide dismutase.[124] One Hap1-dependent genes encodes Rox1 which represses heme synthesis. Heme and $O_2$ levels are correlated. This is partially but not entirely explained by the requirement of $O_2$ in multiple heme synthesis steps. The heme-activated Hap2/3/4/5 complex promotes respiration.

## Mitochondrial matrix

The epicenter of iron metabolism in the cell is the mitochondrial ISU machinery that is used to synthesize $[Fe_2S_2]$ clusters. Cysteine desulfurase (Nfs1) uses a sulfur atom from cysteine to generate a persulfide group at an active-site cysteine residue on the protein.[125] Iron-free Isd11 and Acp1 (the acyl-carrier protein involved in fatty acid biosynthesis) bind and stabilize Nfs1. Scaffold proteins Isu1 and Isu2 bind the {Nfs1:Isd11:Acp1} complex independently and receive the terminal sulfur via a mobile loop that swings the persulfide into position. $[Fe_2S_2]$ clusters are assembled on Isu1/2 using Nfs1-derived sulfur and a mitochondrial $Fe^{II}$ pool called $Fe^{II}_{mit}$. Yeast frataxin homolog 1 (Yfh1) also binds the {Nfs1:Isd11:Acp1:Isu1/2} complex and stimulates the sulfur-transfer activity from Nfs1 to Isu1/2.[126] Yfh1 may also donate $Fe^{II}$ ions to Isu1/2.[127] Under Yfh1-deficient conditions, mitochondria accumulate large quantities of iron in the form of $Fe^{III}$ nanoparticles (NP) while generating large amounts of ROS.[128,129]

The bridging sulfide ions of $[Fe_2S_2]$ clusters are two-electrons more reduced than the sulfur atoms that are transferred onto the scaffold from the persulfide arriving on the mobile loop. The needed electrons are donated by NADPH via the $[Fe_2S_2]$-containing ferredoxin Yah1 and its flavin-containing partner ferredoxin reductase Arh1.[130] Installing the cluster on Yah1 involves an autocatalytic reaction in which Yah1 helps catalyze the ISU-dependent metallation of aYah1, forming Yah1

as the product. Assembled $[Fe_2S_2]$ clusters are transferred from Isu1/2 to apo-glutaredoxin 5 (aGrx5) in a reaction involving the DnaJ-type chaperone Jac1, HSP70-class chaperone Ssq1, and nucleotide exchange factor Mge1.[131] Cluster-containing Isu1/2 bind Jac1 and ATP-bound Ssq1,[132] whereas Mge1 swaps ADP on Ssq1 with fresh ATP in preparation for the next transfer. As with other monothiol glutaredoxins, dimeric Grx5 coordinates a $[Fe_2S_2]$ cluster between its two subunits.[66] Grx5-bound clusters are probably transferred to mitochondrial matrix proteins aYah1, aBol1, aBol3, aSdh2, and aBio2.

Isa1, Isa2, and Iba57 form a complex that helps build $[Fe_4S_4]$ clusters in the mitochondrial matrix.[133] These clusters are likely generated by the reductive coupling of two $[Fe_2S_2]$ clusters donated by holo Grx5 dimers. The reaction probably occurs at the Isa1:Isa2 heterodimer interface. Iba57 doesn't bind clusters; rather it recruits apo-target proteins to the complex and helps transfer assembled $[Fe_4S_4]$ clusters onto them. Target proteins include aAco1, aLip5, aSdh2, aBio2, and aIsa1/2. This last metallation reaction is again autocatalytic. Isa1/2 contain permanent $[Fe_4S_4]$ clusters, and fully metallated Isa1/2 is probably used to metallate apo-Isa1/2. The absence of Isa1, Isa2 or Iba57 causes defects in respiratory complex assembly and lipoic acid biosynthesis indicating that proteins involved in these processes are Isa1/2 targets.[133–137]

Bol1, Bol3, and Nfu1 help the ISA complex transfer $[Fe_4S_4]$ clusters to target proteins.[133–137] Bol1 and Bol3 each contain $[Fe_2S_2]$ clusters bridged between two subunits and coordinated by glutathione, similar to the arrangement in holo-dimeric glutaredoxins.[66,138] These clusters are donated by Grx5. Nfu1 homodimers contain a permanent bridging $[Fe_4S_4]$ cluster which is donated by ISA.[66,138] Bol1, Bol3 and Nfu1 help transfer transient $[Fe_4S_4]$ clusters (also donated by ISA) onto aSdh2 and aLip5. Nfu1 helps ISA transfer a cluster to aAco1, aLip5, and aISA. Nfu1 works somewhat independently of Bol1/3, but all act late in cluster assembly.

The mitochondrial matrix houses two $[Fe_4S_4]$-containing enzymes that are involved in amino acid biosynthesis, namely homoaconitase Lys4[139–141] and dihydroxyacid dehydratase Ilv3.[47,142,143] Ilv3 is involved in valine biosynthesis. Lys4 converts homocitrate to homoisocitrate in the lysine biosynthetic pathway. The Lys4 gene is expressed only under iron-sufficient conditions.

Numerous iron-containing proteins of the matrix are involved in the TCA (tricarboxylic acid) cycle. Both isoforms of aconitase (Aco1 and Aco2) catalyze the conversion of citrate to isocitrate, and both contain $[Fe_4S_4]$ cluster active sites. The Sdh2 subunit of succinate dehydrogenase contains an $[Fe_4S_4]$, $[Fe_3S_4]$, and $[Fe_2S_2]$ cluster, whereas the Sdh3 and Sdh4 subunits together share a heme b center.[66] Although not part of the TCA cycle, biotin synthase (Bio2) participates in a reaction that impacts this cycle – inserting sulfur into dethiobiotin to form biotin.[144] Bio2 contains 1 $[Fe_4S_4]$ and 1 $[Fe_2S_2]$ cluster, the latter of which donates the sulfur used in this reaction. As such, Bio2 is a substrate rather than a catalyst; the cluster needs to be rebuilt after each sulfur transfer. Biotin is a coenzyme of numerous mitochondrial carboxylases[9] which help produce cellular energy, and in certain cases, feed the TCA cycle.

Lipoic acid synthase **Lip5** is a mitochondrial matrix protein that, like biotin synthase, is a substrate in a biosynthetic reaction, in this case of lipoic acid.[145] Lip5 contains a radical-SAM $[Fe_4S_4]$ cluster and a second $[Fe_4S_4]$ cluster that donates the sulfide ion in the reaction. Lipoic acid is a coenzyme for mitochondrial enzymes that catalyze the oxidative decarboxylation of pyruvate, α-ketoglutarate, and branched-chain α-keto acids.

## Mitochondrial inner membrane (IM) and intermembrane space (IMS)

**Mrs3** and **Mrs4** are paralogous high-affinity iron importers on the IM.[146] These iron-free proteins contain a tunnel through which iron passes from the cytosol to the matrix. A membrane potential is required for iron transport. **Rim2** is a low-affinity iron importer on the IM.[147] Cytosolic iron is transported through Rim2 (as are pyrimidine nucleotides). Deleting Mrs3/4 and Rim2 causes defects in ISC and heme biosynthesis.[147,148]

Most iron-related processes in the IM are associated with respiration. Two iron-containing enzymes help synthesize Coenzyme Q ($QH_2$). **Coq6** is a heme-b-containing monooxygenase that hydroxylates a tyrosine ring[149–152] using electrons from NADPH *via* Yah1 and Arh1.[153] The [Fe–O–Fe]-containing 5-demethoxyubiquinone hydroxylase **Coq7** converts 5-demethoxy Q6 to 5-demethyl Q6. Coq7 expression is inhibited during fermentation and stimulated during respiration.[154,155]

Three subunits of respiratory complex cytochrome $bc_1$ contain iron centers. **Cyt1** contains cytochrome $c_1$, **Cob1** contains two heme b centers called $b_H$ and $b_L$, and **Rip1**, the Rieske subunit, contains an unusual $[Fe_2S_2]$ cluster coordinated by two histidine residues and two cysteines.[156] Cytochrome $bc_1$ catalyzes the oxidation of $QH_2$ and reduction of cytochrome $c$. The Rip1 subunit is exposed to the IMS where it reduces cytochrome $c$ during catalysis. **ROS** is generated by one or more of these respiratory complexes.[157]

The only subunit of respiratory complex cytochrome $c$ oxidase that contains iron is **Cox1**; it contains 1 heme a redox center and 1 heme $a_3$:$Cu_B$ active site. The enzyme catalyzes the reduction of $O_2$ by cytochrome $c$. Assembly of cytochrome $c$ oxidase requires three iron-associated proteins. **Cox10** (heme o synthase) binds heme b and converts it to heme o. **Cox15** (heme a synthase), converts heme o into heme a which is then installed into Cox1.[157,158] Cox15 is a $O_2$-dependent monooxygenase that is reduced by NADPH *via* Yah1 and Arh1.[156,159,160] It binds two hemes, including heme b and heme o. **Mss51** is a heme b-containing Cox1 chaperone on the IM which activates Cox1 mRNA for translation. Mss51 is regulated by heme and/or $O_2$ levels and it regulates cytochrome $c$ oxidase assembly.[47]

**Cyc1** and **Cyc7** are isoforms of cytochrome $c$ in the IMS. Their expression is diminished under heme-deficient conditions due to Hap1 regulation.[161] The heme c centers in these proteins are installed by cytochrome $c$ lyase (**Cyc2**) which uses heme b as a substrate. Cytochrome $c$ is a substrate for two other iron-containing enzymes in the IMS, including cytochrome $c$ peroxidase (Ccp1) and L-lactate cytochrome $c$ oxidoreductase (**Cyb2**). The former enzyme *oxidizes* cytochrome $c$ as it reduces $H_2O_2$ to water while the latter *reduces* cytochrome $c$ as it

oxidizes lactate to pyruvate. This allows yeast to respire using lactate.[162] **Dld1** is D-lactate ferricytochrome $c$ oxidoreductase, a heme b-containing enzyme located on the mitochondrial IM. Its expression is regulated by heme levels ala Hap1. Dld1 also catalyzes the oxidation of lactate to pyruvate using cytochrome $c$ as the electron acceptor.[163]

**Hem15** (ferrocheletase) is an IM protein that installs $Fe^{II}$ into porphyrin rings to generate heme b.[164] Its $Fe^{II}$ binding site faces the matrix, implying that $Fe^{II}_{mit}$ is used as substrate in this process. Heme synthesis is inhibited under hypoxic conditions, as two enzymes in the heme biosynthetic pathway require $O_2$ as a substrate.[5,48,50,51,124,161,164]

**Atm1** is an ATP-dependent transporter in the IM that may export the unidentified sulfur-containing X–S species discussed above from mitochondria to cytosol; however, there is some uncertainty regarding its function.[26,165–169] **Mmt1** and **Mmt2** may also export mitochondrial iron but there is also some uncertainty here as well.[170–172]

## Vacuoles

These organelles store iron under iron-replete conditions. **Ccc1** is an iron importer on the vacuolar membrane the expression of which is controlled by Yap5.[58,173,174] The multicopper oxidase **Fet5**, permease **Fth1**, and ferrireductase **Fre6** together function as an $O_2$-dependent iron exporter on the vacuolar membrane.[175,176] These three proteins are paralogs to Fet3, Ftr1, and Fre1, respectively, on the plasma membrane. Expression of both groups of proteins are controlled by the Aft1/2-dependent iron regulon.[13,177]

Vacuoles typically store iron as $Fe^{III}$ polyphosphates.[178] Mobilization of $Fe^{III}$ requires reduction to the $Fe^{II}$ state followed by export *via* the {Fet5:Fth1;Fre6} complex. Electrons used for reduction are provided by NADPH *via* Fre6, which, like Fre1, contains a bis-Heme b center.[14] Fet5 and Fth1 do not house permanent iron centers, but $Fe^{II}$ in the vacuole presumably binds to Fet5 prior to becoming oxidized to $Fe^{III}$ and passing through Fth1. **Smf3**, another iron exporter on the vacuolar membrane, is a member of the Nramp family of metal transporters and is not under the control of Aft1/2.[113,176] Like Fet4, Smf3 expression is stimulated under hypoxia.[179]

## Endoplasmic reticulum (ER)

Most iron-containing proteins in the ER are involved in synthesizing membrane components including ergosterol and sphingolipids. **Ole1** ($\Delta^9$ fatty acid desaturase) is an integral ER membrane enzyme that catalyzes the synthesis of a mono-unsaturated fatty acid (oleoyl-CoA) starting from a saturated fatty acid (stearoyl-CoA). This reaction, which inserts a double bond into a fatty acyl chain, requires NADPH and $O_2$. **Ncp1** (NADP-cytochrome P450 reductase) probably transfers reducing equivalents from NADPH to Ole1. Ole1 contains an [Fe–O–Fe] center and a cytochrome $b_5$ domain which is exposed on the cytosol side of the ER membrane.[180–183,205]

**Sur2** (sphinganine C4-hydroxylase) catalyzes the $O_2$-dependent oxidation of sphinganine.[184–186] Like Ole1, it is an ER membrane protein containing an [Fe–O–Fe] center. However, it lacks a

fused cytochrome $b_5$ domain. Thus, it may use Ncp1 and **Cyb5** (cytochrome $b_5$) to receive electrons from NADPH.[187,188] Cyb5 is bound to the ER membrane but faces the cytosol. It contains a heme b center with two histidine axial ligands.[189]

**Scs7** (sphingolipid α-hydroxylase) is another integral ER membrane enzyme that houses an [Fe–O–Fe] center as well as a fused cytochrome $b_5$ center.[183] Scs7 adds a hydroxyl group to the α-carbon of a ceramide substrate to generate a sphingolipid.

**Mpo1** is an $Fe^{II}$-containing dioxygenase in the ER membrane that catalyzes the oxidation of a 2-hydroxy fatty acid.[190] Unlike the other biosynthetic enzymes just discussed, Mpo1 helps *degrade* a membrane component rather than synthesize one.

Starting from squalene, ergosterol biosynthesis requires four iron-containing enzymes as well as abundant $O_2$ and NADPH.[191–199] **Cyp51** (Cytochrome P450 lanosterol C-14 α-demethylase) catalyzes the synthesis of 4,4-dimethyl fecosterol from lanosterol.[195] It is a key enzyme for ergosterol biosynthesis and is essential for aerobic growth. Cyp51 is tethered on the ER membrane but it faces the cytosol; it contains a heme b center with a cys axial ligand. It is likely metallated by Dap1 (see below). Ncp1 is required for Cyp51 activity.[200–202]

**Erg25** (C-4 methylsterol monooxygenase 1) catalyzes a C-4 demethylation reaction that requires $O_2$ and NADPH. This ER membrane protein contains an [Fe–O–Fe] center.

**Erg3** (sterol C5(6)-desaturase) is in the fatty acid hydroxylase superfamily. It adds a double bond at positions 5 and 6 of Δ-7 sterols in an $O_2$-dependent reaction. Its [Fe–O–Fe] center accesses the cytosol. Like other iron-containing enzymes in the ER, Erg3 is reduced by NADPH *via* Ncp1 and Cyb5.[181,189,203,204]

**Erg5** (sterol C-22(23) desaturase) is a cytochrome P450 enzyme that catalyzes formation of a double bond during ergosterol biosynthesis,[206,207] probably using Ncp1 as a reductant. Ergosterol biosynthesis is inhibited under hypoxic conditions.[208–210]

**Dap1** is a heme-binding protein that activates, stabilizes, and regulates cytochrome P450s in the ER. It lacks a membrane spanning sequence (suggesting that it is not membrane bound) and may be involved more generally in heme trafficking and/or inserting hemes into various target apo-proteins. Dap1 binds heme b using a tyrosine axial ligand.[17,195,196,211] Hemes are synthesized in the mitochondria and must be trafficked to the ER; Dap1 may be involved in this.

**Hmx1** (heme oxygenase) is an ER enzyme that catalyzes the degradation of hemes. This releases $Fe^{II}$ into the cytosol.[12,108,212] The reaction involves NADPH and $O_2$. Hmx1 binds heme b with one axial histidine ligand. The Hmx1 gene is part of the Aft1/2 iron regulon and is activated under iron deficient conditions. This implies that the $Fe^{II}$ released during heme degradation serves to relieve cellular iron deficiency. It also implies that heme-dependent processes are *not* essential for cell survival.

**Yno1** is an NADPH oxidase on the ER membrane.[213] This bis-heme b containing enzyme is related to the Fre proteins, but it is not a ferrireductase. Rather, it catalyzes the synthesis of ROS from NADPH + $O_2$, perhaps in a signaling capacity.

**Grx6** is a monothiol glutaredoxin located in the ER, Golgi, and/or vacuole.[67,214,215] Like other Grx proteins, Grx6 contains an $[Fe_2S_2]$ cluster that bridges the two subunits and coordinates two glutathiones and two cysteinyl protein residues. Grx6 is associated with the early secretory pathway.[193] It has glutathione-S-transferase and glutathione-dependent oxidoreductase activities.[215–219] Grx6 regulates the glutathionylation of proteins in the ER and Golgi, and controls the ratio of oxidized and reduced glutathione to protect the cell against oxidative stress.

## 2. Model components and description

The developed model assumes *S. cerevisiae* cells that are growing exponentially on three nutrients – acetyl-coenzyme A, $O_2$, and non-siderophore environmental iron ($Fe^{III}$ or $Fe^{II}$ depending on the $O_2$ concentration). The modeled cell consists of exactly 40 components including 27 protein groups and 13 nonprotein components (Table S2, ESI†). It produces two waste products ($CO_2$ and iron nanoparticles). Each protein group is named with 3–4 capital letters or numbers whereas individual protein components have the first letter capitalized and others lower case. FET3 is a group; Fet3 is a member of that group. Components are indicated in bold when introduced.

The plasma membrane includes two protein groups, **FET3** and **FET4**, and a nonprotein component called **MEMBRANE** (Fig. 1). FET3 imports nutrient $Fe^{III}_{env}$ *via* the reductive pathway. Some of its members are in the cell wall rather than on the plasma membrane, but the iron content of the wall is insignificant under exponential growth conditions,[10] and so any iron metabolism associated with it is ignored in the model. FET3 expression is controlled by the AFT-dependent iron regulon.
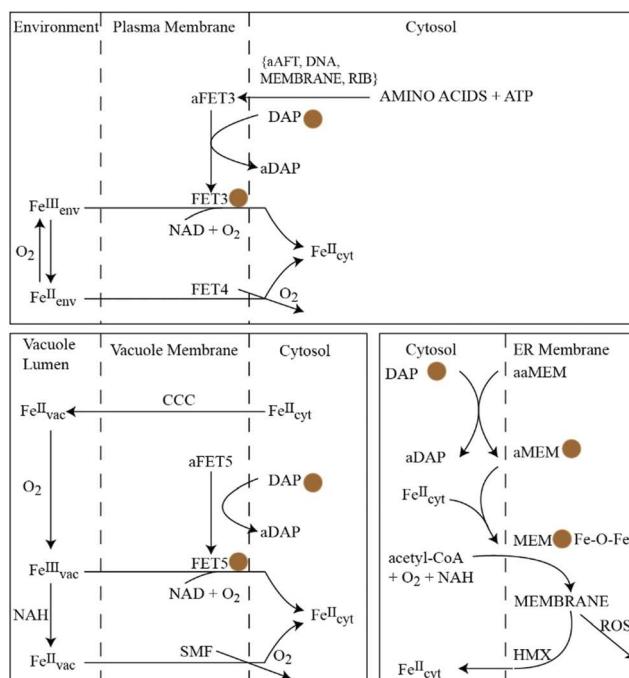


Fig. 1 Model scheme highlighting the iron-related processes in the plasma, vacuolar, and ER membranes. Brown dots represent heme groups. Refer to text for details for Fig. 1–5. The prefix *a* refers to apo- forms.

The product of FET3 activity is $Fe_{cyt}^{II}$. FET3 and other membrane-bound proteins are synthesized from a component called **AMINO ACIDS** at rates that depend on the concentration of: (a) AMINO ACIDS; (b) a model component called **DNA**; (c) a model component called **RIB** (ribosomes) and (d) MEMBRANE. Soluble proteins have the same dependencies except not on MEMBRANE. FET3 contains a bis-heme b center (due to the member Fre proteins) installed by **DAP**. Other substrates for FET3 activity include a model component called **NAH** (representing both NADPH and NADH) and $O_2$. The iron-free group FET4 imports nutrient $Fe_{env}^{II}$, generating $Fe_{cyt}^{II}$. FET4 expression increases under hypoxic conditions when $Fe_{env}^{II}$ dominates.

Vacuoles contain three protein groups called **CCC**, **FET5**, and **SMF**. The latter two are functionally analogous to FET3 and FET4, respectively. CCC is on the vacuolar membrane where it imports $Fe_{cyt}^{II}$ and generates a nonproteinaceous component called $Fe_{vac}^{II}$. CCC is positively controlled by AFT, in that expression is stimulated (rather than inhibited) under iron-replete conditions. FET5 is also on the vacuolar membrane where it reduces $Fe_{vac}^{III} \rightarrow Fe_{vac}^{II}$ and exports iron from the vacuole into the cytosol as $Fe_{cyt}^{II}$. Like FET3, FET5 is regulated by AFT such that expression increases under iron-deficient conditions. Also like FET3, FET5 requires $O_2$ for activity, and it contains a bis-heme b center that is metalled by DAP. SMF is a low-affinity iron exporter that is not controlled by AFT and is repressed under aerobic conditions.[179] It functions similarly to FET4 to generate $Fe_{cyt}^{II}$.

The cytosol includes nine protein groups called **DRE**, **GRX**, **CIA**, **LEU**, RIB, **NUC**, **CAT**, DAP, and **CTH** (Fig. 2). DRE transfers reducing equivalents from NAH to various targets including CIA and NUC. DRE contains two $[Fe_2S_2]$ clusters and is metallated by GRX. GRX receives clusters from the mitochondria *via* ATM, and donates them to aDRE, aAFT, and aCIA. The CIA has permanent $[Fe_4S_4]$ clusters as well as a transient $[Fe_4S_4]$ cluster that is assembled by the reductive coupling of two $[Fe_2S_2]$ clusters donated by GRX. The electrons used in coupling come from NAH *via* DRE. CIA has two apo forms including aaCIA, which is *fully* devoid of iron centers, and aCIA which contains the permanent clusters but not the transient one.

One discounted scenario was to have the CIA use $Fe_{cyt}^{II}$ and a sulfur-only X–S build $[Fe_4S_4]$ clusters *de novo*. Another discounted scenario was to include a second ISU system in the cytosol analogous to that in mitochondria (see below) and to have this system generate $[Fe_2S_2]$ clusters that would be used as substrates for the CIA. Our selection was based on the strength of evidence, popularity in the literature, overall simplicity, and compatibility with known chemistry. Some components of an ISU system are indeed found in the cytosol[220] but their cellular role in that location needs to be better established. Assembling $[Fe_2S_2]$ clusters is an $O_2$-sensitive process such that the low $O_2$ environment of the mitochondrial matrix[221] may be required. The recently reported X-ray diffraction structure of the Cfd1 dimer[222] and the phylogenetic connection of this protein to the iron protein of nitrogenase suggest that the bridging $[Fe_4S_4]$ cluster is assembled from the merger of two pre-formed $[Fe_2S_2]$ fragments bound to each monomer. An analogous $[Fe_4S_4]$
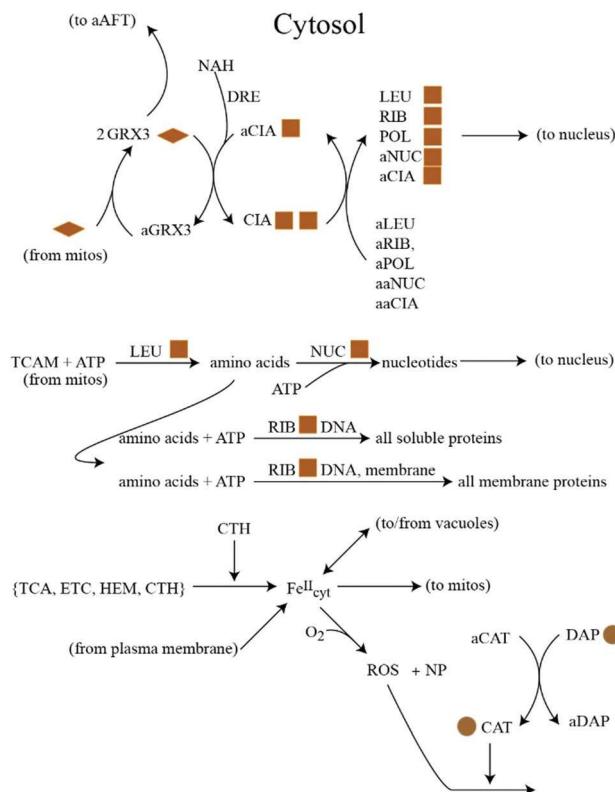


Fig. 2 Model scheme highlighting the iron-related processes in the cytosol. Brown diamonds, $[Fe_2S_2]$ clusters; squares, $[Fe_4S_4]$ clusters. The prefix *aa* refers to apo-forms that are metallated in two steps.

cluster that bridges the two subunits of the nitrogenase iron protein dissociates into $[Fe_2S_2]$ clusters under oxidizing conditions;[223] the same chemistry may operate in reverse for the CIA.

Returning to the model, the CIA transfers its transient $[Fe_4S_4]$ cluster to aLEU, aRIB, aNUC, aPOL, and aaCIA. The requirement for the CIA to metallate itself is an autocatalytic relationship (discussed below). **LEU** catalyzes the synthesis of AMINO ACIDS from the component **TCAM** (the metabolite of the TCA cycle). This reaction is part of anabolism and requires **ATP**. LEU contains $[Fe_4S_4]$ clusters and an iron-bound siroheme. As mentioned above, RIB is required for ribosome biosynthesis and protein translation. It is especially essential because it helps synthesize *all* proteins in the cell, including itself (another autocatalytic relationship). RIB contains $[Fe_4S_4]$ clusters and $Fe^{II}$ centers.

**NUC** catalyzes the synthesis of a model component called **NUCLEOTIDES** from AMINO ACIDS. This protein group contains an $[Fe_4S_4]$ cluster, an $[Fe-O-Fe]$ center, and an $Fe^{II}$ site. According to the model, the cluster is installed by the CIA whereas the other centers are metallated by $Fe_{cyt}^{II}$. Assembling an $[Fe-O-Fe]$ center from the Grx3 $[Fe_2S_2]$ cluster[43,44,51,63,64,191,192] seems unlikely since simple $Fe^{II}$ ions can assemble this center *in vitro*.[224]

**CAT** catalyzes the degradation of **ROS**. It contains heme b centers and an $[Fe_2S_2]$ cluster. The heme centers are installed by DAP, the heme chaperone. To account for the diverse cellular locations of CAT members, the CAT group may degrade ROS generated at any location in the cell.
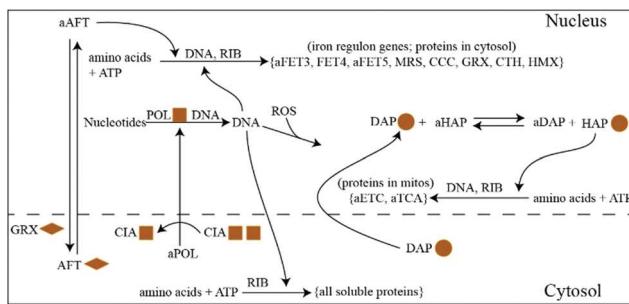
**Fig. 3** Model scheme highlighting the iron-related processes in the nucleus.

**CTH** degrades the respiration-related groups TCA, ETC, HEM, and CTH (promoting its own degradation is autoinhibitory); it also stimulates NUC activity. CTH is a member of the iron regulon; its expression during iron-deficiency helps shift the cell to fermentation mode and economize cellular iron. Cytosolic CTH is presumed to degrade the above proteins regardless of their location.

The nucleus includes protein groups **AFT**, **HAP**, and **POL** (Fig. 3). The first two are involved in homeostatic regulation while the last is involved in DNA replication and repair. Transcription factor AFT regulates iron traffic into the cell, iron movement into and out of vacuoles, and iron movement into mitochondria. It does this by regulating expression of iron regulon genes FET3, FET4, FET5, CTH, MRS, and HMX. AFT also regulates expression of CCC and GRX even though they are not part of the iron-regulon (due to membership of Yap5 in the AFT group). AFT has dual localization in the cytosol and nucleus, depending on whether it is apo (aAFT) or cluster-bound (AFT). Accepting a $[Fe_2S_2]$ cluster from GRX shifts the equilibrium towards the cytosol which prevents expression of the iron regulon. Under iron-deficient conditions, insufficient GRX is present such that most AFT is aAFT; this form translocates to the nucleus and promotes expression of the iron regulon (shutting-down expression of CCC). This homeostatic regulatory system responds to iron deficiency by...

- $\uparrow FET3 \rightarrow \uparrow Fe_{cyt}^{II}$.
(read *increasing FET3 expression causes an increase in $[Fe_{cyt}^{II}]$*)
- $\uparrow FET5 \rightarrow \uparrow Fe_{cyt}^{II}$.
- $\uparrow MRS \rightarrow \downarrow Fe_{cyt}^{II} \rightarrow \uparrow$ mitochondrial $Fe_{mit}^{II}$.
- $\uparrow HMX \rightarrow \uparrow Fe_{cyt}^{II}$.
- $\uparrow CTH \rightarrow$ degrades nonessential respiration-related proteins, economizing cellular iron, and $\uparrow Fe_{cyt}^{II}$ or $\uparrow Fe_{mit}^{II}$.
- $\downarrow CCC \rightarrow \uparrow Fe_{cyt}^{II}$.

Overall, there is a coordinated response of the AFT system to iron-deficiency – namely to increase $Fe_{cyt}^{II}$, even though $Fe_{cyt}^{II}$ is not considered to be "sensed" directly by the cell.[225] Cellular iron deficiency is thought to be sensed by the rate/extent by which ISU-generated $[Fe_2S_2]$ clusters (*i.e.* X–S) exit the mitochondria, bind to aAFT, and deactivate the iron regulon. However, for this mechanism to function properly there must be a causal connection between the concentration of $Fe_{cyt}^{II}$ and the rate/extent of X–S export into the cytosol. I hypothesize that *the rate of X–S exported from mitochondria is controlled by the*

*concentration of $Fe_{mit}^{II}$ which is, in turn, controlled by the concentration of $Fe_{cyt}^{II}$.* These connections close an autoinhibitory loop (see below). Without the causal chain

$$\uparrow Fe_{cyt}^{II} \rightarrow \uparrow Fe_{mit}^{II} \rightarrow \uparrow ISU \rightarrow \uparrow X\text{--}S$$
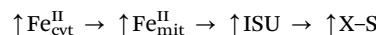
operating in healthy cells, an increase in $Fe_{cyt}^{II}$ could not be sensed by mitochondrial ISC activity or by AFT. In this case, $Fe_{cyt}^{II}$ would increase unceasingly, and the cell would be dysregulated (as is observed in many ISC mutants, in which case the causal connection $\uparrow Fe_{mit}^{II} \rightarrow \uparrow ISU$ is lost).

Returning to the model, HAP is a transcription factor that controls expression of heme-associated proteins involved in respiration. When bound with model component **heme b** (and in the presence of $O_2$), HAP stimulates expression of ETC. Switching from fermentation to respiration in aerobic conditions increases heme levels, and this activates HAP which stimulates expression of heme-requiring respiratory proteins.

POL helps replicate and repair DNA. It contains $[Fe_4S_4]$ clusters which are metallated in the cytosol by the CIA. POL converts NUCLEOTIDES into DNA during both replication and repair. DNA is easily damaged by ROS which is generated mainly as a side-product of respiration.

The mitochondrial matrix contains four protein groups called **ISU**, **ISA**, **TCA**, and **LYS** (Fig. 4). ISU catalyzes the assembly of $[Fe_2S_2]$ clusters using $Fe_{mit}^{II}$ as substrate. ISU contains a permanent $[Fe_2S_2]$ cluster (metallated by ISU in an autocatalytic manner) and a transient $[Fe_2S_2]$ cluster that is transferred to various target proteins.

ISA helps assemble $[Fe_4S_4]$ clusters and deliver them to target proteins. This group contains permanent $[Fe_4S_4]$ and $[Fe_2S_2]$ clusters, as well as a site for two $[Fe_2S_2]$ clusters that are reductively coupled to form a transient $[Fe_4S_4]$ cluster. The $[Fe_2S_2]$ clusters are donated by ISU and the formed transient $[Fe_4S_4]$ cluster is transferred to aTCA, aLYS, and aaISA (another autocatalytic process). TCA represents enzymes of the TCA cycle.
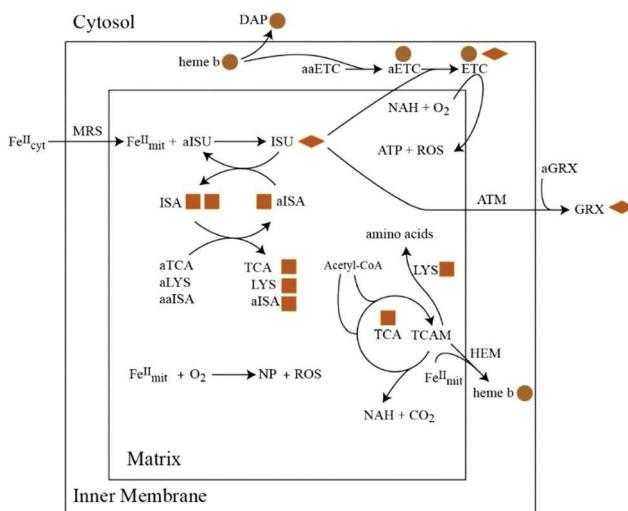


**Fig. 4** Model scheme highlighting the iron-related processes in the mitochondria.

This group receives [Fe$_4$S$_4$] clusters from ISA and [Fe$_2$S$_2$] clusters from ISU. TCA and TCAM both catalyze the reaction

$$\text{acetyl-CoA} \xrightarrow{\text{[TCA][TCAM]}} \text{NAH} + CO_2$$

(Note: model reactions are not balanced). TCA contains [Fe$_2$S$_2$], [Fe$_3$S$_4$] and [Fe$_4$S$_4$] clusters; thus, aTCA is metallated by both ISU and ISA. aLYS receives two [Fe$_4$S$_4$] clusters from ISA; LYS catalyzes the synthesis of AMINO ACIDS from TCAM.

There are four protein groups in the mitochondrial IM, including **MRS**, **ATM**, **ETC**, and **HEM**. MRS imports Fe$_{cyt}^{II}$ into the matrix where it becomes Fe$_{mit}^{II}$. MRS does not contain a permanent iron center. Iron import into mitochondria depends on the mitochondrial membrane potential. Since this property is not included in the model, the concentration of ETC (which should be proportional to membrane potential) is included as a catalytic influence (Table S2, ESI†). ATM is an IM exporter of [Fe$_2$S$_2$] clusters, passing them from ISU in the matrix to GRX in the cytosol. This is an ATP-requiring process.

ETC catalyzes the (unbalanced) reaction

$$\text{NAH} + O_2 \rightarrow \text{ATP} + \text{ROS}.$$
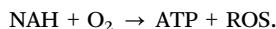
It contains heme centers, an [Fe–O–Fe] center and a [Fe$_2$S$_2$] cluster. Hemes are metallated by heme b, the [Fe$_2$S$_2$] cluster is installed by ISU, and the [Fe–O–Fe] center is metallated by Fe$_{cyt}^{II}$.

HEM catalyzes the (unbalanced) reaction

$$\text{TCAM} + \text{Fe}_{mit}^{II} + O_2 \rightarrow \text{heme b}.$$
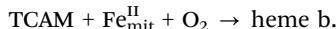
Heme b is used directly to metallate aETC, but it is bound to DAP for transport to other cellular regions.

The ER contains 2 protein groups **MEM** and **HMX** (Fig. 1). MEM is a membrane-bound group that catalyzes the synthesis of MEMBRANE from acetyl-CoA in an NAH- and O$_2$-requiring reaction. MEM contains heme b and [Fe–O–Fe] centers. The heme b sites are metallated by DAP and the [Fe–O–Fe] sites are metallated by Fe$_{cyt}^{II}$.

HMX is heme oxygenase anchored to the ER membrane. Under iron-deficient conditions, it degrades MEM, CAT, and other heme-containing groups (ETC) to generate Fe$_{cyt}^{II}$ and economize iron. HMX is a member of the AFT-dependent iron regulon.

Fe$^{II}$ ions play important roles in the model. Fe$_{cyt}^{II}$ is imported from the plasma membrane and sent to mitochondria and vacuoles. It reacts at the interface between the cytosol and the ER and diffuses into the nucleus and mitochondrial IMS. Fe$_{cyt}^{II}$ metallates proteins that contain either mononuclear Fe$^{II}$ or [Fe–O–Fe] sites. Fe$_{cyt}^{II}$ reacts with O$_2$ to generate ROS and nanoparticles (**NP**). Fe$_{cyt}^{II}$ converts to Fe$_{mit}^{II}$ when it is imported into the mitochondrial matrix. Fe$_{mit}^{II}$ is a substrate for ISC assembly and for heme b biosynthesis.[100,101,111,112,226,227] Like Fe$_{cyt}^{II}$, Fe$_{mit}^{II}$ can react with O$_2$ to generate ROS and NP. When Fe$_{cyt}^{II}$ is imported into vacuoles, it becomes Fe$_{vac}^{II}$, which is oxidized to Fe$_{vac}^{III}$. Mobilization of Fe$_{vac}^{III}$ is initiated by reduction to the Fe$_{vac}^{III}$ state[228,229] which promotes export into the cytosol.

Nutrient acetyl-CoA is a substrate of 3 reactions: the synthesis of MEMBRANES, the TCA cycle, and the synthesis of TCAM (reflecting anaplerotic reactions that serve to fill-up TCA metabolites to counterbalance their utilization in various anabolic processes). This last reaction is required because TCAM is consumed by the synthesis of AMINO ACIDS, NUCLEOTIDES, and hemes. The NAH generated by the TCA cycle is used to synthesize ATP.

Nutrient O$_2$ enters the cell at a defined rate and is used for respiration in mitochondria, and for dioxygenase and hydroxylase reactions in the ER to generate oxidized/unsaturated fatty acids. It is used in the high-affinity iron import pathway on the plasma membrane and in corresponding iron transport pathway from the vacuolar lumen to the cytosol. O$_2$ also reacts with Fe$_{cyt}^{II}$ and Fe$_{mit}^{II}$ to generate ROS and NP. ROS damages the cell by reacting with DNA and MEMBRANES.

# 3. Analysis of the model

Some general conclusions and themes are evident from the model, as listed below.

1. Iron import *via* plasma membrane and iron export *via* vacuoles are synchronized. Both membranes contain reductive Fe$^{III}$ and non-reductive Fe$^{II}$ pathways that are regulated by the iron status of the cell and by O$_2$. The two systems operating on two different membranes must function synchronously. Under iron-deficient aerobic conditions, the reductive systems (FET3 and FET5) dominate and both serve to increase Fe$_{cyt}^{II}$. Under iron-deficient hypoxic conditions, the non-reductive systems (FET4 and SMF) dominate, and both again serve to increase Fe$_{cyt}^{II}$. The opposite would happen under iron-replete conditions.

2. The cell economizes iron by shifting from respiration to fermentation. No iron-containing proteins involved in fermentation were identified whereas respiration uses iron extensively. Thus, the cell could switch from respiration to fermentation to economize iron during period of iron scarcity. Respiration-related iron is *unessential* as long as a fermentable carbon source is available.

3. Essential iron flows from the cytosol, through the mitochondrial ISU system, then through the CIA, and finally into the nucleus (summarized in Fig. 5). This is the most important traffic flow of iron in the cell, as the cell cannot survive without the [Fe$_4$S$_4$]-containing enzymes that help replicate and repair DNA. This is ironic since the biochemical roles of those ISCs are largely unknown. Such clusters might pass charge along the DNA for long-range signaling[230,231] but this is controversial[232] and perhaps simplistic given the diversity of nuclear enzymes that contain these clusters.

4. [Fe$_2$S$_2$] clusters flow out of the mitochondria and into the cytosol *via* the "GRX highway" which splits into CIA and AFT branches. After the split, some [Fe$_2$S$_2$] clusters are trafficked to the CIA (the essential branch) for conversion to [Fe$_4$S$_4$] clusters, while others are sent to the AFT regulatory branch.

5. In the mitochondria, the "ISU highway" splits into an ISA branch and a cytosol branch leading to GRX and CIA highways. The rate of production of mitochondrial [Fe$_2$S$_2$] clusters is regulated by the AFT system.
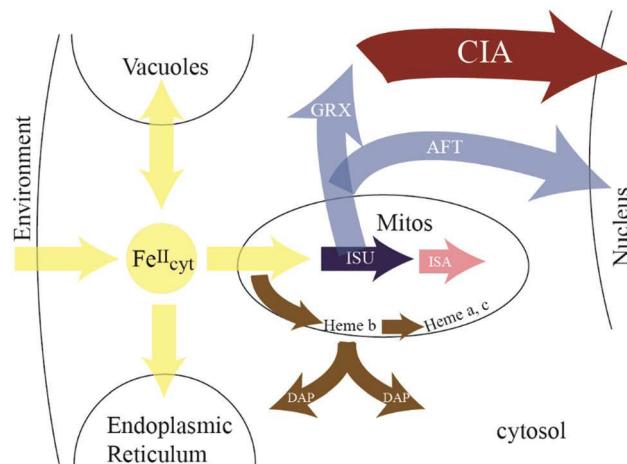
Fig. 5 Major traffic patterns of iron flow in yeast. Cytosolic iron plays a major role in distributing iron the cell; $Fe^{II}_{cyt}$ enters the cell from the plasma membrane and is trafficked to three major locations including the mitochondria, vacuoles and endoplasmic reticulum. Mitocondria play a major role in iron trafficking; the iron the flows into this organelle is converted into $[Fe_2S_2]$, hemes, and $[Fe_4S_4]$ clusters. Much of that iron is installed into mitochondrial respiratory complexes, but some hemes are $[Fe_2S_2]$ clusters are exported through the DAP and GRX "highways". The GRX pathway splits with a portion going to the CIA for conversion into $[Fe_4S_4]$ clusters and passage to the nucleus. This is the most critical flow of iron in the cell. Flow along the GRX pathway branches to AFT which is associated with iron regulation.

6. Heme b originates in mitochondria and splits; one branch converts to hemes a and c which are installed into mitochondrial respiratory proteins. The other branch flows heme b out of the organelle and into the cytosol (the DAP highway) for distribution to non-mitochondrial targets. DAP may be the chaperone used but further evidence is needed. Hemes are regulated by $O_2$, iron content, and metabolic mode (fermentation vs. respiration). Hemes are the major prosthetic groups in respiration-related proteins. Under iron-starved conditions, CTH will shut-down production of heme-containing proteins and HMX will degrade hemes to economize iron for essential DNA-related processes.

7. $Fe^{II}_{cyt}$ is the central iron species in the cell. $Fe^{II}_{cyt}$ is distributed to all other compartments in the cell. It used to metallate proteins with mononuclear iron and [Fe–O–Fe] centers. $Fe^{II}_{cyt}$ is strongly regulated by transport processes in the plasma membrane and vacuoles, which undoubtedly impacts the rate/efficiency of these metallation reactions. $Fe^{II}_{cyt}$ is imported into mitochondria where $Fe^{II}_{mit}$ serves as feedstock for ISC and heme biosynthesis. $Fe^{II}_{cyt}$ is dynamically stored in vacuoles.

8. Autocatalysis is an essential property of growing and dividing cells. Cells are *autocatalysts* because they catalyze their own synthesis from nutrients. This can be translated into a chemical reaction involving nutrient N and cell C (Fig. 6, panel A). One daughter cell can be viewed as the parent and catalyst for the reaction, while the other daughter cell can be viewed as the product. This property causes exponential growth of cells under conditions of unlimited nutrients. The molecular-level mechanisms responsible for it involve biochemicals in the cell
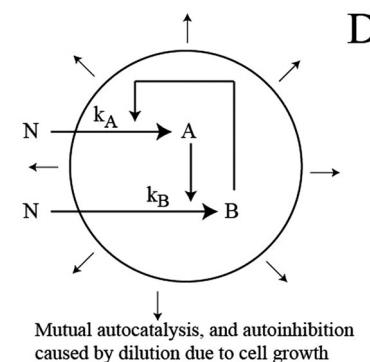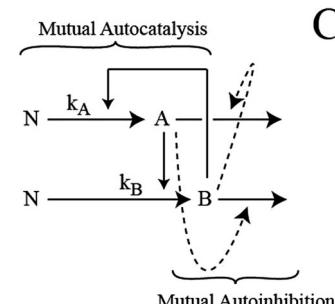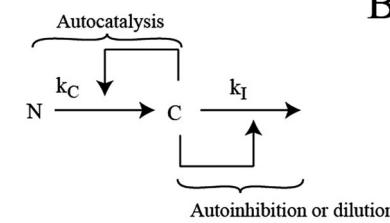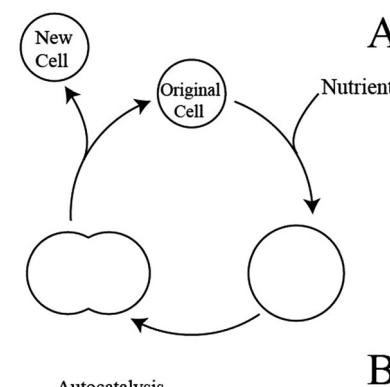


Fig. 6 Simple and mutual autocatalysis and autocatinhibitory mechanisms. Panel A shows a cell growing and dividing, which can be transformed into the simple chemical reaction shown in Panel B. Here, N is nutrient and C represents all components of the cell considered as a group. There may be specific autoinhibitory reactions or nonspecific autoinhibition due to cell growth/dilution. Panel C illustrates a mutual autocatinhibitory reaction in which two components (A and B) are mutual autocatalysts and mutual autoinhibitory agents. In this example, A catalyzes both the synthesis and degradation of B, and vice versa. Panel D illustrates a simple mutual autocatinhibitory system in which the cell grows at an exponential rate α that depends on both [A] and [B] but does not otherwise include specific autoinhibitory reactions.

operating together as a *mutual* autocatalyst. Cell models that possess this property should be more accurate and realistic than those that don't.

Mutual autocatalysis *can be identified by a causal chain that connects arrows head-to-tail, starting with an increase in one component and ending with an increase in the same component.* In these autocatalytic loops (ACLs), horizontal arrows indicate reactions and vertical or U-shaped arrows indicate catalytic influences. To identify an ACL in the system shown in Fig. 6C, follow a causal chain initiated by an increase in either component, *e.g.* A. If the chain eventually leads to a further increase in A, it is an ACL; here ↑A → ↑B → ↑A (read as *an increase in A causes an increase in B which causes a further increase in A*). For B, the ACL is ↑B → ↑A → ↑B. Mutual autocatalysis is lost when the catalytic influence of A or B is removed.

9. Autoinhibition is an essential property of homeostatically regulated cells in which a perturbation in any component from its "set-point" concentration will eventually cause it to return to its set-point. To achieve this property, each component must be part of an *autoinhibitory* loop (AIL). In AILs, a causal chain initiated by an *increase* in one component will eventually cause a *decrease* in the same component. Reaction networks occurring within exponentially *growing* cells are *guaranteed* to have an AIL because the ODE for each component $C_i$ will include a dilution term that is proportional to the particular component,[233,234] as follows.

$$\frac{d[C_i]}{dt} = \underbrace{k[*][*]\ldots}_{\text{chemical terms}} \quad \underbrace{-\alpha[C_i]}_{\text{dilution terms}}$$

The inclusion of the dilution term immediately leads to an AIL of the form ↑$[C_i]$ → ↓$[C_i]$ (this is not technically a loop since it involves just one species). There may be other more specific and more powerful AILs than those associated with dilution.

10. Autocatinhibition is a property of homestatically regulated cells that are growing and dividing: autocatalysis and autoinhibition can occur in the same dynamical system,[235] and we propose that this includes cellular systems. Mutual autocatalysis and mutual autoinhibition *occur simultaneously* in cells. Thus, models of cellular processes occurring within cells should include this property for greater accuracy and realism. In the simplest *autocatinhibitory* system (ACIS) of Fig. 6B, component C is both a catalyst for its own production and a catalyst of its own degradation. If the nutrient concentration is fixed and in excess, the ODE describing time-dependent changes in C might be

$$d[C]/dt = k_C[C] - k_I[C]^2.$$

The second-order dependence of [C] in the negative term arises because C is presumed to be both a first-order substrate and a catalyst for its own degradation. Note that the autoinhibitory chain does *not* loop-back onto C but flows outward from C. The sign of the horizontal reaction arrow must be reversed to create the loop $\left( \xleftarrow{k_2} \right)$ but the direction of causality must also be reversed, becoming ↑C → ↓C.

Either autocatalysis or autoinhibition can dominate depending on the rates of the reactions associated with the chains – or they can be balanced in which case the steady-state concentration of [C] would equal $k_C/k_I$. In the mutual autocatinhibitory system of Fig. 6C, an increase in A will cause an increase in B which could either cause an increase in A (ACL dominates) or a decrease in A (AIL dominates), depending on rates.

Assessing whether a more complicated system is autocatinhibitory is more difficult. The difficulty can be diminished if cell growth is assumed (*e.g.* Fig. 6D); then the only assessment is whether the system is autocatalytic. One necessary condition for a group of chemicals to form a mutual autocatalytic system is that each considered species is related to the others. *A component of the system will influence another component of the system and be influenced by yet another component.* Symbolically, $C_i$ would be a component of the system if

$$↑C_j → (↑ \text{ or } ↓)C_i \text{ and if } ↑C_i → (↑ \text{ or } ↓)C_k$$

where $C_j$ and $C_k$ are known components of the system. Lacking a more rigorous assessment method, ACL's for each component were informally identified by tracing loops mentally. Using these criteria, the 40 components described above appear to be members of an ACIS (see Table S2 for sample influences, ESI†). Nutrients acetyl-CoA, $O_2$, and environmental iron are not components of the ACIS because their rates of synthesis are not influenced by any component of the system. Waste products $CO_2$ and nanoparticles are not components of the ACIS because they do not influence any other components. I have selected FET3 to illustrate an ACL

$$↑FET3 → ↑Fe^{II}_{cyt} → ↑Fe^{II}_{mit} → ↑ISU → ↑GRX → ↑CIA → ↑POL$$
$$→ ↑FET3$$
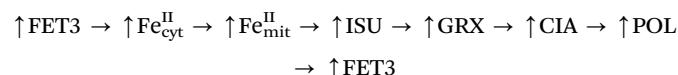
In this loop, an increase in FET3 causes an increase in $Fe^{II}_{cyt}$, which causes an increase in $Fe^{II}_{mit}$, which causes an increase in ISU in mitochondria, which causes an increase in GRX in the cytosol which causes an increase in the CIA which causes an increase in POL which generates more DNA which allows more FET3 (as well as all other proteins in the cell) to be expressed. One AIL is

$$↑FET3 → ↑Fe^{II}_{cyt} → ↑Fe^{II}_{mit} → ↑ISU → ↑GRX → ↓aAFT$$
$$→ ↓FET3$$
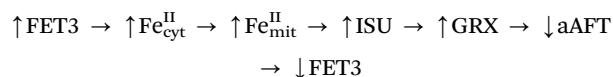
Here, an increase in FET3 has the same effect as above except that an increase in GRX also causes a *decrease* in AFT activity, which causes a decrease in FET3 (since the iron regulation will be turned-off).

Both processes operate simultaneously in the cell. Consider an exponentially growing cell that is just barely iron-sufficient such that both loops are operating at half-capacity. As the cell grows, new FET3 must be generated to maintain a constant concentration in the plasma membrane. If iron is limiting growth, then adding more iron will increase the ACL rate (and the cell will grow faster). Then, after excess iron enters the cell, the AIL rate will increase, shutting down excessive iron import. The opposite would happen if an iron chelator were added.

Only some components of the system will have a specific AIL pathway (*e.g.* the iron regulon) because only some processes are tightly regulated. Likewise, only some components (*e.g.* Yah1, CIA, ISA, RIB) have a direct specific ACL pathway. Perhaps such

components need to be activated or deactivated more quickly (as a burst) than do other components. Such behavior seems to be particularly important for the ISC assembly systems in the cell, implying the need for ultrasensitive regulation for this process.

There has been speculation, including from my lab[236] regarding the species that is "sensed" by the cell in controlling the Aft1/2-dependent iron regulon. Prior to *ca.* 2004, cytosolic iron was presumed to be the sensed signal that controlled the iron regulon. Thereafter, the ISC activity of mitochondria and/ or the rate of X–S export have been viewed as the signal. Cellular regulatory mechanisms are commonly interpreted from the perspective of engineering control theory in which a signal is monitored and compared to a set-point value. Any difference between the two is minimized by adjusting mechanical devices that increase or decrease the signal. However, considering the *chemistry* of AIL and ACL causal loops might be more accurate. This perspective implies that *there is no unique signal in regulatory processes – any member of the causal chain could be a signal.* For example, if the rate of ISC assembly limits the rate of the loop, as occurs in many ISC mutations (*e.g.* Yfh1Δ), *the rate of the entire loop* will affected – similar to how inhibition of any step of an enzyme catalytic cycle can slow the overall rate of catalysis (at steady state). Cytosolic $Fe^{II}$, mitochondrial ISC activity, and X–S are components of the same causal chains. A deletion that affects any component of that chain would cause the same phenotype. In my view, the search for sensed regulatory signals should be replaced by efforts to better understand loop mechanisms and kinetics.

The next step in developing the 40-component model will be to generate a set of ODEs that translate the chemistry developed here into a quantitative description of the flow or iron into the cell and into various cellular compartments. To integrate these ODEs, an entire set of kinetic parameters (rate constants and concentrations) must be employed, but many of these are unknown. If even approximately correct values can be found, the model should be able to simulate (to some degree) the phenotypes observed when *any* of the 139 proteins of the ironome are deleted which would be a novel achievement.

## Conflicts of interest

There are no conflicts to declare.

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