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# Cellular responses to high iron: the critical role of iron-sulfur clusters

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**Abstract**

Iron is a key element required by all eukaryotes. It is utilized by cells in many fundamental biological processes, but it could also damage cells via Fenton chemistry. Therefore cellular iron must be tightly regulated. Cells have developed sophisticated strategies to cope with various iron availabilities. Cellular responses to low iron have been extensively studied and reviewed. The scope of this review is to summarize the current understanding of the cellular responses to high iron. In yeast *Saccharomyces cerevisiae* high iron induces the expression of CCC1, which encodes the main vacuolar iron transporter, thereby promoting the storage of iron into vacuoles for detoxification. The expression of CCC1 is regulated by the transcription factor Yap5p. Recently published results indicate that Yap5-dependent gene induction depends on the mitochondrial iron-sulfur (Fe-S) cluster synthesis and lacks direct correlation with cytosolic iron level. In mammalian cells high-iron responses involve the transcription factor IRP1. High iron converts IRP1 into aconitase, an iron-sulfur cluster containing protein that lacks mRNA binding ability. This decreases iron acquisition and increases iron storage protein ferritin. Iron sulfur clusters seem to play a revolutionarily conserved role in high iron sensing and responses in cells.

## 1. Introduction

Iron is a key element required by all eukaryotes. It is utilized in many fundamental biological processes including biosynthesis of iron-sulfur (Fe-S) cluster and heme groups (1,2). However the free form of iron is reactive and toxic to cells due to its ability to generate reactive oxygen species (ROS) via Fenton chemistry (3-5). Therefore iron must be tightly regulated within cells.

Cells have developed sophisticated strategies to cope with various iron availabilities in the surrounding environments. The best-characterized system is the baker's yeast *Sacchromyces cerevisiae*. Extensive studies have been carried out to study the responses of the yeast *Saccharomyces cerevisiae* to iron deprivation and the results have been reviewed in the recent reviews (6,7). *Saccharomyces cerevisiae* responds to iron deprivation by inducing the expression of approximately 20 genes (collectively referred to as the *iron regulon*). Most of these genes encode cell surface elemental iron transporters (including the high affinity transporter Ftr1) and vacuolar iron transporter, cell surface siderophore transporters. The expression of these genes is regulated largely by the transcription factor Aft1p as well as to some degree by its paralog Aft2p. Aft1/2p are localized in the cytosol and enter nucleus when cells sense low iron. While in the nucleus they occupy the promoters of the iron regulon genes. Studies have suggested that Aft1p's response to cellular iron is indirect: it responds to the changes in mitochondrial iron-sulfur (Fe-S) cluster biosynthesis and export, for which iron serves as an essential substrate (8,9). Defect in mitochondrial Fe-S cluster assembly or export machinery leads to constitutive activation of iron regulon genes, regardless of iron availability in growth medium. However, defect in cytosolic Fe-S cluster assembly machinery does not activate iron regulon genes. The precise role of Fe-S clusters in the Aft1p sensing remains elusive, but studies have suggested that the proteins Grx3p and Grx4p are involved (10,11).

Due to the unstable nature of $Fe^{II}$ in oxygenated solution and low solubility of $Fe^{III}$ at physiological pH, iron deficiency is commonly encountered by modern living organisms. Under some special conditions, e.g. low pH, iron availability can be high. Ancient living organisms, which lived in an environment free of oxygen, needed to develop strategies to cope with high iron environments. The cellular responses to high iron are relatively less extensively studied compared to the studies on iron deprivation. The purpose of this review is to summarize the current understanding of cellular responses to high iron. Recent results clearly indicate that Fe-S cluster status also plays an important role in cellular sensing and responses to high iron.

## 2. High iron responses in yeast

The yeast *Saccharomyces cerevisiae* requires certain amount of iron for its optimal growth. For the growth of the wild-type strain, the optimal iron concentration in the growth medium is 50-100 μM. The growth medium containing iron higher than the optimal concentration may be considered as high iron condition. It is worth noting that wild-type yeast strain is considerably resistant toward high iron, exhibiting little growth defect with up to 5 mM Fe in the medium.

In the yeast *Saccharomyces cerevisiae*, vacuole serves as the major organelle for iron storage and detoxification. Recent biochemical and spectroscopic studies on the isolated yeast vacuole indicate that iron is stored in this organelle mainly at the form of an $Fe^{III}$ species (mononuclear high spin $Fe^{III}$ species and $Fe^{III}$ nanoparticles, possibly containing phosphate) (12-14). The vacuolar membrane protein Ccc1p is the main iron importer and its substrate is thought be an $Fe^{II}$ species (15). Once $Fe^{II}$ enters vacuole, they are oxidized to $Fe^{III}$ and stored within. Vacuolar iron exporters include Smf3p and Fet5p-Fth1p complex (14).

Yeast responds to high iron by activating expression of the vacuolar iron transporter Ccc1p and storing iron in the vacuole. The *Δccc1* strain is sensitive to high iron, exhibiting growth defect with as low as 100 μM iron in the medium. The expression of Ccc1p is regulated by the transcription factor Yap5p (16). Yap5 belongs to an eight-membered ZIP family in yeast (Yap1-Yap8). These homologous Yap proteins are involved in responses to various extracellular stimuli (17). For example, Yap1 and Yap2, are transcriptional activators involved in pleiotropic drug resistance. Scanning the promoter region of the CCC1 gene revealed several Yap binding motifs and further experiments demonstrated that Yap5p is a key transcription factor responsible for CCC1 expression under high iron conditions. Deletion of YAP5 gene renders cells unable to activate CCC1 expression, thus leads to increased sensitivity to high iron. Yap5p is constitutively localized into the nucleus and remains bound to the gene promoter region (16). The activation domain of Yap5p contains seven cysteines which play a key role in iron sensing, as mutation of those cysteines reduce CCC1 transcription. Besides CCC1, Yap5p also regulates the expression of at least other two genes, TWY1 and GRX4 (18).

Early studies proposed that Yap5p was directly sensing the cytoslic iron level (19). This conclusion was mainly based on the seemingly direct correlation between cytosolic iron, medium iron and CCC1 expression level. Accordingly, higher medium iron was thought to lead to higher cytosolic iron and therefore higher CCC1 expression level.

This correlation is true for wild type and *Δccc1* cells. But it does not establish that Yap5p directly senses cytosolic iron. A recent study utilized a well-designed genetic screen to identify the genes involved in the high-iron transcriptional response (20). This screen used two different reporter systems, including CCC1-LacZ and GAL1-HIS3/GAL4-YAP5 CRD (cysteines rich domain). Use of two different constructs ensured that the identified mutation would be in a common iron-sensing system. This screen identified SSQ1 as the gene whose mutation leads to the inability of Yap5p to respond to high medium iron. Ssq1p is a mitochondrial chaperon protein involved in mitochondrial Fe-S cluster assembly. Further

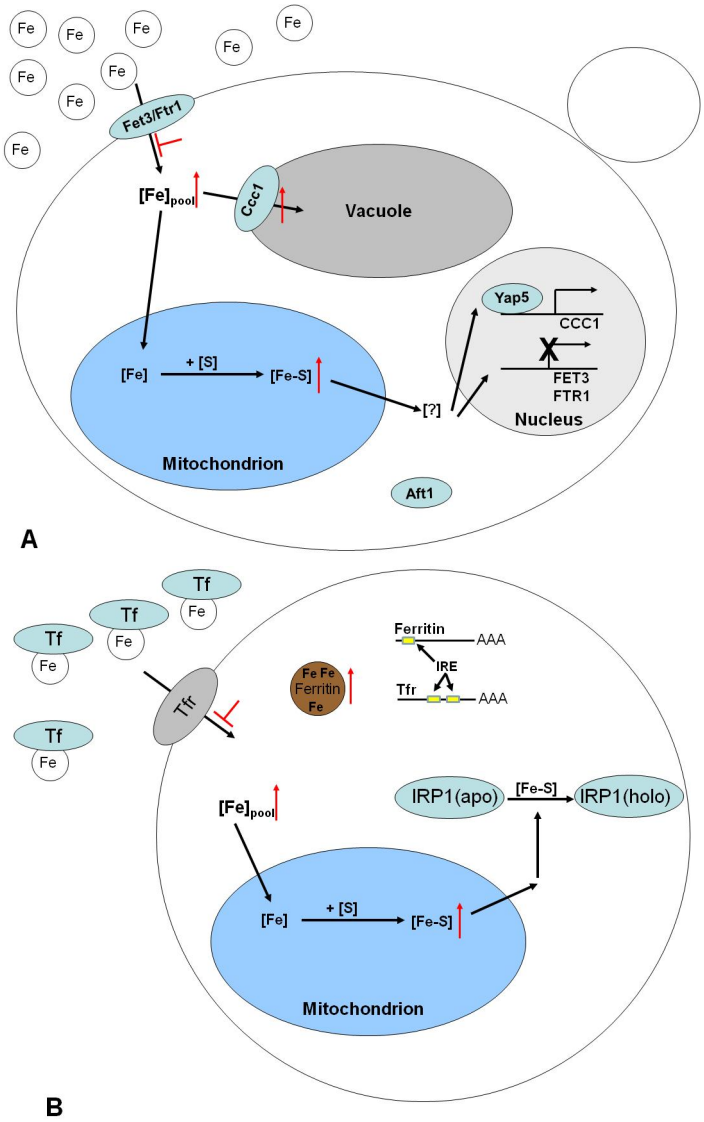


**Figure 1. Schematic of the role of Fe-S cluster in cellular responses to high iron.** A, in yeast *Saccharomyces cerevisiae*, high iron leads to increased cytosolic Fe pool and elevated synthesis of mitochondrial Fe-S cluster. This mitochondrial Fe-S cluster status is transferred out of mitochondria and into nucleus where Yap5 activates the expression of Ccc1p. Higher level of Ccc1p imports extra cytosolic Fe into vacuole. Meanwhile Aft1p senses the Fe-S cluster status and retains in cytosol therefore unable to activate the expression of the iron regulon genes, which leads to decreased cellular iron uptake. B, in mammalian cells, high iron leads to increased cytosolic Fe pool and elevated synthesis of Fe-S cluster. This leads to formation of holo-form of IRP1, which binds to Fe-S cluster and lacks IRE-binding ability. These responses lead to decreased cellular iron uptake and enhanced iron storage into ferritin.

experiments show that deletion or depletion of other key proteins involved in mitochondrial Fe-S assembly (Isu1p, Yfh1p) leads to similar phenotype. This suggests that Yap5p may sense mitochondrial Fe-S cluster status.

However, disruption of mitochondrial Fe-S cluster assembly also causes iron accumulation in mitochondria and this may deplete cytosolic iron. Therefore a reliable method for estimating cytoslic iron is crucial for distinguishing these two possibilities. In the same paper (20) Li et al. reported an novel method to estimate the cytosolic iron level. They expressed a well-characterized bacterial enzyme (gentisate 1,2-dioxygenase, GDO from *Pseudaminobacter salicylatoxidans*) in yeast, which strictly depends on $Fe^{II}$ for activity (21,22). This enzyme has relatively low affinity for $Fe^{II}$ due to the fact that it binds iron with only three ligands (3 His residues) (21). This property enables its use as a readout of the cytosolic iron concentration. In contrast, *E. coli* Fe SOD binds $Fe^{II}$ with 4 ligands and has higher affinity for iron. The SOD activity largely remains unchanged even under iron deprivation conditions (23). The GDO activity is not present in yeast and is easy/inexpensive to assay. Further experiments clearly demonstrated the validity of this method for estimating cytoslic iron level in yeast. Their results show that cytoslic iron level does not decrease even when mitochondrial Fe-S cluster assembly is disrupted, i.e. by mutation of Yfh1p or Isu1p. Therefore Ccc1p expression does not correlate directly with the cytosolic iron level. All these results indicate that Yap5p does not sense cytosolic iron. Instead it probably senses mitochondrial Fe-S status and accordingly regulates Ccc1p expression. Interestingly, disruption of cytosolic iron-sulfur assembly (CIA) machinery does not decrease Yap5p activation, indicating Yap5p activity does not depend upon CIA function. The similar phenotype has been observed for Aft1p, the transcription factor responsible for low iron responses (9).

Similar results have been reported in other studies. Miao et al. reported in their spectroscopic studies (24) that depletion of Yah1p, another key protein involved in mitochondrial Fe-S assembly, leads to evacuation of vacuolar iron and accumulation of iron in mitochondria. Yah1 depletion decreases mitochondrial Fe-S cluster synthesis therefore induces iron regulon genes to increase cellular iron uptake. Cytosolic iron may remain unaltered under such Fe-S cluster defective condition. Cells do not store extra iron into vacuole since low mitochondrial Fe-S cluster synthesis causes Yap5p unable to induce Ccc1p expression. Instead most of iron is imported into mitochondria and precipitate to form $Fe^{III}$ nanoparticles, a common phenotype that has been observed in a number of mitochondrial Fe-S cluster defective mutants (25-28).

Current results clearly indicate that mitochondrial Fe-S cluster status plays a key role in the iron sensing processes. When iron is high, mitochondrial Fe-S cluster synthesis is elevated, as evidenced by higher aconitase activity (23); high Fe-S cluster levels cause Aft1p to localize in the cytosol such that it can not induce the iron regulon genes; Yap5p also senses this change and induces the expression of Ccc1p to store extra iron into vacuole (See Figure 1A). Upon iron deprivation, mitochondrial Fe-S cluster assembly is low since iron is the substrate for Fe-S cluster assembly; low Fe-S cluster levels are sensed by Aft1p, which enters the nucleus and activates iron regulon genes to enhance cellular iron uptake. Low Fe-S cluster levels are also sensed by Yap5p, which inactivates the expression of Ccc1p to diminish iron import into vacuoles.

## 3. High iron response in mammalian cells

In mammalian cells, cellular mechanism for iron sensing is distinctly different from yeast. Iron regulatory proteins 1 and 2 (IRP1 and IRP2) respond to the iron availability and post-transcriptionally regulate the expression of the genes involved in iron metabolism to optimize cellular iron acquisition and utilization (29-31). Under high iron condition, IRPs do not bind to iron responsive elements (IREs), which can be found in the mRNAs of the iron storage protein (ferritin), the transferrin receptor (TfR), the divalent metal ion transporter 1 (DMT1) and other iron metabolism proteins, thereby diminishing cellular iron uptake and enhancing iron sequestration. The position of IREs in the

mRNA of ferrin, TfR and DMT1 determines whether the protein expression is inhibited or allowed (Figure 2B). Upon iron starvation, IRPs bind to IREs and have the opposite responses. The IRE-binding ability of IRP1 has been extensively studied and now it is well accepted that the "iron-sulfur cluster switch" is a critical factor determining whether IRP1 has aconitase or IRE-binding activity (29,32,33). High iron leads to adequate Fe-S cluster synthesis and the apo-form of IRP1 binds a Fe-S cluster becoming the holo-form as aconitase. This Fe-S cluster bound form of IRP1 lacks IREs-binding ability (Figure 1B). Low iron leads to low Fe-S cluster synthesis and IRP1 loses the Fe-S cluster to become the apo-form and binds to IREs of iron-responsive genes.

The importance of Fe-S cluster status in determining the IREs-binding ability of IRP1 is supported by other studies. Knockdown of ISCU (the mitochondrial scaffold protein on which nascent Fe-S cluster is assembled) in mammalian cells leads to defective mitochondrial Fe-S cluster assembly and markedly decreases mitochondrial aconitase activity. Under this condition IRP1 loses the Fe-S cluster and acquires IRE-binding ability (34). These results indicate the mitochondrial Fe-S cluster assembly machinery is required for IRP1 function.

Fe-S cluster assembly in IRP1 also depends on the cytosolic iron-sulfur assembly (CIA) machinery. Nbp35 depletion in human cells by RNA interference (RNAi) led to defective cytosolic Fe-S cluster biogenesis and impaired the conversion of IRP1 to cytosolic aconitase (35). Similarly, depletion of Nar1 in cultured animal cells led to an increase in the IRE-binding activity of IRP1 due to defective cytosolic Fe-S assembly (36). This is in contrast to yeast, where both high iron (Yap5) and low iron (Aft1) sensing appear to be independent of CIA machinery (20,37). It is noted that another regulatory protein (IRP2) exhibits little dependence upon Fe-S cluster status, as it does not bind Fe-S cluster (38).

It has been observed that an increase in cellular iron in mammalian cells leads to an enhanced level of conversion of IRP1 to cytosolic aconitase (38). This indicates that elevated iron stimulates Fe-S cluster biosynthesis (Figure 1B). It has been therefore suggested that the mammalian cells have an excess capacity for Fe-S cluster assembly and this process is being rate-limited by Fe availability.

Recent discovery of the high-resolution crystal structures of IRP1 and its RNA complex have provide key insights into the nature of this unusual conversion process (39,40). Like other members in the aconitase family, IRP1 has a four-domain organization (41). The first three domains are tightly associated and the fourth domain is connected through a long linker. Under high iron situation, IRP1 acquires a Fe-S cluster and exists as the holo-form. The conformation of the aconitase form of IPR1 has four domains tightly associated due to the bridging effect of Fe-S cluster. Upon iron starvation, IRP1 loses the bridging Fe-S cluster and binds IRE with high affinity. Upon binding to IRE, IRP1 undertake considerable conformational changes. Most noticeably, the domain 3 and 4 open up by ~25Å (relative to aconitase form) to form an open "L" shop conformation. More interestingly, the IRE binding sites coincide well with the Fe-S cluster-binding region in the aconitase form, using many same amino acid residues. This explains the mutually exclusive nature of these two functions of IRP1. In summary, the IRE-binding ability of IRP1 depends on Fe-S cluster status and accordingly regulates iron acquisition and iron storage to optimize the cellular iron distribution and utilization.

## 4. Conclusions

Cells have developed sophisticated strategies to cope with various iron availabilities. Recent studies indicate the importance of Fe-S cluster status in the cellular response to both low and high iron. In yeast, high iron leads to adequate Fe-S cluster assembly and induces expression of Ccc1p (via the transcription factor Yap5p) to enhance iron storage into vacuole and repress the expression of iron regulon genes (via the transcription factor Aft1p) to diminish cellular

iron uptake; low iron leads to insufficient Fe-S assembly thus have the opposite responses. In mammalian cells, under high iron condition IRP1 binds a Fe-S cluster thus lacks IREs-binding ability and this leads to diminished iron uptake and enhanced iron sequestration. Under low iron conditions, mammalian cells show low Fe-S cluster assembly levels and IRP1 existing as an apo-form without Fe-S cluster which binds to IREs and have opposite responses.

The detailed molecular mechanism of the iron sensing processes is still not well understood. For example, how mitochondrial Fe-S cluster status is transferred to and sensed by Yap5p, a transcription factor protein localized in nucleus, remain to be determined. Such research will lead to a more complete and accurate understanding of iron metabolism.

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